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In Immunoassays for Residue Analysis; Beier, R., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

ACS SYMPOSIUM SERIES **621**

Immunoassays for Residue Analysis

Food Safety

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Developed from a symposium sponsored
by the Division of Agriculture and Food Chemistry
at the 209th National Meeting
of the American Chemical Society,
Anaheim, California,
April 2–7, 1995

American Chemical Society, Washington, DC 1996

TX 549 .I66 1996 Copy 1

Immunoassays for residue
analysis

Library of Congress Cataloging-in-Publication Data

Immunoassays for residue analysis: food safety / Ross C. Beier, Larry H. Stanker, editors.

p. cm.—(ACS symposium series, ISSN 0097–6156; 621)

"Developed from a symposium sponsored by the Division of Agriculture and Food Chemistry at the 209th National Meeting of the American Chemical Society, Anaheim, California, April 2–7, 1995."

Includes bibliographical references and indexes.

ISBN 0–8412–3379–9

1. Immunoassay. 2. Pesticide residues in food. 3. Food—Analysis.

I. Beier, Ross C., 1946– . II. Stanker, Larry H., 1948– . III. American Chemical Society. Division of Agriculture and Food Chemistry. IV. American Chemical Society. Meeting (209th: 1995: Anaheim, Calif.) V. Series.

TX549.I66 1996
334′.07—dc20 95–50870
CIP

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Foreword

THE ACS SYMPOSIUM SERIES was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of this series is to publish comprehensive books developed from symposia, which are usually "snapshots in time" of the current research being done on a topic, plus some review material on the topic. For this reason, it is necessary that the papers be published as quickly as possible.

Before a symposium-based book is put under contract, the proposed table of contents is reviewed for appropriateness to the topic and for comprehensiveness of the collection. Some papers are excluded at this point, and others are added to round out the scope of the volume. In addition, a draft of each paper is peer-reviewed prior to final acceptance or rejection. This anonymous review process is supervised by the organizer(s) of the symposium, who become the editor(s) of the book. The authors then revise their papers according to the recommendations of both the reviewers and the editors, prepare camera-ready copy, and submit the final papers to the editors, who check that all necessary revisions have been made.

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Preface

RESIDUE ANALYSIS IN FOOD SAFETY is a rapidly growing field using a wide array of analytical methods. Immunoassay of small molecules is becoming an important method within many methods of analysis. The use of immunoassays in food safety is relatively new, but they are currently being used worldwide. Because of the ease of use, immunoassay will no doubt become a method of high use in the areas of food safety, food manufacturing, and quality assurance.

This volume is based upon a symposium that focused on the application of immunoassays for residue analysis in food safety. Our challenge, at the outset of planning this symposium, was to bring together researchers who were applying immunoassay at the forefront of residue analysis in foods worldwide. This challenge was met when, in fact, experts in the area of immunoassay from all parts of the globe attended the symposium, and many of them have contributed chapters to this volume. We have come to learn through this endeavor that not only are more reports of new immunoassays occurring in the literature, but many new companies are handling immunoassay kits worldwide.

Recently, there has been an explosion in the number of symposia and meetings encompassing the field of immunoassay, and also the number of books that have been published as a result of these meetings. This book is different, in that it focuses on the basic concepts of immunoassay and the application of immunoassay analysis in food safety.

The book is divided into seven sections. The first, Introduction to Immunoassays, looks at the concept of immunoassays, their formats, and how they may be adapted to the analysis of foods. Also discussed is the detection and removal of sample matrix effects in immunoassays. The section ends with an overview of the use of rapid test methods in regulatory programs. The second section, Immunoassay of Veterinary Drugs, concentrates primarily on immunoassay of chemicals used in large-animal production, for example, coccidiostats in poultry and β-agonistic drugs used as growth promoters, as well as antibiotics. Approaches to the synthesis of haptens for organophosphate and pyrethroid insecticides, including the detection of pesticides in human milk and dairy products and the application of commercial immunoassay kits to plant and water samples, are shown in Immunoassay of Insecticides, Fungicides, and Pesticides, the third section. The fourth section includes examples of immunoassay of natural toxicants and phytoprotectants in food, naturally occurring alkaloids in foods, staphylococcal enterotoxins, and insect contaminants. Immunoassay of Mycotoxins, the fifth section, reviews immunoassay development for mycotoxins and covers immunoassay detection of acetylated deoxynivalenol, citrinin, fumonisins, and the *Alternaria alternata* mycotoxin. Immunoassays for the detection of mercury in seafood and animals; the toxins that cause paralytic shellfish poisoning; and a comparison of immunoassay, cellular, and classical bioassay methods for detection of neurotoxic shellfish toxins are discussed in the sixth section, Immunoassay of Residues in Fish. The final section, Applications of New Immunoassay Methods and Protein Interactions, presents new applications of immunoassay methods. These chapters describe coupling supercritical fluid extraction and immunoassay, solid-phase fluorescence immunoassay, surface plasmon resonance immunosensor assay, and the use of recombinant antibodies for immunoassay.

We hope that the basic concepts of immunoassay and the many applications of immunoassay in food safety presented here will provide a stimulus for those interested in this topic.

We thank the authors for completing and finalizing their chapters in a timely manner. We express our thanks and appreciation to the ACS Division of Agricultural and Food Chemistry for supporting the symposium, and to the Biotechnology Secretariat for coordinating our segment of the symposium.

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November 1, 1995

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Chapter 1

Introduction to Immunoassays for Residue Analysis

Concepts, Formats, and Applications

Larry H. Stanker and Ross C. Beier

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Modern agriculture has benefited from the development and use of agrochemicals including insecticides, herbicides, and veterinary drugs. In addition to these exogenous chemicals, many undesirable compounds (e.g., plant toxicants and mycotoxins) are known to occur naturally. Thus, there exists a need for monitoring residue levels in foods, other agricultural commodities and environmental samples. Immunoassay (IA) represents an additional chemistry that is rapidly becoming available to the analysts for residue analysis. An ever increasing number of manuscripts that describe individual immunoassays for detection of different residues in specific matrixes have appeared. In addition many excellent reviews recently have been published. It is not the aim of this chapter to duplicate these reviews or tabulate all known immunoassays. Such a task is beyond the scope of this chapter or even a single volume. Instead, we seek to present some of the salient features of IA and to provide the reader a general introduction to the principles, formats, and applications of IA to residue analysis, particularly to analysis of foods. When ever possible, examples to illustrate immunoassay principles are taken from chapters in this book.

Traditionally, residue analysis has relied upon classical analytical methods such as chromatography, either gas or liquid, coupled to various detectors, such as UV absorbency, mass spectrometry, or fluorescence detection. Such methods require highly trained individuals to operate sophisticated instruments and interpret complex chromatograms or spectral results. These features make most traditional residue methods highly accurate, but they also make them time consuming, costly, and generally not adaptable to use in the

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field. An alternative approach is to utilize immunochemical methods, such as immunoassays, for residue analysis. A number of excellent reviews (*1–3*) discussing the use of immunoassay in residue analysis have recently appeared. In addition to residue analysis, immunoassays have applications in a number of areas of the food industry including i) quality assurance, ii) estimation of naturally occurring products such as flavor constituents, mycotoxins (reviewed by Chu in this volume), and seafood toxins (*4*, see chapters by Dietrich *et al.*, and Garthwaite *et al.*, this volume), and iii) detection of microbial or insect contamination (see chapter by Kitto *et al.*, this volume).

Antibody Molecules

The central component of any IA is the antibody molecule. Antibody molecules are serum proteins that have the ability to specifically bind a related group of antigens. Antigens recognized by antibodies are most commonly proteins, glycoproteins or glycolipids found naturally on the surface of invading microorganisms. Antibodies that bind with high specificity to small organic chemicals can be isolated. However, small molecules of less than a few thousand daltons in molecular size are not immunogenic. In order to produce antibodies to these small molecules, they must be covalently linked to a larger carrier molecule, usually a protein. Immunization with this complex antigen results in production of antibodies to the carrier protein, as-well-as antibodies to the small molecule of interest. Molecules with this property, (i.e., that can be bound by antibodies, but need to be coupled to a carrier protein in order to act as an immunogen) are referred to as haptens. These principles were demonstrated early in this century and are exemplified by the pioneering work of Landsteiner (*5*) who developed antibodies capable of binding *m*-amino benzene arsonic acid. Furthermore, Landsteiner demonstrated that antibody binding was sensitive to substitutions on the benzene ring. These studies and others clearly demonstrate that immunoassays are ligand binding assays, and are not bioassays that depend on a biological process such as cell growth, enzyme activation, or cell division.

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Antibody molecules are complex biomolecules composed of heavy (H) and light (L) chain polypeptides. A typical immunoglobulin-G antibody molecule is constructed of two identical H-chains and two identical L-chains. The H- and L-chains are held together by disulfide bridges resulting in the formation of a divalent molecule (Figure 1). The H- and L-chains are organized into variable and constant regions, and the antigen binding site, or combining site, is created by the association of the variable region portions (located at the amino end) of the H- and L-chains. Amino acid analyses (*6*) indicate that the variable regions of both H- and L-chains are organized into three hypervariable or complementarity determining regions (CDR) separated by four framework regions (see Figure 1, lower panel). The greatest amino acid sequence variation occurs within the CDRs where as the framework regions are more conserved. It is generally thought that association of the

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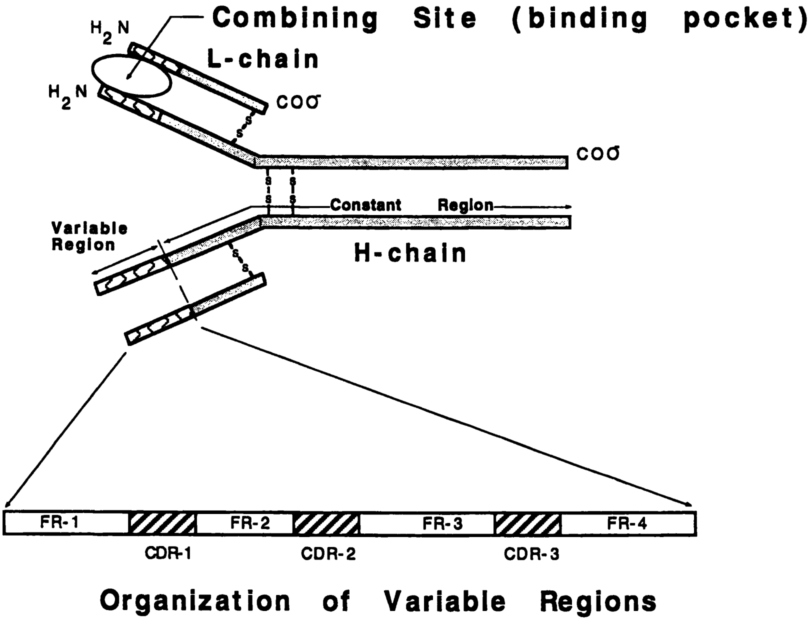
Combining Site (binding pocket)
H_2N
L-chain
H_2N
COO⁻
COO⁻
S S
Constant Region
Variable Region
H-chain
FR-1
FR-2
FR-3
FR-4
CDR-1
CDR-2
CDR-3
Organization of Variable Regions

Figure 1. Diagrammatic representation of a typical Immunoglobulin-G molecule. The organization of the variable portions of the heavy- and light-chains depicting the framework regions (FR) and the complimentarity determining regions (CDRs) is shown in the lower portion of the figure.

CDR regions, three contributed from the H-chain and three from the L-chain, form the antibody combining site.

The ability of an antibody molecule to specifically bind an antigen, or ligand molecule, is controlled by structural and chemical interactions that occur within the antibody combining site. The antigen-antibody interaction is a reversible interaction and does not involve formation of covalent bonds (*7*). Antigen binding instead is the result of a variety of interactions including hydrophobic, ionic, H-bonding, π-π electron interaction, and van der Waals forces. Thus, the antigen-antibody interaction is analogous in many respects to the interactions that occur between an enzyme and its substrate, and it follows the same rules of physical chemistry. Clearly, antibody binding is a complex process involving a steric component (i.e., the analyte must fit properly into the combining site), as-well-as formation of specific chemical interactions between the antibody and antigen. It is generally thought that the greater the number of specific chemical interactions that occur between an analyte and the amino acid residues in the antibody combining site, the greater the binding energy (relative affinity of the antibody). Thus, the fundamental properties of an immunoassay, its specificity and sensitivity are controlled by the precise nature of the antigen-antibody binding process.

Often the user or potential user of an immunoassay is confronted with having to make decisions based, in part, on whether a particular assay uses a monoclonal antibody or polyclonal antiserum. Since the performance of immunoassays ultimately depends on the properties of the antibodies that are used as the detectors, the nature of the antibody is of importance. However, both monoclonal antibodies and polyclonal antisera can be formatted into assays with similar if not identical performance. As outlined above, production of antibodies to small molecules, whether polyclonal or monoclonal, requires that the analyte first be covalently conjugated to a large carrier protein. The nature of this conjugation has a direct effect on the properties of the resulting antibodies. Many excellent reviews (*8–10*) discuss the importance of the position on the molecule where linkage occurs, the length of the linker, and the nature of the chemistry used, e.g., linkage via a nitrogen versus a sulfur atom). The chapter by Skerritt and Lee, this volume, discusses various approaches to the synthesis of haptens for organophosphate and synthetic pyrethroid insecticides and provides many examples of the above considerations. In general, the less the perturbation of the three dimensional structural conformation and of the electronic properties of the analyte as a result of linkage, the better will be the chances of producing high quality antibodies. Application of energy minimized molecular modeling methods to hapten design holds great promise for evaluating a range of chemistries before valuable time and resources are spent completing complex, multistep, chemical syntheses. Energy minimized molecular models of the haptens used in production of monoclonal antibodies to ceftiofur, a cephalosporin antibiotic, and models of related compounds are presented in the chapter by Rose *et al.* Their studies clearly demonstrate the usefulness of molecular modeling for hapten design, and also for clarifying the cross reactivity patterns observed with related compounds for the

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subsequently produced antibodies. The chapter by Beier *et al.* presents molecular models for various fumonisins. Their studies demonstrate the usefulness of being able to visualize a molecule in three dimensions and speculate on the relationships of antibody sensitivity versus the site of hapten coupling for fumonisin B_1. As molecular modeling software becomes more available and more user friendly, its application should be the first step in the development of a new immunoassay.

In summary, the immunization process for production of antibodies to small molecules relies upon the use of an analog of the analyte, the hapten-protein conjugate. The details of the chemistry to produce the analog may not be of great interest to the end user of an immunoassay kit, however, understanding of the basic principles will aid the user in interpreting their results and in trouble-shooting specific applications.

Following immunization with a hapten protein conjugate, antibodies are produced by the animal's immune system. Populations of antibodies will be found in the serum that bind the carrier protein, these will often represent the majority of the antibody molecules produced as a result of the immunization and are of no interest for development of an immunoassay for a particular analyte. Antibodies also will be produced that bind specifically with the linkage chemistry, and/or with the hapten-linkage chemistry complex. Finally, antibodies that specifically bind the hapten with little or no influence of the linkage chemistry also will be present in the serum. These latter antibodies will in fact represent a collection of antibodies, each binding the hapten in a slightly different manner. Thus, even though all of this group of antibodies bind the hapten, they may each bind with different affinities and have different cross-reactivities. Therefore, an antiserum is a complex mixture of antibody molecules. In addition, the exact nature and composition of the serum antibody populations will vary over the time-course of immunization and certainly will vary with individual animals. Thus, each "lot" of antiserum (every bleed of the animal or bleeds from different animals) that is collected needs to be extensively characterized because differences in antibody specificity and sensitivity may occur as the populations of antibodies vary in the animals serum. In contrast, monoclonal antibodies are produced *in vitro* by cell lines referred to as hybridomas. Instead of isolating a group of antibodies with a specific binding profile from a complex serum mixture, the cells that produce a specific antibody are isolated and are immortalized by fusion of the antibody producing lymphocyte with a transformed cell. Thus, a hybrid cell, a hybridoma is produced. The hybridomas can then be propagated indefinitely in culture and they will continue to produce the antibody of the lymphocyte parent. Since an individual lymphocyte produces only a single antibody type, all of the antibody molecules produced by a hybridoma cell line, that has arisen from a single hybrid cell, are identical. Thus all of the antibodies produced by a hybridoma cell line have the same amino acid sequence. Therefore, they all have the same binding properties. For example, let us assume that compound A (a veterinary drug) is metabolized in three steps in the animal, compound A is metabolized to A1, then A2, and finally A3. If we have

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produced a monoclonal antibody that binds the parent molecule (A) and the first metabolite A2, but not the second or third metabolites, then all of the antibodies produced by that hybridoma cell line will bind only the parent and metabolite A1. Furthermore, the antibodies produced by a hybridoma cell line are highly consistent from batch to batch. Thus, unlike different batches of antisera, antibody produced by a given hybridoma cell line will have the same cross reactivity and affinity regardless of when the antibody lot was produced. Therefore, in our example, antibody produced two years after the first production from our hybridoma above will still recognize only the parent compound A and its first metabolite A1.

A criticism of monoclonal antibodies is that they have lower affinities than do polyclonal antisera. This is indeed often the case and antibody affinity and assay specificity are usually critical considerations in residue detection. In our laboratory it appears that the majority of monoclonal antibodies isolated from a cell fusion experiment are of intermediate affinity and a screening strategy must be employed that identifies only the higher affinity antibodies. A casual survey of the commercial immunoassay kits for residue analysis suggests that the majority use polyclonal antibodies. Thus, polyclonal antibodies that have been properly produced and characterized are valuable immunoreagents and form the basis of useful immunoassays with excellent performance specifications.

Recently, the genes coding for a specific antibody molecule have been cloned and expressed in bacterial systems. The reader is directed to recent reviews (*13–15*) and to the chapters by Kramer and Hock and by Kamps-Holtzapple and Stanker, this volume. These molecular genetic techniques will ultimately allow for customization of antibodies by genetic modifications resulting in substitutions in the amino acid sequence of the combining site. Thus, the potential exists to alter affinity or specificity of an antibody to meet a specific analytical need.

Assay Formats

A number of different immunoassay formats have been described. One of the most popular is the enzyme-linked immunosorbent assay (ELISA). In this assay one component, either the analyte protein conjugate or the antibody itself is immobilized on the bottom of a 96-well microwell assay plate, or to the walls of plastic tubes, or onto filter membranes. As the name implies, an enzyme system is used as the reporter signal. The antibody-antigen binding event is measured either directly in those cases where the antibody molecule is modified to contain a reporter system (e.g., an enzyme or fluorescent probe is conjugated to the antibody molecule), or binding is detected indirectly using a second antibody that specifically binds to the original antibody and the second antibody carries the reporter system. Many clinical immunoassays are based on a modification of the above in which the antigen is sandwiched between two antibodies, one a capture antibody and the second antibody is used as the reporter molecule.

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A modification of the ELISA format that incorporates a competition or inhibition step has been almost exclusively used for development of immunoassays to small molecules. Two basic types of competition ELISA (cELISA) are most often utilized for small molecule detection. These are referred to as a competitive direct ELISA (cdELISA) or a competition indirect ELISA (ciELISA). A schematic representation of these two methods is shown in Figures 2 and 3. In the cdELISA, the analyte-specific antibody is immobilized in the wells of a microassay plate or on the wall of an assay tube. Next, the unknown sample along with a fixed amount of enzyme-labeled analyte is added to the antibody-coated tube. The sample is then incubated for between 5 min to over an h, during which time the labeled and unlabeled analyte (in the test sample) compete for binding sits on the antibodies. Afterwards, the unreacted material is washed away and the amount of labeled analyte bound by the immobilized antibody is quantified by addition of an enzyme substrate that forms a chromatic product. The amount of color developed is inversely proportional to the amount of unlabeled analyte in the original sample. For comparison, a ciELISA is shown in Figure 3. Here, the analyte, usually coupled to a carrier protein such as bovine serum albumin, is immobilized on the bottom of a microassay plate well or to the walls of an assay tube. Next, the unknown sample is added to the microassay well immediately followed by addition of a fixed amount of analyte-specific antibody and the microassay plate is then incubated. Thus, the antibody in solution is allowed to partition between the "free" analyte present in the unknown sample and the analyte immobilized on the bottom of the microassay well. Again, unreacted materials are washed away after an appropriate incubation and the amount of the analyte-specific antibody that has bound to the bottom of the microassay well or to the walls of the assay tube is quantified by addition of a second antibody that specifically binds the first antibody. After a second incubation, the unreacted materials are washed away and an appropriate substrate is added. As with the cdELISA, the amount of color developed (the optical density) in an competition indirect ELISA is inversely proportional to the amount of analyte in the original sample. Both cELISA formats can result in highly specific and sensitive assays often that are able to detect an analyte in the picogram range. The chapter by Usleber *et al.*, this volume, makes a comparison of the direct and indirect ELISA for the detection of the mycotoxin citrinin.

Typical data, recorded as the optical density, from a competition ELISA is shown in Figure 4 (top panel). Competition ELISA data is often reported as the percent optical density or B/Bo value where Bo is the optical density in a sample known not to contain the analyte and B the optical density of the unknown sample (or analytical standard). Likewise, the data may be further transformed and reported as a percent inhibition of control activity observed in the absence of analyte using the following equation

$$(1-(B/Bo))*100$$

(Figure 4, bottom panel). However, the data from all competition immunoassays results in generation of a standard curve with a sigmoidal shape. The linear portion of these curves usually span only a few logs of

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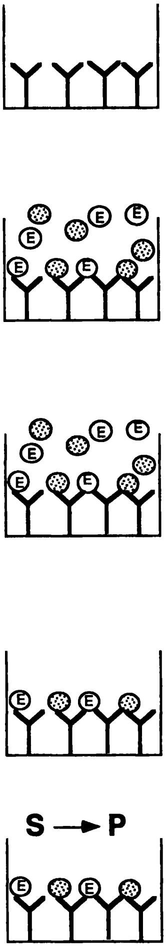

step 1

Immobilize the ligand-specific antibody

step 2

Add enzyme-conjugated analyte and unknown sample

step 3

Allow enzyme-conjugated analyte and the "free" analyte in unknown sample to compete for binding to the immobilized antibody

step 4

Remove reactants not bound to the immobilized analyte

step 5

Add substrate

Figure 2. Schematic representation of a competition direct ELISA

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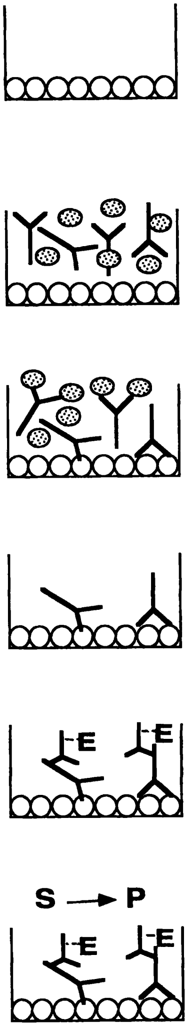

step 1
Immobilize the analyte-protein conjugate

step 2
Add analyte specific antibody and unknown

step 3
Allow antibody to partition between immobilized analyte and the analyte in the unknown

step 4
Remove reactants not bound to the immobilized analyte

step 5
Add enzyme conjugated antibody

step 6
Add substrate

Figure 3. Schematic representation of a competition indirect ELISA

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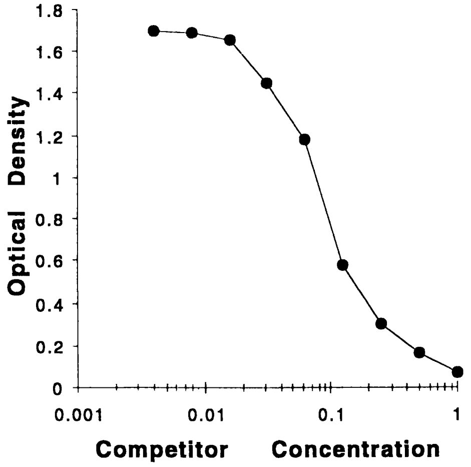

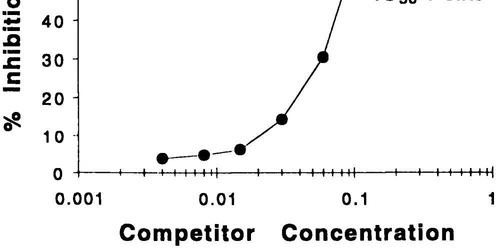

Figure 4. A typical standard curve (log-linear plot) obtained with a competition ELISA (top panel) where analyte concentration is plotted versus the optical density observed. The same data transformed and expressed as a percent inhibition of control (lower panel).

analyte concentration. A sensitive point on such a curve is the 50% activity or 50% inhibition of control (IC_{50}) point (indicated by the * in Figure 4). When large numbers of antibodies are analyzed or different antibodies are studied to determine cross reactivity, it is often only the IC_{50} concentration that is reported in a table.

Determination of the analyte concentration in unknown samples can be accomplished using various methods. For example, if the data is plotted as a log-linear plot, a simple linear regression can be applied for those points on the linear portion of the curve. Other authors have fitted the standard curve using 4-parameter curve fitting methods. Should the concentration of the analyte fall beyond the linear portion of the standard curve, the sample must be either further diluted or concentrated to bring the observed optical density value onto the linear portion of the curve. Many commercial kits have avoided the need for multiple sample dilutions by requiring that the test results, the B/Bo value, fall between predetermined values (e.g., 25–75%) that are known to be on the linear portion of the standard curve. The reader is referred to a series of excellent papers by Rocke (*16*), Karu *et al.*, (*17*), and Ndlovu *et al.*, (*18*) that review statistical methods in immunoassay, four-parameter curve fitting, and parallel curve fitting.

Many parameters effect the performance of a cELISA assay. In an competition indirect ELISA, the amounts of immobilized hapten protein conjugate and the concentration of the primary anti-analyte antibody will affect assay sensitivity. Likewise, in a competition direct ELISA, the concentration of labeled analyte used and of immobilized antibody will affect the sensitivity of the assay. Increased assay sensitivity is usually observed when lower amounts of reagents are used in these assays. Since the antibody is most often in limiting amounts, assay sensitivity is controlled by the absolute affinity of the antibody for the analyte or enzyme-labeled analyte and by the sensitivity of the reporter system used to measure antibody binding.

Sample Preparation

Isolation of an analyte specific antibody represents only the first step in production of a useful immunoassay. The next major hurdle that must be overcome is development of an extraction and cleanup method for the matrix of interest. These tasks are often formidable for analysis of foods because of the complex nature and variability of food matrices (see chapter by Jourdan *et al.*, this volume). In addition, the chemical properties of the analyte (e.g., its lipophilicity) and the properties of the antibody will influence the complexity of the extraction/cleanup method. However, some general principles can be identified. First, in order to take full advantage of the simplicity of an immunoassay, an equally simple extraction/cleanup method must be devised. Because immunoassays are extremely sensitive, often in the picogram/mL range, simple dilution of the sample can often remove the need for extensive sample cleanup. With most food matrices however, some form of extraction of the sample is needed. Since antibodies function in aqueous environments under physiological conditions (near neutral pH, 0.15 N salt), the method of

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analyte extraction is of great importance. Analytes that are water soluble and can be efficiently extracted in an aqueous buffer, for example many antibiotics, will have the most direct extraction method and eliminate the need to use organic solvents. However, many compounds of interest are not readily water soluble, for example many pesticides, and they must be extracted with an organic solvent. This latter case represents a greater challenge to the immunochemist. Usually following extraction in such cases, the solvent must be exchanged for a solvent system compatible with antibody function. It may be necessary to substitute a completely new solvent (the least desirable case, since more steps are introduced), or it may be possible to simply dilute the extraction solvent with a buffer. The ability of an immunoassay to tolerate various levels of mixed solvent, (e.g., low percentages of methanol, acetonitrile, or dimethylformamide), is controlled by the specific antibody used in any given immunoassay. An exciting approach in sample preparation is the application of supercritical fluid extraction. These methods generally employ CO_2 or CO_2 containing various modifiers. The chapters by King *et al.*, and by Lopez-Avila *et al.*, describe the use of supercritical fluid extraction and chromatography, coupled with immunochemical detection.

Regardless of the extraction cleanup method, the user should clearly recognize that assays using antibodies obtained from different sources will not necessarily demonstrate comparable performance specifications. An assay developed for analysis of a specific compound in a specific matrix may use an antibody that was selected to have optimal characteristics in the extraction system used for the matrix. Therefore, use of the same immunoassay with a different matrix, even if the same extraction methods are used, may not result in equivalent performance since different materials may be coextracted in the new matrix. Likewise, substitution of antibodies from different sources may drastically alter assay performance. Thus, it is critical for users to closely adhere to all instructions supplied with a commercial kit or follow a published method as closely as possible to insure that quality data is obtained.

Rapid sample cleanup methods compatible with immunoassays have been reviewed in a number of excellent papers including those of Jourdan *et al.*, and of Skerritt and Rani, this volume. As with any analytical method, the purpose of sample cleanup is to remove coextracting materials that interfere with subsequent analysis. In traditional analytical methods such compounds may be structurally similar compounds that co-chromatograph with the analyte of interest, e.g., separation of PCB's from dioxins. Developers of immunoassays often attempt to utilize traditional sample extraction and cleanup methods. There are valid reasons for so doing, including the wealth of information that is usually available and the desire to be able to quickly confirm the results from samples that have tested positive with an immunoassay with a more traditional method. However, the nature of the interferences associated with a traditional analytical method may be very different than the interferences associated with an immunoassay. This is due to the fundamental differences that exist between immunoassays and

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chromatographic based analyses. For example, the immunoassay that we developed for measurement of the polychlorinated dibenzo-*p*-dioxins (*19*) does not bind PCBs. Thus, there is no need to separate the PCBs from the dioxins when the immunoassay is used. Because of the hydrophobic nature of the dioxins, however, they will partition into any lipid material that is present in the sample before analysis. Therefore, these (fats and/or oils) must be removed inorder for the antibody to be able to bind the dioxins. Likewise, the immunoassay we developed for measurement of dimetridazole in turkey (*20*) incorporates a cleanup method that removed interferences associated with the immunoassay. However, use of the cleanup method designed for HPLC analysis of dimetridazole did not remove the interferences that compromised the ELISA.

Applications

We can expect an ever increasing number of immunoassays for residue analysis. This is driven, in part, by the need for more cost-effective, field portable assay systems that can be conducted and interpreted by users that are as close to the source of contamination as possible. This volume contains numerous chapters describing specific immunoassays for particular food matrices. However immunoassays must meet certain standards in order to be useful for their application in a regulatory program. The chapter by Ellis outlines these criteria and considerations that must be incorporated into an assay in order for it to be applied in a regulatory mode. Application of new immunoassay methods will enhance the application of immunoassay for both field and laboratory based testing. Even though most immunoassays utilize an ELISA format, there is a general desire to develop assay formats that are even more user friendly than is ELISA. These include biosensors, flow immunoassay devices, and dry chemical assays where all of the reagents are impregnated into an assay cassette and the sample is simple applied and the reaction develops with no further input from the user (e.g., many of the commercial home pregnancy tests). The readers are referred to the chapters by Sternesjö *et al.* (this volume), where they describe an immunoassay for sulfamethazine in milk using a surface plasmon resonance based immunosensor, and Kumar *et al.* (this volume), where the use of a solid-phase fluorescence immunoassay for detecting antibiotic residues in milk is described. The development of biosensors and uniquely new formats will ultimately simplify the look and expand the application of immunoassays.

Conclusions

Immunochemistry clearly represents a new tool for the analyst. The number of manuscripts describing new assays, the number of immunochemistry symposia at major meetings, and the number of commercial kits are all increasing at an astounding rate. The potential to incorporate genetic engineering techniques to customize the antibody molecule offers further possibilities for immunoassay development. Finally, the development of

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new assay formats, such as antibody-based biosensors will speed application of this technology. This volume is an attempt to highlight the application of immunoassay methods to residue analysis in foods. The manuscripts in this volume clearly demonstrate that the future is bright for application of immunoassay in many areas of the food industry including food safety and quality assurance.

References

1. Samarajeewa, U.; Wei, O. I.; Huang, S.; Marshall, M. R. Application of Immunoassay in the Food Industry; *Crit. Rev. Food Sci. Nutr.* 1990, *29*, 403-434.
2. *Food Safety and Quality Assurance Applications of Immunoassay Systems;* Morgan, M. R. A.; Smith, C. J.; Williams, P. A., eds.; Elsevier Applied Science: New York, NY, 1992; 496 p.
3. Kaufman, B. M.; Clower, M., Jr. *J. AOAC Int.* **1995**, 78, 1079–1090.
4. Stanker, L. H. In *Analysis of Contaminants in Edible Aquatic Resources*; Kiceniuk, J. W. and Ray, S., (eds); VCH Publishers Inc.: New York, NY, 1994; pp 523-537.
5. Landsteiner, K. *The Specificity of Serological Reactions*; Harvard University Press: Cambridge, MA, 1945.
6. Kabat, E. A.; Wu, T. T.; Perry, H. M.; Gottesman, K. S.; Foeller, C. *Sequences of Proteins of Immunological Interest*, 5th edition Vol. I-III, U.S. Department of Health and Human Services: Washington, DC, 1991, NIH Publication No. 91–3242.
7. Golub, E. S. *Immunology: A synthesis*; Sinaver Assoc. Inc.: Sundrland, MA, 1987; 551 p.
8. Harrison, R. O.; Goodrow, M. H.; Gee, S. J.; Hammock, B. D. In *Immunoassays for Monitoring Human Exposure to Toxic Chemicals in Foods and the Environment;* Vanderlaan, M.; Stanker, L. H.; Watkins, B.; Roberts D. W., eds.; American Chemical Society: Washington, DC, 1991, ACS Symposium Series No. 451; pp 14–27.
9. Goodrow, M. H.; Sanborn, J. R.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. In *Immunoanaysis of Agrochemicals, Emerging Technologies*; Nelson, J. O.; Karu, A. E.; Wong, R. B., eds.; American Chemical Society: Washington, DC, 1995, ACS Symposium Series No. 596; pp 119–139.
10. Szurdoki, F.; Bekheit, H. K. M.; Marco, M.; Goodrow, M. H.; Hammock, B. D. In *New Frontiers in Agrochemical Immunoassay;* Kurtz, D. A.; Skerritt, J. H.; Stanker, L. H., eds.; AOAC International: Arlington ,VA, 1995; pp 39–64.
11. Skerritt, H. J.; Hill, A. S.; McAdam, D. P.; Stanker, L. H. *J. Agric. Food Chem.* **1992**, *40*, 1287–1292.
12. Stanker, L. H.; Watkins, B.; Vanderlaan, M.; Ellis, R.; Rajan, J. 1991. In *Immunoassays for Monitoring Human Exposure to Toxic Chemicals in Foods and the Environment;* Vanderlaan, M.; Stanker,

L. H.; Watkins, B.; Roberts D. W., eds.; American Chemical Society: Washington, DC, 1991, ACS Symposium Series No. 451; pp 108–123.

13. Choudary, P. V.; Lee, H. A.; Hammock, B. D.; Morgan, M. R. A. In *New Frontiers in Agrochemical Immunoassay;* Kurtz, D. A.; Skerritt, J. H.; Stanker, L. H., eds.; AOAC International: Arlington,VA, 1995; pp 171–186.
14. Ward, V. K.; Hammock, B. D.; Maeda, S.; Choudary, P. V. In *New Frontiers in Agrochemical Immunoassay;* Kurtz, D. A.; Skerritt, J. H.; Stanker, L. H., eds.; AOAC International: Arlington ,VA, 1995; pp. 217-236.
15. Lee, H. A. R. G.; Garrett, S. D.; Yanguela, M. C.; Morgan, M. R. A. R In *Immunoanalysis of Agrochemicals, Emerging Technologies*; Nelson, J. O.; Karu, A. E.; Wong, R. B., eds.; American Chemical Society: Washington, DC, 1995, ACS Symposium Series No 596; pp 22–30.
16. Rocke, D. M. In *New Frontiers in Agrochemical Immunoassay;* Kurtz, D. A.; Skerritt, J. H.; Stanker, L. H., eds.; AOAC Int.: Arlington ,VA, 1995; pp 251–260.
17. Karu, A. E.; Perman, M.; McClatchie, I. R. T.; Speed, T. P.; Richman, S. J. In *New Frontiers in Agrochemical Immunoassay;* Kurtz, D. A.; Skerritt, J. H.; Stanker, L. H., eds.; AOAC International: Arlington, VA, 1995; pp. 261-282.
18. Ndlovu, P.; Kurtz, D. A.; Rosenberger, J. L. In *New Frontiers in Agrochemical Immunoassay;* Kurtz, D. A.; Skerritt, J. H.; Stanker, L. H., eds.; AOAC Int.: Arlington ,VA, 1995; pp 283–303.
19. Watkins, B. E.; Stanker, L. H.; Vanderlaan, M. *Chemosphere* **1989**, *19*, 267–270.
20. Stanker, L. H.; McKeown, C.; Watkins, B. E.; Vanderlaan, M.; Ellis, R.; Rajan, J. *J. Agric. Food Chem.* **1993**, *41*, 1332–1336.

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Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0621.ch001

Chapter 2

Adapting Immunoassays to the Analysis of Food Samples

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Immunoassays are powerful tools which augment current monitoring and measurement capabilities in residue testing of food samples. Immunoassay technology can minimize cleanup in analysis and provide a rapid screen for the analysis of large sample loads when conventional analytical methods are too costly or cumbersome. Many immunoassay test kits are sensitive enough to directly analyze crude extractions with few or no steps involved in extract cleanup. Often extracted samples can be diluted to remove matrix interferences in the immunoassay. To adapt an immunoassay to food samples, potential problems can be systematically investigated. Studies can be easily performed to evaluate the matrix interferences and method performance. In this paper, common problems encountered in this process will be reviewed using examples from the literature and our experience.

The application of immunoassay to foods has been widely reported and reviewed (*1–3*). Over the past five years our group has developed over 25 immunoassays and, in addition to using them to detect and measure residues in water and soil, they have been adapted for whole fruits and vegetables, juices, oils, dairy products and grains. This paper will review the approach we have been taking to the application of immunoassay to foods.

Based on our experience the most important factors when developing a method are: 1) the required detection levels, 2) the choice of an extraction technique, and 3) matrix effects. We hope that sharing our experience will aid other investigators in their application of immunoassays to foods.

Detection Levels

When applying an immunoassay to a food matrix, detection levels required for the testing and achieved by the immunoassay must be determined. This may sound trivial,

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but this consideration probably dictates the ultimate method more than any other factor.

The first issue to consider is the concentration required for detection. A number of sources may dictate this requirement or can provide guidance. They include tolerances published by the US EPA in the Federal Register, the regulations of the European Community, the Codex Commission of the World Health Organization, or internal organizational requirements.

Next, it is important to understand the sensitivity of the immunoassay. Typically, immunoassays are more sensitive than is required. However, performance aspects of an immunoassay differ across its working range. Detecting analytes at the least detectable dose (LDD) is not generally the most reliable way of employing immunoassays since the optimum performance of these methods is found in the center of their working range. The point of optimum performance is estimated to be at the estimated dose at 50% B/Bo (ED_{50}). The ED_{50} or inhibitory concentration at 50% (IC_{50}) in a small molecule assay is the concentration of analyte required to inhibit the signal in the assay 50% compared to the signal at zero analyte concentration.

Based on the detection requirements and the sensitivity of the immunoassay the degree to which dilution or concentration step(s) will be required can be calculated. It should be recognized that, while dilution steps will dilute potential interferences, concentrations will often concentrate these inferences. Consequently it is advantageous to begin with an immunoassay with greater sensitivity than will be required.

Extraction Techniques

Once the detection level required is understood, an extraction technique can be developed. There is an obvious need to extract the residue from the food stuff since the antibody-antigen reaction is typically optimum in an aqueous environment. The current literature is rich with examples of extraction methods. To fully capitalize on the value of an immunoassay it is important that the investigator recognize that the immunoassay can often tolerate much less cleanup than traditional chromatographic methods due to the selectivity of antibodies.

In some cases the residue is known to be confined to the surface of a food. In these cases the dislogible foliar residues (DFRs) can be obtained by simple methods which wash the surface of the food. This technique also has been useful in monitoring worker reentry into pesticide treated fields (*4–5*). Washes often employ solvents or water-based detergents.

When the residue is found throughout the food, it becomes necessary to blend or homogenize the food and extract the residue in an organic solvent. The methods of Luke (*6*) and Mills (*7*) are examples of these techniques that were developed for traditional chromatographic methods. Sometimes the residue is more difficult to remove from the food tissue and requires more strenuous extraction. An example is paraquat which can be extracted by acid digestion aided by refluxing or sonication. (*8*).

Another extraction technique that has been applied to immunoassay is supercritical fluid extraction (SFE) (*9–10; see chapters by King and Nam, and Lopez-Avila et al., this volume*). This technique is based on the solvating properties of a compound such as carbon dioxide used in its liquid state by applying it under increased pressure. SFE is becoming more widely applied as equipment becomes more commonly available. Examples of applying this extraction method to immunoassays for foods is described in greater detail by King and Nam, and by Lopez-Avila et al. in this volume.

Evaluating Matrix Effects

Once an extraction method is chosen, it is important to evaluate the effect of the extracted sample on the immunoassay. All immunoassays rely on antibodies as the critical analytical reagent. Anything in the sample presented to the immunoassay that effects the antibody binding event can have a negative effect on the accuracy of the immunoassay. Antibodies are large protein molecules known as immunoglobulins. The properties of antibodies vary widely and a concentration of organic solvent that has no effect on one immunoassay can dramatically increase or decrease the sensitivity of other immunoassays.

One step in determining potential matrix effects is to examine the effect of the extraction solvent on the results by validating that the addition of known quantities of the analyte can be accurately recovered. Potential solvent effects can be further investigated by validating that dilutions of positive samples behave as expected. Once the development of the method is complete it is always important to fully validate it including correlation to a traditional method.

Solvent Tolerance. The simplest approach to determining solvent tolerance in an immunoassay is to evaluate the effect of the solvent on the calibration curve. An enzyme-linked immunosorbent assay (ELISA) based on a magnetic particle solid phase has been used in our work. Further details of this technique have been given elsewhere (*11–13*). This type of immunoassay is known as a competitive heterogeneous immunoassay where the antibody is immobilized. The "bound" or antibody fraction of the enzyme label is measured. Consequently the resulting color produced is inversely related to concentration.

Figure 1 illustrates the effect of methanol on an atrazine immunoassay (*11*). In our experience, methanol is generally one of the best tolerated solvents in immunoassays. In this case as the methanol concentration is increased, the calibration curve is depressed, i.e., the methanol seems to inhibit the antigen-antibody binding. This phenomenon is particularly pronounced at low atrazine concentrations.

Figure 2 shows the same atrazine immunoassay in the presence of various concentrations of acetone. These data demonstrate that in this system acetone is less tolerated than methanol. This is consistent with our overall experience.

Figure 3 demonstrates the effect of acetonitrile on the calibration curve of the same atrazine immunoassay. It should be noted that at a concentration of 2.5% acetonitrile the calibration curve is nearly identical to the control containing no acetonitrile. In most cases, it would be recommended to dilute out the acetonitrile to this concentration before introducing the sample into the immunoassay. At 10% acetonitrile there is still an adequate dose response to distinguish atrazine concentrations but at higher concentrations, 25–50%, the response is unusable.

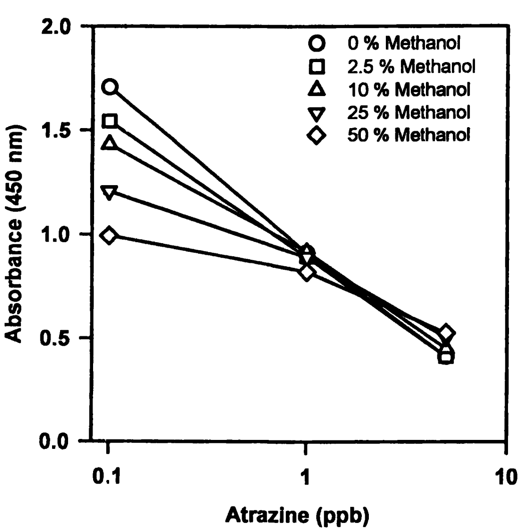

Figure 1. Tolerance of an atrazine immunoassay to various concentrations of methanol in the sample.

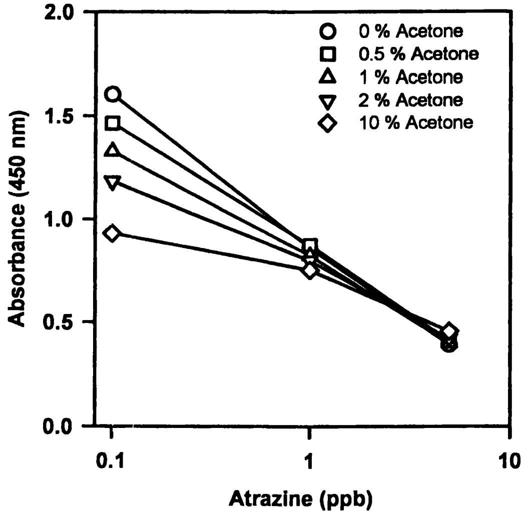

Figure 2. Tolerance of an atrazine immunoassay to various concentrations of acetone in the sample.

Figure 4 again shows the calibration curves from the same atrazine immunoassay in the presence of hexane. The results from this experiment are misleading since hexane is not miscible in the aqueous buffers used in the immunoassay. As a result, the performance would prove to be unacceptable in terms of spike recovery, although the calibration curve is unaffected. This illustrates the need for validation of the method beyond solvent tolerances.

In Figure 5 results from a 2,4-D immunoassay (*13*) demonstrate an effect from methanol that is distinctly different than that observed in the atrazine immunoassay. In this case increasing the methanol concentration increases the observed absorbance as opposed to decreasing it as in atrazine. In other words, the methanol seems to increase the rate of the antigen-antibody binding event. This is not unique to 2,4-D and has been observed with other analytes. Typically it has been observed when an analyte is particularly hydrophobic and may be due to improved solubility of the analyte in the presence of the solvent.

Spike Recovery. The next experiment would be to fortify blank samples and determine recovery. The data shown in Table I is from protocols used to measure atrazine in corn oil and grain. The method for corn oil utilized a simple acetonitrile phase extraction of the oil followed by a 1:50 dilution in buffer. The method for the grain required grinding the corn, extraction in 80% methanol, storing overnight in the cold followed by a 1:50 dilution in buffer.

Table I. Spike Recovery of Atrazine in Corn Oil and Grain

	Corn Oil		*Corn Grain*	
Atrazine spike (ppb)	*Mean (ppb)*	*% Recovery*	*Mean (ppb)*	*% Recovery*
0	0	---	0	---
50	54	108	56	112
100	105	105	122	122
200	---	---	228	114
250	247	99	---	---
500	---	---	566	113

Good recovery of the analyte from the fortified samples indicates accuracy of the extraction method and the immunoassay. Failure to accurately recover added analyte may indicate the presence of interfering substances in the sample matrix. It is preferable to prepare spiked samples by adding analyte to the food prior to extraction. Alternatively, the spiked sample can be prepared by adding analyte after extraction, directly to the extracted sample prior to immunoassay analysis. Samples should be analyzed without any analyte addition (neat) and at several spike levels in the immunoassay.

Linearity. Another experiment to determine whether there are matrix interferences present has been referred to as "parallel dilution" or "linearity." In the absence of interfering substances, the standard curve of an immunoassay is parallel to the curve obtained by diluting the sample (*14*). The dilutions of samples containing non-specific

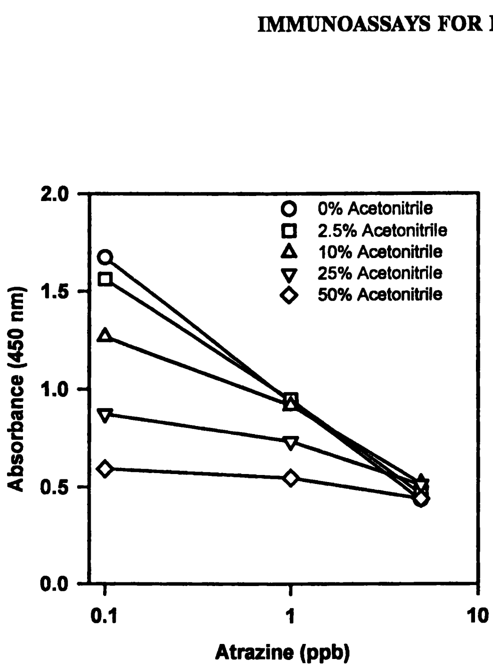

Figure 3. Tolerance of an atrazine immunoassay to various concentrations of acetonitrile in the sample.

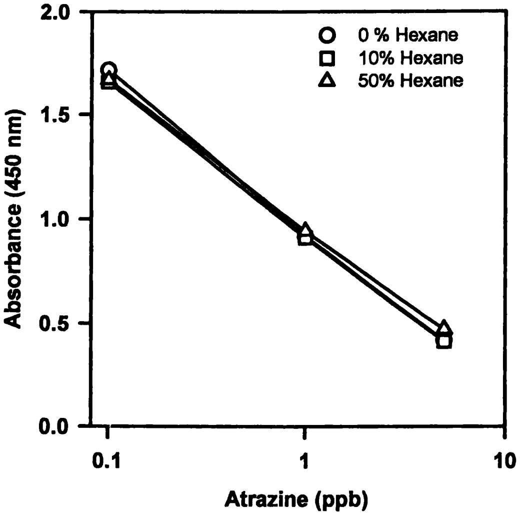

Figure 4. Tolerance of an atrazine immunoassay to various concentrations of hexane in the sample.

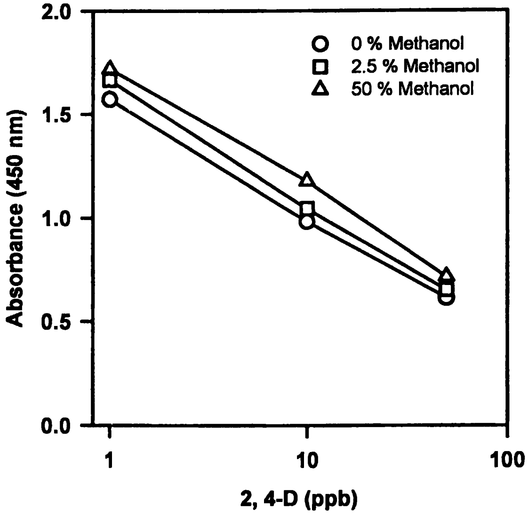

Figure 5. Tolerance of a 2,4-D immunoassay to various concentrations of methanol in the sample.

reactants are not parallel to the standard curve due to the difference in affinity for the antibody that these interferences exhibit. The data shown in Table II demonstrates the results of a linearity experiment for the corn oil and grain methods previously described.

Table II. Linearity of Atrazine Dilution in Corn Oil and Grain

Sample	*Neat*	*1:2*	*1:4*	*1:8*
Corn Oil:				
Assayed Conc. (ppb)	247	128	65	32
Expected Conc. (ppb)	250	125	63	31
Recovery (%)	99	102	104	102
Corn Grain:				
Assayed Conc. (ppb)	566	290	147	77
Expected Conc. (ppb)	500	250	125	63
Recovery (%)	113	116	118	122

Resolving Matrix Effects

There are a number of ways to approach resolving a matrix effect once it has been identified. They can be divided into two groups, those that attempt to remove the matrix effect, e.g. cleanup of the extract, and those that attempt to live with the effect, e.g. standardization in the presence of the matrix. The preferred approach is to remove the effect. Although this approach may be somewhat more difficult to acheive, it generally results in a more robust method.

Dilution. Table III reports data that was obtained while developing a method for atrazine in sugar cane juice. The first column represents the dilution of the sugar cane juice tested. The second column shows the absorbance of the immunoassay result for an unspiked sample and the third and fourth columns give the absorbance and the recovery for samples spiked with 1 ppb atrazine. The last row of the table reports the results for a buffer control without any added matrix.

Table III. Dilution of Sample Matrix Interference in Sugar Cane Juice (SCJ)

	Neat	*SCJ + 1 ppb Atrazine*	
Dilution of SCJ	*Abs. (450 nm)*	*Abs. (450 nm)*	*% Recovery*
1:2	1.467	1.233	0
1:50	0.965	0.433	72.5
1:100	0.930	0.398	84.1
1:200	0.919	0.380	90.6
1:400	0.915	0.367	96.4
Sample Diluent	0.919	0.357	100

In this system, the matrix effect did not dilute out until the matrix was diluted 1:400. In this case the sensitivity required for the application allowed this dilution. To further investigate the phenomenon, the reactivity of the sample directly with the color reagent used in the assay was examined. In this immunoassay the color reagent consists of peroxide and tetramethylbenzidine which in the presence of peroxidase activity will produce the colored product that is measured photometrically. By adding 20 µL aliquots of diluted sugar cane juice directly to the color reagent the results shown in Table IV were observed. Apparently high levels of peroxidase activity are present in the juice. This suggests that peroxidases in the sample were adhering to the tube and/or the solid phase during the assay and creating a false reaction.

Table IV. Reactivity of Diluted Sugar Cane Juice (SCJ) with Color Reagents

Dilution of Sugar Cane Juice	*Absorbance (450 nm)*
1:2	4.048
1:50	1.293
1:100	0.675
1:200	0.326
1:400	0.165
Sample Diluent	0.011

Solvent Exchange. If the matrix effect is a result of the extraction solvent, the problem can be addressed by moving the extract into a solvent more compatible with immunoassay. The easiest way to accomplish this, if the solvent is volatile, is to evaporate it and then redissolve the extract with an aqueous buffer. This approach is not recommended if the analyte is volatile since it will be lost during the blow-down. Loss of analyte can sometimes be overcome by avoiding evaporation to dryness with the use of a "keeper" solvent such as octanol.

A solid phase extraction (SPE) column also can be used for this purpose if the analyte can be absorbed to the column in the extracting solvent and then eluted with another solvent. Generally the goal is to obtain a sample in an aqueous system. Consequently the eluting solvent is generally evaporated and the extract redissolved in a buffer. These approaches can often afford an opportunity to concentrate the sample when greater sensitivity is required.

Cleanup. When the sample cannot be diluted adequately to eliminate the matrix effect, it is recommended that the interference be removed with a cleanup step. Since immunoassays use small sample sizes and are quite sensitive, small scale liquid/liquid extractions, such as that previously described for corn oil, can be very effective. SPE separations provide a convenient means of cleaning up an extract. As previously mentioned, this approach often provides an opportunity to concentrate the analyte when sensitivity is an issue. Solid adsorbents added directly to the extract to remove interferences also have been used successfully. The use of diatomaceous earth to remove interfering fats from raw milk has been employed in methods for atrazine and 2,4-D (*15*).

Figure 6 represents a protocol was been found to be widely applicable to fruits and vegetables (*16*). It includes both a phase separation step and a proprietary bulk adsorbent to remove many of the colored compounds such as flavonoids and phenols that often interfere with immunoassays. Table V shows the results of an experiment in which strawberry and grape samples were extracted using the protocol as described in Figure 6 except the cleanup step using the adsorbent was omitted. The resulting extract was analyzed with a carbendazim immunoassay (*17*).

Table V. Analysis of Strawberries and Grapes for Carbendazim without Adsorbent Treatment.

Commodity	*Spike (ppb)*	*% Recovery*
Strawberries	0	*fp*[a] (83 ppb)
	25	500
	100	290
	300	216
Grapes	0	*fp* (25 ppb)
	25	248
	100	178
	300	143

[a]*fp* false positive result

The results clearly indicate the presence of interferences that cause a false positive result in the absence of carbendazim and elevated recoveries as the blank food was spiked with carbendazim. Table VI shows the results from the same commodities using the cleanup adsorbent step. The results of the spike recovery have been dramatically improved with this treatment.

Table VI. Analysis of Strawberries and Grapes for Carbendazim with Adsorbent Treatment.

Commodity	*Spike (ppb)*	*% Recovery*
Strawberries	0	*nd*[a]
	25	92
	100	87
	300	88
Grapes	0	*nd*
	25	116
	100	98
	300	100

[a]*nd* non-detect, e.g., results below the detection limit of the method

Standardization and Calculation. An alternate technique employed by some investigators is to standardize the immunoassay in the matrix being tested. Figure 7 shows the calibration curves that were obtained using the buffer-based standards provided with the atrazine immunoassay and standards prepared in raw milk. Using the raw milk standards, accurate results could be obtained from raw milk samples as long as the level of interference was relatively constant between samples.

Another approach used by some investigators is to correct for the presence of interferences by subtraction of blank matrix results from the result obtained with samples. This assumes that the interference is constant as a concentration result across the range of the immunoassay. This is frequently not the case as can be observed in the solvent tolerances (Figures 1–5).

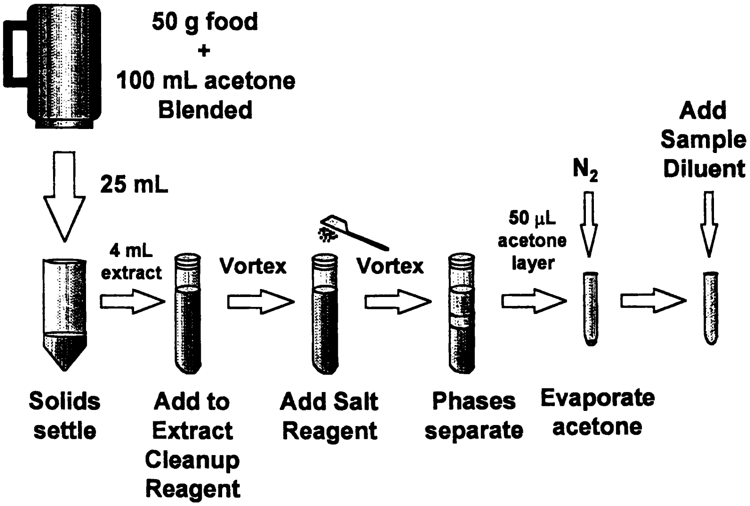
50 g food
+
100 mL acetone
Blended
25 mL
4 mL
extract
Vortex
Vortex
50 µL
acetone
layer
N2
Add
Sample
Diluent
Solids
settle
Add to
Extract
Cleanup
Reagent
Add Salt
Reagent
Phases
separate
Evaporate
acetone

Figure 6. Schematic outline of Food Prep™ protocol for sample preparation of fruits and vegetables..

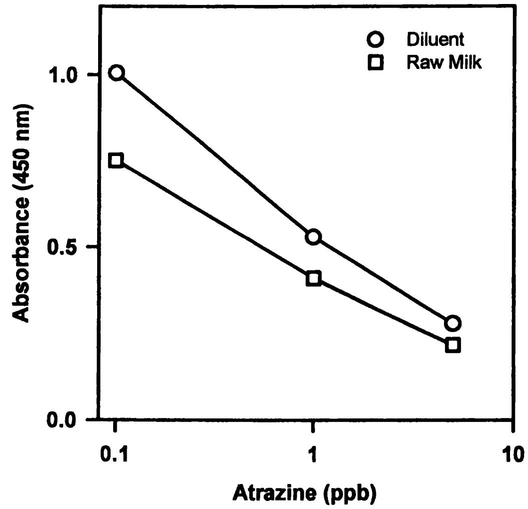

Figure 7. Atrazine calibration curves developed using standards prepared in aqueous buffer and raw milk.

Richman and Lee (*18*) have proposed another approach using a variation on the standard addition method. In this technique samples are tested before and after the addition of a known amount of analyte. Results are classified as positive or negative based on the amount of displacement that occurs with the analyte spike.

Summary

In this paper the methods used to adapt immunoassays to analysis of food samples have been reviewed. The approach recommended is as follows: 1) determine the required detection levels; 2) choose an extraction technique; 3) determine if any matrix effects are present; 4) address any matrix effects; and 5) fully validate the method against accepted analyses. Using this approach a wide variety of foods have been analyzed for a number of different analytes by immunoassay. Many of these are available from the authors upon request.

Literature Cited

1. Newsome, W. H. *J. Assoc. Off. Anal. Chem.* **1986**, *69*, 919–923.
2. Kaufman, B. M.; Clower, M., Jr. *J. Assoc. Off. Anal. Chem.* **1991**, *94*, 239–247.
3. Bushway, R. J.; Fan, T. S. *Food Tech.* **1995**, *49*, 108–115.
4. Gunther, F. A.; Westlake, W. E.; Barkley, J. H.; Winterlin, W.; Langbehn, *Bull. Environ. Contam. Toxicol.* **1973**, *9*, 243–249.
5. Iwata, Y.; Knaak, J. B.; Spear, R. C.; Foster, R. J. *Bull. Environ. Contam. Toxicol.* **1977**, *18*, 649–655.
6. Luke, M. A.; Froberg, J. E.; Matsumoto, H. T. *J. Assoc. Off. Agric. Chem.* **1975**, *58*, 1020–1026.
7. Mills, P. A.; Onley, J. H.; Gaither, R. A. *J. Assoc. Off. Anal. Chem. Int.* **1963**, *46*, 186–191.
8. Sellero, S.; Lopez-Rivadulla, M.; Cruz, A.; Bermejo, A.; Fernandez, P. *Anal. Lett.* **1993**, *26*, 1891–1904.
9. Nam, K.; King, J. W. *J. Agric. Food Chem.* **1994**, *42*, 1469–1474.
10. Lopez-Avila, V.; Charan, C.; Van Emon, *J. Environ. Lab*, **1993**, *5*, 30–33.
11. Rubio, F. M.; Itak, J. A.; Scutellaro, A. M.; Selisker, M. Y.; Herzog, D. P. *Food Agric. Immunol.* **1991**, *3*, 113–125.
12. Itak, J. A.; Selisker, M. Y.; Herzog, D. P. *Chemosphere* **1992**, *24*, 11–21.
13. Lawruk, T. S.; Hottenstein, C. S.; Fleeker, J. R.; Hall, J. C.; Herzog, D. P.; Rubio, F. M. *Bull. Environ. Contam. Toxicol.* **1994**, *52*, 538–545.
14. Jung, F.; Gee, S. J.; Harrison, R. O.; Goodrow, M. H.; Karu, A. E.; Braun, A. L.; Li, Q. X.; Hammock, B. D. *Pestic. Sci.* **1989**, *26*, 303–317.
15. Ohmicron Application Procedure T00012, Detection of atrazine and 2,4-D in raw milk.
16. Jourdan, S. W.; Scutellaro, A. M.; Hayes, M. C.; Herzog, D. P. California Pesticide Residue Workshop, Sacramento, CA, February, 1994.
17. Itak, J. A.; Selisker, M. Y.; Jourdan, S. W.; Fleeker, J. R.; Herzog, D. P. *J. Agric. Food Chem.* **1993**, *41*, 2329–2332.
18. Richman, S. J.; Karthikeyan, D. Bennet, D. A.; Chung, A.; Lee, S. M. Eighth IUPAC Congress of Pesticide Chemistry, Washington, DC, July 4-9, 1994.

RECEIVED August 16, 1995

Chapter 3

Detection and Removal of Sample Matrix Effects in Agrochemical Immunoassays

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With the completion of the initial development of assays for many of the most important agrochemicals, there has been an increased focus on actual applications of agrochemical immunoassays to food and environmental matrices. A major prerequisite to assay utilization has been the need to identify and remove matrix interferences, which may result in: 1. residue-free samples appearing positive, or else samples containing residues appearing negative; or 2. changes in the sensitivity of residue detection, from either or both shifts in the assay absorbance values in the absence of pesticide or in the standard curve. Matrix effects are best detected by analysis of spikes of pesticide standards into a solvent extract of pesticide-free matrix and comparison of this concentration-response curve with that obtained using standards prepared in solvent alone. A number of approaches to the removal of matrix interference is reviewed, with particular reference to examples from the analyses of insecticide residues in plant-derived foods, including grain and fruit products.

Over the last 10–15 years, antibody-based tests have been developed for many of the most important agrochemicals (*1,2*). The motivation for such development has been production of very sensitive assays that are simple to perform, high in sample throughput and can potentially reduce the large volumes of solvent that are often used in conventional analysis, such as liquid-liquid extraction. Most application work for agrochemical immunoassay to date has been on the development and validation of assays (usually of herbicides) for environmental matrices, such as water and soil. Analysis of agrochemical residues in food matrices (in this case, usually of insecticides and fungicides applied for pre- or postharvest product protection) has been demonstrated to be feasible, but the work has been less thorough, with most emphasis on fruits and their juices (*3–8*), grain products (*9–14*) and limited work with meats (*15,16*). Food matrices are especially challenging as they have a wide range in

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composition. They can have low (e.g., citrus juices, beer) or high pH (e.g., Asian yellow noodles), or can vary in sugar or lipid content. Protein components and tannins or polyphenols can bind or otherwise interfere with reactions involving antibodies and enzyme conjugates.

Despite these potential complications, there are many potential advantages in being able to use immunoassay for the analysis of agrochemical residues in plant-derived foods. However, whatever data is generated, considerations of accuracy and precision are important, since the methods are to be used in either a regulatory or commercial manner. Identification and removal of matrix effects is the major impediment to the wider application of pesticide immunoassay to foods. Methods need to be developed that do provide reliable data but they need to use a minimum of sample processing, or otherwise many of the potential advantages of immunoassays, such as low cost, speed and simplicity, would be lost.

Several residue/matrix combinations are of particular interest in our work, which has a focus on plant-derived foods. These include:

- For organophosphates: grain and grain products, grapes and dried vine fruits, vegetables and spices;
- For synthetic pyrethroids: grain and grain products;
- For endosulfan: tea, coffee, rice and cottonseed;
- For other organochlorines: grapes and dried vine fruits, rice, root vegetables and nuts;
- For selected fungicides: grapes and dried fruits, tropical fruits.

Approaches that we are taking towards developing a set of "universal" clean-up methods will be reviewed, together with research by other groups on immunoassay with food matrices. This work has largely been restricted to combinations of single pesticides and a few commodities, including fruits, grain, meat and milk. Our aim is to eventually develop a limited number of generic extraction and clean-up methods that can readily be coupled with immunoassay. In this way, the routine analyst would be able to interface specific extraction and clean-up methods, based on the chemical nature of the target pesticide/s and on a knowledge of the fat and sugar content of the food matrix. This is similar in philosophy to the extraction and clean-up approaches that form part of instrumental multiresidue methods (*17–20*).

Materials and Methods

The majority of examples have utilized immunoassays for cyclodienes, which were based on endosulfan derivatives as haptens for conjugates and antibody production (*21*). The immunogen used in assay one was hapten II (4-oxobutanoic acid, 4-(4,5,6,7,8,8-hexachloro-3a,4,7,7a-tetrahydro-4,7-methano-1H-indenyl-1-oxy) succinimidyl ester) coupled to keyhole limpet hemocyanin while the enzyme-hapten conjugate was based on hapten III (i.e., 4-oxybutanoic acid, 4-(1,3,4,5,6,7,8,8-octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindanyl-2-oxy) succinimidyl ester). The immunogen used in assay two was hapten I of ref. *21*, in which one of the hydroxy groups of endosulfan diol was esterified with succinic anhydride to provide a hemisuccinate spacer arm coupled to keyhole limpet hemocyanin, while the enzyme-hapten conjugate used in assay two was hapten II coupled to horseradish peroxidase.

Two assays were developed because they have slightly different specificities for cyclodienes and their metabolites (*21*). Assay one was used routinely, unless otherwise shown. The assays were all performed at room temperature (18–23 °C), using immobilized antibody. Briefly, 1 µg antibody per 100 µL well was immobilized overnight from 50 mM sodium carbonate buffer, pH 9.6. After three washes with PBS (50 mM sodium phosphate—0.9% NaCl, pH 7.2), with added 0.05% Tween 20, 100 µL per well of 1% bovine serum albumin in PBS was then incubated 1 h to decrease non-specific binding of conjugates.

Food samples (rice, wheat grain, grapes, dried sultana grapes and tea) were extracted by blending with 150 mL of the indicated solvent per 30 g food, for 2 min at full speed using a Waring blender. The extracts were allowed to settle 10 min, before an aliquot was diluted (1 in 5, unless otherwise indicated) with PBS containing 0.5% *Teleostean* fish skin gelatin (FG, Sigma, St Louis, MO). Agrochemical standards were prepared in solvent and diluted in a similar manner. One hundred µL of either standard or sample were applied per well, followed by 100 µL enzyme conjugate, diluted in FG-PBS. After 1 h, the wells were washed 5 times with PBS-Tween, then 150 µL 3,3',5,5'-tetramethylbenzidine–hydrogen peroxide substrate–chromogen mixture (*10*) was added and incubated for 30 min. Color development was stopped using 50 µL 1.25 M sulfuric acid and absorbances read at 450 nm, and the background at 650 nm was subtracted.

Results and Discussion

What are Matrix Effects? Matrix effects (or "matrix interference") can be loosely described as any change in assay performance that can be attributed to effects of components in the sample. The usual means of detection of matrix effects is to compare the standard curves generated using a series of dilutions of pesticide standards prepared in an extract of a specific sample of the food that is known to be pesticide-free with the absorbance values for the same set of standard concentrations prepared in solvent only; clearly, the same extraction solvent should be used for both curves and the analyses run together, preferably on the same microtiter plate.

Matrix effects can manifest themselves in several ways (Figure 1). The most common is as a change in assay sensitivity, with the curve for standards prepared in matrix extract usually being shifted to the right of the curve for standards prepared in solvent; an example is shown for dried sultana grapes in Figure 1. Such a decrease in assay sensitivity, if not corrected for, could give rise to significant under estimates in calculated residue contents in the sample extract, and make some residue-containing samples appear residue-free (false negatives). Sometimes, however, assay sensitivity can actually be increased in the presence of a matrix extract. An example is an assay for the insect growth regulator, methoprene, in wheat grain (*10*). Another common matrix effect is a decrease in assay absorbance from the matrix; in the absence of pesticide residues in the sample, this can provide false positives in the assay. This effect is often accompanied by a decrease in the limit of detection of the assay (e.g., tea extract in Figure 1).

In some other cases, the curves for the pesticide standards in the matrix extract and in solvent alone samples may be superimposable, but the assay may suffer from poor precision and/or accuracy, such that poor correlations are obtained between analyte spike and recovery data. To be sure that this is the case, there is a need to distinguish poor extraction of residues (especially incurred residues) from actual matrix interference.

An example of the implications of not properly accounting for matrix effects in an assay for endosulfan in rice is shown in Figure 2. In this assay, extracts are diluted 1:10 in FG-PBS before analysis, so the concentrations in the solvent extract of the rice are ten-fold greater than those shown on the Figure. In the presence of rice extract the standard curve is shifted to the right, but the matrix interference can be removed by passage of the extract through octadecylsilica and partitioned with phosphate buffer (see below). A 50 ppb spike of endosulfan into rice extract provided a recovery of only 20–25 ppb when the result was read with reference to standards prepared in acetonitrile solvent only. When the same spike was read with reference to a standard curve prepared in rice extract, the correct result was obtained, but the limit of detection of the assay was only about 20 ppb (in solvent extracts). Since the sample was extracted in 5 mL acetonitrile per g rice, this equates to a limit of detection of 0.1 ppm endosulfan in rice. Greater sensitivity than this is required for most applications. After the clean-up, the spike also gave a 50 ppb (in solvent extract) result, indicating that no pesticide was lost through the clean-up process, but the sensitivity of the assay was restored to 2 ppb in the solvent extract, equivalent to 0.01 ppm in rice. Thus, the main advantages of performing cleanup resulted in allowing the sample to be analyzed with reference to standards prepared in the extraction solvent and providing the assay with the necessary sensitivity.

What Factors Determine the Extent of Matrix Interference? A commonly-cited advantage of pesticide immunoassays is that they exhibit fewer matrix interferences than instrumental methods, because of the specificity and high affinity of the antibody-antigen interaction. This is often true, but sometimes instrumental methods can be less subject to matrix effects. Often, pesticide samples are extracted with non-polar, immiscible solvents such as hexane, which extract fewer matrix components but are compatible with direct instrumental analysis (these sometimes also incompletely extract the target compound (*17,18,20*). The availablity of selective detectors (e.g., electron capture) or detection methods (e.g., mass spectroscopy) also aid in distinguishing responses due to pesticide from those due to matrix components. Removal of matrix interference also can be more readily monitored using chromatographic methods, since there is considerably greater information content of the chromatogram compared with an ELISA, which simply provides for each sample an absorbance value relative to a control. In chromatography, the pesticide and the matrix components may have different retention times such that specific determination of the pesticide is not affected.

In immunoassay, matrix effects are highly dependent on the nature of the food sample being tested; i.e., the content of lipids, proteins and polyphenols that may be coextracted during sample preparation. Matrices that require more clean-up during instrumental analysis also usually require more sample cleanup for immunoassay. For example, we have found that black tea gives major matrix interference in immunoassays for both organochlorines and organophosphates, whereas the interference produced by red grapes is limited in the same assays.

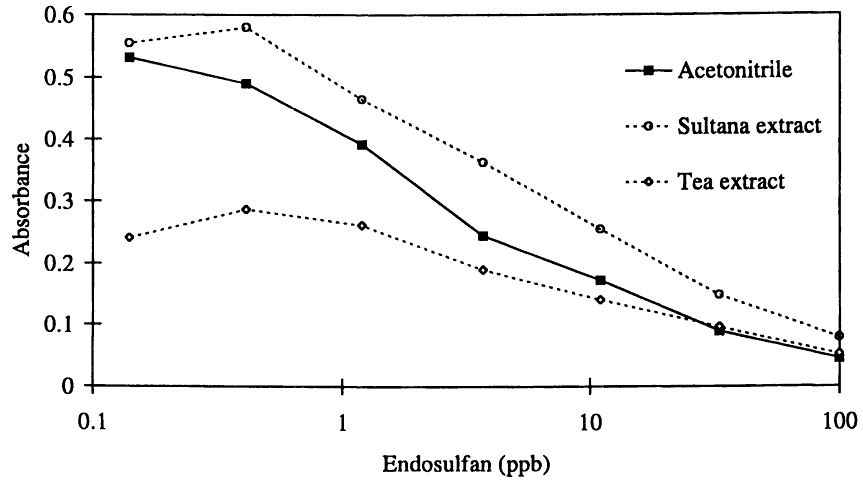

Figure 1. Standard curves for immunoassay of endosulfan in acetonitrile and in acetonitrile extracts of black tea and dried sultana grapes. Data shown in Figures 1 to 6 are from single experiments, which were repeated with similar results.

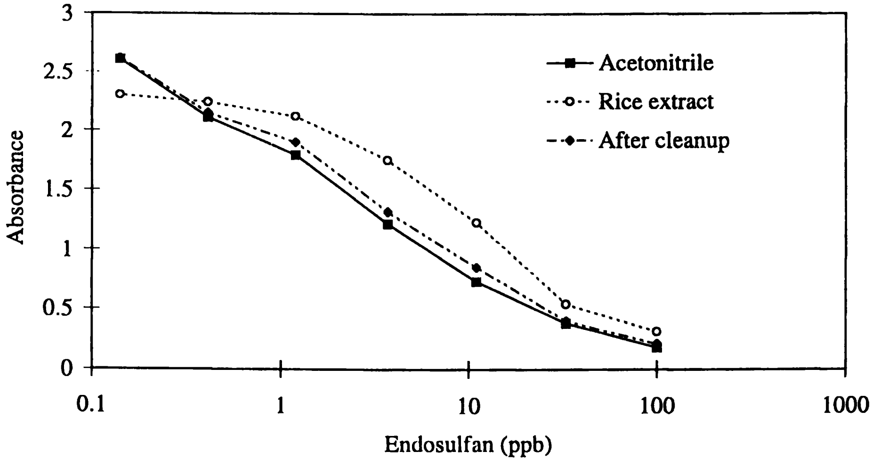

Figure 2. Implications of analysing samples against the "wrong" standard curve; standard curves for analysis of endosulfan residues in acetonitrile, rice extract and rice extract, after cleanup by chromatography on octadecylsilica and partitioning of interfering compounds into phosphate buffer.

It is important to realise that the lack of interference by a particular matrix in one or more immunoassay does not imply that other assays using that matrix would be interference-free. For example, curves of pesticide standards prepared in methanol and a methanol extract of wheatmeal are superimposable for each major grain organophosphate, including fenitrothion, chlorpyrifos-methyl and pirimiphos-methyl, as assessed using different polyclonal and monoclonal antibodies (*13,23*). However, there is a two to threefold loss in assay sensitivity when standards of the synthetic pyrethroid, bioresmethrin are assayed in a similar methanol wheatmeal extract (*14*). Sometimes even when the same antibody, pesticide conjugate and extraction solvent are used, different pesticide standards can exhibit different types of interference with the one matrix. For example, Figure 3 shows an assay of endosulfan and heptachlor using a broad-specificity cyclodiene antibody and conjugate. In an acetone extract of black tea, there is an 8–10-fold loss of endosulfan assay sensitivity, but a definite concentration-dependent inhibition curve, roughly parallel to that for standards which had been prepared in solvent, is observed. In contrast, a standard curve is not developed for heptachlor in tea extract, with no inhibition observed at 100 ppb. The difference in behaviour of the two pesticides may be related to a difference in their polarity (endosulfan possesses a cyclic sulfite group) and is more water-soluble. The less soluble heptachlor may be bound by tea matrix components more tightly. It follows that a clean-up strategy that fully removes matrix interference in the assay of endosulfan may not fully remove interference in the assay of heptachlor or other cyclodienes.

The solvent used for extraction of the pesticide residue from the food matrix not only affects the extent of extraction of the target compound, but also the degree of matrix interference. The simplest approach is to extract the sample using a water-miscible polar organic solvent, as these potentially allow for no further sample manipulation other than dilution, and are usually good extractants of pesticides from foods. For example, different instrumental multiresidue methods have recommended acetone (*18,19*), acetonitrile *(17)*, and methanol (*24*) as initial extractants for plant food matrices. Using the immunoassay for endosulfan, the sensitivities are similar for standards prepared in methanol, acetone or acetonitrile, with 50% inhibition of antibody binding (IC_{50}) values of 2–3 ppb (in the sample extract after dilution in buffer). However, the extent of matrix interference, shown by decreases in assay sensitivity, differed considerably for various solvents. For example (Figure 4), with methanol extracts, a decreased sensitivity of detection was only seen for the rice extract, while with acetone, dried sultana grapes provided the least matrix interference.

Only a few groups have studied the role of assay format in matrix interference. In assays using immobilized antibodies, which are often more sensitive and form the bulk of commercial assays, the sample extract comes into contact with both antibody and conjugate. In an indirect (two-step) assay using an immobilized hapten-protein conjugate, the enzyme conjugate does not contact the sample extract, so it is possible that this format is less affected by matrix interference (*25*). Finally, processing of the sample also can affect the degree of matrix interference (*22*). In a study on the determination of several organophosphate and pyrethroid residues using immunoassay

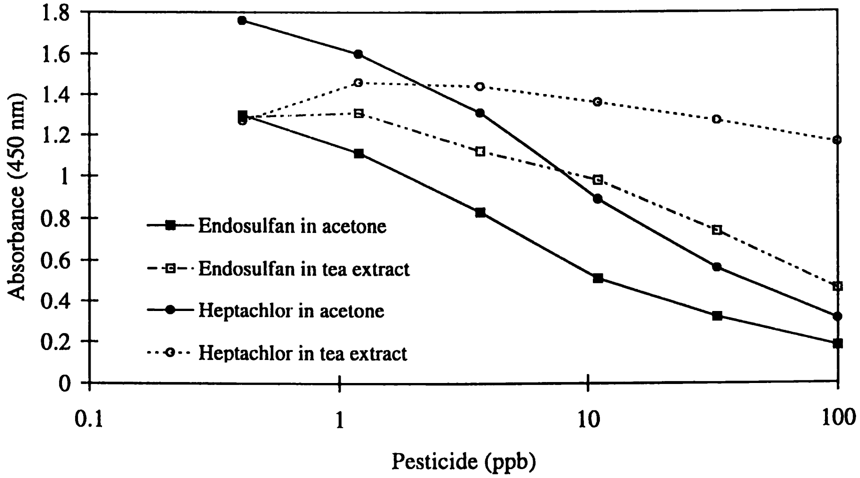

Figure 3. Different pesticides (endosulfan and heptachlor) manifest different levels of matrix interference in tea extract, using a cyclodiene-binding antibody.

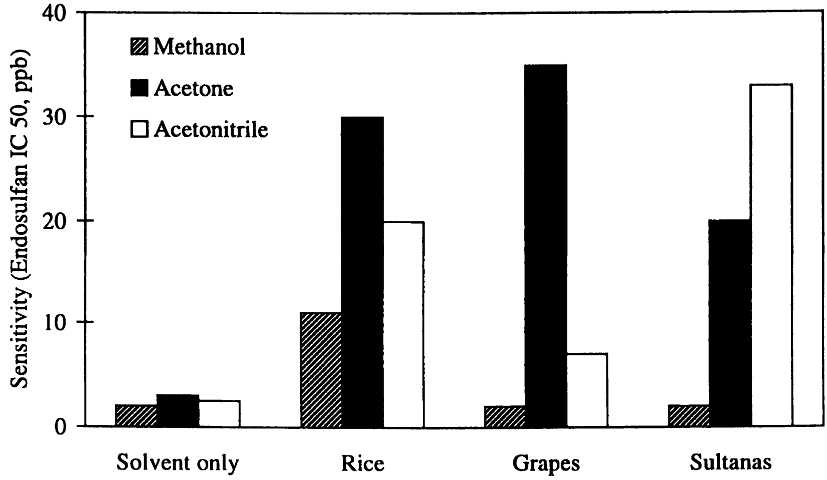

Figure 4. Influence of extraction solvent on endosulfan assay sensitivity. Data shown is the endosulfan concentration required for 50% inhibition of antibody binding in extracts of rice, grapes and dried sultana grapes.

with wheat milling fractions and baked goods, matrix interference was more prevalent in breads and noodles than in the flours used to derive these products. For the breads, this could probably be attributed to the other materials used in baking, such as yeast and lipids, while the use of alkaline salts in processing yellow Asian noodles could be responsible for matrix interference observed with some of the assays.

Performing Immunoassays Once Matrix Interferences Have Been Detected

Accounting for Matrix Effects. Sometimes it is possible to avoid matrix cleanup and analyse the samples with respect to a standard curve prepared in an extract of pesticide-free matrix. We have found this approach satisfactory in situations where matrix effects are present in extracts of grain products, such as breads (*22*) and for pesticides such as bioresmethrin, where matrix effects are found for ground wheat and barley grain and milling fractions (*14*). It seems to be most reliable when there is a simple parallel shift of the standard curve in the presence of the matrix. However, the approach requires a standard sample of pesticide-free matrix, and the composition of the pesticide-free standard sample must be the same as test samples. Even if these criteria are met, the detection sensitivity for the target compound may be much lower than in matrix-free samples (Figure 2).

Another approach, developed by workers at the California Department of Food and Agriculture, and applied to the detection of 2,4-D residues in fruits (*26*), is based on spiking extracts of each sample with a constant level of pesticide, then analyzing both spiked (constant level) and unspiked extracts in the assay. The principle of this approach relies on the ratios of absorbances for spiked extracts to non-spiked extracts being similar for most matrices and for solvent standards. Thus if the ratio is depressed, there must be an incurred residue of the same pesticide in the sample. The disadvantage of this approach, apart from requiring two analyses of each sample to be performed, is that it provides qualitative (screening) data rather than quantitative data.

Methods That do Not Use Sample Treatment or Cleanup. Often it is possible through judicious choice of sample extraction or dilution techniques, to reduce or remove potential matrix inteferences. A comparison of the three major water-miscible extraction solvents (methanol, acetone and acetonitrile) may demonstrate markedly different interferences with extracts of the same foodstuff (Figure 4). However, the efficiency of extraction of residues also must be studied before a particular solvent is decided upon. Some solvents may reduce matrix effects but be poor extractants of residues, especially incurred residues. An even simpler approach, which takes advantage of the high sensitivity of many immunoassays, is to simply dilute the food extracts more extensively before the assay is performed. This has been used by us to remove interferences from assays of parathion residues in juices and milk. Others also have used this method to remove interferences in assays of carbendazim in fruit juice (*27*) and thiabendazole in liver (*28*).

Addition of protein or detergents to the assay diluent can overcome matrix interferences. The standard curves for permethrin in methanol extract of grain and in methanol standards are superimposable if the extracts are diluted 10-fold in 1% bovine serum albumin-containing buffer (*12*); whereas, there is a significant difference in assay sensitivities if the corresponding extracts are diluted in buffer alone. Similarly, analysis of procymidone in wine could be performed without interferences, providing a sample diluent containing protein and detergent is used (*29*). Unfortunately, agrochemical immunoassays differ in their compatibility with detergents, thus limiting the potential for using detergents to reduce matrix interference.

Inclusion of a wash step between the sample and the conjugate incubation steps also can reduce potential matrix effects. This approach is commonly used in assays that employ immobilized hapten-protein conjugates and unlabeled pesticide-specific antibody, and specific antibody binding is quantified through the use of a labeled second antibody. However, there are few systematic comparisons of the likelihood of this format, and the corresponding format using immobilized antibody, to display matrix effects. Laboratories tend to develop one or other format, only developing both if one displays a sensitivity problem. It also is possible to have a wash step between the sample and conjugate incubation steps in immunoassays involving immobilized antibody. This is the norm for "sandwich" assays involving two antibodies and macromolecular antigens, but it also can be done prior to addition of a hapten-enzyme conjugate, for example, in separate commercial assays for polychlorinated biphenyls in soil (*30*). However, in some assays we have noted an unacceptable loss in sensitivity; the approach also introduces an additional incubation and wash step.

Simple Treatments of Extracts Made in Water-Miscible Solvents. Sometimes it is possible to selectively remove interfering components, without the need for solvent exchange, through adsorption of these components onto silica, alumina, charcoal, octadecylsilica, or by partitioning interfering components into salt-saturated solutions. The main advantages are that there is no dilution of the extract, or need for evaporation or solvent exchange steps. We have found, for example, that passage of methanol or acetonitrile extracts of grain through basic alumina columns significantly improves the accuracy and precision of data in an immunoassay for permethrin in wheat grain (*12*), however, a corresponding cleanup was not observed when another pyrethroid, bioresmethrin, was analyzed in the same matrix (*14*). The need to ensure that the residue also is not retained by the column would suggest that the method is only useful for some solvent/matrix combinations.

A more-widely used approach with extracts made in water-miscible solvents is to partition the extract to either hexane, light petroleum or dichloromethane, usually after addition of water or brine to the sample. The addition is to improve the partitioning of the hydrophobic residue into the less polar solvent, and to improve the phase separation between the water-miscible and immiscible organic solvent. After partitioning, the immiscible solvent is evaporated off, and the residue redissolved in methanol or detergent-buffer for immunoassay. Again, there is a need to check that the residue under analysis is hydrophobic enough to partition quantitatively and that losses do not occur during the evaporation step. Examples of the successful use of this method include partitioning of: urea herbicides from methanol extracts of root vegetables into dichloromethane (*31*), carbendazim from fruits and vegetables into dichloromethane (*32*), the captan metabolite, tetrahydrophthalimide from methanol extracts of fruit or baby foods into 10% diethyl ether in hexane (*33*), and cyclodienes from acetone extracts of apple, lettuce and tomato into hexane:dichloromethane (*34*). Foods that are already in liquid form can be directly partitioned; for example, carbendazim from fruit juices (*35*) and diflubenzuron from milk (*36*).

Gel permeation chromatography on polystyrene beads, using immiscible solvents is a widely used method for clean-up of fatty matrices in conventional instrumental analysis (*37*). The large lipid aggregates and proteinaceous components of the matrix are separated from the target pesticide on the basis of size. Over twenty years ago, a similar approach was described, using other column matrices and alcohol or acetonitrile as developing solvents; e.g., for the cleanup of ethanol extracts of grain (*38*). Only some chromatography media are compatible with neat miscible organic solvents, these include cellulose and hydroxypropylated dextran (Sephadex LH-20 (TM)). We have evaluated the method for the clean-up of endosulfan residues in a variety of matrices, but with limited success. The chromatographic separation of the pesticide from interfering compounds seems to vary with the compound and matrices, the chromatography time is somewhat slower than chromatography on adsorbents such as Florisil and use of the method results in dilution of the sample. Several groups have utilized adsorption of the residues onto octadecylsilica (ODS, also known as "C_{18}" or reverse-phase columns). This approach is widely used in water analysis for residue extraction and concentration, but it also must be regarded as a clean-up technique for foods. A miscible solvent extract is diluted in buffer, applied to the column, and the column washed. The agrochemical is then eluted in neat or concentrated methanol or acetonitrile, and can be analyzed directly by immunoassay. Examples of its application include picloram in soil and plant matrices (*39*), captan in peaches (*40*) and abamectin in strawberries (*41*). The main dangers are precipitation of the pesticide after dilution in buffer, or that the matrix components may bind or precipitate on the column.

We have utilized octadecylsilica columns in another manner. Since in reverse phase chromatography, virtually all pesticides are eluted from ODS at acetonitrile concentrations below 75%, it follows that only interfering substances should bind to ODS at this concentration. Thus some cleanup should be possible by passing extracts of food matrices, prepared in 75% acetonitrile through ODS. Indeed, this forms an early step in a published instrumental multiresidue method (*42*). We have successfully adapted the initial parts of this method to immunoassay of organochlorines and organophosphates in rice, grapes and dried sultana grapes. In this method, the food is blended in acetonitrile, after addition of water to 25% (if the food is low in moisture content). The extract is then passed through a C_{18} cartridge, such that hydrophobic interfering substances bind. The cleaned-up extract is then shaken with saturated sodium chloride in 2 M potassium phosphate, pH 7 (hydrophilic interfering substances partition out), and an aliquot of the upper acetonitrile layer assayed in the ELISA. Advantages of this method are that solvent exchange by evaporation is not needed, it is relatively simple and fast, the method can be used in field situations, and if required, the same extract can be further processed for confirmatory instrumental analyses. The ability of this method to cleanup acetonitrile extracts of analyses of dried sultana grapes is shown in Figure 5. Spike and recovery data for analyses of heptachlor in extracts of rice (cleaned up using this method) using the two cyclodiene immunoassays are shown in Figure 6.

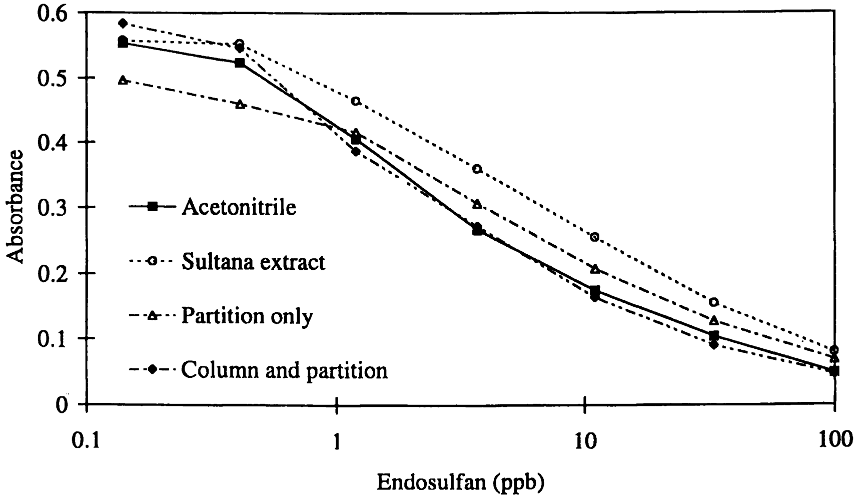

Figure 5. Removal of matrix interference from dried sultana grapes in an immunoassay for endosulfan by octadecylsilica column treament and partitioning into a phosphate buffer.

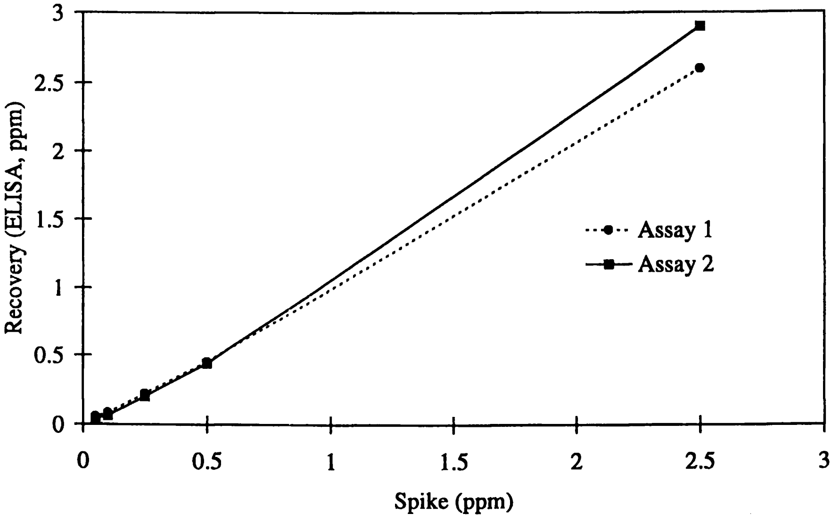

Figure 6. Spike and recovery of heptachlor in rice using clean-up by octadecylsilica column treament and partitioning into a phosphate buffer. Grain was spiked and mixed 24 h before extraction and analysis.

Using Extracts Prepared in Water-Immiscible Solvents. The sample is extracted in a water-immiscible solvent. A clean-up step may or may not be applied, and can utilize any of the range of commonly used instrumental residue analysis, such as adsorption on alumina or silica, or gel permeation chromatography. The main practical difference in the application of this approach to immunoassay is the miniaturization of sample and solvent volumes and column sizes, which is possible because of the higher sensitivity of immunoassay. If alumina or Florisil is used, the residue may be selectively eluted using a more polar solvent. Prepacked cartridges are especially useful here. The final step of the method requires removal of the solvent by evaporation and transfer of the residue to detergent/buffer or a water-miscible solvent. The approach has been used in immunoassays of methoprene in grain (Guihot, S. L. and Skerritt, J. H., the CSIRO Division of Plant Industry at Canberra, Australia, unpublished results), permethrin in meat (*15*) and cyclodienes in fat (*16*). In other cases, it is possible to simply evaporate off the extractant and redissolve the residue in a water-miscible one; an example is analysis of the fungicide, iprodione in fruits and vegetables (*43*).

Disadvantages of the use of non-polar solvents are that a multistep procedure is required with the risk of residue loss through evaporation and the chromatography step also requires optimization for each matrix. However, there is a wealth of clean-up methods for instrumental analyses in the analytical literature, and these can be used to develop a smaller-scale and/or simple method for immunoassay.

Supercritical Fluid Extraction. Supercritical fluid extraction (SFE) is being actively explored by residue analysis laboratories as an alternative to conventional solvent extraction of residues. Advantages of the technique include speed and minimization of solvent waste. SFE of a variety of pesticide residues from meat matrices has recently been investigated (*44*; see chapters by King and Nam, and by Lopez-Avila *et al.*, this volume). The extracts still required some clean-up before immunoanalysis, but it was relatively simple, involving one of either solvent partitioning, solid-phase extraction or microfiltration.

Conclusions

Identifying and removing matrix interference is the largest barrier to the more widespread application of pesticide immunoassay to food analysis. Assays for a number of analytes have been reported to be free of matrix interferences, enabling solvent (typically methanol) extracts to be simply diluted for the assay. These include assays for organophosphate insecticides in grain (*11,13,23*), the fungicides, metalaxyl and triadimefon in vegetable and fruit matrices (*45,46*), atrazine in cornmeal (*47*), and aldicarb sulfone and carbofuran in meats (*48*). Thus, the appearance of such interferences can be hard to predict from theory, as it depends on a number of factors, including the nature and type of processing of the food sample, the extraction solvent used for the sample, as well as the particular antibody and conjugate used. In class-specific assays, the ability to detect different cross-reacting compounds can vary due to matrix interference, as well as relative affinity of the antibody. Finally, the assay format (direct competitive assay using immobilized antibody or assay using immobilized hapten-protein conjugate) can affect the degree of matrix interference. The only approach, therefore, is that during assay development matrix interference and clean-up strategies need to be studied for each assay, food and extractant combination. Nonetheless, ample evidence has accumulated that would suggest that immunoassay is applicable to the analysis of agrochemical residues in quite complex food matrices, and in most cases the method should be simpler and higher in throughput than instrumental methods. To obtain accurate results in the immunoassays, clean-up is often needed, but it can usually be kept simple. It is important to validate an immunoassay method against the standard instrumental method using residues extracted with the same solvent and extraction method, and ideally with incurred residues as well as spiked samples.

The ultimate aim of this work is the development of 3 or 4 methods that are readily coupled to immunoanalysis for residue extraction and cleanup from major food types (high or low in moisture, fat or sugar content). While this will certainly simplify the use of immunoassay methods by the residue analyst, during the development of the method, the effectiveness of the "generic" method will need to be confirmed for each antibody and matrix combination. It is likely, that specialized cleanup methods may be needed for complex matrices such as tea and coffee.

Acknowledgments

The authors are grateful for the collaboration of A. Hill, N. Lee, H. Beasley and S. Guihot, and to the Australian Centre for International Agricultural Research, the Dried Fruits R&D Council of Australia and Millipore Pty Ltd for financial support.

Literature Cited

1. Jung, F.; Gee, S. J., Harrison, R. J.; Goodrow, M. H.; Karu, A. E.; Braun, A. L; Li, Q. X; Hammock, B. D. *Pestic. Sci.* **1989**, *26*, 303–317.
2. Harrison, R. O.; Goodrow, M. H.; Gee, S. J.; Hammock, B. D. In *Immunoassay for Trace Chemical Analysis: Monitoring Toxic Chemicals in Humans, Food and the Environment*; Vanderlaan, M.; Stanker, L. H.; Watkins, B. E; Roberts, D. W.. eds.; ACS Publications: Washington, DC, 1991, ACS Symposium Series No. 451; pp 14–27.
3. Newsome, W. H.; Collins, P. G. *J. Assoc. Off. Anal. Chem.* **1987**, *70*, 1025–1027.
4. Bushway, R. J.; Savage, S. A.; Ferguson, B. S. *Food Chem.* **1990**, *35*, 51–58.
5. Brandon, D. L.; Binder, R. G.; Wilson, R. E.; Montague, W. C. *J. Agric. Food Chem.* **1993**, *41*, 996–999.
6. Itak, J. A.; Selisker, M. Y.; Herzog, D. P.; Fleeker, J. R.; Bogus, E. R.; Mumma, R. O. *J. AOAC Int.* **1994**, *77*, 86–91.
7. Bushway, R. J.; Young, B. E. S.; Paradis, L. R.; Perkins, L. B.; Martin, S. K.; Brown, M. P. *J. AOAC Int.* **1994**, *77*, 1237–1243.
8. Bushway, R. J.; Young, B. E. S.; Paradis, L. R.; Perkins, L. B. *J. AOAC Int.* **1994**, *77*, 1243–1248.

9. Newsome, W. H.; Collins, P. G. *Bull. Environ. Contam. Toxicol.* **1989**, *47*, 211–216.
10. Hill, A. S.; Mei, J. V.; Yin, C-M.; Ferguson, B. S.; Skerritt, J. H. *J. Agric. Food Chem.* **1991**, *39*, 1882–1886.
11. Hill, A. S.; Beasley, H. L.; McAdam, D. P.; Skerritt, J. H. *J. Agric. Food Chem.* **1992**, *40*, 1471–1474.
12. Skerritt, J. H.; Hill, A. S.; McAdam, D. P.; Stanker, L. H. *J. Agric. Food Chem.* **1992**, *40*, 1287–1292.
13. Skerritt, J. H.; Hill, A. S.; Thwaites, H. L.; Edward, S. L.; McAdam, D. P. *J. AOAC Int.* **1992**, *73*, 519–528.
14. Hill, A. S.; McAdam, D. P.; Edward, S. L.; Skerritt, J. H. *J. Agric. Food Chem.* **1993**, *41*, 2011–2018.
15. Stanker, L. H.; Bigbee, C.; Van Emon, J.; Watkins, B.; Jensen, R. H.; Morris, C.; Vanderlaan, M. *J. Agric. Food Chem.* **1989**, *37*, 834–839.
16. Stanker, L. H.; Watkins, B. E.; Vanderlaan, M.; Ellis, R.; Rajan, J. In *Immunoassays for Trace Chemical Analysis: Monitoring Toxic Chemicals in Humans, Food and the Environment*; Vanderlaan, M.; Stanker, L. H.; Watkins, B; Roberts, D, eds.;. American Chemical Society: Washington, DC, 1990, ACS Symposium Series No. 451; pp 108–123.
17. Storherr, R. W.; Ott, P.; Watts, R. R. *J. Assoc. Off. Anal. Chem.* **1971**, *54*, 513–516.
18. Luke, M. A.; Froberg, J. E.; Doose, G. M.; Masumoto, H. T. *J. Assoc. Off. Anal. Chem.* **1981**, *64*, 1187–1195.
19. Hernandez Herandez, F.; Grases, J. M.; Beltran, J.; Sancho, J. V. *Chromatographia* **1990**, *29*, 459–466.
20. Leoni, V.; Caricchia, A. M.; Chiavarini, S. *J. AOAC Int.* **1992**, *75*, 511–518.
21. Lee, N.; Skerritt, J. H.; McAdam, D. P. *J. Agric. Food Chem.* **1995**, *43*, 1730–1739.
22. Edward, S. L.; Hill, A. S.; Desmarchelier, J. M.; Gore, P. J.; Skerritt, J. H. *Proc. 43rd. Australian Cereal Chem. Conf.*, Wrigley, C. W., ed.; Royal Aust. Chem. Inst.: Melbourne, Australia, pp 123–127.
23. Edward, S. L.; Skerritt, J. H.; Hill, A. S.; McAdam, D. P. *Food Agric. Immunol.* **1993**, *5*, 129–144.
24. Sharp, G. J.; Brayan, J. S.; Dilli, S.; Haddad, P. R.; Desmarchelier, J. M. *Analyst* **1988**, *113*, 1493–1507.
25. Reck, B.; Frevert, J. In *Immunochemical Methods for Environmental Analysis*; Van Emon, J. M.; Mumma, R. O., eds.; American Chemical Society: Washington, DC, 1990, ACS Symposium Series No. 442; pp 193–198.
26. Richman, S. *Eighth IUPAC International Congress on Pesticide Chemistry*; Washington, DC, July, 1994, Abstract 56.
27. Itak, J. A.; Selisker, M. Y.; Jourdan, S. W.; Fleeker, J. R.; Herzog, D. P. *J. Agric. Food Chem.* **1993**, *41*, 2329–2332.
28. Brandon, D. L.; Binder, R. G.; Wilson, R. E.; Montague, W. C. *J. Agric. Food Chem.* **1993**, *41*, 996–999.
29. Ferguson, B. S.; Kelsey, D. E.; Fan, T. S.; Bushway, R. J. *Sci. Total Env.* **1993**, *132*, 415–428.

30. Baek, N. H. *Bull. Environ. Contam. Toxicol.* **1993**, *51*, 844–851.
31. Newsome, W. H.; Collins, P. G. *Food Agric. Immunol.* **1990**, *2*, 75–84.
32. Bushway, R. J.; Young, B. E. S.; Paradis, L. R.; Perkins, L. B. *J. AOAC Int.* **1994**, *77*, 1243–1248.
33. Yeung, J. M.; Newsome, W. H. *J. AOAC Int.* **1993**, *76*, 1225–1229.
34. Wigfield, Y. Y.; Grant, R. *Bull. Environ. Contam. Toxicol.* **1992**, *49*, 342–347.
35. Bushway, R. J.; Young, B. E. S.; Paradis, L. R.; Perkins, L. B.; Martin, S.K .; Brown, M. P. *J. AOAC Int.* **1994**, *77*, 1237–1243.
36. Wie, S.; Hammock, B. D. *J. Agric. Food Chem.* **1984**, *32*, 1294–1301.
37. Walters, S. M. *Anal. Chem. Acta.* **1990**, *236*, 77–82.
38. Horler, D. F. *J. Sci. Food Agric.* **1968**, *19*, 229–231.
39. Deschamps, R. J.; Hall, J. C. *Food Agric. Immunol.* **1991**, *3*, 127–134.
40. Itak, J. A.; Selisker, M. Y.; Herzog, D. P.; Fleeker, J. R.; Bogus, E. R.; Mumma, R. O. *J. AOAC Int.* **1994**, *77*, 86–91.
41. Karu, A. E.; Schmidt, D. J.; Clarkson, C. E.; Jacobs, J. W.; Swanson, T. A.; Egger, M. L.; Carlson, R. E.; Van Emon, J. M. In *Immunochemical Methods for Environmental Analysis*; Van Emon, J. M.; Mumma, R. O., eds.; American Chemical Society: Washington, DC, 1990, ACS Symposium Series No. 442; pp 95–111.
42. Lee, S. M.; Papathakis, M. L.; Feng, H-M. C.; Hunter, G. F.; Carr, J. E. *Fresenius J. Anal. Chem.* **1991**, *339*, 376–383.
43. Newsome, W. H. In *Pesticide Science and Biotechnology*; Greenhalgh, R.; Roberts, T.R., eds.; Blackwell Scientific: Oxford, England, 1987; pp 349–352.
44. Nam, K-S.; King, J. W. *J. Agric. Food Chem.* **1994**, *42*, 1469–1474.
45. Newsome, W. H. *J. Agric. Food Chem.* **1985**, *33*, 528–530.
46. Newsome, W. H. *Bull. Environ. Contam. Toxicol.* **1986**, *36*, 9–14.
47. Wigfield, Y. Y.; Grant, R. *Bull. Environ. Contam. Toxicol.* **1993**, *51*, 171–177.
48. Lehotay, S. J.; Argauer, R. J. *J. Agric. Food Chem.* **1993**, *41*, 2006–2010.

RECEIVED September 7, 1995

Chapter 4

Rapid Test Methods for Regulatory Programs

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A major component of food safety programs is to assure compliance with regulatory limits for pesticides, environmental contaminants and veterinary drugs. While the Food and Drug Administration and the Environmental Protection Agency have primary responsibility for establishing regulatory limits for these substances, the U.S. Department of Agriculture (USDA) has the daily responsibility for determining compliance with these residue limits in meat and poultry products. This food safety responsibility is accomplished through USDA's National Residue Program, complimented on occasions with special residue studies. Meeting the objectives of a statistically designed residue control program that traditionally examines 10 or more classes of xenobiotics and more than 75 individual compounds requires a wide variety of analytical and microbiological methods and screening tests in inspection facilities and laboratories using animal tissue, biological fluids or other matrices as a test media. Environmental, economic, regulatory and evolving public health considerations will require new strategies with more focus on screening methods to complement traditional quantitative and confirmatory laboratory methods.

Immunochemistry based assays are emerging as promising screening test methods. Test systems for a wide variety of organic residues in soil, water, food, plant and animal tissues are being developed by scientists in the public and private sectors in the United States. Examples of new immunochemistry based tests are presented in other chapters of this book. In addition, organizations such as AOAC International have developed and implemented test kit evaluation programs (e.g., AOAC Research Institute) to assure test performance with the sponsor's labeling claims. These tests are being developed in rapid, very sensitive, easy and usually highly specific formats.Immuno-based assays presented at this ACS meeting show promise as rapid non-laboratory qualitative assays, while some are now being designed for fast, quantitative, laboratory tests. Their antibody design specificity, which is commonly very high, generally permits use of relatively simple

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sample preparation procedures for test materials and makes immunochemisry methods attractive for use in laboratory as well as non-laboratory environments. Generally, the cost of these assays is less than that of traditional analytical laboratory methods even though most of these test systems are dependent on some sample preparation. Nevertheless, per sample cost for such assays is usually less than 25 percent (including administrative costs) of costs for instrumental methods for similar analyses. The major constraint for many of these assay systems is their relatively high cost of development. An Australian study (*1*) estimates that development of test kits for pesticide residue screening becomes practical when markets for 100,000 test kits per year or more are anticipated.

Method Performance Concepts

An analysis scheme that can simultaneously quantify the presence of all compounds or classes of compounds of interest in foods, animal tissue or fluid with acceptable accuracy and correctly identify the analyte or analytes would be a desirable, unified approach for regulatory control agencies. However, at the present time there are very few analytical procedures available to regulatory agencies that can simultaneously quantitate and confirm the identity of such residues. Until universal methods are available, regulatory programs will have to employ methods with individual attributes of presumptive presence, quantification and positive identification. To satisfy this goal, methods with different attributes must integrate well with each other for a highly effective residue control program regardless of individual regulatory mandates.

Terms such as confirmatory, reference, quantitative, or rapid methods are well known but may mean different things to different people. An alternative to categorizing methods with the potential paradigm (e.g., every one knows what confirmation means) associated with these descriptive terms, is to define the methods according to the attributes or qualities of method performance. Attributes and qualities for three types of analytical methods are relevant to support regulatory programs. While the focus is on screening methods, a brief description of the method types is intended to help clarify their interrelationship. A detailed description has been published recently in a Codex document developed by the Codex Committee on Residues of Veterinary Drugs in Food (*2*). Recently, the Codex Committee on Methods of Analysis and Sampling began considering this approach for other commodities.

Type I methods have the ability to quantify the amount of a specific analyte or class of analytes and provide positive identification in a single analytical process. These are assays with the highest level of credibility for providing quantitation and unequivocal identification at the level of interest. They may be single procedures that determine the concentration and identity of the analyte, or combinations of procedures for determining analyte concentration and confirming its structure. Few such methods currently exist for use in regulatory programs. These methods often include a mass spectrometry component.

Type II methods are not unequivocal for identification of an analyte at the concentrations of interest but are useful for determining the concentration of an analyte and providing some structural information. For example, these methods may employ structure, functional groups or biochemical properties (e.g., mechanism of action) as the basis for the analytical scheme. These methods are generally reliable enough to be used as regulatory reference methods and provide a very acceptable approach for residue control programs. These methods may also be used to corroborate the presence of a compound or class of compounds. Thus, two Type II methods may provide information suitable for Type I attributes providing they employ different chemical technologies. There are two fundamental applications for analytical methods in regulatory analysis schemes. For situations where there is a regulatory tolerance or action limit, quantification around the regulatory limit is the primary consideration. Where no regulatory limit has been established for an analyte (the compound is not approved for use in food producing animals or birds) the primary issue is identification rather than quantification of the analyte. The more common scenario is quantification of an analyte. In the latter case, confirmation of sturctural identity is a critical concern. The majority of analytical methods presently available and used by regulatory control agencies are Type II methods used for quantification.

Type III methods are those that generate less specific though useful information. These testing procedures, for example, detect the presence or absence of a compound or class of compounds at some designated level of interest and often are based on non-instrumental techniques for analytical determination. Screening methods are typically Type III methods. An important consideration for Type III methods is their use to rule out the presence of the analyte at or above a concentration of interest. Assuming that a Type III method has an acceptable limit of false negative performance, no further analysis may be required. Samples that yield a positive response using a Type III method require additional analysis before a regulatory disposition can be made. Thus, Type III methods are a powerful tool for cost and time effective regulatory programs. Many microbiological inhibition and immunoassay test systems fall into this category.

Results on a given sample may not be as definitive as Type I and II methods without corroborating information. These methods may, for example, provide reasonably good quantitative information but poor compound or class specificity or identity, or may provide strong or unequivocal structural identification with very little quantitative information. Type III methods must have defined operating characteristics of reliable performance. They are useful because of their convenience and potential suitability to non-laboratory environments, analytical speed, sample efficiency through batch analysis, portability to differing environments, sensitivity, and the ability to detect classes of compounds. A property of Type III methods is that regulatory action based on positive results requires verification using Type I or II methods based on the uncertainty of the Type III individual result. However, epidemiological information may provide substantive data reducing the uncertainty of individual Type III results.

The applicability of Type III methods should be measured, in part, by their performance characteristics, their ability to process relatively large numbers of samples within a given time frame, low use of organic solvents and their robust nature. This latter characteristic encourages the use of these qualitative and semi-quantitative methods in non-laboratory surroundings where tests may often be performed by individuals not experienced in analytical chemistry techniques. However, methods performed in non-laboratory surroundings place constraints and needs on certain types of methodology. It a) limits use of certain types of equipment, instruments, and reagents; b) requires methods to be written in simple, unambiguous instructions that will enable a tester to correctly prepare the test material, conduct the analysis, interpret and report test findings; and c) requires developing simple, rugged process controls defining critical steps in the test procedure to enhance their performance and reliability. These are critical because a common response to an adverse regulatory action is to challenge the test procedure and analytical result.

Performance Characteristics

To ensure analytical reliability for regulatory programs, performance characteristics ought to be determined by multi-laboratory evaluation for those methods primarily intended for laboratory use, and multi-analyst studies in non-laboratory settings for methods designed for non-laboratory uses. Minimum standards should fit the needs of specific program requirements. The principal attributes considered relevant for all types of analytical methods are its specificity, precision, systematic error, accuracy, and sensitivity. The sensitivity desired in a method is its ability to discriminate between small differences in analyte concentration.

Specificity is the response of a method to the substance being measured. This characteristic is often a function of the measuring principle used or analyte functionality — key factors for rapid test methods. Methods should be able to qualitatively differentiate the analyte from analogues or metabolic products of the compound(s) of interest under the experimental conditions or use employed.

Precision is the closeness of agreement between independent test results obtained from repeated measurements using separate portions of homogeneous test material under the stipulated conditions of use. It may be applied to conditions of repeatability (the same method on identical test material in the same laboratory by the same analyst using the same equipment during short time intervals) or reproducibility (the same method on identical test material in different laboratories with different analysts using different equipment). Precision is usually expressed as a standard deviation. A useful term in a regulatory program is the relative standard deviation, or coefficient of variation, because it is generally constant over a considerable concentration range (an order of magnitude, for example), ideally covering the concentration of interest. It may be reported as a percentage by dividing the standard deviation by the absolute value of the arithmetic mean and multiplying by 100. Precision is sometimes used, particularly in the European Union, to describe other method characteristics such as limit of detection and limit of determination. Using an analysis of a minimum of 20 blank samples, the limit of detection is expressed as the mean value for the blank determinations plus three times the standard deviation. The limit of determination is the lowest analyte content for which the method has been validated with specified degrees of accuracy and repeatability. It is commonly calculated as the mean value for the blank determinations plus six times the standard deviation (*3*).

Precision limits agreed upon for analytical methods within the Codex Committee on Residues of Veterinary Drugs in Food, as a function of concentration, are presented in Table I (*2*). Within the Food Safety and Inspection Service, comparable values are used. The values listed take into consideration the wide variety of methods, analytes, matrices, and species and are usually applied in consideration of a broad-based residue control program.

Table I. Precision Guidelines for Analytical Methods.

Concentration	Coefficient of Variation (Repeatability, %)
≤ 1 μg/kg	35
≥1 μg/kg ≤ 10 μg/kg	30
≥10 μg/kg ≤ 100 μg/kg	20
≥ 100 μg/kg	15

Systematic error (analytical method bias) is the difference of the measured value from the true, assigned or accepted value (mean value). It is often expressed as the percent recovery of added analyte to a sample blank. At relatively high concentrations, recoveries are expected to approach one hundred percent. At lower concentrations, and particularly with methods involving a number of steps, recoveries may be lower but need to have low variability.

Accuracy refers to the closeness of agreement between the true value and the measured result. The accuracy requirements of different types of methods will vary with the use being made of the results. Accuracy requirements will vary with the objective of the test procedure. In general, methods should have their greatest accuracy at the regulatory residue limit. The accuracy requirement of confirmatory methods may not be as great as is required for quantification methods because in most instances these methods are only performed after a residue concentration greater than the regulatory limit has been determined by a quantification method. Suggested accuracy requirement for methods in the Codex Committee on Residues of Veterinary Drugs in Food (*2*) are given in Table II, and are based upon the previously stated considerations of a broad-based residue control testing program.

Table II. Accuracy Guidelines for Analytical Methods.

Concentration	Acceptable Range (%)
≤ 1 μg/kg	50-120
≥ 1 μg/kg ≤ 10 μg/kg	60-120
≥ 10 μg/kg ≤ 100 μg/kg	70-110
≤ 100 μg/kg	80-110

For screening methods, two key characteristics requiring definition include the percent false positives (reporting a positive response when no analyte is present) and percent false negatives (reporting a negative response when the analyte is present) when measured against a validated quantitative assay in a statistically designed protocol. The percent false negatives must be quite low at the concentrations of interest (less than 5 percent, ideally zero) while some latitude may be acceptable for false positives. Residue detection limits can be described based on a balance between these two parameters.

Rapid test systems ought to achieve parallel curves for standard solutions of an analyte and extracts of analyte added to a test sample. One benefit of this information is that it provides a degree of confidence that the matrices of interest do not cause adverse interferences with the test system. This may be difficult to determine when the rapid test system is in a card format or does not use a spectrophotometer to measure the test result.

Sensitivity for a regulatory method is a measure of the ability to discriminate between small differences in analyte concentration. A common practice is to define sensitivity as the slope of the calibration curve with known standards at the concentrations of interest.

In addition to these basic characteristics are a number of collateral criteria particularly suitable for Type III methods for regulatory control programs. Methods should be rugged or robust, cost-effective, relatively uncomplicated, portable, handle a set of samples simultaneously in a time effective manner, and have rugged and practical quality assurance/quality control procedures.

Ruggedness of a method refers to its performance capability to be relatively unaffected by small deviations from the established protocol for the use of reagents, the quantities of reagents used, time factors for extractions or reaction or temperature. Use of Type III methods in non-laboratory environments means giving consideration to extreme temperature and humidity effects, stability of reagents to these environments, choices in solvents so that they are sufficiently non-volatile to minimize safety and disposal concerns yet maintain acceptable solvating properties. Portability for non-laboratory tests, in particular, is associated with ruggedness characteristics. It is the characteristic that enables the method to be transferred from one location to another without losing its established performance characteristics.

Cost-effectiveness, particularly for laboratory based Type III methods refers to practical use of common reagents and supplies, minimizing use of reagents and non-disposable items, and for example, employing instrumental techniques commonly used for trace environmental analyses. The capability to analyze a set of test samples simultaneously reduces the analytical time requirements of sample analysis. This is particularly important where large numbers of samples are to be analyzed in short or fixed time frames. For non-laboratory applications

To easily describe test methods in training manuals, Type III methods, among others, must make use of simple, straightforward, mechanical or operational procedures throughout the method. This is important, in part, because of the requirements to develop unambiguous training materials so that practitioners, industry and farm personnel, or others that may be non-technical individuals, perform the test and correctly interpret the results in a consistent manner.

Recognizing the need for simplicity in a Type III method is obvious. Making tutorials available for non-technical individuals requires making self-instructional materials that are clear and unambiguous. Putting this type of information into pictorial and narrative training materials is very important and may be a complex process. The tutorial must address precautions to be observed, test sample preparatory steps to be followed, application of the test material to the testing system and any sequential steps necessary for addition of reagents, expected observations, what to do if the expected response(s) does not occur, how to interpret and report results, and instructions for appropriate disposal of used materials. Self instructional guidance materials need to be field tested to demonstrate their unambiguity and effectiveness.

For Type III methods primarily intended for non-laboratory use, other parameters considered include the methods' simplicity, versatility, reliable availability of commercial equipment and reagents, well defined quality assurance and quality control procedures, safety and well characterized critical control points. It is essential that systems, particularly of this nature, assure that the reagents, materials and disposable supplies be readily and commercially available. Logistics in acquiring these testing items should not hamper the potential utilization of immunochemical (or any other) screening method technologies.

Any test (or test system) has to be versatile in several ways. It ideally should be applicable to a class of analytes (or a defined sub-group of analytes), a variety of matrices such as plasma, urine, feeds, multiple tissue matrices and species. In addition, the method must perform in an acceptable manner in a wide variety of environments as noted above. Where limitations occur, they need to be clearly stated so that test systems are applied correctly.

Type III non-laboratory methods are often intended for broad audiences in urban or rural situations. The materials used (supplies, reagents and other disposable materials, *et cetera*) have another important consideration. They must demonstrate sufficient shelf life to be practical. It is important to avoid situations where a tester may compromise the integrity of a test system by having to substitute other equipment or reagents for performing the test. The sponsor of the proposed test systems has to be responsible for the ready availability and reproducible performance (quality) of any proprietary reagent or other test material at a reasonable cost.

Rapid tests (in fact, all analytical tests) used in a regulatory program to support food safety initiatives require well defined quality assurance (QA) and quality control (QC) programs. It is critically important for the manufacturer or sponsor of the rapid test to assure reliable and reproducible quality for reagents, supplies and other disposable items from one batch to another when tested against a standard reference challenge, as well as having a rugged QC procedure for evaluating the performance of the rapid test itself by users of the test system. Challenges on test results are a common response to a regulatory finding. Rapid tests that cannot withstand rigorous peer review weaken or may invalidate use of any test for a regulatory program.

An important component to good QA-QC procedures is having well defined critical control points and stopping points. A knowledge of these parameters help define the ruggedness of a rapid test system and provide important information to accompany written instructions (e.g., label inserts) to advise end users of necessary precautions when performing the test.

Safety issues for Type III tests may transcend common considerations when using chemical or microbiological reagents particularly in non-laboratory environments. Significant consideration has to be given for end users that are not skilled in the art. What may be a common sense response by an analytical chemist may not be to other users. In addition, whereas there are suitable waste disposal procedures in laboratories, in non-laboratory environments waste disposal considerations are generally a greater concern. Type III tests that are intended for use in work environments such as a government inspected food manufacturing or processing establishment, other environmental health requirements may have to be considered as well as concerns of contamination of food products from inadvertent contact with test materials or reagents. Further, places set aside for performing rapid tests may not have the same air quality handling capabilities as a laboratory and result in other worker safety concerns. Perhaps a final consideration is that the instructions accompanying rapid test systems must be sufficient to advise end users of simple and adequate safety procedures. Liability considerations for sponsors or manufacturers of test systems are not a trivial matter.

Advantages of Immunoassays as Screening Tests

The big advantage of such systems is often their simplicity, freedom from complex or sophisticated instrumentation and minimal use of (organic) solvents and reagents, allowing tests to be performed by individuals that may not be highly skilled in diagnostic or analytical procedures. A disadvantage voiced on occasion is that they are designed specifically for only one compound and require separate tests for other compounds or class of compounds. In some instances, sufficient cross reactivity to a class of compounds will allow other analytes to be detected in the sample matrix though usually at higher concentrations. Thus, there is some trade off in methods development plans and end use by producers, industry and laboratories in regulatory programs.

Reduced analysis and analytical time is a major attribute of immuno-based (or other) screening test technologies. Most laboratory-based regulatory methods are relatively longer and more complex, require more extensive sample preparation, highly skilled laboratory personnel, and dedication of relatively expensive and complex instruments. Collecting samples, processing and shipping samples to regulatory laboratories are not insignificant expenses and neither are the costs for waste disposal. Collecting these samples and the associated costs prior to actual sample analysis are significant. Screening tests that can be used in federally inspected facilities to help separate products that do not contain residues of public health significance and identify potential residue contaminated products requiring additional regulatory analysis helps reduce overall program shipping and handling costs and makes laboratory analysis more cost effective by focusing analysis on a larger percentage of potentially residue positive or contaminated product requiring some additional regulatory action.

Waste disposal issues are of growing importance. Federal laboratories managed either by government personnel or contract organizations, are vigorously working to meet the objectives of Executive Order 12856 to reduce the use of 17 EPA priority chemicals as part of the Federal government sector pollution prevention strategy. This Executive Order calls for a 50% reduction at all government owned-contractor operated facilities, and a 33% reduction at a selected subset Federal facilities by the end of the decade for these 17 chemicals. This list includes benzene, toluene, xylene, methyl- and ethyl isobutyl ketones, chlorinated organic solvents including carbon tetrachloride, chloroform, dichloromethane, tri- and tetrachloroethylene, and 1,1,1-trichloroethane. The list includes the heavy metals cadmium, chromium, lead, mercury, nickel as well as cyanide. It also calls for a 20% reduction in energy use. Use of alternative methodologies obviously includes immunochemistry tests as part of the strategy to reach these goals and emphasizes one aspect of the FSIS long range research objectives.

Results from national residue surveys and programs, such as the Food Safety and Inspection Service's National Residue Program (*4,5*), designed to determine compliance with FDA and EPA food safety tolerance and action levels in meat and poultry products, indicate a low percentage of positive and even smaller percentage of samples containing violative amounts of residues collected as part of FSIS's statistically based, random sampling residue monitoring program. As a consequence, the cost for identifying those relatively small number of residue violative samples is relatively high. In times of limited budgets, effective ways of assuring compliance with food safety regulatory limits for those agricultural products and substances used in food production are a major consideration, particularly in those situations where food safety inspection is mandatory. Analyzing large numbers of samples with reliable Type III tests at the producer level, federally inspected facilities and laboratories leads to more effective use of laboratory resources.

It is now possible to develop reliable and effective Type II methods using the same technology. Traditionally, quantitative regulatory residue assays have required more complex or state-of-the-art instrumentation, performed by analysts in well equipped laboratories where quantitation is necessary (*6*). Adoption of qualitative or semi-quantitative immunochemical assays to quantitative analysis in a regulatory program may take some time before confidence and recognized legal status for such methods are attained. Though the winds of change are evident, it may still require considerable understanding, experience and familiarization with the technology by regulatory agencies, producers, and others to use test systems containing unknown reagents ("black box" test systems) to gain confidence in assuring themselves that public health protection is not compromised. As noted earlier, third party evaluation programs are being established to evaluate performance of test kits and memoranda of understanding between the AOAC Research Institute with FDA's Center for Veterinary Medicine and FSIS indicate movement in using these new test systems in regulatory programs. Past history with other screening tests (e.g., radio-immunoassays for DES) indicate that an unambiguous structure identification or confirmation procedure is vitally important for enforcement purposes and withstanding legal challenges where laboratory results are used for making regulatory decisions and other food safety purposes (e.g., developing guidelines or regulatory initiatives).

Some limitations and concerns still exist regarding use of immunology based assays. Early in the evolutionary development and design of immunochemistry based ready-to-use tests the reliability and performance of the test systems varied from lot-to-lot because reagent strength and other quality issues were not always adequately controlled. Quality control for production is improving and will likely continue to improve with gained experience. Sponsors and users of these test systems recognize the need to employ good quality control/quality assurance protocols to ensure test performance. Establishing criteria or guidelines for sponsors, either by the industry itself or regulatory agencies planning to use such tests, will help facilitate their acceptance.

Another limitation that regulatory agencies must address is that some of these immunochemistry assays may be capable of measuring smaller amounts of analytes than the current or traditional regulatory laboratory quantification and confirmation methods, making it difficult for these existing methods to verify the qualitative or quantitative results. In the near term, this may limit further regulatory use. This is expected to lead to technology development of new Type I and II methods to match the performance limits of detection and quantification of the immunochemistry screening methods. Regulatory agencies need to be able to confirm what they have the capability to detect.

A third limitation for analytes in meat and poultry products is the heavy reliance on aqueous media for performing immunochemical based tests. For certain food types aqueous media extracts may be of little consequence, such as use with fresh vegetables where the analyte may be readily extracted (surface rinse), but for meat and poultry it has been a measurable deterrent. For example, most residues in meat and poultry products are the result of ingestion of pesticides through treatment on grasses, grains and other foraging materials or veterinary drugs used prophylactically in animal production via animal feed or injection. Thus, analytes become endogenous materials requiring extraction from an animal matrix. This puts heavy reliance on use of organic solvents to extract the analyte of interest from the test sample matrix. In addition, because of the mechanism(s) of animal or bird treatment or ingestion and metabolism, parent substance may not be available — rather, metabolic products may be the major residue(s). This often requires developing extraction systems that include steps to transfer the analyte of interest from an organic solvent to one compatible (not destroying or deactivating the immunochemical reagents) with the test system. Technical advances are being made in this area. For example, an immunoassay system has been developed for chlorinated triazines that allows low ng/g (ppb) detection using selected aqueous organic solvent systems (*7*). In instances where a residue marker (a compound that relates to total residues in a reliable and consistent manner and is a measure of residues of food safety concern) is the parent compound, immunoassays are valuable options. However, if safe residue limits are based on or include metabolites as part of the regulatory limit, immunoassays may be of more limited value unless the immunoassay has sufficient cross-reactivity to measure all the analytes (conjugates or other metabolites) of concern.

A recent report that has broad potential for residue analysis of meat and poultry products as well as other food commodities is the use of organic polymers that act as substrates for residue analysis (*8*). The potential for use with residues in food producing animals is intriguing. The benefit is the ability to use small volumes of organic or aqueous organic solvent systems having sufficient solvating properties to extract compounds of interest without necessarily relying on aqueous media for extracting and isolating the organic molecules of interest as noted above.

Other Rapid Test Systems

Although the focus is on immunological based test systems, microbiological methods are excellent examples of screening tests for non-laboratory environments. They include antimicrobial inhibition tests for antibiotic and sulfa residues (*9*), enzymatic tests such as the catalase test to check canned foods and cooked meat for adequacy of heat treatment (*9*), cholinesterase enzyme inhibition assays for organophosphate pesticides (*10*) and an enzyme-linked immunosorbent assay for detecting natural toxins and a variety of other analytes (*11*). Tests have been developed for food animal species identification that are immunological based tests used for compliance and other food safety programs (e.g., the

Overnight Rapid Beef Identification Test — ORBIT) (*9*). This test was used in the mid 1980's for species identification purposes after finding kangaroo meat labeled as beef. These tests also help prevent species substitution in processed meat and poultry products, a potential important health and ethnic or cultural consideration.

Current USDA Agricultural Research Service research programs are developing immunological reagents for potential assays to detect animal diseases, microbiological toxins, and chemical residues for many veterinary drugs. Examples of veterinary drugs and pesticides include salinomycin, halofuginone, hygromycin B, benzimidazoles, pirlimycin, ceftiofur, carbadox sulfonamides and monensin (it is premature to determine which analytes may eventually be successfully developed for residue control programs). Other immunochemistry related research includes antibodies for bovine haptoglobin (acute phase reactants for early identification of animal diseases or other pathological condition), immunochemical sensors on vitreous carbon electrodes for salmonella detection, antibody binding to piezoelectric crystals and aminoglycoside immunoaffinity columns (on polystyrene beads).

Integrating Screening Methods Into Multi-Residue Regulatory Programs

Applications of screening methods for residue control programs depend on a number of factors. The first scenario covers instances, for example, where data indicate a low prevalence of residues above tolerance for an approved pesticide or veterinary drug that may be used in a large number of animal classes. A second scenario applies to situations with a relatively high prevalence of residue violations for an approved pesticide or veterinary drug (or class of compounds) regardless of the number of animal classes (or other appropriate sampling unit) of interest. A third scenario would be for detecting, quantifying or confirming residues from an unapproved use or veterinary practice. A fourth scenario would be where very high statistical confidence is required for food safety purposes. In all these situations (described below) it is assumed that the intended screening (Type III) methods have well defined and acceptable performance characteristics for the intended purpose.

In the first example above, where a history of low violation prevalence from statistical based random sampling programs has been demonstrated, screening methods are particularly attractive because they permit testing large numbers of samples to more readily separate negative samples from positive or potentially violative samples without expending significant laboratory resources. A requirement for this regulatory approach where residue limits have been established is that the test method has acceptable performance characteristics at the concentrations of interest. Results from the Food Safety and Inspection Service (FSIS) National Residue Program, for example, indicate that in the statistical based random sampling program, more than 95% of the samples, across animal classes, contain non-detectable residues or concentrations of residues below a regulatory limit (*5*). This provides the opportunity to test a large number of samples in a cost effective manner and pass these animal carcasses into commerce where no residues or residue concentrations below a level of interest may be identified. The presumptive or suspect positives from the screening (Type III) methods are detained for laboratory analytical procedures. Use of Type III methods in this situation permits more effective use of expensive laboratory facilities and human resources.

Where Type III methods can be designed for safe and reliable use at the initial point of inspection (e.g., an abattoir or other inspected food establishment) obvious benefits accrue. A very strong argument can be made that this scenario is the best and most practical application of Type III immunological based or similar test procedures because they are being effectively used to separate out those animals with no food safety residue issues) while retaining presumptive positives for further, more refined analytical testing. Laboratory quantitative analysis methods and valuable personnel resources may then focus on analyzing the presumptive positive test samples for making better informed regulatory decisions. The major net effect is lower inspection costs through reduced use of laboratory resources, improved timeliness of results, improved cost effectiveness without compromising food safety, and reduced laboratory hazardous waste disposal.

An available option in the above scenario is to allow well defined sample compositing schemes to be employed in homogeneous populations of food producing animals where the test material from one sample unit is too small for reliable analysis. Compositing is somewhat less attractive where potentially high residue violation rates may occur because it calls for reanalysis of individual samples when a positive test result occurs in a test material composite.

In the second scenario where a relatively high residue violation prevalence may occur, immuno-based test systems or others (such as enzyme inhibition assays and thin layer chromatographic systems primarily designed for rapid testing in laboratory environments) become very attractive. These methods provide needed data to take expedient regulatory action where violative or suspect product may be more effectively controlled to prevent its distribution into commercial food channels. Further, use of an alternative analytical technique (another Type II method as discussed earlier), using an independent assay system or technology for the analyte of interest, is generally suitable for sample disposition purposes in a regulatory program. An alternative to immuno-based Type III methods is to develop improved laboratory based methods using more sensitive analytical detectors, new spectrophotometric or fluorescent derivatizing reagents or other biochemical/biosensor systems that match the sensitivity, specificity and other screening method performance characteristics — a potentially costly alternative.

In the third scenario, the qualitative analysis and structural confirmation that identifies the presence of residues of unapproved pesticides or veterinary drugs, Type III tests are very attractive because determination of any amount of an unapproved substance in specific products is an unapproved use and, therefore, a residue violation. This assumes, as in all other cases, performance characteristics of the employed methods are well defined and that false negative and false positive results are negligible. Regulatory action normally requires support by a Type I or II procedure. In this scenario, quantitation is not a specific requirement although administrative procedures or policies may have to be defined (e.g., does the analyte have to be detected in an edible tissue or would a positive result in a biological fluid be sufficient) before initiating other regulatory action. In this scenario, epidemiological information may be incorporated into the regulatory strategy to design effective subsequent field or laboratory analysis programs.

Where increased levels of statistical confidence may be required, Type III methods or procedures are almost essential. Table III describes the relationships between residue prevalence and statistical confidence.

Using this statistical table, for example, to detect a 1 percent violation rate with 95% confidence in a homogeneous sampling population, requires 299 samples to make this determination. If it were necessary to determine a violation rate of 0.1 percent at the 95% confidence level, it would require 2995 samples. In applying this statistical approach to the FSIS National Residue Program, a homogeneous population is usually described as a specific slaughter class within an animal or bird species. This means, for example, that residue testing for pigs would include market hogs, boars, sows, etc., as separate homogeneous populations of animals. Thus, a request for a statistical confidence for 0.1 percent prevalence versus a 1 percent prevalence means thousands of additional tests must be performed within an animal species. With limited analytical resources, the cost effective and practical approach to this situation is to broadly use Type III methods to identify the presumptive positives from the non-detect samples as described above. Without relying on rapid Type III methods, it would be necessary to limit the number or classes of analytes in the annual National Residue Plan and potentially compromise a portion of the intended objective of this food safety program.

Tabel III. Samples Required to Detect Violations With Predefined Probabilities.

Prevalence Rate (% in a Population)	Minimum Number of Samples Required to Detect a Violation with a Confidence Level of		
	90%	95%	99%
10	22	29	44
5	45	59	90
1	230	299	459
0.5	460	598	919
0.1	2302	2995	4603

Potential Impediments

Type III testing procedures may have some legal or program related limitations. A perceived constraint in some programs with Type III tests is that results may not be unambiguous and may consume too large a portion of valuable resources to sufficiently quantitate and specifically identify the analyte of interest. One possible resolution is development of more selective Type III assays or test methods using different chemistry to improve the selectivity of a multi-residue or single residue analysis method. That is, develop a hierarchy of test methods based on current and emerging technologies focusing on the concept of simplicity in design and application, on automation technologies and on commercially available systems and equipment with potential for broad application to regulatory analytical needs.

Type III test systems may be compromised by other constraints. As noted earlier, a critical factor that may limit application of these tests is sample preparation. Improved sample preparation techniques or systems amenable to practical use of Type III test systems in non-laboratory and laboratory environments for residues of agricultural chemicals and veterinary drugs in food producing animals is essential for more rapid acceptance of immunoassays and research in this area ought to be a high priority. At the present time, most rapid test systems are designed to use aqueous systems (e.g., using a water wash system to extract a pesticide residue from a vegetable crop). The nature of the antibody and detection reagents in an immunoassay generally are more compatible with aqueous or aqueous organic solvent systems because these solvent systems do not inactivate the immunochemical reagents or significantly reduce their performance. It is not uncommon to see a 10-fold reduction in analyte sensitivity when using an aqueous organic solvent system compared with a completely aqueous media (for example, see reference *7*). Although some commercial test kit systems for antibiotics use aqueous solvents because of their relatively polar nature, residue programs for other neutral and non-polar analytes must rely on isolating the incurred residue from animal tissue using an organic solvent as an initial isolation step. Though it may be possible to transfer the purified extract to an aqueous system through a number of individual steps, if that is the case, the end result is a procedure that defeats the main purpose of a Type III method. In these situations, these methods become as rigorous and detailed as a laboratory based quantitative procedure and may reduce the immunochemistry analytical system to little more than another analytical detector. An option is to develop tissue–fluid relationships through metabolism studies that would allow regulatory programs to use a biological fluid as the test sample. Laboratory analysis with a Type I or II method would likely be required for making a final regulatory disposition of the animal carcass that gives a presumptive positive result with the Type III method.

A third potential impediment may occur when specific methodology is cited in national or state legislation or Federal regulation that binds regulatory authorities on the type of testing or specific methods that must be employed. Change in regulatory statutes to provide broader regulatory options may be a slow or difficult process.

Another issue that may limit use of Type III tests is the relative high cost of development either by government laboratories or a private enterprise. Markets must be sufficient to support the cost of development and yield a corresponding return on investment. To encourage government or commercial organizations to develop the methodology needed for rapid testing systems requires development of a global perspective where potential users — domestic and international regulatory organizations, the food industry, trade associations, producers and others, realize a mutual benefit of application of reliable screening tests for food safety programs. Hazard Analysis Critical Control Point programs may provide some incentive as well. Public health, economic benefits and regulatory requirements are prerequisites for organizations to underwrite the development and commercialization of Type III tests.

Future Perspectives

The food safety regulatory climate shows clear indication of moving to more Hazard Analysis Critical Control Programs (HACCP). The National Marine Fisheries Service, the Food and Drug Administration, and the USDA Food Safety and Inspection Service have all advocated HACCP for their respective food safety responsibilities through proposed regulations. Within FSIS, as in other food safety programs and Congress, the trend is away from restrictive regulations and movement towards empowering the food industry and producers to take their appropriate role in providing safe foods for consumers. For example, FSIS recently noted that it would assess ways or mechanisms for Federal establishments and producers to be more responsible for the primary determination of animals being free of unacceptable residues of pesticides, environmental contaminants and veterinary drugs — consistent with a critical control point for animals being safe for human consumption when entering a federally inspected abattoir or food processing establishment. This industry and producer focus for primary residue control will, with a high probability, require the availability of simple, reliable and rugged screening tests for veterinary drugs or other agricultural chemicals frequently used by food producers. The joint effort of state milk marketing programs and FDA for bulk testing of raw, liquid milk for antibiotic residues is a landmark for the successful use of screening tests. Hopefully, other food safety programs employing Type III tests will follow.

It seems clear that technology, the regulatory climate, and economic outlooks for food safety programs are moving in the same general direction — simpler, less costly and more pragmatic food safety programs. Success in fulfilling food safety mandates rests with putting the promising new technologies into practical use for the benefit of all consumers.

Literature Cited

1. McCausland, I.; Healy, K.; Wells, R. *Emerging Technologies for Testing Chemical Residues in Meat;* Report of the Australian Meat and Livestock Research and Development Corporation: Sydney, NSW, Australia, 1987.
2. Codex Alimentarius Commission. *Residues of Veterinary Drugs in Food;* Joint FAO/WHO Food Standards Programme, Second Edition, Food and Agriculture Organization of the United Nations: Rome, Italy, 1993.
3. Commission of the European Communities. *Veterinary Drug Residues — Residues in Food-Producing Animals and Their Products: Reference Materials and Methods;* Heitzman, R. J., ed.; Directorate-General for Agriculture Final Report EUR 14126 EN: Brussels, Belgium, 199; pp 27–41.
4. USDA, FSIS. *National Residue Program Plan 1994*; Food Safety and Inspection Service: Washington, DC, 1994.
5. USDA, FSIS. *1993 Domestic Residue Data Book National Residue Program*; Food Safety and Inspection Service: Washington, DC, 1993.
6. USDA, FSIS. *Analytical Chemistry Laboratory Guidebook — Residue Chemistry;* Food Safety and Inspection Service: Washington, DC, 1991.
7. ImmunoSystems, Inc.: Scarborough, ME.
8. Vistakie, G.; Anderssen, L.; Muller, R.; Mosbach, K. *Nature* **1993,** *381*, 645–647.
9. USDA, FSIS. *Microbiology Laboratory Guidebook (and Laboratory Communication updates);* Food Safety and Inspection Service: Washington, DC, 1974.
10. *Product Bulletin, Pesticide Detector Ticker;* EnyzTech, Inc.: Lenexa, KS.
11. ACS. in *Immunoassays for Trace Chemical Analysis, Monitoring Toxic Chemicals in Humans, Food, and the Environment;* Vanderlaan, M.; Stanker, L.; Watkins, B.; Roberts, D., eds.; American Chemical Society: Washington, DC, 1991, ACS Symposium Series 451; pp 140–185.

RECEIVED October 3, 1995

Chapter 5

Immunochemical Approaches to the Analysis of β-Agonistic Drugs

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β-Agonistic drugs are the most frequently found growth promoting agents in the European Union (E.U.). For tracing the illegal use of β-agonists, several types of sample materials (cattle feed, urine, feces, liver, kidney, blood, muscle, bile and retina) are used. For screening of the presence of a variety of β-agonists, three enzyme immunoassays (Clenbuterol-, Salbutamol- and Fenoterol-EIA) were developed. The Clenbuterol-EIA was found suitable for the detection of at least five β-agonists (clenbuterol, cimbuterol, bromobuterol, mapenterol and mabuterol) in all the sample materials applying the direct method for urine or a simple liquid-liquid (water-isobutanol) extraction. The detection limits depend on the β-agonist and the sample material, and varied between 0.1 ng/mL for plasma and 5 ng/g for feed samples. The detectability of β-agonists can be improved to at least eight compounds by applying the Salbutamol-EIA. The Fenoterol-EIA is specific to fenoterol and ractopamine; however, matrix effects prevent the application for routine analysis.

The use of growth regulators (anabolic agents), i.e., naturally occurring steroid hormones, synthetic hormones, xenobiotic compounds and other growth-promoting compounds, in food-producing ruminants (cattle, calves, sheep) causes a shift from fat to lean, an increase in growth rate, and reduced feed requirements for growth (*1*). The United States Food and Drug Administration (FDA) has maintained that hormones approved for use (estradiol, progesterone, testosterone, trenbolone-acetate and zeranol) are safe when used according to label directions (*2*). Within the European Union (EU) the use of all growth-promoters in livestock breeding is forbidden (*3*). For the last five years, β-agonistic drugs have become serious competitors of the anabolic steroids. β-Agonistic drugs are chemically modified adrenalin (epinephrin) derivatives with a profound β_2-effect. They are originally used as bronchospasmoytics in humans, but administered to animals at a higher dosage, a positive effect on growth as well as the

0097–6156/96/0621–0060$15.00/0

protein/fat tissue ratio also is observed. Several investigations have demonstrated that the β-agonists ractopamine (in pigs (*4-7*)), clenbuterol (in rats (*8*) and calves (*9,10*)) and cimaterol (in pigs (*11*) and lambs (*12*)) increase muscle mass and reduce fat deposition. In Europe, despite the EU ban on all growth-promoting agents, β-agonists are the most frequently found growth promoting agents. There have been reports of food poisoning related to the consumption of illicit β-agonists in liver in which clenbuterol exerts toxicity at a relatively high level (200-300 ng/g (*13,14*)). These reports and an increasing misuse of β-agonists have led to increased control. The number of samples analyzed within the Dutch Ministry of Agriculture Nature Management and Fisheries for the presence of β-agonists increased from around 200 in 1988 to almost 20,000 in 1994. Urine is still the most frequently analyzed sample material, however, other materials are used for different reasons. In farmhouses, urine, feces and cattle feed can be sampled. Sampling of feces is much easier and faster than sampling urine, and the residue levels for β-agonists are comparable (*15*). At slaughter, edible tissues (liver, kidney and muscle) can be sampled next to body fluids (plasma, urine and bile) and eye samples.

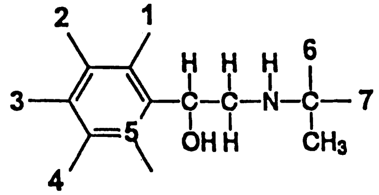
Bile is one of the most suitable sample material for the control on misuse of anabolic steroids and would, if possible, be preferred for the control of both steroids and β-agonists. Clenbuterol accumulates in the choroid/pigmented retinal epithelium tissue of the bovine eye (*16,17*), and even after a withdrawal period of 140 days clenbuterol can still be detected (*18*), which makes this material extreemly suitable for the control on misuse of clenbuterol. Besides the increased amount of different samples, other β-agonists such as salbutamol, bromobuterol, mabuterol, mapenterol, cimaterol and terbutaline (see Table I) have been included in the control system.

In order to detect all these β-agonists in different sample materials, three antisera (anti-clenbuterol, -salbutamol and -fenoterol) were raised in rabbits. These antisera were used to develop microtiter plate EIA's (*19*) and on-site tests (a strip test (*20*) and a tube test (*21*)). Immunoaffinity chromatography (IAC), using immobilized anti-clenbuterol, was used to simplify the sample preparation prior to confirmatory analysis (*22,23*). All these assays were mainly focussed on the analysis of urine samples. Applications of the microtiter plate EIA's in detecting β-agonists in different biological samples are described.

Experimental

Materials. *Helix Pomatia* digestive juice (containing a minimum of 40 U/mL β-glucuronidase and 20 U/mL arylsulphatase) and isobutanol were obtained from Merck (Darmstadt, Germany). Sheep anti-rabbit immunoglobulin G (IgG; whole molecule), Tween-20, bovine serum albumin (BSA), Pronase E (5.5 units/mg), clenbuterol hydrochloride and fenoterol hydrobromide were obtained from Sigma (St. Louis, MO, U.S.A.). Salbutamol sulfate and terbutaline sulfate were obtained from Bufa-Chemie (Castricum, The Netherlands). Robert Schilt (RIKILT-DLO, Wageningen, The Netherlands) supplied us with standards of mabuterol, mapenterol, bromobuterol, cimbuterol, tulobuterol, pirbuterol and cimaterol. Horseradish peroxidase grade I (E 1.11.1.7) was obtained from Boehringer (Mannheim, Germany). Flat bottom microtiter ELISA plates (96-well) were obtained from Greiner (Nurtingen, Germany). Tetramethylbenzidine

Table I. Structures of β-agonists



	Group at position						
β-Agonist	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Clenbuterol	H	Cl	NH_2	Cl	C	CH_3	CH_3
Mabuterol	H	Cl	NH_2	CF_3	C	CH_3	CH_3
Bromobuterol	H	Br	NH_2	Br	C	CH_3	CH_3
Mapenterol	H	Cl	NH_2	CF_3	C	CH_3	CH_2-CH_3
Tulobuterol	Cl	H	H	H	C	CH_3	CH_3
Cimaterol	H	CN	NH_2	H	C	CH_3	H
Cimbuterol	H	CN	NH_2	H	C	CH_3	CH_3
Salbutamol	H	CH_2OH	OH	H	C	CH_3	CH_3
Pirbuterol	H	CH_2OH	OH	H	N	CH_3	CH_3
Carbuterol	H	NH-CO-NH_2	OH	H	C	CH_3	CH_3
Isoproterenol	H	OH	OH	H	C	CH_3	H
Terbutaline	H	OH	H	OH	C	CH_3	CH_3
Fenoterol	H	OH	H	OH	C	H	CH_2-C_6H_6-OH
Ractopamine	H	H	OH	H	C	H	CH_2-CH_2-C_6H_6-OH

(TMB) peroxidase substrate was obtained from Kirkegaard and Perry Labs (Gaithersburg, MD, USA).

ELISA Equipment. A Wellwash Model 4 microplate washer (Denley Instruments, Billinghurst, U.K.) and an Argus 400 microplate reader (Canberra Packard, Downers Grove, Il, U.S.A.) were used.

Antibody Preparation. Clenbuterol was conjugated to BSA (molar ratio 7:1) after diazotization, as described by Yamamoto and Iwata (*24*). Salbutamol-hemisuccinate was prepared according to Beaulieu (*25*). For the coupling of salbutamol-hemisuccinate to BSA (molar ratio 30:1), the procedure described by Kyrein (*26*) was used. Fenoterol was coupled to BSA (molar ratio 30:1) using 1,4-butane diglycidyl ether as a spacer (*27*). The BSA conjugates (ca. 0.4 mg) were mixed with Freund's complete adjuvant. The emulsions were subcutaneously injected into New Zealand white rabbits. After four weeks the rabbits were subcutaneously injected every six weeks with ca. 0.4 mg of conjugate in Freund's incomplete adjuvant and blood samples were taken one and two weeks after the respective immunizations. The collected sera were stored at -20 °C.

Preparation of Horseradish Peroxidase Conjugates. For the coupling of salbutamol-hemisuccinate and fenoterol to HRP (molar ratio 1:1), the procedures described by Kyrein (*26*) and Lommen (*27*) were used, respectively.

Enzyme Immunoassays. Microtiter plates were coated overnight with 100 µL aliquots of sheep-anti rabbit IgG (10 µg/mL in 50 mM sodium-carbonate [pH 9.6]) at 4 °C. Plates were washed 3 times with PBST (5.4 mM Na-phosphate/1.3 mM K-phosphate/150 mM NaCl [pH 7.4], 0.05% Tween-20). In case of the Clenbuterol-EIA (results expressed as clenbuterol-equivalents), aliquots of 50 µL of diluted clenbuterol standard solutions (0.05-5 ng/mL) or 50 µL sample (extract) were added to the wells. In case of the Salbutamol-EIA (results expressed as salbutamol-equivalents), 50 µL of salbutamol standard solutions (0.05-5 ng/mL) were added. In case of the Fenoterol-EIA (results expressed as fenoterol-equivalents), 50 µL of fenoterol standard solutions (0.02-5 ng/mL) were added. Next, 25 µL of appropriately diluted salbutamol-HRP (Clenbuterol- and Salbutamol-EIA) or fenoterol-HRP (Fenoterol-EIA) and finally 25 µL of diluted antisera (all in PBST) were added. Raw sera were used in the three EIA's. The sera and enzyme-conjugates were diluted in glycerol (1:1; v/v) and stored at -20 °C. In case of the Clenbuterol-EIA, the serum and the salbutamol-HRP were used at final dilutions of 10,000 and 150,000, respectively. In case of the Salbutamol-EIA, the serum and the salbutamol-HRP were used at final dilutions of 1,000 and 60,000, respectively. In case of the Fenoterol-EIA, the serum and the fenoterol-HRP were used at final dilutions of 40,000 and 20,000, respectively. The plates were incubated for 1 h at 4 °C and after washing (3 times with PBST), the bound peroxidase was assessed by adding 100 µL of a tetramethylbenzidine (TMB) peroxidase substrate system. After incubation in the dark for 20-30 min at 20 °C, the reaction was stopped by adding 100 µL aliquots of 1 M phosphoric acid and the colored product of the peroxidase reaction was measured at 450 nm.

Origin of Samples. Lyophilized blank bovine urine samples (n = 20) were obtained from the bank of reference blank samples prepared by the European Community Reference Laboratory (CRL) the National Institute of Public Health and Environmental Protection (RIVM, Bilthoven, The Netherlands). Bovine urine (n = 1,730) and feed samples (n = 114) were taken by the General Inspection Service (Kerkrade, The Netherlands) in 1994 at 122 farm houses. Blank calf feces samples (n = 28) and calf feed samples (milk replacers; n = 28) were obtained from TNO Nutrition and Food Research (Zeist, The Netherlands). Urine, plasma, feces, liver, kidney and muscle samples from treated animals were obtained from an animal experiment in which two bulls were orally treated with low doses of clenbuterol (0.8 µg/kg b.w. for 28 days) and two bulls were treated with high doses of clenbuterol (8 µg/kg b.w. for 28 days). No withdrawal period was applied. This animal experiment was performed at the Research Institute for Livestock Feeding and Nutrition (IVVO-DLO, Lelystad, The Netherlands) in February 1993. Sample materials from treated calves were obtained from an animal experiment in which calves (n = 20) were orally treated with a mixture of clenbuterol, mabuterol and mapenterol (each 0.4 µg/kg b.w.) for a period of 6 weeks. Urine, feces, liver, bile and retina samples were taken directly after treatment (no withdrawal period). This animal experiment was performed at IVVO-DLO in June 1993.

Sample Preparation. ***Urine***. Method I (direct method). The pH of the sample was adjusted to 7 ± 0.5 by adding a few drops of 1 M acetic acid and/or 1 M sodium hydroxide. The sample was diluted five times in PBST and 50 µL were pipetted into the microtiter plate (0.2 mL of sample/mL). Method II (hydrolysis). The pH of 1 mL of the urine sample was adjusted to 4.8 by adding a few drops of 1 M acetic acid. Afterwords, *Helix Pomatia* Juice (25 µL) was added and the mixture was incubated for 2 h at 55 °C or overnight at 37 °C. To the hydrolyzed urine, PBST (4 mL) was added and the pH was adjusted to 7 ± 0.5 by adding a few drops of 1 M sodium hydroxide and 50 µL were pipetted into the microtiter plate (0.2 mL of sample/mL). Method III (liquid-liquid extraction). The pH of the urine sample was adjusted to 9.5 ± 0.5 by adding a few drops of 1 M sodium hydroxide. To 1 mL of urine, isobutanol (2 mL) was added and after mixing (Vortex) for 1 min and centrifugation for 10 min at 1,500 X g, 1 mL of the isobutanol was evaporated at 50 °C under a stream of nitrogen. The residue was dissolved in PBST (0.5 mL) and 50 µL were pipetted into the microtiter plate (1 mL of sample/mL). Method IV (hydrolysis + liquid-liquid extraction). Urine was hydrolyzed according to method II. The pH was re-adjusted to 9.5 ± 0.5 and 1 mL of the hydrolyzed sample was extracted with isobutanol according to method III (1 mL of sample/mL).

Feed. To 5 g of feed, methanol (25 mL) and 0.2 M phosphoric acid (25 mL) were added. The extraction was performed using a mechanical shaker for 30 min. After centrifugation for 15 min at 3,000 X g at 4 °C, 0.25 mL of the supernatant was pipetted into 1 mL of PBST. The mixture was vortexed, centrifuged for 5 min at 3,000 X g and 50 µL were pipetted into the microtiter plate (0.02 g of feed/mL).

Feces, Liver, Kidney, Bile and Plasma. To 1 g of sample, 0.1 M hydrochloric acid (5 mL) was added. The sample was homogenated with a Sorvall Omni-Mixer (Model 17106, Dupont, Newtown, USA) and placed in an ultrasonic bath for 15 min. After centrifugation for 10 min at 4,000 X g at 4 °C, the pH of the supernatant was re-adjusted to 9.5 ± 0.5 by adding a few drops of 10 M sodium hydroxide. After centrifugation for 5 min at 1,500 X g, 2 mL of the supernatant was pipetted into 4 mL of isobutanol. The mixture was vortexed and centrifuged for 5 min at 1,500 X g. In case of feces, liver, kidney and bile, 1 mL of the supernatant was evaporated at 50 °C under a stream of nitrogen and the residue was dissolved in 500 µL of PBST (0.2 g of sample/mL). In case of plasma, 2.5 mL of the supernatant was evaporated and the residue dissolved in 250 µL PBST (1 mL of plasma/mL). 50 µL were pipetted into the microtiter plate.

Choroid/Retina and Muscle. To 1 g of the sample, 4 mL of a pronase containing Trisbuffer (pH 8.0 + 1.25 mg pronase E) were added. After an overnight incubation at 55 °C and centrifugation for 10 min at 1,500 X g, 2 mL of the supernatant was pipetted into a test tube and the pH was adjusted to 9.5 ± 0.5 by the addition of a few drops of 10 M sodium hydroxide. To this mixture, isobutanol (5 mL) was added and after vortexing and centrifugation for 5 min, at 1,500 X g, the isobutanol was pipetted into a test tube. In case of the retina, 1 mL of the isobutanol was evaporated and the residue dissolved in 500 µL of PBST (0.2 g of sample/mL). In case of muscle, isobutanol (2.5

mL) was evaporated and the residue dissolved in 500 µL PBST (0.5 g of muscle/mL) and 50 µL were pipetted into the microtiter plate.

GC-MS. The presence of β-agonists was confirmed by the procedure described before (*28*).

Results and Discussion

Under the conditions described in the experimental, the dose response curves showed measuring ranges of 0.1 to 5 ng/mL for the Clenbuterol-EIA, 0.05-5 ng/mL for the Salbutamol-EIA and 0.02 to 5 ng/mL for the Fenoterol-EIA (see Figure 1). The Clenbuterol-EIA showed high cross-reactivity towards mapenterol, mabuterol, bromobuterol and cimbuterol (Table II).

Table II: Cross-reactivity of the EIA's towards β-agonists and recovery (%) of the β-agonists after extraction in isobutanol

β-agonist	*Percentage of cross-reactivity*			*Isobutanol*
	Clenbuterol-EIA	*Salbutamol-EIA*	*Fenoterol-EIA*	*extraction recovery (%)*
clenbuterol	100	130	<0.1	70
cimbuterol	60	70	<0.1	80
salbutamol	6	100	<0.1	70
bromobuterol	100	100	<0.1	-[a]
mapenterol	80	70	<0.1	80
mabuterol	70	70	<0.1	80
tulobuterol	2	30	<0.1	70
carbuterol	5	10	<0.1	50
terbutaline	4	20	<0.1	70
pirbuterol	4	20	<0.1	20
cimaterol	6	10	<0.1	80
fenoterol	<0.1	<0.1	100	20
ractopamine	<0.1	<0.1	20	20

[a] - not determined

However, the cross-reactivity towards salbutamol was low (6%). The tertiary butyl group of the β-agonists plays an important role in the interaction between antigen and antibody of both antisera (anti-clenbuterol and anti-salbutamol). Changing this butyl group into a pentyl group as in mapenterol does not have a major effect on the cross-reactivity. However, a propyl group (as in cimaterol) lowers the cross-reactivity strongly. When a second phenyl group was added to the aliphatic side (as in fenoterol and ractopamine), the antibodies showed no cross-reactivity at all. For that reason we developed a specific EIA for these compounds (*19*). All the β-agonists have a phenylethylamine base structure in common (see Table I). Next to differences on the aliphatic side, differences at the phenyl group also occur. The primairy phenylamine group seems to be very important to the anti-clenbuterol. In case of a lack of such a group (as in tulobuterol, salbutamol, pirbuterol, carbuterol and terbutaline) the cross-reactivity was low (2-6%). Replacing the chloride for a bromide (as in bromobuterol) or CF_3 (as in mabuterol and mapenterol) or a nitrile (as in cimbuterol) does not have a great influence on the cross-reactivity of both antisera. Compared with the clenbuterol antiserum, the salbutamol antiserum improved the sensitivity of the screening for at least five β-agonists (tulobuterol, salbutamol, pirbuterol, carbuterol and terbutaline) which are all compounds with no primary phenylamine.

The reference blank bovine urine samples (n = 20) were analyzed with the three EIA's applying four sample preparations (direct, using extraction and hydrolysis with and without extraction). Hydroxylated β-agonists (as for instance salbutamol) are excreted in urine mainly as glucuronide and or sulphate and, prior to an extraction procedure, hydrolysis to the free compound is necessary. The results obtained with the Clenbuterol-EIA are presented in Figure 2. The urine samples with the lowest blank values (no.s 5, 13, 14, 19 and 20) were obtained from veal calves.

The other samples originated from older cows and bulls. Thus, blank values depend on the age of the sampled animals, but also on the applied sample preparation. Compared with the direct method (method I), higher values were obtained after hydrolysis (method II). The lowest blank values were obtained after extraction in isobutanol (method III). The results obtained with the three EIA's and the four sample preparation methods are summarized in Table III.

The Clenbuterol- and Salbutamol-EIA showed acceptable mean blank values with all four sample preparation methods. Extraction in isobutanol resulted in acceptable extraction efficiencies for most of the β-agonists detected with these EIA's (see Table II). The Fenoterol-EIA gave low blank values (0.6-1.6 ng/mL for method II) only in urine from calves (sample no.s 5, 13, 14, 19 and 20). The mean blank values obtained with the Fenoterol-EIA in the 20 bovine urine samples, applying all four sample preparation procedures, were too high to apply this screening method for routine control on the misuse of fenoterol and ractopamine. Next, the extraction efficiency of fenoterol and ractopamine in isobutanol also was too low (Table II). Further research must be done with respect to sample preparation prior to application of the Fenoterol-EIA.

Table III. Mean blank values (ng/mL) and standard deviations obtained in 20 blank bovine urine samples applying three EIA's and four sample preparation methods.

method	*Clenbuterol-EIA* *mean ± SD*[a]	*Salbutamol-EIA* *mean ± SD*[a]	*Fenoterol-EIA* *mean ± SD*[a]
I	0.74 ± 0.48	0.59 ± 0.39	11.4 ± 7.8
II	1.24 ± 0.63	0.68 ± 0.32	18.4 ± 7.1
III	0.25 ± 0.12	0.10 ± 0.02	3.8 ± 1.7
IV	0.43 ± 0.18	0.26 ± 0.28	4.7 ± 0.7

[a] SD = standard deviation.

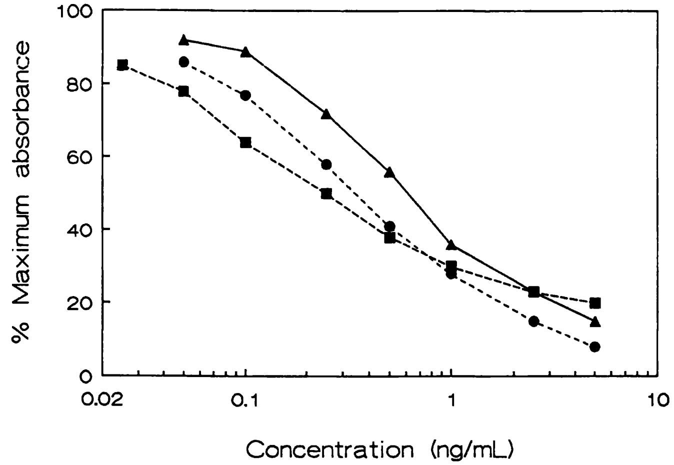


Figure 1. Dose response curves of the three EIA's.
–▲– Clenbuterol-EIA, –●– Salbutamol-EIA, and –■– Fenoterol-EIA

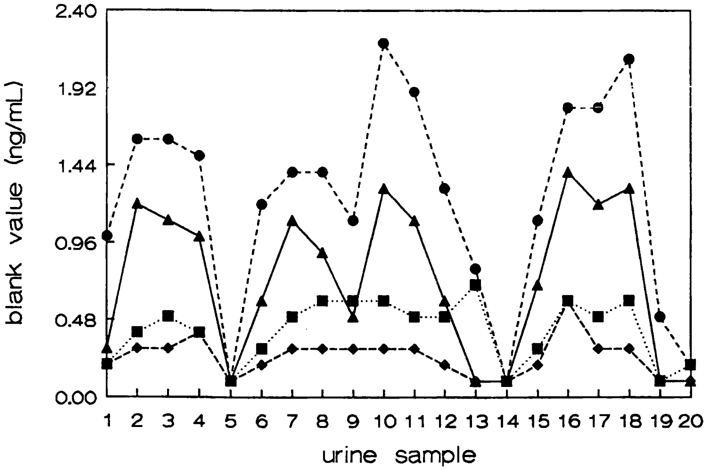


Figure 2. Blank values (ng/mL) of the reference blank urine samples analyzed with the Clenbuterol-EIA and four sample preparation methods. –▲– Method I, –●– Method II, –◆– Method III, and –■– Method IV.

In 1994, 1,730 bovine urine samples, taken by the General Inspection Service at 122 farm houses which were suspected of the presence of treated animals, were analyzed by the Clenbuterol-EIA (Method I). Almost all of the samples were taken from older bulls and cows and a decision level of 3 ng/mL was applied. In total 435 urine samples were found positive by this EIA (> 3 ng/mL) and were re-analyzed by GC-MS (*28*). In 366 of the EIA-positive samples (84%) the presence of β-agonists could be confirmed. Clenbuterol was found in 327 samples with levels in the range of 0.8 to 220 ng/mL. Both, clenbuterol and mabuterol were found in 4 samples. Mabuterol was found in 34 samples with concentrations in the range of 1.0 to 20 ng/mL and one sample contained salbutamol (35 ng/mL). In the Clenbuterol-EIA, a dose response curve of 0.05 to 5 ng/mL was used. Urine samples were diluted five times which resulted in a measuring range of 0.25 to 25 ng/mL. Of the 435 EIA-positive samples, 213 (49%) contained a concentration >25 ng/mL. The results from the samples that were in the measuring range (201 samples containing clenbuterol and 20 containing mabuterol) were plotted against the GC-MS results (see Figure 3). In most of the urine samples (96.4%) the concentrations found with the EIA were higher compared with GC-MS. These higher results can be partly explained by the measured background using the direct EIA. Another reason might be the presence of metabolites of clenbuterol.

Twelve of the positive urine samples were analyzed with the Clenbuterol-EIA using method I and III (see table IV). After extraction in isobutanol (method III) the concentrations were much lower compared with the direct method (method I). These lower values are the result of a reduced matrix effect and by the lack of (hydrophilic) metabolites of the β-agonists that are not extracted using this method. Compared with the GC-MS results, the concentrations found with the Clenbuterol-EIA, applying the isobutanol extraction, were somewhat lower due to the extraction efficiency and the cross-reactivity (see Table II).

The Clenbuterol-EIA was used for the detection of β-agonists in cattle feed samples. The mean blank value of the milk replacer samples (n = 28) was 2.5 ± 0.7 ng/g. Of the 114 cattle feed samples taken by the General Inspection Service, 36 samples were found positive by the EIA applying a decision level of 10 ng/g. The presence of clenbuterol could be confirmed by GC-MS in 13 samples (16–410 ng/g) while 4 samples contained clenbuterol below the GC-MS action level of 10 ng/g (2.7–8.5 ng/g). Mabuterol was found in 1 sample (12 ng/g). Thus, out of the 114 cattle feed samples, the Clenbuterol-EIA showed a response >10 ng/g in 18 samples in which no β-agonists could be found (16% false positives).

Blank calf feces samples (n = 28) were analyzed with both the Clenbuterol- and the Salbutamol-EIA and the mean blank values were low (0.15 ± 0.13 ng/g and 0.16 ± 0.08 ng/g, respectively). The mean recovery of clenbuterol added to feces at the 1 and 5 ng/g level was 75 ± 5% (n = 8).

The Clenbuterol-EIA also was used to determine the concentration of β-agonists in different sample materials obtained from treated animals (see table V and VI). Of the calf samples, the retina/choroid samples contained the highest concentrations of β-agonists due to accumulation as described before (*16–18*). Using the direct method (method I) for urine, the level will be higher compared with GC-MS due to matrix effects and the possible presence of metabolites. As shown in Figure 3, the direct EIA resulted in 1.5–3.5 (level depended) times higher concentrations. Thus, the concentration of the

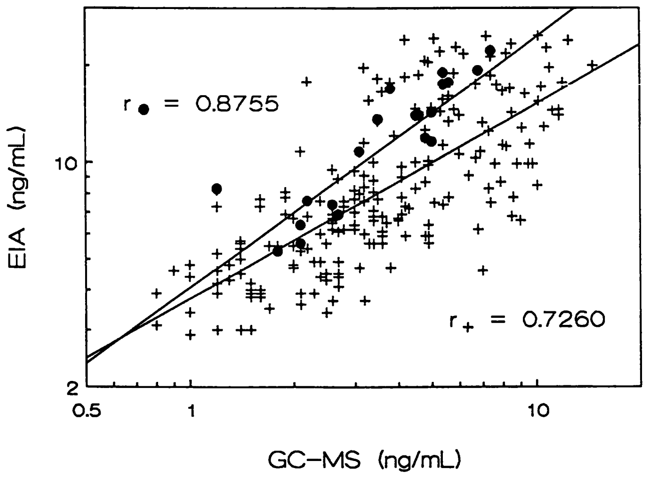


Figure 3. Comparison of the results obtained by the Clenbuterol-EIA (method I) and GC-MS for clenbuterol (n = 201) and mabuterol (n = 20) positive urine samples. + Clenbuterol and ● Mabuterol

parent compounds in urine will be at the same level as in feces which also was found by Courtheyn *et al.* (*15*). The concentrations in liver and bile were at about the same level. In the samples obtained from the treated bulls (see Table VI), the lowest concentrations were found in plasma samples which makes this sample material less suitable for the control on misuse. Somewhat higher concentrations were found in the muscle samples but, liver and kidney samples contained much higher concentrations. The mean concentration in the liver samples of the bulls treated with the high dose was still below the toxic level (200–300 ng/g). The urine samples, applying the direct method, contained the highest concentrations of clenbuterol.

Table IV. Concentrations found with the Clenbuterol-EIA using the direct method (method I) and the method applying isobutanol extraction (method III) and with GC-MS in positive urine samples

β-agonist	*EIA results (ng/mL) Clenbuterol-equivalents*		*GC-MS results (ng/mL)*
	Methode I	*Method III*	
Clenbuterol	17.0	6.0	8.4
Clenbuterol	20.0	7.0	9.8
Clenbuterol	7.0	1.6	2.4
Clenbuterol	10.0	3.9	5.5
Clenbuterol	> 25.0	8.3	8.8
Mabuterol	11.6	3.0	5.0
Mabuterol	6.9	2.0	2.7
Mabuterol	5.6	2.2	2.1
Mabuterol	7.6	1.2	2.2
Mabuterol	6.4	2.2	2.2
Mabuterol	7.4	1.0	2.6
Mabuterol	14.0	3.3	4.6

Table V. Determination of β-agonist concentration in sample materials obtained from calves (n = 20) treated (orally) with a mixture of clenbuterol, mabuterol and mapenterol (low doses; each 0.4 μg/kg body weight). No withdrawal period.

Sample material	*Clenbuterol equivalents (ng/g/mL)*	
	Mean concentration	*SD*[a]
Urine (method I)	24.0	19.4
Feces	10.8	3.5
Liver	8.8	4.5
Bile	7.7	4.3
Retina/choroid	61.2	41.6

[a] SD = standard deviation.

Table VI. Determination of clenbuterol in sample materials obtained from bulls (n = 4) treated (orally) with a low dose (0.8 µg/kg body weight) and a high dose (8 µg/kg body weight) of clenbuterol. No withdrawal period.

Sample material	*Mean clenbuterol equivalents (ng/g/mL)*	
	Low dose	*High dose*
Urine (method I)	14.7	124
Feces	2.6	35
Kidney	7.4	116
Liver	5.8	110
Plasma	0.4	3.4
Muscle	0.9	9.2

Conclusions

Due to matrix effects, the Fenoterol-EIA is not suitable for application in routine control on the misuse of fenoterol and ractopamine. Futher experiments will be focussed on the development of a suitable sample preparation procedure. The Clenbuterol-EIA is suitable for the screening of at least five β-agonists in all sample materials. Urine samples can be used for screening of misuse of β-agonists. The direct method (without sample preparation) results in higher values compared with GC-MS results which are caused by matrix effects but probably also by the presence of hydrophilic metabolites. Identification of these metabolites in the urine of treated animals will be one of the subjects of our future work. For all sample materials, the isobutanol extraction seems to be an easy and a good clean-up procedure with high recoveries for most of the important β-agonists. Next to urine, feces seems to be a suitable material for sampling in farm houses. In slaughter houses, sampling of eyes seems to be the best choice for detecting misuse of clenbuterol. Bile can be a suitable material for the detection of both, anabolic steroids and β-agonists. Plasma and muscle samples are less suitable because of the low concentrations of β-agonists. Currently, the Clenbuterol-EIA is used for routine control. In the future, this EIA will be replaced by the Salbutamol-EIA because of the detectability of more β-agonists (at least eight compounds)

Acknowledgments

The authors thank Martin Essers (RIKILT-DLO, Wageningen, The Netherlands) for providing the data concerning the urine and feed samples taken by the General Inspection Service, and Robert Schilt (RIKILT-DLO) for supplying the β-agonist standards. Cor Arts of TNO Nutrition and Food Research (Zeist, The Netherlands) is thanked for supplying the calf samples and Leen van Ginkel (CRL; RIVM, The Netherlands) for supplying the blank calf reference urine samples.

Literature Cited

1. Berende, P. L. M.; Ruitenberg, E. J. In *Domestication Conservation and Use of Animal Resources*; Peel, L. J.; Tribs, D. E., eds.; Elsevier: Amsterdam, The Netherlands, 1983; pp 191–233.
2. *Animal Drugs and Human Health*; Crawford, L. M.; Franco, D. A., eds.; Technomic Publishing Company: Lancaster, U.S.A., 1994.
3. Council of the European Communities, Council Directive 86/469/EEC of 16 September 1986, *Off. J. Eur. Commun.* 1986, L275, 36.
4. Jones, D. J.; Waitt, W. P.; Mowrey, D. H.; Anderson, D. B. *J. Anim. Sci.* **1988,** *66* (Suppl. 1), 324.
5. Adeola, O.; Darko, E. A.; He, P.; Young, L. G. *J. Anim. Sci.* **1990,** *68,* 3633.
6. Adeola, O.; Ball, R. O.; Young, L. G. *J. Nutr.* **1992,** *122,* 488.
7. He, P.; Aherne, F. X.; Thompson, J. R.; Schaefer, A. L.; Merrill, J. K. *Can. J. Anal. Sci.* **1993,** *73,* 169.
8. Reeds, P. J.; Hay, S. M.; Dorwood, P. M.; Palmer, P. M. *Br. J. Nutr.* **1986,** *56,* 249.
9. Williams, P. E. V.; Pagliani, L.; Innes, G. M.; Pennie, K.; Harris, C. L.; Garthwaite, P. *Br. J. Nutr.* **1987,** *57,* 417.
10. *Beta-Agonists and Their Effects on Animal and Carcass Quality*; Hanrahan, J. R., ed.; Elsevier: Amsterdam, The Netherlands, 1987.
11. Jones, R. W.; Easter, R. A.; McKeith, F. K.; Dalrymple, R. H.; Maddock, H.; Betchel, P. J. *J. Anim. Sci.* **1985,** *61,* 905.
12. Beerman, D. H.; Butler, W. R.; Hogue, D. E.; Fischell, V. K.; Dalrymple, R. H.; Ricks, C. A.; Scanes, C. G. *J. Anim. Sci.* **1987,** *65,* 1514.
13. Martinez-Navarro, J. F. *Lancet* **1990,** *336,* 1311.
14. Pulse, C.; Lamaison, D.; Keck, G.; Bostvironnais, C.; Nicolas, J.; Descotes, J. *Vet. Hum. Toxicol.* **1991,** *33,* 480–481.
15. Courtheyn, D.; Bakeroot, V.; de Volder, F.; Vercammen, J. *Food Agric. Immunol.* **1994,** *6,* 131–139.
16. Meyer, H. H. D.; Rinke, L. M. *J. Anim. Sci.* **1991,** *69,* 4538–4544.
17. Sauer, M. J.; Limer, S. In *Proceedings of the Euroresidue II Conference*; Haagsma, N.; Ruiter, A.; Czedik-Eysenberg, P. B., eds.; University of Utrecht, Faculteit der Diergeneeskunde: Utrecht, The Netherlands, 1990; pp 617–621.
18. Elliott, C. T.; Crooks, S. R. H.; Mcevoy, J. G. D.; Mccaughey, W. J.; Hewitt, S. A.; Patterson, D.; Kilpatrick, D. *Vet. Res. Commun.* **1993,** *17,* 459–468.
19. Haasnoot, W.; Stouten, P.; Lommen, A.; Cazemier, G.; Hooijerink, H.; Schilt, R. *Analyst* **1994,** *119,* 2675–2680.
20. Ploum, M. E.; Haasnoot, W.; Paulussen, R. J. A.; van Bruchem G. D.; Hamers, A. R. M.; Schilt, R.; Huf, F. A. *J. Chromatogr.* **1991,** *564,* 413–427.
21. Haasnoot, W.; Streppel, L.; Cazemier, G.; Salden, M.; Stouten, P.; Kemmers-Voncken, A.; van Wichen, P. *RIKILT-DLO report 95.06,* 1995.
22. Haasnoot, W.; Ploum, M. E.; Paulussen, R. J. A.; Schilt, R.; Huf, F. A. *J. Chromatogr.* **1990,** *519,* 323–335.
23. Hooijerink, H.; Schilt, R.; van Bennekom, E. O.; Huf, F. A. *J. Chromatogr.* **1994,** *660,* 303-313.

24. Yamamoto, I.; Iwata, K. *J. Immunoassay* **1982**, *3*, 155.
25. Beaulieu, N. *J. Pharm. Anal.* **1985**, *3*, 575–579.
26. Kyrein, H.J. *Z. Lebensm.-Unters.-Forsch.* **1983**, *177*, 426.
27. Lommen, A.; Haasnoot, W.; Weseman, J. M. *Food Agric. Immunol.* **1995**, *7*, 123–129.
28. van Rhijn, J.A.; Traag, W. A.; Heskamp, H. H. *J. Chromatogr.* **1993**, *619*, 243–249.

RECEIVED September 7, 1995

Chapter 6

Immunochemical Detection of Streptomycin in Honey

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A sensitive method was developed for the detection of streptomycin residues in natural honey. Streptomycin was recovered from honey samples using a solid phase extraction (SPE) procedure, and determined by polyclonal antibody-based competitive direct enzyme immunoassay (EIA). SPE extracts were further purified using monoclonal antibody-based immunoaffinity (IA) chromatography columns, and these SPE/IA extracts were reanalyzed by EIA. The detection limits for streptomycin in honey using SPE and SPE/IA extracts were 32 and 11.5 ng/g, the recoveries at the 50 ng/g level were 100% and 93%, respectively. In a first series of analyses, streptomycin was detected in several honey samples obtained from retail shops in Southern Germany, maximum values found were in the range of 100 ng streptomycin per gram honey.

The aminoglycoside antibiotic streptomycin (Figure 1) is intensively used in several countries to control fire blight, a devastating bacterial (*Erwinia amylovora*) disease affecting apple and pear trees. Recommended streptomycin concentrations for field applications are in the range of 100 μg/mL (*1*). High streptomycin contamination levels of the orchard environment imply the possibility of an inadvertent co-contamination chain: pollen, nectar, honey bees, and finally "carry-over" of streptomycin into the honey.

This paper is the first report of the detection of streptomycin in honey, and describes a simple and sensitive analytical approach, using a direct competitive EIA (*2*) to detect streptomycin after sample clean-up on reversed-phase (C_{18}) extraction cartridges. Further confirmation of the results obtained by this method was achieved employing a selective immunoaffinity chromatography procedure (*3*) for streptomycin.

0097–6156/96/0621–0074$15.00/0

Materials

Honey Sample Materials. Streptomycin-negative samples of natural honey were kindly supplied by F. Neumann, Tierärztliches Untersuchungsamt Aulendorf, Germany. Commercial honey (n=18) was purchased from retail stores in Southern Germany, mostly in the Munich area.

Solid Phase Extraction (SPE). Throughout the study, Sep-Pak C_{18} cartridges (Waters-Millipore, Milford, MA, U.S.A.) were used for SPE. The honey extraction buffer (modified from the method described by Kurosawa *et al.* (*4*)) was 1-heptanesulfonic acid (50 mM) and tribasic sodium phosphate ($Na_3PO_4 \bullet 12\ H_2O$; 25 mM) in distilled water. The pH of this solution was adjusted to 2.0 with orthophosphoric acid. For elution of streptomycin from SPE cartridges, methanol (analytical grade) was used. The SPE extracts were diluted with phosphate buffered saline (PBS; 0.01 M phosphate buffer containing 0.1 M NaCl, pH 7.2−7.3), to give a methanol concentration of 10% for EIA analysis. Further dilutions of this extract were made with PBS containing 10% methanol.

Immunoaffinity (IA) Chromatography. The IA columns consisted of monoclonal antibodies against streptomycin/dihydrostreptomycin (Strep II 4E2), coupled to CNBr-activated Sepharose 4B (*3*), with a gel volume of 200 µL per minicolumn (Mobitec, Göttingen, Germany). The streptomycin elution buffer for IA was glycine (0.1 M)/HCl (0.2 M) buffer, pH 2.5. The neutralisation buffer for IA eluates was carbonate-bicarbonate buffer, 0.05 M, pH 9.6.

Immunoreagents for EIA. Sheep anti-rabbit immunoglobulin G (Anti-rabbit IgG), purified by immunoaffinity chromatography, was used as described earlier (*5*). Rabbit antiserum against streptomycin, and streptomycin-horseradish peroxidase (HRP) conjugate were used in direct competitive EIA as reported previously (*2*). This EIA is specific for streptomycin and dihydrostreptomycin, having relative cross-reactivities of 100% and 150%, respectively. Streptomycin sulfate was purchased from Sigma-Aldrich Vertriebs GmbH, Deisenhofen, Germany. Microtiter plates (Maxisorp) were from Nunc GmbH, Wiesbaden, Germany.

Methods

Sample Preparation. Approximately 1 g of honey was weighted into a 100 mL beaker. The extraction buffer was added to give a tenfold weight, thus 1 mL of buffer corresponded to 0.1 g of honey. The mixture was stirred for 10 min on a magnetic stirrer.

Ten mL of this solution was passed through a SPE cartridge which had been conditioned with methanol (20 mL) and distilled water (20 mL). The cartridge was washed with distilled water (2 mL), and then dried by passing air (5 mL) through the cartridge with a syringe. Streptomycin was eluted with methanol (2 mL), the solvent was completely recovered from the cartridge with air (5 mL). The eluate was diluted with PBS (18 mL), and 300 µL of this mixture was directly used for EIA analysis (SPE extract).

The remaining diluted extract (19.7 mL) was further purified by IAC. An IA minicolumn was rinsed with glycine/HCl buffer (5 mL), followed by PBS (10 mL). Then the extract was passed through the column at a flow rate of 2–3 mL/min. The effluent waste was collected and assayed for presence of unretained streptomycin by EIA. The column was washed with PBS (5 mL), then streptomycin was eluted with glycine/HCl buffer (2 mL). The pH of the eluate was raised to near neutral with 850 μL carbonate-bicarbonate buffer prior to EIA analysis (IA extract). The IA minicolumn was rinsed with 10 mL PBS for immediate reuse, or rinsed with 10 mL of PBS containing 0.05% sodium azide for storage at 4 °C.

Competitive Direct Enzyme Immunoassay. Microtiter plates were coated with anti-rabbit IgG (10 μg/mL carbonate-bicarbonate buffer; 100 μL/well) overnight in a humid chamber. Free protein binding sites of the plate were blocked with PBS containing sodium caseinate (Sigma-Aldrich; 20 g/L) for 30 min at room temperature, then the plate was washed three times with Tween 20 solution (0.25 mL/L of 0.15 M sodium chloride solution). To each well, 35 μL of streptomycin standard or sample extract solution was added. To analyse SPE extracts, standards were prepared in 10% methanol/PBS, for IA extracts the standards were performed in 100% PBS. Then, antiserum dilution (1:6000 in PBS; 35 μL/well) and streptomycin-HRP solution (1:5000 in 1% sodium caseinate/PBS; 35 μL/well) were added and incubated for 2 h at room temperature. The plate was washed again, and enzyme substrate/chromogen (*6*) solution (1 mmol 3,3',5,5'-tetramethylbenzidine and 3 mmol H_2O_2 per liter potassium citrate buffer, pH 3.9) was added (100 μL/well). After 15 min, the color development was stopped with 1 M H_2SO_4 (100 μL/well) and the absorbance was measured at 450 nm. The tests were evaluated using a competitive EIA calculation software (*7*).

Evaluation of Test Parameters. All standard and sample solutions were analysed in quadruplicate throughout the study. Mean detection limits and 50% inhibition values were calculated from streptomycin standard curves (Figure 2) obtained over a period of 2 months (n=84).

Repeated (n=38) analysis of streptomycin-free honey samples, using both SPE and SPE/IA purification, was performed to study sample extract variability and its influence on the EIA response. For SPE extracts as well as for effluent waste of IA columns, two independent dilutions of each honey extract were analyzed, corresponding to final sample dilution factors of 20 and 40, respectively. For IA-purified extracts, three independent dilutions were assayed, corresponding to final sample dilution factors of 2.89, 5.78, and 11.56.

For SPE extracts (absorbance values 70–120% B/B_0), the detection limit was derived from the mean relative absorbance value ($B/B_0 x100$) minus three standard deviations obtained for streptomycin-negative sample extracts (Figure 3). For SPE/IA purified extracts (absorbance values 70–100% B/B_0), the detection limit was defined as the mean calculated concentration for these samples (ng/g) plus three standard deviations (Figure 4).

To check recoveries obtained by either sample preparation procedure, honey was artificially contaminated with streptomycin at levels of 50 and 100 ng/g. Additionally, on each day of analysis (10–20 samples per day), a spiked honey

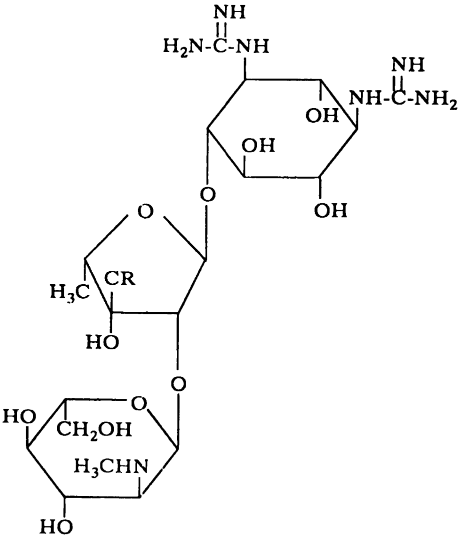

Figure 1. Structure of streptomycin (R: =O) and dihydrostreptomycin (R: −OH)

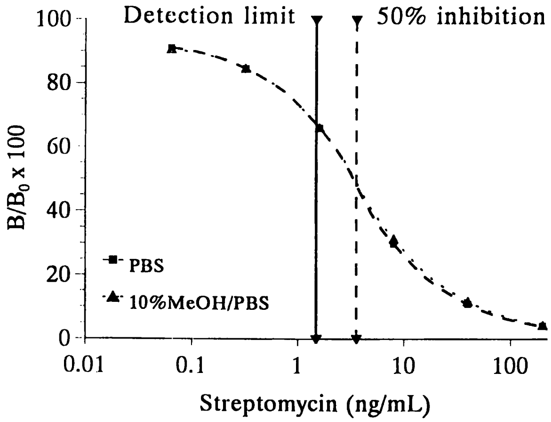

Figure 2. Comparison of typical standard curves of the EIA for the detection of streptomycin, using standards in PBS or in 10% methanol/PBS. The 50% inhibition value (3.5 ng/mL) and the detection limit (1.5 ng/mL) are indicated by arrows. Absolute B_0 values were routinely 0.8−1.0 absorbance units. Intraassay coefficients of variation (n=4) were between 1.5 and 7.5%.

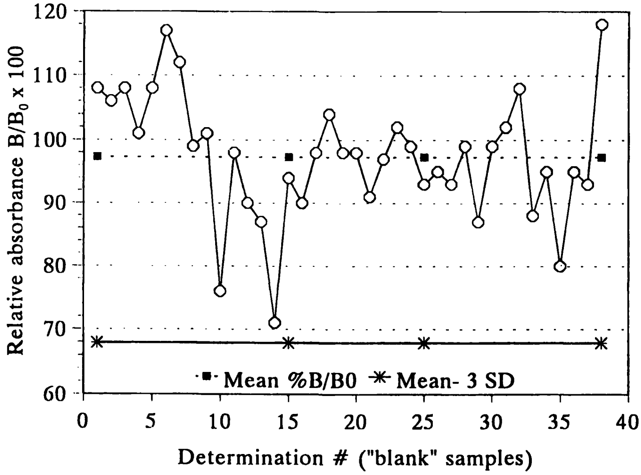

Figure 3. Evaluation of the detection limit of the streptomycin EIA after SPE, using repeated analysis of streptomycin negative honey sample extracts. Blank extracts gave %B/B_0 values ("noise") ranging from 70% to 118%. The mean response was 97.3±9.8 %B/B_0. Subtracting three standard deviations from the mean value, the limit of detection was calculated to be at 67.9 %B/B_0, corresponding to a streptomycin concentration of 1.6 ng/mL. Considering a minimum sample dilution factor of 20, the detection limit for streptomycin in honey is 32 ng/g.

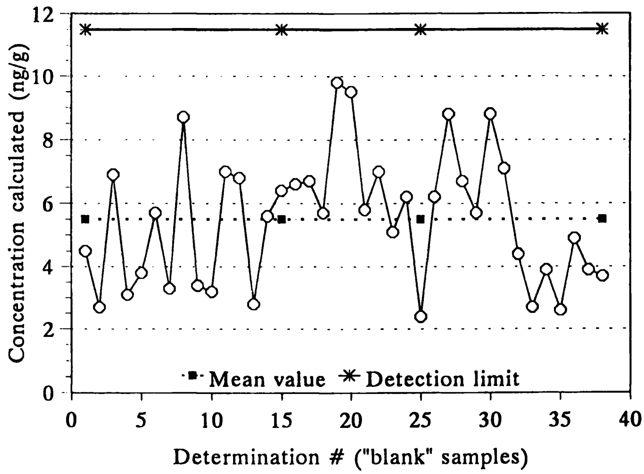

Figure 4. Evaluation of the detection limit of the streptomycin EIA after combined SPE/IAC cleanup. All blank extracts gave %B/B_0 values ≤100% (70−100%). Mean "noise" calculated for negative samples was 5.5±2.0 ng/g. Adding three standard deviations to the mean value, the detection limit for streptomycin in honey was calculated to be 11.5 ng/g.

sample (50 ng streptomycin/g) was extracted and analysed to check sample preparation performance, in particular that of the IA column.

Results and Discussion

This is the first one description of an immunoassay method for the detection of trace levels of streptomycin in honey. Streptomycin neither has fluorescence nor sufficient UV absorbance properties to allow sensitive detection with physicochemical techniques. Additionally, being a sugar-like compound, selective detection of streptomycin in natural honey presents an analytical challenge for physicochemical methods.

Microbiological inhibition tests in general have poor sensitivity for aminoglycosides, in particular for streptomycin. Moreover, natural honey contains compounds with original antibiotic activity (*8,9*), which implies the risk of false positive results when using tests which are based on inhibition of bacterial growth.

In such a situation, an immunochemical approach was chosen to check the possibility of a contamination of honey with streptomycin. As false positive EIA results may occur when inappropriate sample extracts are analysed, emphasis was given to a careful evaluation of EIA test parameters and sample preparation performance. The competitive direct EIA for streptomycin has been successfully used in our laboratory to detect this antibiotic compound in defatted milk samples (*10*), without any sample cleanup required. For natural honey however, initial tests using dilutions of streptomycin-negative samples in PBS, as described for the analysis of sulfathiazole in honey by EIA (*11*), indicated strong and variable matrix interference (data not shown).

Therefore, a solid phase extraction procedure was developed, using C_{18} cartridges and 1-heptanesulfonic acid as an ion-pairing reagent, adapting a method for streptomycin analysis in human serum (*4*). As no second, independent method for streptomycin detection was available, IAC was used as a second extract purification step. The basis of IAC are monoclonal antibodies which exclusively bind to streptomycin and dihydrostreptomycin. Combining SPE and IA for sample purification, a highly reliable selective isolation of the analyte could be achieved, making false positive results very unlikely. A major advantage of the IA minicolumns was that due to the mild elution conditions, a single column could be re-used at least 50 times when SPE-prepurified extracts were applied, making the application of this technique very cost-efficient. For direct purification of diluted honey samples the IA technique was less appropriate, because some blocking of the column was observed after a few uses.

For standard solutions, the mean 50% inhibition dose and detection limit of the streptomycin EIA were at 3.5 ng/mL and 1.5 ng/mL, respectively. No differences in sensitivity were observed between standard curves performed in 10% methanol/PBS and in 100% PBS (Figure 2).

For SPE extracts (Figure 3), the mean (n=38) percent absorbance value (B/B_0) of streptomycin negative samples was 97.3±9.8%, with absolute B_0 values ranging from 0.8−1.1 units. Subtracting three standard deviations from the mean, the detection limit of the standard curve was at 67.9% relative absorbance, corresponding to a streptomycin concentration of 1.6 ng/mL. This value obtained for sample extracts was in excellent agreement with the calculated mean detection limit of the EIA standard curve for streptomycin buffer solutions (1.5 ng/mL). Considering a minimum sample dilution factor of 20, the detection limit for streptomycin in honey after SPE cleanup was found at 32 ng/g.

For sample extracts prepared by the sequential SPE/IAC procedure, some absorbance reduction (70−100% B/B_0) by zero streptomycin extracts compared with zero streptomycin standards was observed. This was due to differences in buffer composition between standards and extracts. Although these percent absorbance values were outside the detection limit of the standard curve (ca. 68% B/B_0), a noise level could be derived from of these extracts, which was used for calculation of the detection limit after SPE/IAC cleanup (Figure 4). The mean noise value (three independent dilutions per sample) calculated for blank samples was 5.5 ±2.0 ng/g. Adding three standard deviations to the mean value, the detection limit for streptomycin in honey extracts after IA purification was found to be 11.5 ng/g.

The mean recovery for streptomycin at levels of 50−100 ng/g was at 100% for SPE alone, and 93% for combined SPE/IAC. For spiked honey samples, no unretained streptomycin could be detected in effluent waste of IA columns, further indicating that the antibiotic was completely bound to the solid-phase antibodies in the IA column.

When the method was used to analyse a total of 18 commercial honey samples, 9 samples were found to be streptomycin-positive by EIA analysis of SPE extracts at levels between 32 ng/g and 102 ng/g. Using combined SPE/IAC sample purification, 11 samples were streptomycin-positive in a range from 14−92 ng/g (Table I). In general, the EIA results obtained for both sample preparations were in excellent agreement. For the nine samples found positive after analysis of SPE-extracts, the correlation coefficient between results for SPE extracts and SPE/IAC extracts was 0.92.

Table I. Comparison of results for commercial honey samples obtained by streptomycin EIA, using either solid phase extraction (SPE extract) or combined SPE/immunoaffinity chromatography (SPE/IAC extract) for cleanup.

Test parameter	SPE extract	SPE/IAC extract
Method detection limit (ng streptomycin per g honey)	32	11.5
# of honey samples analysed	18	18
# of streptomycin positive samples	9 (50%)	11 (61%)
Mean streptomycin concentration of positive samples	66 ng/g	42 ng/g
Concentration range	32-102 ng/g	14-92 ng/g

As the detection limit for streptomycin was lower after SPE/IAC, two samples which had less than 32 ng/g after EIA analysis of the SPE extract were found to be positive at levels of 16 and 22 ng/g. However, no sample which was found streptomycin-negative after SPE/IAC gave a positive result for the corresponding SPE extract, indicating that the solid phase cleanup of honey samples alone eliminated interfering sample matrix constituents very efficiently.

The frequency of streptomycin occurrence in commercial honey found in this study indicates that more intense analytical and control measures should be taken, both to clarify the route of contamination and to protect the consumer from ingestion of honey containing streptomycin residues. Further efforts to develop practicable and sensitive physicochemical reference methods for streptomycin in honey would be desirable. Immunoaffinity chromatography columns as used in this study are ideally suited for sample purification and analyte concentration, and therefore could greatly improve existing methods (*12,13*). For an inexpensive and rapid screening of honey samples however, the use of SPE cartridges for sample purification and detection by EIA was found to be a satisfying and reliable analytical procedure, especially when considering the results obtained after confirmational purification of extracts by IAC.

Acknowledgment

We thank M. Straka for excellent technical assistance.

Literature Cited

1. McManus, P. S.; Jones, A. L. *Phytopathology* **1994**, *84*, 627−633.
2. Schnappinger, P.; Usleber, E.; Märtlbauer, E.; Terplan, G. *Food Agric. Immunol.* **1993**, *5*, 67−73.
3. Dietrich, R.; Schnappinger, P.; Usleber, E.; Terplan, G. *Milchwiss.* **1993**, *48*, 588.
4. Kurosawa, N.; Kuribayasi, S.; Owada, E.; Ito, K.; Nioka, M.; Arakawa, M.; Fukuda, R. *J. Chromatogr.* **1985**, *343*, 379−385.
5. Märtlbauer, E.; Gareis, M.; Terplan, G. *Appl. Environ. Microbiol.* **1988**, *54*, 225−230.
6. Gallati, H.; Pracht, J. *J. Clin. Chem. Clin. Biochem.* **1985**, *23*, 453−460.
7. Märtlbauer, E. In *Enzymimmuntests für antimikrobiell wirksame Stoffe.* Ferdinand Enke Verlag: Stuttgart, Germany, 1993; pp 206−222.
8. Bentler, V. W.; Frese, E. *Arch. Lebensmittelhyg.* **1981**, *32*, 130−135.
9. El-Sukhon, S. N.; Abu-Harfeil, N.; Sallal, A. K. *J. Food Prot.* **1994**, *57*, 918−920.
10. Usleber, E.; Hensler, E.; Dötsch, C.; Märtlbauer, E.; Terplan, G. *Arch. Lebensmittelhyg.* **1994**, *45*, 80−83.
11. Sheth, H. B.; Sporns, P. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 871−874.
12. Gerhardt, G. C.; Salisbury, C. D. C.; Macneil, J. D. *J. AOAC Int.* **1994**, *77*, 334−337.
13. Gerhardt, G. C.; Salisbury, G. D. C.; Macneil, J. D. *J. AOAC Int.* **1994**, *77*, 765−767.

RECEIVED August 30, 1995

Chapter 7

Molecular Modeling Studies of Ceftiofur

A Tool for Hapten Design and Monoclonal Antibody Production

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Competitive indirect enzyme-linked immunosorbent assays (ciELISA's) are described for the detection of ceftiofur using different strategies for hapten design, use of immunogens, and plate-coating antigens. Molecular modeling data are presented to demonstrate investigation of hapten design. Mice were immunized using an extended immunization protocol to develop the required immune response. Serum antibody levels were evaluated for each conjugation method using both homologous and heterologous conjugates as plate-coating antigens. A heterologous assay system proved to be more sensitive than a homologous system. Thus a heterologous assay system was employed for the production of monoclonal antibodies for the detection of ceftiofur. On the basis of preliminary experiments, desfuroylceftiofur proved to be the most advantageous hapten. Desfuroylceftiofur was prepared *in situ* and linked to maleimide-activated carrier proteins, i.e., BSA and KLH. Three antibodies were isolated from mice immunized with the KLH conjugate. The limit of detection by the antibodies Cef-36, Cef-68, and Cef-116 for ceftiofur was in the range of 0.33–32.33 ppb. Results from antibody characterization demonstrate that certain structural features are necessary for antibody binding. Cross-reactivity studies with structurally related cephalosporins, as well as penicillins were performed using the monoclonal antibodies Cef-68 and Cef-116.

Ceftiofur (Figure 1) is an FDA approved veterinary cephalosporin for the treatment of respiratory diseases in cattle, horses, and swine (*1–3*). In a recent paper we discussed immunization strategies focusing on different aspects of hapten-design and the advantage of using heterologous plate assay systems (*4*). Current publications on the detection of ceftiofur mainly include HPLC and microbiological assays as illustrated in Table I. However, detection methods for ceftiofur which are specific, deliver faster results and do not require the use of expensive sophisticated equipment have not yet

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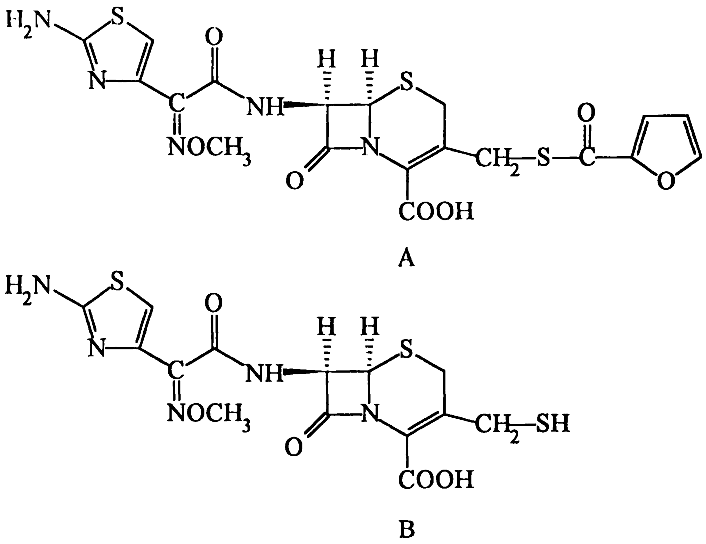

H2N
S
N
O
C
NOCH3
NH
H H
S
N
O
COOH
CH2—S—C
O
O
A
H2N
S
N
O
C
NOCH3
NH
H H
S
N
O
CH2—SH
COOH
B

Figure 1. Structures of ceftiofur (A) and desfuroyl ceftiofur (B).

Table I: Methods of Detection for Ceftiofur.

Method	Medium	Reference
HPLC	body fluids, tissues	(*5*)
HPLC	body fluids, tissues	(*6*)
HPLC	plasma	(*7*)
HPLC	plasma, urine	(*8*)
HPLC	milk	(*9*)
Charm Test II[a]	milk	(*9*)
MA[b]	milk	(*9,10*)
Delvotest-P[c]	milk	(*9,11*)
HPLC	milk, mammary tissue	(*11*)
HPLC	blood, plasma	(*12*)
HPLC	bovine serum, milk	(*13*)
LC/EMS[d]	milk	(*14*)
LC/EMS[d]	milk	(*15*)
HPLC	serum	(*16*)
HPLC	blood	(*17*)
HPLC	serum, urine	(*18*)
Agar gel[e]	serum, urine, milk	(*18,11*)
MA[f]	milk	(*19*)
MA[f]	serum, milk	(*20*)
LC, MS	blood	(*21*)
Colorimetry	blood	(*22*)
Agar gel[e], MIC[g]	mice	(*23*)
Agar gel[e]	blood	(*24*)

[a] Receptor-binding assay.

[b] Cylinder-plate microbiological assay.

[c] Colorimetric bacterial inhibition test.

[d] Liquid chromatography with electrospray mass spectrometry.

[e] Agar gel diffusion.

[f] Microbiological assay.

[g] Minimal inhibitory concentration.

been reported. Therefore, we chose to develop a monoclonal antibody against ceftiofur to provide a simple, alternative method of detection. It is conceivable that the production of monoclonal antibodies directed against ceftiofur sodium has so far been difficult because the enzyme-catalyzed cleavage of the thioester bond results in the formation of desfuroylceftiofur (Figure 1) and furoic acid metabolites in animal tissues (*7*). Desfuroylceftiofur has been detected in the plasma of calves treated with labeled ceftiofur. In species such as rats and dogs, desfuroylceftiofur is presumably bound to macromolecules *via* disulfide bonds (*8*). We describe the production and characterization of three cell-lines that produce monoclonal antibodies specific for ceftiofur sodium. The recently developed competitive indirect enzyme-linked immunosorbent assay (ciELISA) has proven to be useful in the detection of ceftiofur by using its major metabolite desfuroylceftiofur as the immunogen.

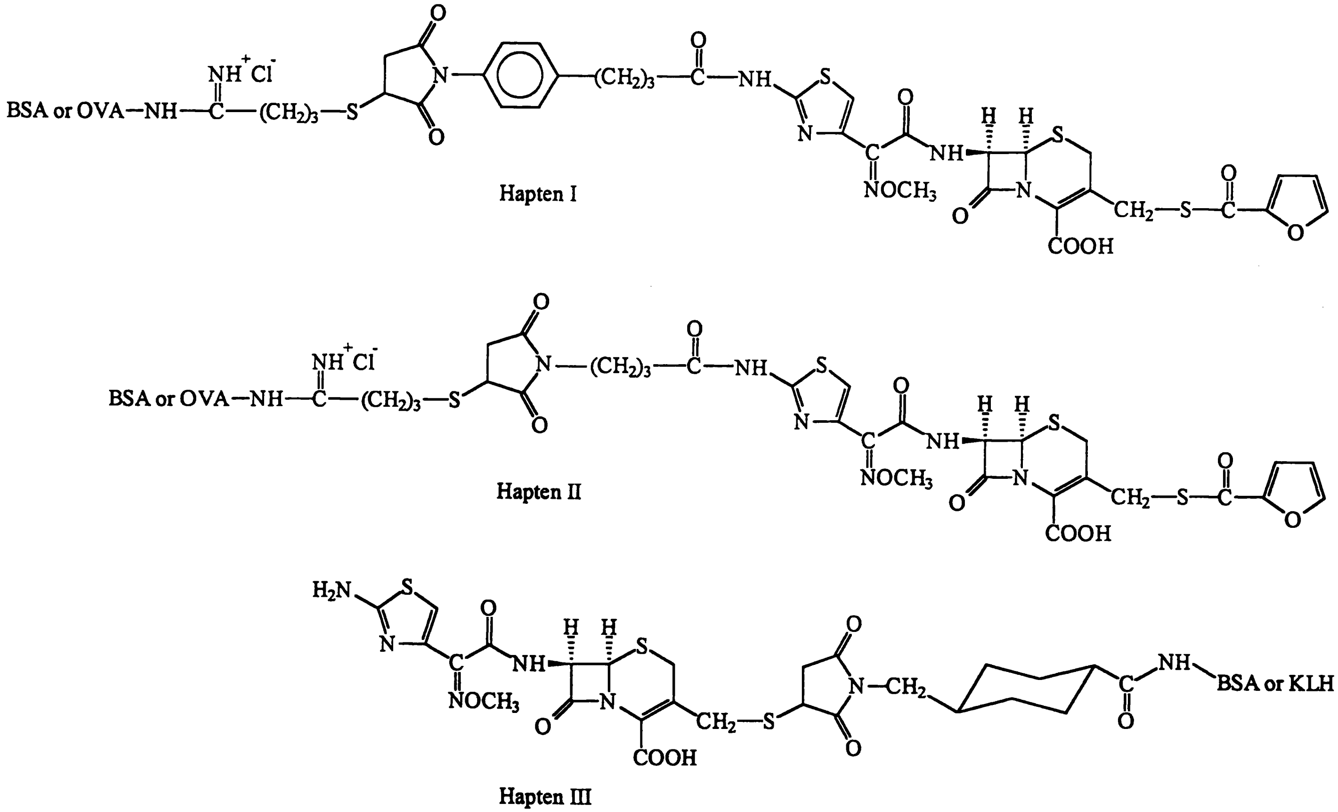
Materials and Methods

Hapten Design. Details on strategies for ceftiofur hapten design can be found in the report by Rose *et al.* (*4*). Briefly, haptens were conjugated to thiolated carrier proteins, bovine serum albumin, and ovalbumin, using the amine-specific heterobifunctional cross-linkers, sulfosuccinimidyl-4-(*p*-maleimidophenyl) butyrate (*s*-SMPB) and *N*-γ-maleimido-butyryl-oxy-sulfosuccinimide ester (*s*-GMBS). A third hapten was prepared by the hydrolysis of ceftiofur to produce desfuroylceftiofur *in situ*. Structures of the three haptens are shown in Figure 2. The free thiol was conjugated to maleimide-activated BSA and keyhole limpet haemocyanin (activated by sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate, *s*-SMCC). Mice were immunized with conjugates of the three haptens dissolved in either Freund's or Ribi adjuvants. The resulting polyclonal antibodies were screened for spacer recognition and specificity for the parent compound, ceftiofur, using both homologous and heterologous plate assay systems.

For the production of monoclonal antibodies, the best immunogen–plate coating antigen combination was chosen from the preliminary polyclonal blood serum study. Antibodies raised against ceftiofur were subsequently tested for cross-reactivity by a competitive indirect enzyme linked immunosorbent assay (ciELISA). Details on the production and characteristics of Mabs and optimization of the immunoassays will be published separately and can be found in a recent publication (Rose *et al.*, submitted for publication in *J. Agric. Food Chem.*).

Molecular Modeling. Molecular modeling was used as a theoretical tool to predict which of the three haptens would provide the most advantageous immunogen. Molecular modeling studies of ceftiofur and derivatives, were performed using a CAChe WorkSystem running on a Macintosh Quadra 700 equipped with a RP88 coprocessor board and a CAChe stereoscopic display (CAChe Scientific, Inc.; Beaverton, Oregon).

Minimum energy conformations of all the structures were calculated using Allinger's standard MM2 force field augmented to contain force field parameters for cases not addressed by MM2 (*25*). Calculations were performed on isolated gas-phase systems void of solvent, ions, and other materials. Following the initial optimization in the CAChe Mechanics application, utilizing the block-diagonal


BSA or OVA—NH
BSA or KLH
NH⁺Cl⁻
(CH₂)₃
NOCH₃
COOH
CH₂—S
H₂N

Hapten I

Hapten II

Hapten III

Figure 2. Diagram of ceftiofur conjugated to carrier proteins *via* *s*-SMPB (Hapten I) and *s*-GMBS (Hapten II), and desfuroyl ceftiofur conjugated to carrier proteins *via* *s*-SMCC (Hapten III).

Newton–Raphson optimization technique, a sequential search for low energy conformations was performed by rotating all dihedral angles through 360 ° in 15 ° increments. The global energy minima obtained from this search were used in all further calculations.

Molecular orbitals were calculated in the CAChe Extended Hückel application using the Alvarez Collected Parameters (*26*). The CAChe Extended Hückel application determines the electronic properties of a molecule by solving the electronic Schrödinger equation with the extended Hückel approximation (*27*).

The wave function data were converted into three-dimensional coordinates for visualizing electron densities and electrostatic potentials using the CAChe Tabulator application. Electron densities were calculated by superimposing the electrostatic potential onto the three-dimensional isosurfaces according to the multipole expansion method. The multipole expansion method computes the electrostatic potential from the sum over potentials from each atom using several terms for an atom rather than a simple monopole. The isosurfaces display two components of the frontier molecular orbital interaction in a single image, (i) the geometric or steric term giving the overall three-dimensional shape of the molecule, and (ii) the electrostatic or electronic term represented by shaded areas.

Results and Discussion

Hapten Design. A multi-hapten approach was chosen to test the efficacy of length and composition of the cross-linker, and the position of attachment to the target compound. Preliminary experiments showed limited antibody maturation for ceftiofur using *s*-SMPB and *s*-GMBS as cross-linkers. For this reason an extended immunization and bleeding protocol was followed as illustrated in Table II.

Several reports have shown that the sensitivity of both monoclonal and polyclonal immunoassays was significantly improved by using site, cross-linker, or hapten heterologous assay systems between the conjugation methods of the immunogen and the plate-coating antigens (*28–32*). We adopted this approach to analyze the polyclonal blood sera. Significant differences in assay performances were observed between the three plate coating antigens tested. Table III shows the results obtained by varying the test sera from the three immunogens with different plate coating antigens. For all three haptens, the heterologous plate assay system works best for the analysis of ceftiofur. The most sensitive assay was obtained when using hapten III, the hydrolyzed form of ceftiofur as the immunogen, and testing it against hapten II, the ceftiofur–protein conjugate linked *via* *s*-GMBS as the plate-coating antigen. By using a heterologous assay system one can eliminate the immune response recognition of a determinant on the cross-linker. Further improvements in sensitivity can be observed if the functional groups of the target molecule are left in their original state. In hapten III the cross-linker is furthest from the thiazolyl ring fragment. Points of contact in this fragment are thus free to interact intermolecularly (H-bonding, hydrophobic properties, van der Waals interactions) with the antibody. The presentation of this structural feature to the immune system is maximized and the production of low-affinity antibodies is avoided.

Table II. Immunization and Bleeding Protocol

Day of Immunization or Bleed	OVA-I[a] 0.1 mg/0.1 mL	OVA-II[a] 0.05 mg/0.1 mL	KLH-III[b] 0.1 mg/0.2 mL
1	0.05 mL/footpad	0.1 mL/footpad	0.2 mL ip
14			0.2 mL ip
23	0.1 mL ip	0.1 mL ip	
28			0.2 mL ip
37	0.1 mL ip	0.1 mL ip	
43			0.2 mL ip
50			bleed[c]
149	0.1 mL tail vein	0.1 mL tail vein	
156	bleed[c]	bleed[c]	
163			0.2 mL ip[d]
170			bleed[c]
260	0.1 mL ip[d]		
267	bleed[c]	0.1 mL ip[e]	
274		bleed[c]	

[a] Immunogen prepared in Freund's adjuvant.
[b] Immunogen prepared in Ribi adjuvant.
[c] Tail vein bleed.
[d] Solution prepared in phosphate-buffered saline.
[e] Solution prepared in saline.
Source: adapted from Rose *et al.*, *J. Bioconj. Chem.*, in press.

As the mice aged, the antisera appeared to deteriorate and later bleeds gave less satisfying results. This is not surprising since the immune response passes its peak level with prolonged exposure to the hapten.

Molecular Modeling. Computer molecular modeling studies were performed to determine the structural and electronic properties of ceftiofur and its three haptens: i.e., ceftiofur attached to the cross-linker *s*-SMPB (hapten I), ceftiofur attached to the cross-linker *s*-GMBS (hapten II), and desfuroylceftiofur attached to *s*-SMCC (hapten III). These studies were performed in order to establish which of these haptens possessed the ideal properties for presentation to the immune system, keeping in mind that the desired hapten should closely simulate the target molecule structurally and electronically. These features are important for antibody binding.

Low energy conformations of the three haptens were calculated by applying multiple sequential searches to afford global energy minima for each conformation. The calculated minimum energy conformations for ceftiofur, desfuroylceftiofur, hapten I, hapten II, and hapten III are illustrated in Figure 3. The global energy minima were used to determine the electronic properties. Figures 4, 5, and 6 show the electrostatic potential energies displayed on electron density surfaces (shaded

Table III. Percent Inhibition of Control (IC_{50}/ppb) observed for ceftiofur sodium antisera tested against different plate coating antigens.

		IC_{50} (ppb)[a] for Plate Coating Antigens		
Immunogens	Day of Bleed[b]	BSA-I	BSA-II	BSA-III
OVA–I[c]	156	96	40	e
	267	—	—	>1000
OVA–II[c]	156	58	560	e
	274	>1000	160	—
KLH–III[d]	50	350	72	e
	170	>1000	3	—

[a] Values are an average of 2–5 determinations.

[b] Antisera were diluted to represent approximately 50% maximum activity in a titration ELISA. These dilutions were used in the ciELISA experiments and tested on the corresponding plate coating antigens.

[c] Immunogen prepared in Freund's adjuvant.

[d] Immunogen prepared in Ribi adjuvant.

[e] Experiment not done due to insufficient material.

— Indicates that no inhibition was observed at a concentration of 10 μg/mL in the ciELISA.

areas represent an increase in negativity from white to gray to dark gray). The negative charge introduced by the furan ring fragment in the ceftiofur molecule is not significant since this ring is hydrolyzed once in the animal tissue, thus desfuroylceftiofur should be considered as the actual analyte (the free thiol is denoted by the arrow in Figure 4B). Notice that addition of *s*-SMCC in hapten III does not alter the electronic profile of the desfuroylceftiofur portion (Figure 4C). Ceftiofur, desfuroylceftiofur and hapten III are virtually identical (Figure 4) with respect to three-dimensional structure and to surface charge, particularly in that portion of the molecule containing the thiazolyl ring fragment (denoted by the arrow in Figure 4A). Hapten I and hapten II differ in this region since the cross-linkers introduce additional electronegative charges due to their functional groups (Figure 5). Also, the three-dimensional shape is significantly altered by the folding of the cross-linkers parallel to the cephem nucleus and the rotation of the thiazolyl ring fragment towards the furan ring fragment. However, as we found in the blood sera study, these differences do not rule out an immune response, but the models explain why the sensitivity is affected when comparing homologous and heterologous assay systems.

We make the presumption that, like ceftiofur, hapten I and hapten II lose the furan ring as a result of thioesterase hydrolysis. Additionally, haptens I and II were modeled without the furan ring to represent the immunizing hapten more closely. Figure 6 shows the three-dimensional isosurfaces for haptens I and II without the furan ring (compare to structure of desfuroylceftiofur in Figure 4). Replacement of the furan ring by a thiol group projects an overall positive charge on the cephem nucleus and the thiazolyl ring fragment contrasting the negatively charged portion of

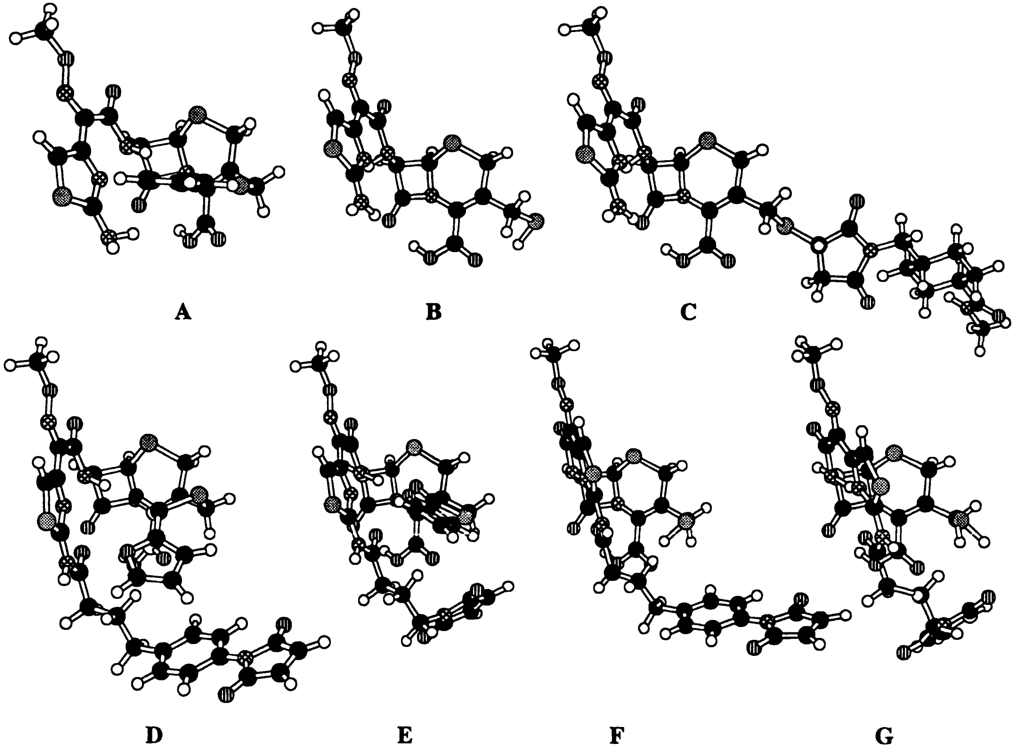


Figure 3. Spatial orientations of minimum energy conformations of A: ceftiofur, B: desfuroyl ceftiofur, C: hapten III, D: hapten I, E: hapten II, F: hapten I without furan ring, and G: hapten II without furan ring (carbons are represented by solid spheres, hydrogens are represented by open spheres, oxygens are represented by spheres with vertical lines, nitrogens are represented by spheres with cross-hatches, and sulphurs are represented by dotted spheres).

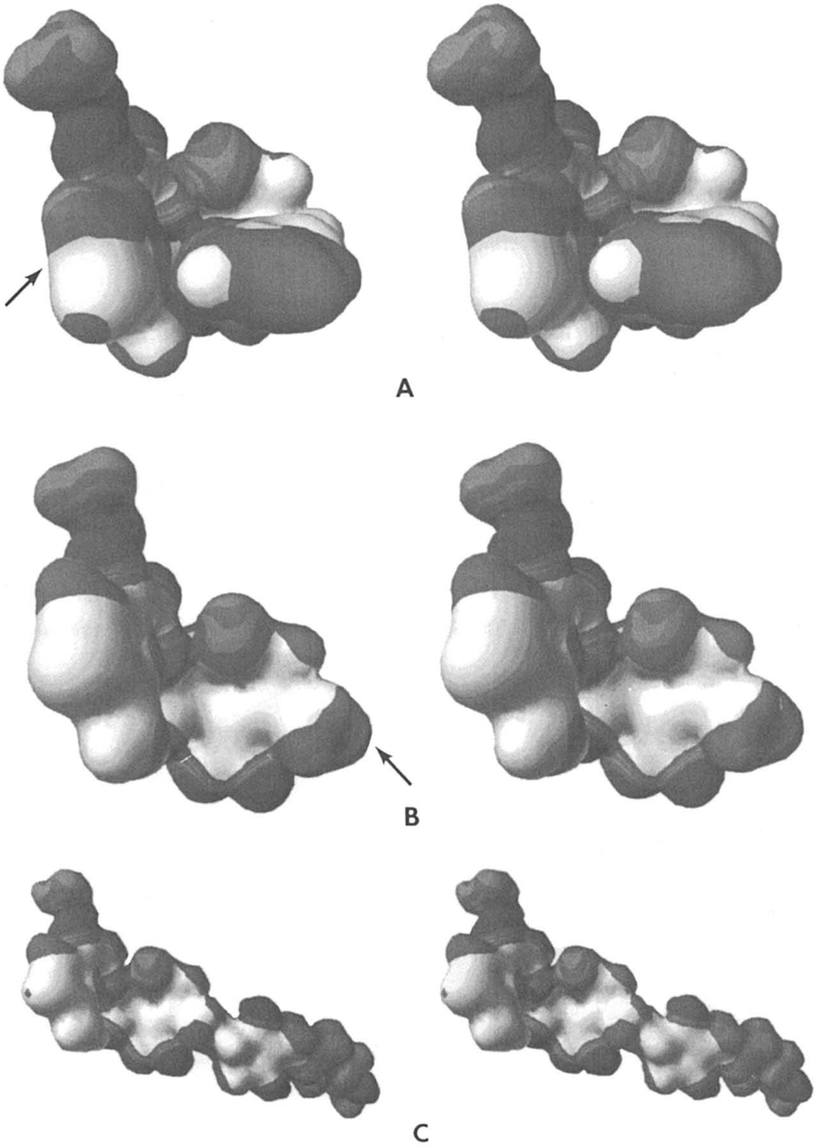


Figure 4. Three-dimensional stereoscopic views of electron density diagrams shaded with electrostatic potential isosurfaces for plate A: ceftiofur, plate B: desfuroyl ceftiofur, and plate C: hapten III.

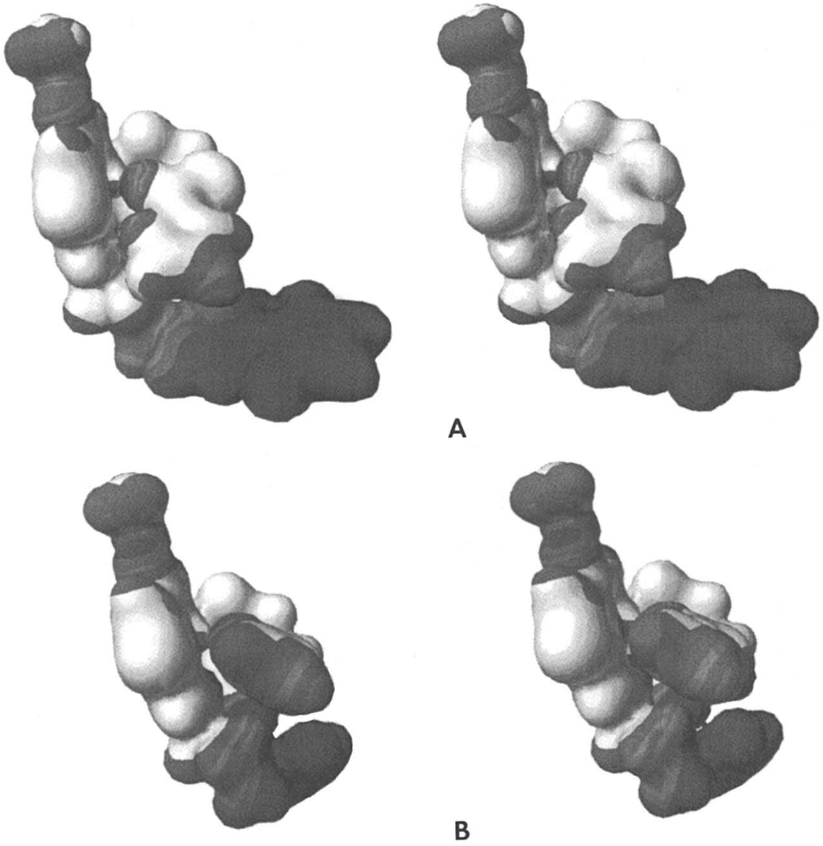


Figure 5. Stereoscopic views of electron density diagrams shaded with electrostatic potential isosurfaces for plate A: hapten I and plate B: hapten II.

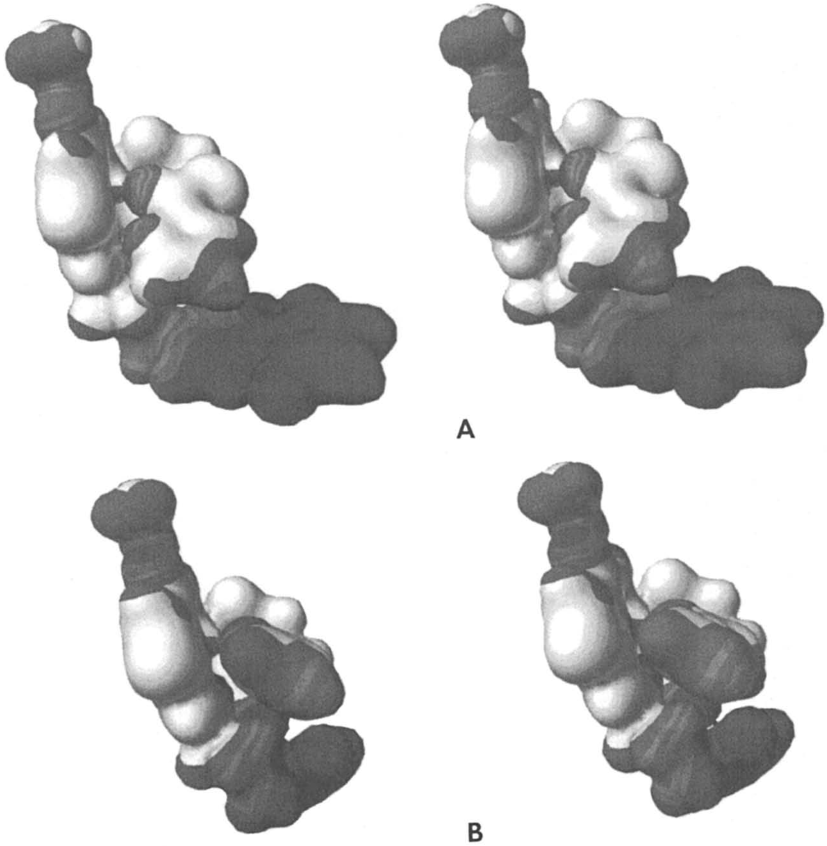


Figure 6. Stereoscopic views of electron density diagrams shaded with electrostatic potential isosurfaces for plate A: hapten I without furan ring and plate B: hapten II without furan ring.

the cross-linker. The thiazolyl ring fragment is twisted even further towards the cephem nucleus than it is in desfuroylceftiofur.

From these calculations it appears that the electrostatic interactions required for antibody binding may be altered enough to affect the immune response when bulky functional groups are substituted at the site of binding. These structural and electronic differences explain why differences in sensitivity can be observed when analyzing homologous and heterologous assay systems. In the heterologous assay the antibody preferentially binds the free analyte, presumably since the bound antigen is electronically and sterically different to the antibody binding pocket. Such differences in structure and electronics have been reported in recent publications using molecular modeling as a tool for hapten design and antibody characterization (*33–37*; chapter by Beier *et al.*, this volume).

ciELISA of Monoclonal Antibodies. Both the blood sera study and the molecular modeling results showed that using the hydrolyzed form of ceftiofur as the immunogen would probably provide the most sensitive hapten for the production of monoclonal antibodies. The procedure was followed as described in (*21*). Out of ten clones, three active antibodies were isolated. The three antibodies Cef-36, Cef-68, and Cef-116 were further subcloned. Using the ciELISA method, the anti-ceftiofur antibodies were tested against unconjugated ceftiofur. The results are shown in Table IV with antibody Cef-116 having the highest affinity for free ceftiofur. Table V illustrates the cross-reactivity between ceftiofur and related cephalosporins, including penicillins for the two antibodies Cef-68 and Cef-116. Three of the cephalosporins tested are similar to ceftiofur in that they contain the 2-(2-aminothiazol-4-yl)-2-methoxy-iminoacetamide fragment (thiazolyl ring fragment) at the C-7 position of the cephem nucleus. The anti-ceftiofur antibodies recognized all cephalosporins containing the thiazolyl ring fragment. Only the structurally related cephalosporins, ceftriaxone, cefotaxime, and cefteram showed cross-reactivity. Ceftazidime contains the thiazol ring, but not the acetamide oxime group and could not be detected. Cefuroxime containing a furan ring instead of the thiazol ring could be detected by Cef-116, albeit to a lesser extent. Chemical structures of ceftiofur and the related cephalosporins are represented in Figure 7. None of the penicillins were detected by either Cef-68 or Cef-116. It appears that both the thiazol ring and the acetamide oxime portions are important for antibody binding and the cephem nucleus plays a minor role. This fact is corroborated by the molecular modeling studies.

Conclusion

The model which best fits the conjugation pattern necessary to detect ceftiofur in biological fluids should be one in which immunogens and plate coating antigens are varied. More sensitive results can be obtained when using a heterologous assay system. Ideally, one would like the antibodies to recognize antibiotics such as ceftiofur in meat and dairy products. We now have a sensitive antibody which detects ceftiofur and certain related cephalosporins. Useful information about hapten design and the characteristics necessary for antibody binding was gained by molecular modeling studies. The three-dimensional structural and electronic calculations showed which functionalities of the ceftiofur molecule are recognized by the antibody and thus provide a reasonable comparison to actual experimental results.

Table IV. Competitive inhibition results for antibodies Cef-36, Cef-68, and Cef-116.

	Inhibition at 50% (ppb) for Antibodies[a]		
	Cef-36	Cef-68	Cef-116
Ceftiofur	15.00	32.33	0.33

[a] Values are an average of six determinations.

Table V. Cross-reactivity of ceftiofur, related cephalosporins, and penicillins for antibodies Cef-68 and Cef-116.

	% Cross-Reactivity[a]	
Compound	Cef-68	Cef-116
Ceftiofur	100	100
Ceftriaxone	84	98
Cefotaxime	41	69
Cefteram	16	9
Ceftazidime	0	0
Cefuroxime	0	<1
Cephalothin	0	0
Cefoxitin	0	0
Cefazolin	0	0
Cefadroxil	0	0
Cefamandole	0	0
Cephradine	0	0
Cephapirin	0	0
Cefaclor	0	0
Cefsulodin	0	0
Cefoperazone	0	0
Ampicillin	0	0
Amoxicillin	0	0
Cloxacillin	0	0
Penicillin G	0	0

[a] The percentage cross-reactivity was determined relative to ceftiofur; i.e., (inhibition of ceftiofur at 50% / inhibition of related cephalosporin at 50%) x 100. A value of 0 indicates that no cross-reactivity was observed at the 50% level and below.

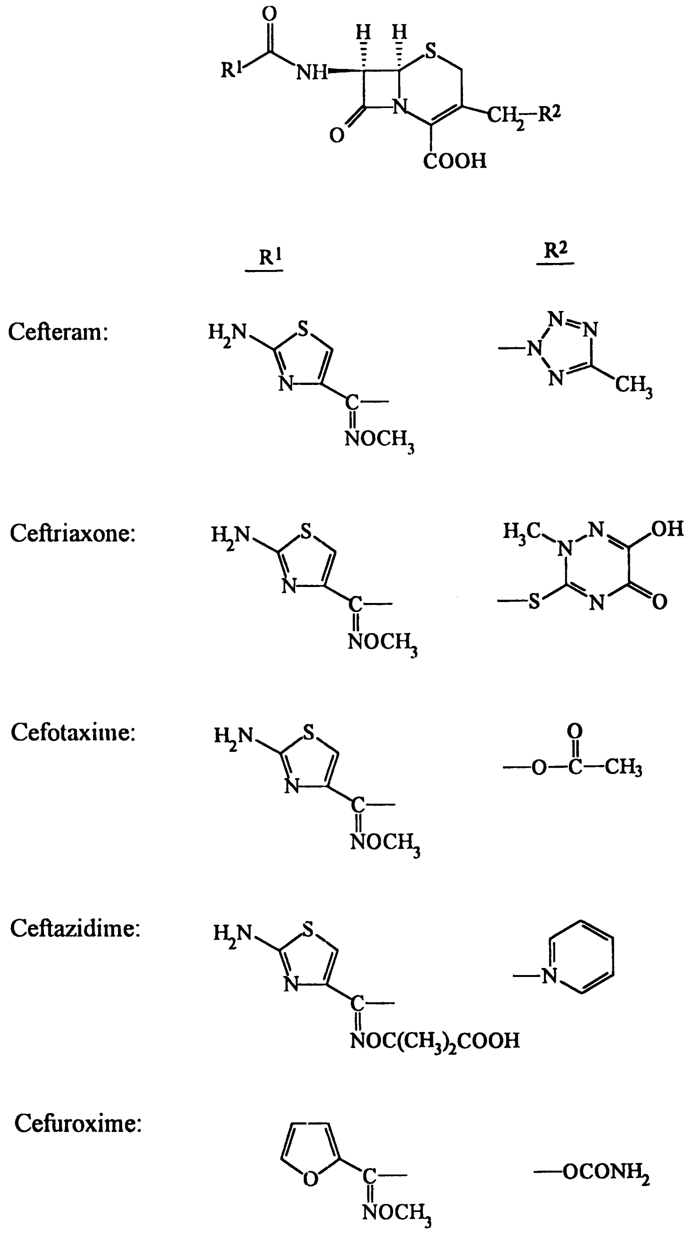

O
R1
NH
H H
S
N
O
CH2—R2
COOH
R1
R2
Cefteram:
H2N
S
N
C—
NOCH3
N=N
—N
N
CH3
Ceftriaxone:
H2N
S
N
C—
NOCH3
H3C
N
N
OH
—S
N
O
Cefotaxime:
H2N
S
N
C—
NOCH3
O
—O—C—CH3
Ceftazidime:
H2N
S
N
C—
NOC(CH3)2COOH
—N
Cefuroxime:
O
C—
NOCH3
—OCONH2

Figure 7. Chemical Structures of the cephalosporins, cefteram, ceftriaxone, ceftriaxone, cefotaxime, ceftazidime, and cefuroxime.

Acknowledgments

We thank The Upjohn Co. (Kalamazoo, MI) for the supply of ceftiofur sodium, Hoffmann-La Roche Inc. (Nutley, NJ) for cefteram sodium and ceftriaxone sodium, and Glaxo Manufacturing Services Ltd. (Barnard Castle, County Durham, England) for cefuroxime sodium and ceftazidime pentahydrate.

Literature Cited

1. Food and Drug Administration, *Fed. Regist.* **1988**, *53*, 5369–5370.
2. Food and Drug Administration, *Fed. Regist.* **1991**, *56*, 12–119.
3. Food and Drug Administration, *Fed. Regist.* **1992**, *57*, 41862.
4. Rose, B. G.; Kamps-Holtzapple, C.; Stanker, L. H. *J. Bioconj. Chem.*, in press.
5. Gilbertson, T. J.; Roof, R. D.; Nappier, J. L.; Zaya, M. J.; Robins, R. H.; Stuart, D. J.; Krzeminski, L. F.; Jaglan, P. S. *J. Agric. Food Chem.* **1995**, *43*, 229–234.
6. Beconi-Barker, M. G.; Davison, K. L.; Hornish, R. E.; Arnold, T. S.; Craigmill, A. L.; Gilbertson, T. J.; Smith, E. B.; Vidmar, T. J.; Hoffman, G. A.; Gatchell, C. L. *J. Agric. Food Chem.* **1995**, *43*, 1589–1597.
7. Jaglan, P. S.; Cox, B. L.; Arnold, T. S.; Kubicek, M. F.; Stuart, D. J.; Gilbertson, T. J. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 26–30.
8. Jaglan, P. S.; Kubicek, M. F.; Arnold, T. S.; Cox, B. L.; Robins, R. H.; Johnson, D. D.; Gilbertson, T. J. *J. Agric. Food Chem.* **1989**, *37*, 1112–1118.
9. Jaglan, P. S.; Yein, F. S.; Hornish, R. E.; Cox, B. L.; Arnold, T. S.; Roof, R. D.; Gilbertson, T. J. *J. Dairy Sci.* **1992**, *75*, 1870–1876.
10. Gilbertson, T. J.; Hornish, R. E.; Jaglan, P. S.; Koshy, K. T.; Nappier, J. L.; Stahl, G.; Cazers, A. R.; Nappier, J. M.; Kubicek, M. F.; Hoffman, G. A.; Hamlow, P. J. *J. Agric. Food Chem.* **1990**, *38*, 890–894.
11. Owens, W. E.; Xiang, Z. Y.; Ray, C. H.; Nickerson, S. C. *J. Dairy Sci.* **1990**, *73*, 3449–3456.
12. Jaglan, P. S., Roof, R. D.; Yein, F. S.; Arnold, T. S.; Brown, S. A.; Gilbertson, T. J. *J. Vet. Pharmacol. Ther.* **1994**, *17*, 24–30.
13. Tyczkowska, K. L.; Voyksner, R. D.; Anderson, K. L.; Aronson, A. L. *J. Chromatogr.* **1993**, *614*, 123–134.
14. Tyczkowska, K. L.; Voyksner, R. D.; Straub, R.; Aronson, A. L. *J. AOAC Int.* **1994**, *77*, 1122–1131.
15. Straub, R.; Linder, M.; Voyksner, R. D. *Anal. Chem.* **1994**, *66*, 3651–3658.
16. Halstead, S. L.; Walker, R. D.; Baker, J. C.; Holland, R. E.; Stein, G. E.; Hauptman, J. G. *Can. J. Vet. Res.* **1992**, *56*, 269–274.
17. Mahrt, C. R. *Am. J. Vet. Res.* **1992**, *53*, 2201–2205.
18. Cervantes, C. C.; Brown, M. P.; Gronwall, R.; Merritt, K. *Am. J. Vet. Res.* **1993**, *54*, 573–575.
19. Louhi, M.; Inkinen, K.; Myllys, V.; Sandholm, M. *Zentralbl. Veterinärmed. [B]* **1992**, *39*, 253–262.
20. Soback, S.; Bright, S., Paape, M. *Acta Vet. Scand. Suppl.* **1991**, *87*, 93–95.

21. Brown, S. A.; Jaglan, P. S.; Banting, A. *Acta Vet. Scand. Suppl.* **1991**, *87*, 97–99.
22. Jacobs, R. M.; Lumsden, J. H.; Taylor, J. A.; Grift, E. *Can. J. Vet. Res.* **1991**, *55*, 150–154.
23. Yancey Jr., R. J.; Kinney, M. L.; Roberts, B. J.; Goodenough, K. R.; Hamel, J. C.; Ford, C. W. *Am. J. Vet. Res.* **1987**, *48*, 1050–1053.
24. Meyer, J. C.; Brown, M. P.; Gronwall, R.; Merritt, K. *Equine Vet. J.* **1992**, *24*, 485–486.
25. Allinger, N. L. *J. Am. Chem. Soc.* **1977**, *99*, 8127–8134.
26. Alvarez, S., Universidad de Barcelona, 1989. Alvarez Collected Parameters for extended Hückel calculations.
27. Hoffmann, R. *J. Chem. Phys.* **1963**, *39*, 1397–1412.
28. Goodrow, M.; Sanborn, J. R.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D.; In *Immunoanalysis of Agrochemicals. Emerging Technologies*; Nelson, J. O.; Karu, A. E.; Wong, R. B., eds.; American Chemical Society: Washington, D.C., 1995, *ACS Symposium Series Number 586*; pp 119–139.
29. Goodrow, M.; Harrison, R. O.; Hammock, B. D. *J. Agric. Food Chem.* **1990**, *38*, 990–996.
30. Harrison, R. O.; Goodrow, M.; Hammock, B. D. *J. Agric. Food Chem.* **1991a**, *39*, 122–128.
31. Harrison, R. O.; Goodrow, M.; Gee, S. J.; Hammock, B. D. In *Immunoassays for Trace Chemical Analysis; Monitoring Toxic Chemicals in Humans, Food, and the Environment*; Vanderlaan, M.; Stanker, L. H.; Watkins, B. E.; Roberts, D. W., eds.; American Chemical Society: Washington, D.C., 1991b, *ACS Symposium Series Number 451*; pp 14–27.
32. Schneider, P.; Hammock, B. D. *J. Agric. Food Chem.* **1992**, *40*, 525–530.
33. Carlin, R. J.; Kamps-Holtzapple, C.; Stanker, L. H. *J. Mol. Immunol.* **1993**, *31*, 153–164.
34. Beier, R. C.; Elissalde, M. H.; Stanker, L. H. *Bull. Environ. Contam.* **1995**, *54*, 479–487.
35. Stanker, L. H.; Recinos III, A.; Linthicum, D. S.; In *Immunoanalysis of Agrochemicals. Emerging Technologies*; Nelson, J. O.; Karu, A. E.; Wong, R. B., eds.; American Chemical Society: Washington, D.C., 1995, *ACS Symposium Series Number 586*; pp 72–88.
36. Elissalde, M. H.; Kamps-Holtzapple, C.; Beier, R. C.; Plattner, R. D.; Rowe, L. D.; Stanker, L. H. *Food Agric. Immunol.* **1995**, *7*, 109–122.
37. Kamps-Holtzapple, C.; Carlin, R. J.; Rose, B. G.; Kubena, L. F.; Stanker, L. H. *J. Mol. Immunol.*; in press.

RECEIVED October 6, 1995

Chapter 8

Immunochemical Detection of the Anticoccidial Salinomycin in Poultry Feed

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Salinomycin is a coccidiostat approved for use in poultry feed at the manufacturers recommended dose of 60 ppm (ca. 55 g/ton). Neogen Corporation has developed a rapid (10-min) quantitative ELISA which allows on-site monitoring of salinomycin medicated feed. Using a <salinomycin> monoclonal antibody, the assay employs a five-level standard curve covering a range of 0–100 ppm. Intra- and inter-assay precision and accuracy at the 60 ppm level was determined to be 60.9 ± 1.47, CV = 2.8% and 60.9 ± 0.64, CV = 2.0% (n = 10), respectively. The analytical sensitivity of the assay is approximately 2.8 ppm. The salinomycin ELISA correlated well with the manufacturer's (Pfizer Inc., New York, NY) HPLC reference method as evidenced by the following: slope = 0.975, r = 0.952, and y-int = 6.04 ppm. The ELISA employs a simplified extraction method of 5 min manual shaking instead of the 1 h method used for the HPLC. This permits conducting a test easily applicable in the laboratory or on-site. The assay also can be used in a semi-quantitative format using 50 and 75 ppm controls as low and high concentrations to screen samples if quantitative results are not required. The kit is stable for six months when stored at 4 °C.

Salinomycin is a polyether antibiotic first isolated from *Streptomyces albus* by Miyazaki *et al.* (*1*). Polyether antibiotics, known as ionophores, are active against gram positive bacteria, mycobacteria, and some fungi. Additionally, they have high activity against certain parasites such as coccidia (*2*). Coccidiosis causes substantial losses to the poultry industry and to a lesser extent to the swine and cattle industries

0097–6156/96/0621–0099$15.00/0

In Immunoassays for Residue Analysis; Beier, R., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

unless controlled by preventative therapy (i.e., coccidiostat). Even slight infections in the poultry industry can result in negative effects in growth and feed conversion (*3*).

Salinomycin was introduced as a coccidiostat in the early 1980's. It soon became a major competitor to the then number one coccidiostat, monensin, and it also is an ionophore. The most common method for dispensing a coccidiostat in the poultry industry is in medicated feed. Commonly, this is done by using a concentrated form of the drug to produce a premix which then is mixed with ton quantities of complete feed. In general, the therapeutic range for ionophores is relatively narrow (i.e., therapeutic index is slightly > 1.0). Thus, it is very important to mix coccidiostats into feed at the correct level. The therapeutic dose for salinomycin is 60 ppm (ca. 55 g/ton) with the acceptable range being 48 to 72 ppm (*4*).

Assurance of proper mixing can be determined only by analyzing the final mixed feed. Currently, this is accomplished by sending feed samples to a reference laboratory for analysis, commonly HPLC is the method of analysis. The salinomycin levels are then reported to the feed producer. This process frequently takes two or more days. Frequently this information is of little use to the poultry producer because of the long turnaround time and trends to use 'just-in-time' supply practices. Suspected problems are remedied by switching feed to eliminate further risk. Considering that the U.S. alone produces over 110 million chickens weekly, the economic losses associated with this practice can be significant.

An ELISA has been developed by Neogen Corporation to allow rapid onsite monitoring of salinomycin in poultry feed. The monoclonal antibody-based competitive direct immunoassay provides quantitative results in as few as 10 min. This rapid feedback of feed status, to both the poultry producer and manufacturer's technical representative, allows informed decisions to be made as soon as a problem is diagnosed or, indeed, it can even prevent problems from occurring.

Materials and Methods

Chemicals and Supplies. The sodium salt of salinomycin, salinomycin 70% (v/v) methanolic stock solution and medicated poultry feed samples were obtained from Pfizer Canada (Cornwall, Ontario). Hydrazine was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was purchased from Sigma Chemical Company (St. Louis, MO) and horseradish peroxidase (HRP) was purchased from Boehringer Mannheim (Indianapolis, IN). Tris (hydroxymethyl) aminomethane was obtained from Fisher Scientific (Fair Lawn, NJ). The single component HRP-tetramethylbenzidine-substrate (K-Blue Substrate®, Neogen Corporation, Lexington, KY) and sodium fluoride stop solution were components of the ELISA test kit produced by Neogen Corporation (Lansing, MI). Immulon II® microtiter strips were obtained from Dynatech Laboratories, Inc. (Chantilly, VA). ELISA data was generated using a Bio-Tek EL301 microwell reader purchased from Bio-Tek Corporation (Winooski, VT) equipped with a 386 laptop computer obtained from Zenith Data Systems Corporation (St. Joseph, MI). All other chemicals and biochemicals utilized were reagent grade or better.

<Salinomycin> Monoclonal Antibody Production. The <salinomycin> monoclonal antibody was obtained from a mouse cell line (hybridoma) licensed from the Food Animal Protection Laboratory, Agricultural Research Service, U.S. Department of Agriculture (College Station, TX). A detailed account of the development and characterization of this antibody is described by Elissalde *et al.* (*5*). Ascites fluid was utilized for microwell immobilization studies without further purification.

Salinomycin Hydrazide. The *N*-hydroxysuccinimide (NHS) ester of salinomycin was prepared using the combination dicyclohexyl carbodiimide (DCC) and NHS in dimethylformamide (DMF). The active ester derivative was separated from the dicyclohexyl urea by filtration and reacted with hydrazine to form the salinomycin hydrazide.

Salinomycin-Horseradish Peroxidase Conjugate. A salinomycin-HRP conjugate was prepared using salinomycin hydrazide and *N*-hydroxysuccinimide activated HRP. A solution of salinomycin hydrazide in dimethylformamide (DMF) was added to the activated HRP solution while vortexing. The reaction mixture was incubated at ambient temperature. The conjugate reaction mixture was dialyzed against three changes of physiological saline. The dialysate was purified by size exclusion chromatography using Sephadex G-25. The concentrated conjugate solution was diluted 1:3000 in a 50% fetal bovine serum/phosphate buffer (containing preservative) prior to use in the ELISA.

<Salinomycin> Microtiter Well Coating. Ascites fluid containing monoclonal <salinomycin> antibody was diluted to an appropriate level in Tris buffer. Microtiter wells are filled with 100 µL aliquots each of diluted ascites fluid and dried. Antibody coated strips were foil pouched with desiccant and stored at 4 °C.

Salinomycin Competitive Enzyme-Linked Immunosorbent Assay. The salinomycin standards used in the assay were 0, 25, 50, 75 and 100 ppm. [Note: in order to account for an initial 1:50 sample dilution, the standards were provided in a pre-diluted [70% (v/v) methanol] ready-to-use form]. Samples (10 g of poultry feed) were extracted with 50 mL methanol in Whirl-Pac® bags. Following a 5-min manual shaking step, the extract was filtered using a syringe containing glass wool. The filtered sample extract was diluted 1:10 (v/v) with 67% (v/v) methanol and mixed manually by shaking. The resulting filtrate was analyzed for salinomycin content.

A 100 µL aliquot of salinomycin HRP conjugate was added to each mixing well followed by 100 µL of standard or diluted sample. Using a multichannel pipettor, the conjugate/sample solutions were mixed. Aliquots (100 µL) of these mixtures were transferred to the antibody-coated wells and incubated for 5 min at ambient temperature. After incubation, the antibody wells were washed 5 times with deionized water and the wells were turned upside down and tapped on a piece of paper towel until the remaining water was removed.

Using a multichannel pipettor, 100 µL aliquots of K-Blue Substrate were added to each antibody well. The substrate containing antibody wells were allowed to incubate for 5 min at ambient temperature.

After incubation, the assay was "stopped" by the addition of 100 µL aliquots of sodium fluoride to all wells.

The assay absorbance of each well was read spectrophotometrically at 650 nm using the Bio-Tek EL301 microwell reader. Absorbance data were transported to a laptop computer for analysis. The standard curve was generated using a log/logit model and salinomycin values calculated therefrom.

Salinomycin HPLC Reference Assay. All HPLC analysis of feed samples was performed by the QC laboratory of Pfizer Canada in Cornwall, Ontario, Canada using the method of Goras and Lacourse (*6*). Sample extraction involved the addition of 100 mL of methanol to 20 g of poultry feed and shaking the mixture reciprocally 1 h at ambient temperature. The methanol extract was filtered using filter paper. The resulting filtrate was evaporated to dryness, or to an oily residue by rotary evaporation. The residue was quantitatively transferred to a volumetric flask and diluted to 50 mL with mobile phase [ethyl acetate-isooctane-acetic acid-triethylamine (750:250:4:2)]. Samples were analyzed for salinomycin by HPLC (Waters HPLC system) equipped with Zorbax® Sil (4.6 mm x 25 cm) chromatography column. Following separation, salinomycin was reacted with vanillin reagent at 95 °C to generate a reaction product that absorbs visible light. The reaction product was detected using a variable wavelength spectrophotometric detector at 527 nm (Waters model 450). The chromatograms were recorded using a HP model 3390A integrator.

Salinomycin ELISA Kit Validation. Salinomycin ELISA kit performance was validated using the following parameters: analytical sensitivity, intra-assay and inter-assay variability (same d), correlation versus the HPLC reference procedure, extraction efficiency and accelerated and real time stability. Analytical sensitivity, intra- and inter-assay variability were determined by running 10 strips each having a single standard curve and 7 replicates of the 60 ppm control. The analytical sensitivity or limit of detection was calculated using the mean signal of the zero standard minus two standard deviations. The intra-assay (within strip) precision and accuracy were determined for the 60 ppm standard using the mean of the coefficient of variation within each run. The inter-assay variability was determined by the coefficient of variations of the standards and 60 ppm control across 10 assays.

Correlation data were generated by analyzing seven replicates of 10 unknown field samples using ELISA and HPLC methods. These samples were extracted with methanol and split into equal portions and run over a period of 2 d by both methods.

Extraction efficiency was determined by analyzing 40 field samples. Three replicates of each sample were extracted for 5 and 60 min. All sample extracts were analyzed in duplicate by both ELISA and HPLC.

Shelf life stability was determined using packaged ELISA test kits stored at three temperatures, 4, 25 and 40 °C. These kit performances (color loss and standard degradation) were evaluated at various intervals during a six-month period.

Results and Discussion

Figure 1 shows a typical standard curve for the salinomycin ELISA. Using the mean value of the 0 ppm standard minus two standard deviations analytical sensitivity was determined from the standard curve. The calculated analytical sensitivity of 2.8 ppm is well below the therapeutic range (i.e., 48–72 ppm).

The results of the intra- and inter-assay precision and accuracy at the 60 ppm salinomycin level are shown in Tables I–III. The reproducibility of the standard curve (n = 10) was excellent as demonstrated by inter-strip coefficient of variations of 4.3% or less at all standard concentrations. The overall inter-assay precision and accuracy of the 60 ppm control (7 replicates/run, 10 runs or n = 70) is 60.9 ppm ± 1.47 ppm with a % CV of 2.8. The intra-assay variability of the 60 ppm control (n = 7) was very good as evidenced by a % CV of 3.0 or less in all 10 runs.

The correlation data for 10 salinomycin medicated feed samples analyzed by ELISA and HPLC is depicted as a bar graph in Figure 2. Based on the results, eight of the feeds were in the effective range (i.e., 48–72 ppm), whereas, two samples were determined to be ~ 50% lower than the approved dose of 60 ppm ± 20%. The two methods correlated well as evidenced by the linear regression data (i.e., slope = 0.975 and R = 0.952). Another method utilized to determine how well two methods correlate involved calculation of the grand means, SDs, and CVs for both methods. These calculations revealed that the ELISA yields resulted in values that were ~ 9.0% higher than the HPLC method (i.e., X ELISA = 58.14 ppm vs. X HPLC = 53.34 ppm). The CVs for both methods were essentially identical (i.e., CV ELISA = 25.3% vs. CV HPLC = 27.2%).

Table IV shows the results of the extraction efficiency as a function of time for both the HPLC and ELISA methods. Sample values for ELISA range from 40.2 to 75.0 ppm at a 5 min extraction and from 47.8 to 82.7 ppm at a 60 min extraction time. HPLC sample values ranged from 35.0 to 72.6 ppm at 5 min and from 41.3 to 70.7 ppm at 60 min. The shorter extraction time exhibited a 10% decrease in ELISA results whereas only a 5% decrease was observed when the HPLC method was utilized on the same extracts. However, the HPLC results contained several samples over 100% efficiency which is likely biasing the results. Based on the extraction efficiency results, an adjustment was made to the ELISA standards lowering the concentration of salinomycin 10% to compensate for the observed recoveries.

A minimum real time stability requirement for most commercial ELISA kits is six months when stored at 4 °C. Accelerated stability testing is performed by thermally stressing kits at elevated temperatures in order to determine labile kit components. Kit stability of 4, 25 and 40 °C as a function of time (56 d) was assessed using the accuracy of the 60 ppm control as shown in Figure 3. These data demonstrate that the salinomycin ELISA kit is stable at all three temperatures. Accelerated stability data at 25 °C (56 d) and 40 °C (28 d) suggest that the kit will have 4 °C real time stability greater than or equal to 18 months. Additional 4 °C real time stability data (not shown) shows no loss of activity after 6 months thereby meeting our minimum requirements.

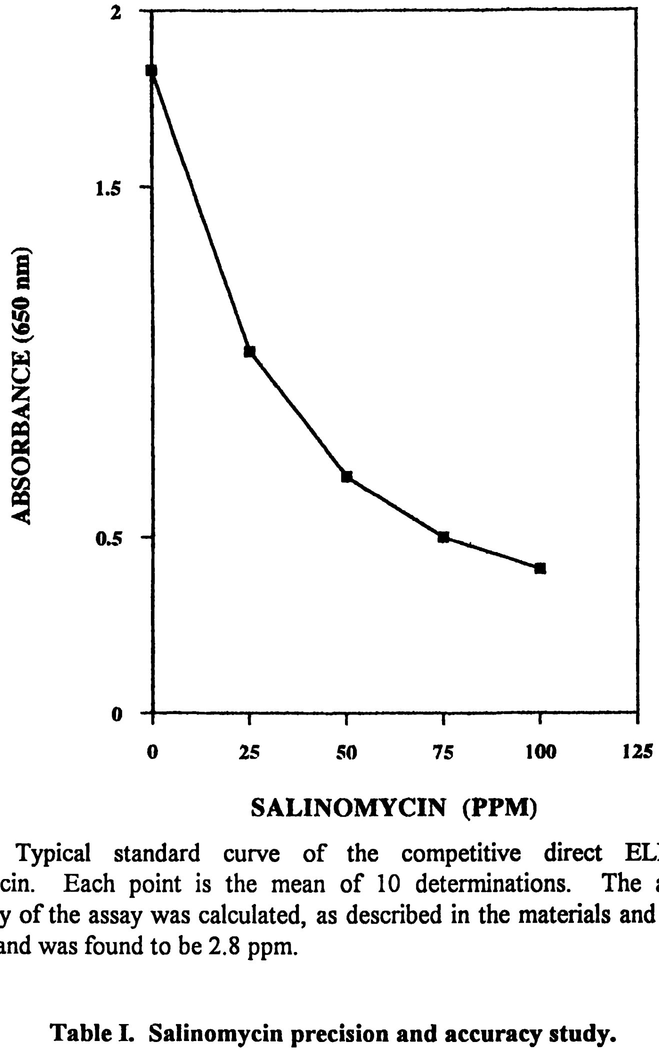

Figure 1. Typical standard curve of the competitive direct ELISA for salinomycin. Each point is the mean of 10 determinations. The analytical sensitivity of the assay was calculated, as described in the materials and methods section, and was found to be 2.8 ppm.

Table I. Salinomycin precision and accuracy study.

Standard (ppm)	Standard Curves[a] Mean	Standard Curves[a] % CV[b]	Calculated (ppm)
0	1.829	3.4	
25	1.031	2.9	24.7
50	0.675	3.8	50.9
75	0.500	3.9	76.2
100	0.407	4.3	97.9

[a] N = 10

[b] Static method used.

Table II. Salinomycin precision and accuracy study.

	Inter-Assay Variability 60 ppm Control[a]		
Sample Replicate	Mean	% CV[b]	Calculated (ppm)
1	0.601	3.5	59.9
2	0.595	2.3	60.8
3	0.589	1.8	61.6
4	0.599	3.5	60.2
5	0.596	2.7	60.6
6	0.587	2.9	61.9
7	0.593	3.1	61.1
X	(2.8)	60.9	

[a] N = 10

[b] Static method used.

Table III. Intra-assay variability 60 ppm control.[a]

Assay Run	% CV	Calculated (ppm)
1	3.0	59.1
2	1.6	62.4
3	2.7	60.7
4	1.5	63.9
5	1.8	59.3
6	2.9	59.9
7	1.4	62.4
8	1.7	57.6
9	1.5	60.5
10	1.5	63.2
X	2.0	60.9

[a] N = 7.

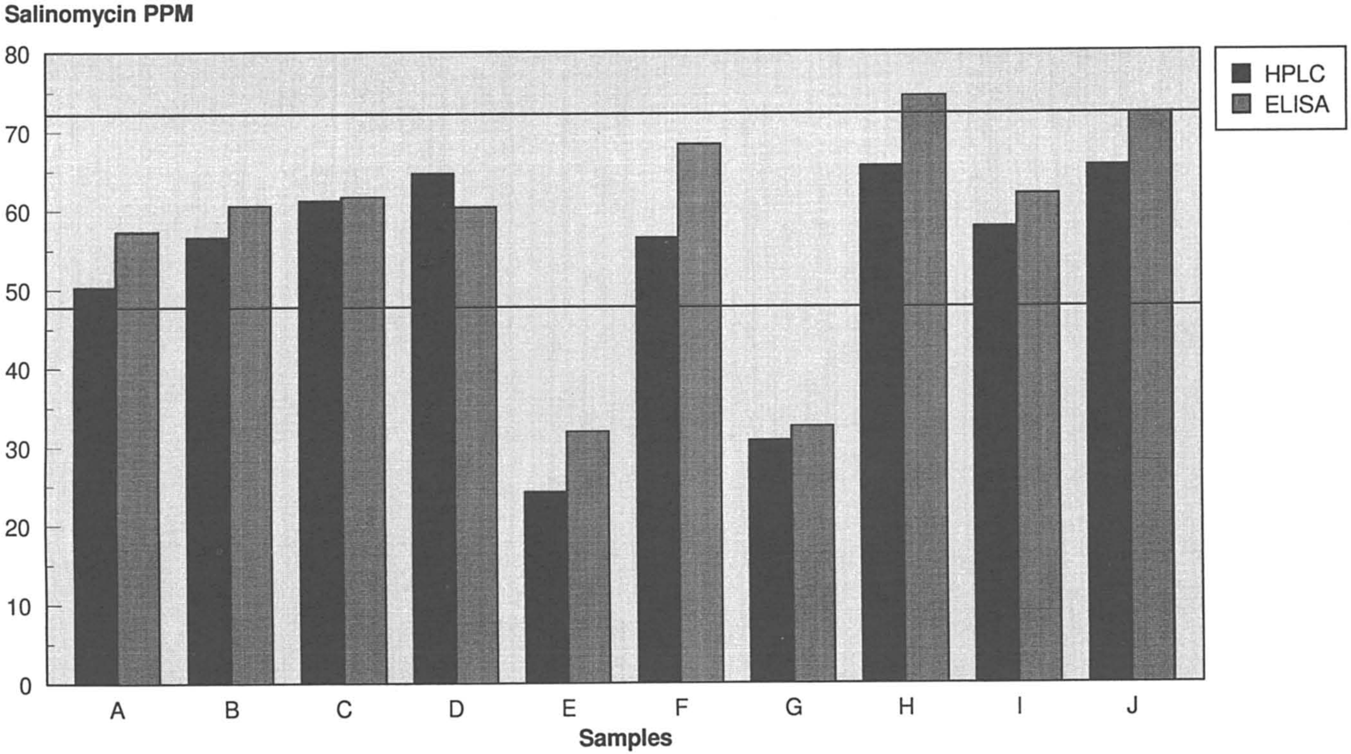

Figure 2. Salinomycin feed sample correlation study. Results shown are the averages of seven replicates of 10 salinomycin medicated feed samples extracted and split into equal portions analyzed by HPLC and the Salinomycin ELISA. The two horizontal lines present the therapeutic range of salinomycin in poultry feed.

Table IV. Salinomycin extraction efficiency as a function of time.

ELISA	X Value (ppm)^a	X^b	% Efficiency
	63.11	56.12	89.3
HPLC	**X^a**	**X^b**	**% Efficiency**
	56.14	53.22	95.0

[a] Extractions were performed in 100% methanol for 60 min using a wrist action shaker.

[b] Extractions were performed in 100% methanol for 5 min with manual shaking.

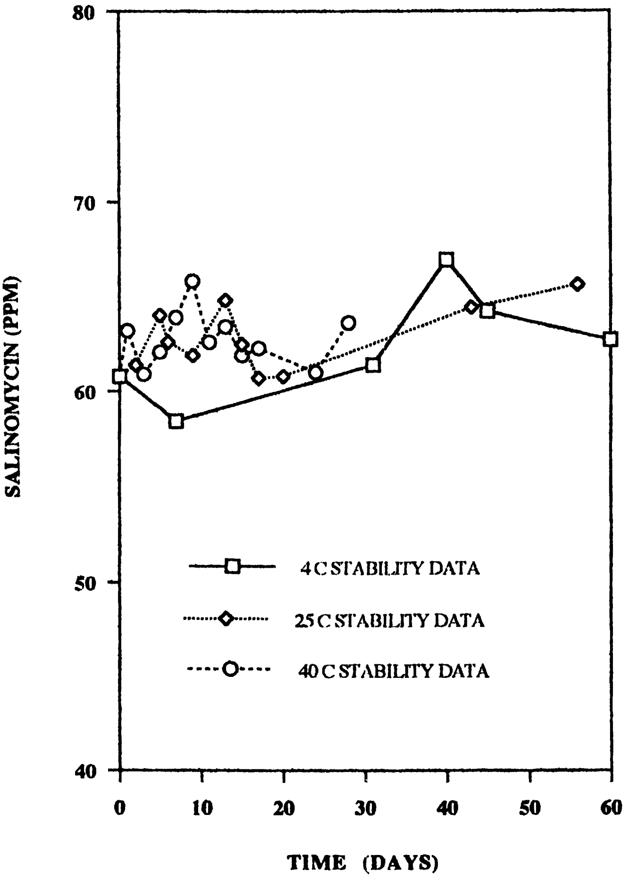

Figure 3. Salinomycin 60 ppm control thermal stability. Points shown are ELISA results of 60 ppm controls stored at 4, 25, and 40 °C over a period of 60 d.

Conclusions

A rapid ELISA has been developed to monitor salinomycin in poultry feed. This assay can be used to both screen feeds for the presence of salinomycin and to obtain quantitative results with a high degree of correlation to the HPLC reference method. The ELISA is very stable even at elevated temperatures enabling it to survive a wide variety of extremes while still maintaining its accuracy. This application of immunotechnology should allow accurate informed decisions of feed status to be made within an h after collection of a sample on site. Cost is a concern in any industry, and the ELISA has an advantage over HPLC in this respect. Combined with its ease of use, these reasons make the salinomycin ELISA applicable in almost any feed testing situation.

Literature Cited

1. Miyazaki, Y.; Shibuya, M.; Sugawara, H.; Kawaguchi, O.; Hirose, C.; Nagatsu, J.; Esumi, S. *J. Antibiot.* **1974**, *27*, 814–821.
2. Miller, G. A.; Clark, R. C.; Jessee, E. *J. Hybridoma* **1986**, *5*(4), 353–360.
3. Suls, L. *Int. Poult. Prod.* **1995**, *3*(1), VII–XI.
4. *Feed Additive Compendium*; The Miller Publishing Company: Minnetonka, MN 1991; pp 322–328.
5. Elissalde, M. H.; Beier, R. C.; Rowe, L. D.; Stanker, L. H. *J. Agric. Food Chem.* **1993**, *41*(11), 2167–2171.
6. Goras, J. T.; Lacourse, W. R. *J. Assoc. Off. Anal. Chem.* **1984**, *67*, 701–706.

RECEIVED October 11, 1995

Chapter 9

Detection and Quantification of Salinomycin in Chicken Liver Tissue

Comparison of Enzyme-Linked Immunosorbent Assay and High-Performance Liquid Chromatography Methods

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Salinomycin is one of the most widely used coccidiostats in the U.S. poultry industry. A rapid and accurate analytical method for this drug would be valuable to producers and users as an effective management tool. We have developed an enzyme-linked immunosorbent assay (ELISA) coupled to a simple aqueous extraction procedure for the analysis of salinomycin in chicken liver, since it is the target tissue in this species. Recoveries from spiked chicken liver homogenates were quantitative in the range from 5.0 to 0.05 ppm. The ELISA was used to monitor fractions from reversed-phase high performance liquid chromatography (HPLC) in order to characterize non-specific matrix effects on the assay. Results from the analysis of incurred salinomycin residues in chicken livers obtained by both the ELISA method and a HPLC method were highly correlated ($p < 0.0001$). The ELISA method could detect 20 ng of drug in a 100 μL sample and has a limit of quantification of 50 ppb in chicken liver tissue. The limit of quantification was lower with the ELISA method than with the HPLC method.

The polyether ionophore antibiotics, produced by various species of *Streptomyces*, possess broad spectrum anticoccidial activities (*1*). Members of this class of compounds include salinomycin, monensin, narasin, and lasalocid A (Figure 1). The mode of action of the ionophores is attributed to their ability to form complexes with mono- and divalent cations disrupting cell membrane function (*2*). In the United States, salinomycin (SAL) is registered for use as a feed additive at concentrations of 44 to 66 ppm to control coccidiosis in broiler chickens. In 1990, it was the most widely used coccidiostat in agriculture (*3*). SAL toxicity in broilers can occur when

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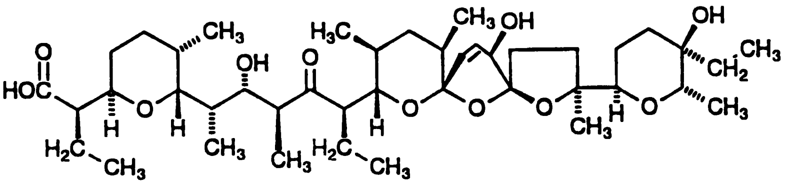
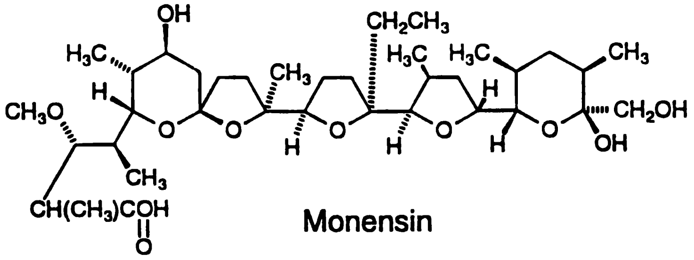
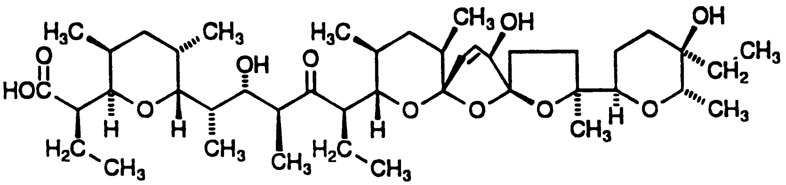


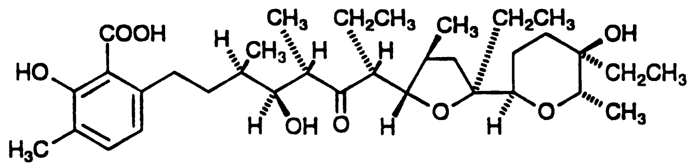
Salinomycin



Monensin



Narasin



Lasalocid A

Figure 1. Structures of various polyether ionophore antibiotics.

birds are fed the drug above the recommended therapeutic levels. However, the therapeutic levels in broilers can be toxic to turkeys and horses (*1*). Because of its importance in poultry production, rapid and sensitive analytical methods for SAL in animal tissues and feeds would provide useful management tools (See Shimer *et al.*, this volume).

The ionophores are commonly used in the form of non-volatile sodium salts, and thus are not amenable to gas chromatographic analysis. In addition, they are not fluorescent and do not possess a useful chromophore. Therefore, they are not readily detected spectrophotometrically without prior derivatization. To circumvent these limitations, analytical methods developed for the detection of the ionophores have utilized either the compounds' biological activity (bioassays), or more commonly, employed derivatization procedures to convert the analyte to a chromophoric or fluorescent species which can be more readily measured. Table I summarizes several methods used for the detection of SAL (and in most cases the method also is used for other ionophores) in various sample matrices. The detection limit of each method is expressed as the amount detected (e.g., µg or ng), as well as the sample concentration. Early analytical methods employed bioautographic detection following thin-layer chromatography (TLC). These methods were very sensitive but extremely time consuming, requiring over 18 h for their completion. Vanillin has been used to convert various ionophores to chromophoric derivatives which absorb in the visible range. This was performed following either TLC or high performance liquid chromatography (HPLC). The advantage of the HPLC methods was the ability to perform a post-column derivatization step on-line. These methods were most commonly applied to the analysis of feed grains where high sensitivity is not critical and performed using a dilute sample extract, hence high sample detection limits are obtained. Useful chromophoric derivatives have been obtained via pre-column pyridinium dichromate oxidation resulting in UV-absorbing species. This method has been employed for the analysis of various animal tissues. Fluorescent derivatives using 9-anthrydiazomethane (ADAM) and 1-(bromoacetyl)pyrene were made for the detection of various ionophores in animal tissue and feed grains, respectively. These pre-column derivatization procedures, although sensitive, require extensive sample purification prior to derivatization. Additional purification of the derivatized mixture was necessary prior to separation by HPLC and either UV or fluorescence detection.

Recently, we reported the development of a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for SAL (*15,16*). Salinomycin was coupled to carrier proteins via the carboxylic acid moiety and sixteen monoclonal antibodies were produced which equally recognized both salinomycin and narasin. Neither monensin nor lasalocid A were recognized by the antibodies. Using a homologous indirect ELISA, less than 0.3 ng of SAL could be detected in a 100 µL sample. Preliminary results showed the assay to be suitable for analyzing buffer extracts of chicken livers spiked with SAL at concentrations from 1.25 to 5.0 ppm. The ELISA method was further optimized which lowered the detection limit of the assay to 20 pg SAL (Table I) and made it more suitable for residue monitoring (*14*). It was applied to the analysis of chicken liver tissues from an incurred residue study. These results were validated using conventional HPLC methodology. This paper summarizes these results and discusses further applications of the method.

Table I. Analytical Methods for the Determination of Salinomycin in Various Sample Matrices.

Sample Matrix	Method Description	Detection Limit amount / sample concentration	Reference
Various edible rabbit tissues	Organic extraction, TLC, bioautographic detection.	0.5 ng / 10 ppb	4
Chicken liver	Organic extraction, TLC, bioautographic detection	50 ng / 25 ppb	5
Feed grains	Organic extraction, TLC, derivatization, visualization	1.5 μg / 3 ppm	6
Feed grains	Organic extraction , HPLC, post-column derivatization, spectrophotometric detection	20 ng / 1 ppm	7
Feed grains	Organic extraction, HPLC, post-column derivatization, spectrophotometric detection	10 ng / 0.25 ppm	8
Beef liver tissue	Organic extraction, purification, derivatization, purification, HPLC, fluorometric detection	30 ng / 150 ppb	9
Various chicken tissues	Organic extraction, purification, derivatization, purification, HPLC, spectrophotometric detection	13 ng / 5 ppb	10
Feed grains	Organic extraction, HPLC, post-column derivatization, spectrophotometric detection	10 ng / 1 ppm	11
Human plasma	Organic extraction, purification, derivatization, HPLC, spectrophotometric detection	4 ng / 5 ppb	12
Feed grains	Organic extraction, purification, derivatization, purification, HPLC, fluorometric detection	not indicated / 25 ppm	13
Chicken liver	Organic extraction, HPLC, post-column derivatization, spectrophotometric detection	25 ng / 100 ppb	14
Chicken liver	Aqueous extraction, immunochemical detection	20 pg / 50 ppb	14

Methods

Competitive Inhibition ELISA. The ELISA procedure was adapted from the method reported by Elissalde *et al.* (*15*) and further modified by Muldoon *et al.* (*14*). Briefly, wells of microtiter plates were coated with BSA-SAL in 100 µL of coating buffer (carbonate, pH 9.6), incubated 18 h at 4 °C, and washed (0.05% (v/v) Tween 20). Wells were blocked with 200 µL of blocking buffer (0.5% (w/v) BSA in phosphate buffered saline containing 0.05% (v/v) Tween 20, pH 7.0 incubated 60 min at ambient room temperature, washed, and stored at -20 °C until used. For the ELISA, 100 µL of sample diluted in assay buffer (Tris, pH 7.75) was added to the microtiter plate well and mixed with purified anti-salinomycin monoclonal antibody (2 ng in 100 µL of assay buffer). The mixture was incubated at room temperature for 60 min and then the plate was washed. Horse radish peroxidase labelled goat anti-mouse IgG (100 µL of 1:500 dilution in assay buffer) was added to each well, incubated at room temperature for 60 min, and then the plate was washed. K-Blue enzyme substrate (ELISA Technologies, Lexington, KY) (100 µL) was added and plate optical density (OD) measurements (655 nm) were taken at 30 min.

High Performance Liquid Chromatography. The HPLC method was adapted from previously described methods (*8,11*). Modifications and details of the method are described in detail elsewhere (*14*). Briefly, the HPLC method used a Dionex (Sunnyvale, CA) microbore system with a post-column reaction system. and a variable wavelength detector set at 520 nm. The column was a 5 µm Supelcosil LC-18 (15 cm X 2.1 mm) from Supelco (Bellefonte, PA). Samples (25 µL) were injected onto the system using a Spectra-Physics (San Jose, CA) SP 8880 autosampler. The isocratic solvent system was 95% methanol/acetic acid (1% v/v, water). The gradient solvent system was 80% methanol/acetic acid (1% v/v, water), maintained for 1 min post injection, a gradient to 100% methanol reached at 4 min post injection and maintained until 18 min. Then, 80% methanol/acetic acid gradient was reached at 20 min post injection. The solvent flow rate was 0.25 mL/min for both solvent systems. The derivatization reagent consisted of 10% (w/v) vanillin in methanol containing 2% (v/v) sulfuric acid delivered at 0.5 mL/min.

Aqueous Extraction of Salinomycin from Chicken Liver Tissue. Sixty gram portions of control chicken liver, obtained from a local grocery store, were homogenized using a tissue homogenizer. Four grams of the homogenized liver was weighed into a 50 mL polypropylene centrifuge tube. Assay buffer (40 mL) was added and the sample was vortexed to suspend the homogenate. The sample was centrifuged and the supernatant was further diluted in assay buffer prior to ELISA analysis.

Methanolic Extraction of Salinomycin from Chicken Liver Tissue. The extraction method was an adaptation of a previously described method used for the analysis of SAL in beef liver tissue (*9*). Ten grams of homogenized liver were weighed into a 50 mL polypropylene centrifuge tube and extracted twice with 25 mL portions of 80% (v/v) methanol/water. The methanolic liver extract was back-extracted with methylene chloride. The methylene chloride extract was evaporated to dryness, the residue redissolved in 1 mL of methanol, filtered, and analyzed by HPLC.

Evaluation of the Aqueous Liver Extract on ELISA. In order to determine the effects of the aqueous liver extract on the ELISA, SAL standard curves made in assay buffer and various dilutions of aqueous liver extracts (1:10, 1:50, and 1:100) were compared. Results were analyzed using either OD values or B/B_0-transformed data, where B is the OD value of the sample and B_0 is the OD value of the sample without competitor (assay buffer or the appropriate dilution of unspiked liver extract). IC_{50} values (concentration of inhibitor which produces a 50% decrease in signal of the no competitor control) were derived using a 4-parameter log-logistic curve fitting function (SOFTmax 2.01, Molecular Devices Corp., Menlo Park, CA) for each standard curve.

Analysis of Spiked and Non-Spiked Control Liver Extracts by HPLC-ELISA. Methanolic extracts from 2 aliquots of non-spiked control liver homogenate were obtained as previously described and pooled. In addition, a solvent blank was obtained in which the entire extraction procedure was carried out with the omission of a liver sample. The pooled liver extract sample was split (2 x 1 mL) and one was spiked to 10 ppm SAL. The 3 samples were analyzed by HPLC using the isocratic solvent system and post-column derivatization (vanillin) with eluent monitored at 520 nm. In addition, samples were injected onto the HPLC system under the same solvent conditions but without post-column derivatization. In this case, 100% methanol was pumped through the post-column reaction solvent pump (0.5 mL/min) and thirty 15 s fractions were collected by hand for each injection. The fractions were evaporated to dryness, reconstituted in assay buffer and analyzed by ELISA. During these runs, HPLC eluant was monitored at 220 nm.

Comparison of ELISA and HPLC Methods for the Determination of Salinomycin in Spiked Chicken Liver Tissue. Control liver tissue was homogenized and aliquots (4 g for ELISA; 10 g for HPLC) were spiked with various amounts of SAL to give tissue concentrations of 5.0, 1.0, 0.5, 0.25, 0.1, 0.05, and 0.0 (no SAL) ppm. The samples were extracted and analyzed by either the ELISA or HPLC methods as described above.

For the ELISA analysis, several dilutions of each sample extract were made on the plate. Raw OD values were transformed to B/B_0 values (as described above). Concentrations of SAL in the extracts were calculated based on the standard curve analyzed on each plate using the 4-parameter log-logistic curve fitting function in SOFTmax. The lowest extract dilution which resulted in a B/B_0 value in the linear, quantitative region of the standard curve (B/B_0 = 0.70 to 0.20) was used for determining SAL in the sample. Three sets of samples were prepared and analyzed immediately in triplicate wells of a microtiter plate.

For the HPLC analysis, three sets of spiked samples were prepared one set at a time and analyzed immediately. All samples were analyzed in duplicate. The isocratic HPLC solvent system was used in this study. Sample concentrations were determined based on SAL standards ranging from 0.41 to 100 ppm in methanol analyzed concurrently with the sample extracts.

Comparison of ELISA and HPLC Methods for the Determination of Salinomycin in Chicken Liver Tissues from an Incurred Residue Study. Livers were obtained from chickens which were fed, from 6 weeks of age, either a control diet (no SAL), a diet containing the recommended therapeutic dose of salinomycin (66 ppm), or a diet containing 2 times the therapeutic dose of salinomycin (132 ppm). The birds were maintained on these diets for 2 weeks at which time 5 birds from each treatment group (No Dose, 66 ppm, or 132 ppm) were killed and their livers removed and frozen at -70 °C until processed. The remaining 10 birds in each of the 3 treatment groups were immediately given control feed and sacrificed 18 and 72 hours later (5 birds/group/time period).

Livers were thawed at room temperature, homogenized, and stored at 4 °C prior to analysis (within 18 h). Livers were extracted and analyzed by either ELISA or HPLC as described above. For the ELISA, the OD values of the aqueous control liver extract was used for calculating B_0 for data transformation of the samples. For each set of samples (one bird from each treatment group per withdrawal time (9 samples)), the ELISA analysis and extraction of SAL into methylene chloride for subsequent HPLC analysis were performed on the same day. On the next day, samples were further processed and analyzed by HPLC using the gradient solvent system.

Results and Discussion

ELISA Optimization. Several modifications were made in the ELISA procedure as reported by Elissalde *et al.* (*15*) which resulted in improvements in sensitivity and precision. These are described in detail in Muldoon *et al.* (*14*). Most importantly was the coating conditions used for immobilizing the ELISA antigen and the use of purified anti-salinomycin antibody (derived from ascites fluid). In the previous method, antigen was dissolved in distilled water and plates were coated overnight at 37 °C. In the current method we dissolved the coating antigen in carbonate buffer (pH 9.6) and plates were coated overnight at 4 °C. This had the effect of increasing the OD values of the no competitor control samples from a reading of 0.6 units using the previous coating method to 1.3 units using the current coating method. In addition, there was an improvement in well to well variation to below 10%. All modifications considered, there was an improvement in assay sensitivity from an IC_{50} of 3.30 ng/mL (interassay CV = 30.3%) for the previous method to an IC_{50} of 0.52 ng/mL (interassay CV = 20.7%) for the current procedure.

Evaluation of the Aqueous Liver Extract on ELISA. Salinomycin standard curves prepared in assay buffer and in various dilutions of the aqueous liver extract were evaluated in order to detect any matrix effects on the assay. Maximum optical densities of 1.38, 0.85, 0.72, and 0.66 units were observed when the ELISA was performed in assay buffer or aqueous liver extracts diluted 1:100, 1:50, and 1:10 in assay buffer, respectively. Due to these effects, it was necessary to transform OD measurements to B/B_0 values where B_0 was the mean OD measurement of the no competitor control in buffer for the standards, and the appropriate dilution of control liver extract for the samples. Figure 2 shows the standard curves made in assay buffer and in the various dilutions of aqueous liver extract following B/B_0-transformation of the OD measurements. The standard curves prepared in buffer and the 1:100 dilution of aqueous liver extract overlapped. The IC_{50} value obtained from the standard curve made in assay buffer (0.63 ng/mL, CV = 5.65%) was not different from the standard curve made in liver extract diluted 1:100 (0.98 ng/mL, CV = 7.52%) ($p > 0.05$). Therefore, it was possible to use a B/B_0-transformed standard curve prepared in buffer for extrapolating B/B_0-transformed sample data, with the requirement that a valid matrix control be used for the sample B_0 and the samples diluted to a minimum of 1:100.

Analysis of Spiked and Non-Spiked Control Liver Extracts by HPLC-ELISA. After observing the apparent non-specific interference of control liver extract on the ELISA, we were interested in further characterizing these effects by separating the sample by HPLC and analyzing column fractions by ELISA since this detection system is over 1000 times more sensitive than the spectrophotometric detector (see Table I). Aliquots of control liver extract (spiked and non-spiked), a standard solution containing 10 ppm SAL, and a solvent extract blank were injected onto the HPLC system without post-column derivatization. Column eluent fractions were collected, evaporated, reconstituted in assay buffer, and analyzed by ELISA. Figure 3 shows the HPLC-ELISA chromatogram from the analysis of unspiked control liver extract, control liver extract spiked to 10 ppm SAL (tissue concentration equivalent of 1 ppm), and the 10 ppm SAL standard. No inhibition was observed in any of the fractions from the solvent extract control blank (data not shown). The total SAL equivalents (ng) recovered in the unspiked control liver, the control liver extract spiked to 10 ppm SAL, and the 10 ppm SAL standard were 5.26, 222.3, and 204.8 ng/25 µL, respectively. The amounts which eluted at the same retention time as SAL (as determined by the 10 ppm standard) were 0.83, 217.5, and 204.8 ng/25 µL, respectively. For the control liver extract, this corresponded to a tissue concentration of 3.32 ppb SAL. Most of the inhibition observed with the ELISA in this sample (84.2%) was associated with fractions collected between 3 and 4.5 min and was not attributed to SAL (6 to 8 min). In addition, the UV chromatograms (220 nm) for both the unspiked and spiked extracts were identical (data not shown); each showed multiple peaks with a large, broad peak from 5 to 7 min, where SAL eluted. The UV chromatogram (220 nm) for the 10 ppm SAL standard gave no peaks which was not surprising since post-column derivatization was not performed. It is possible that the inhibition observed with the unspiked control liver extract in the fraction near the retention time of SAL is in fact residual drug, however, since it was associated with high non-specific UV absorbance, it may be non-specific inhibition. Nevertheless, the use of a sample extract dilution of 1:100 for ELISA analysis alleviates the influence of such low background levels of interference on the ELISA measurement. In addition, since the amount is over 10 times less than the lower limit of detection of the HPLC method (approximately 50 ppb) it should not influence the results using this method.

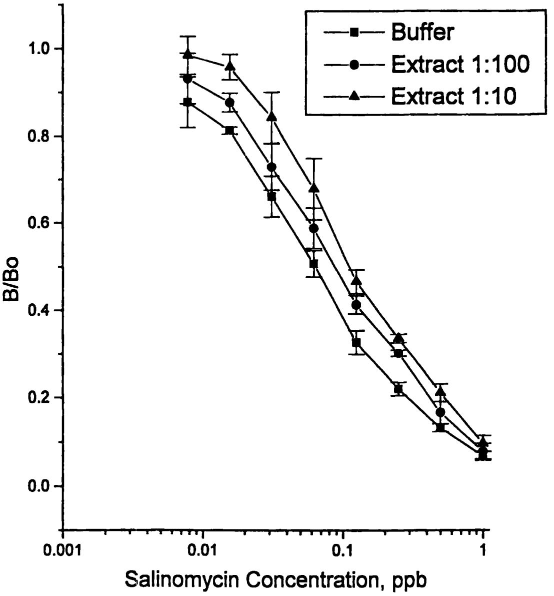


Figure 2. Salinomycin ELISA standard curves made in assay buffer and aqueous liver extract diluted in assay buffer after B/B_0 transformation of OD readings. Mean IC_{50} values derived from standard curves made in either assay buffer or liver extracts diluted 1:10, 1:50, or 1:100 in assay buffer were 0.63, 0.98, 1.00, and 1.25 ng/mL, respectively. Mean IC_{50} values derived from standard curves made in liver extract diluted 1:10 and 1:50 were significantly different from the value obtained in assay buffer alone ($p > 0.05$). (Reprinted with permission from ref. *14*).

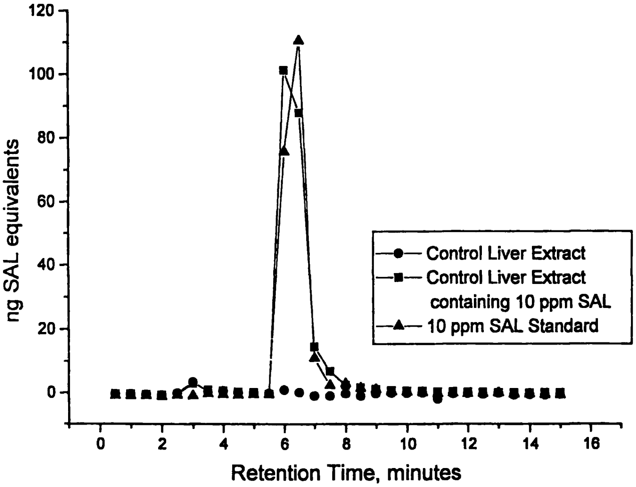


Figure 3. HPLC-ELISA chromatograms of 10 ppm SAL standard, unspiked, and spiked (10 ppm SAL) control chicken liver extracts.

Comparison of ELISA and HPLC Methods for the Determination of Salinomycin in Spiked Chicken Liver Tissue. Chicken liver tissue homogenate was spiked with various levels of SAL and analyzed by ELISA and HPLC. The results from this study are shown in Figure 4. Linear regression showed that for both the ELISA and the HPLC methods, the amount detected was highly correlated to the amount added ($R^2 = 0.999, p < 0.0001$). The ELISA method was more accurate than the HPLC method, particularly at the lower tissue concentrations. In addition, mean recoveries for the ELISA method were quantitative over the entire range tested. This was not the case with the HPLC method which showed a large deviation from linearity below 100 ppb. Variability between triplicate extraction and analyses by ELISA (CV = 19.9%) was higher than when the HPLC method was used (CV = 4.11%). The average CV observed for individual sample determinations was 8.4% (n = 3) for the ELISA, and 4.13% (n = 2) for the HPLC method.

The lower limit of quantification by the ELISA method was approximately 50 ppb SAL in liver tissue. Figure 2 shows that the analysis of a 1:100 dilution of aqueous liver extract spiked at a level corresponding to 50 ppb SAL in liver (0.5 ppb) gave B/B_0 values which were within the linear, quantitative range of the assay (B_0 values between 0.7 and 0.2). Acceptable recoveries (83%) were obtained from control liver samples spiked at this level and analyzed by ELISA. The lower limit of quantification for the HPLC method, defined as 10 times the variance (10σ) (*17*), was also approximately 50 ppb. However, as shown in Figure 4, there was a large deviation from linearity below 100 ppb resulting in an over estimation of analyte using this method. This effect limited the accuracy of the HPLC method to levels at or above 100 ppb. Therefore, we report the limit of quantification for the HPLC method as 100 ppb. This limit of quantification is similiar to that reported for other instrumental methods developed for the analysis of SAL in animal tissues using either pre- or post- column derivatization (see Table I).

Determination of Salinomycin in Chicken Liver Tissues from the Incurred Residue Study. Table II shows the results from the incurred residue study. Results from the analysis of incurred residue samples were highly correlated ($p < 0.0001$). When compared to HPLC results (all 45 samples) only 1 false negative and 2 false positives were produced by ELISA. However, these occurred near the limit of quantification for the HPLC method (100 ppb) and also could be interpreted as being 1 false positive and 2 two false negatives by HPLC. Nevertheless, these results indicated that the ELISA method is a reliable screening tool for SAL in chicken liver tissue. There was good agreement between the two methods in the samples where the SAL concentrations obtained by the two methods were above the limits of quantification for the two methods. Results from bird numbers 2 and 3 in the 132 ppm, 2 h withdrawal time group were exceptions, and the concentration estimates by ELISA were underestimated for bird number 2 and overestimated for bird number 3. Inconsistencies such as these can be caused by sample matrix effects which can occur with both methods.

Table II. ELISA and HPLC Results from the Analysis of Salinomycin in Chicken Liver Tissues from an Incurred Residue Study.

Treatment	Bird No.	ELISA Concentration, ppb			HPLC Concentration, ppb		
		0 hr	18 hr	72 hr	0 hr	18 hr	72 hr
No Dose	1	-[a]	-	-	-	-	-
	2	-	-	-	-	-	-
	3	-	-	-	-	-	-
	4	-	-	-	-	-	-
	5	-	-	-	-	-	-
66 ppm	1	101.2	-	-	-	-	-
	2	459.0	-	-	339.2	-	-
	3	153.0	-	-	-	-	-
	4	146.4	-	-	93.8	-	-
	5	155.3	-	-	168.1	-	-
132 ppm	1	317.9	-	-	278.0	-	-
	2	181.6	-	-	364.4	-	-
	3	688.0	-	-	157.6	-	-
	4	315.3	-	-	245.5	111.6	-
	5	251.5	-	-	238.6	-	-

[a] Below the limits of quantitation: 50 ppb for ELISA; 100 ppb for HPLC. ELISA and HPLC results were highly correlated, $p < 0.0001$. (Reproduced with permission from reference 14. Copyright 1995 American Chemical Society).

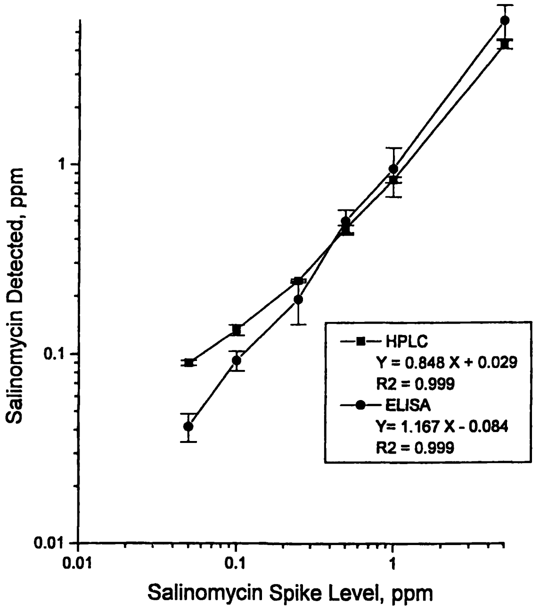


Figure 4. Results from HPLC and ELISA analysis of salinomycin in spiked chicken liver homogenates. (Reprinted with permission from ref. *14*).

Both methods showed that SAL residues were present immediately following withdrawal from medicated feed and are undetectable after 18 h. These results are similiar to the results reported by Atef *et al.* (*18*). It is important to note that either of the methods used alone would have provided the same pharmacokinetic data.

Conclusions

Most current analytical methods for the determination of SAL require organic solvent extraction followed by sample cleanup, chromatography, and pre- or post-column derivatization. These procedures are laborious and this limits the number of samples that can be effectively processed. We have developed a procedure which uses an aqueous buffer extraction coupled to an ELISA for the analysis of SAL in chicken liver tissue. The method was more sensitive and accurate than a HPLC method. ELISA and HPLC results from the analysis of incurred residue liver samples showed a rapid dissappearance of SAL from the tissue. The advantages of the ELISA method were reduced organic solvent use and increased sample throughput which should save time and expense in residue monitoring.

The ELISA was coupled to HPLC as a sensitive detection system. This alleviated the need to derivatize the drug prior to detection. The HPLC-ELISA system was used to characterize non-specific matrix effects, but in the future, it should provide a general analytical strategy in situations where an ultra-sensitive detection system is required. This strategy is particularly interesting for the analysis of compounds which otherwise require derivatization for detection and ones in which the required derivatization is particularly difficult. Further work will focus on utilizing this hybrid technology in other applications.

Literature Cited

1. McDougald, L. R.; Roberson, E. L. In *Veterinary Pharmacology and Therapeutics;* Booth, N. H., McDonald, L. E., eds.; Iowa State University Press: Ames, IA, 1988; pp 950–968.
2. Pressman, B. C.; Fahim, M. *Ann Rev. Pharmacol. Toxicol.* **1982**, *71*, 480–484.
3. SRI International. *Chemical Economics Handbook;* Dialog Information Services: Menlo Park, CA, 1992, File 359; CEH Accession 201.8002B-2.
4. Heil, K.; Peter, K.; Cieleszky, V. *J. Agric. Food Chem.* **1984**, *32*, 997–998.
5. Dimenna, G. P.; Walker, B. E.; Turnbull, L. B.; Wright, G. J. *J. Agric. Food Chem.* **1986**, *34*, 472–474.
6. Owles, P. J. *Analyst* **1984**, *109*, 1331–1333.
7. Goras, J. T.; LaCourse, W. R. *J. Assoc. Off. Anal. Chem.* **1984**, *67*, 701–706.
8. Blanchflower, W. J.; Rice, D. A.; Hamilton, J. T. G. *Analyst* **1985**, *110*, 1283–1287
9. Martinez, E. E.; Shimoda, W. *J. Assoc. Off. Anal. Chem.* **1986**, *69*, 637–641.
10. Dimenna, G. P., Lyon, F. S.; Thompson, F. M.; Creegan, J. A.; Wright, G. J. *J. Agric. Food Chem.* **1989**, *37*, 668–676.
11. Lapointe, M. R.; Cohen, H. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 480–484.
12. Karnes, H. T.; Wei, A. T.; Dimenna, G. P. *J. Pharm. Biomed. Anal.* **1993**, *11*, 823–827.
13. Asukabe, H.; Murata, H.; Harada, K.; Suzuki, M.; Oka, H.; Ikai, Y. *J. Agric. Food Chem.* **1994**, *42*, 112–117.
14. Muldoon, M. T.; Elissalde, M. E.; Beier, R. C.; Stanker, L. H. *J. Agric. Food Chem.* **1995**, 43, 1745–1750.
15. Elissalde, M. H.; Beier, R. C.; Rowe, L. D.; Stanker, L. H. *J. Agric. Food Chem.* **1993**, *41*, 2167–2171.
16. Stanker, L. H.; Elissalde, M. H.; Rowe, L. D.; Beier, R. C.; Nasr, M. I. A. *Food Agric. Immunol.* **1994**, *6*, 45–54.
17. Keith, L. H. In *Environmental Sampling and Analysis. A Practical Guide*; Lewis: Chelsea, MI, 1991.
18. Atef, M; Ramadan, A.; Youssef, S. A. H.; Abo El-Sooud, K. *Res. Vet. Sci.* **1993**, *54*, 179–183.

RECEIVED October 6, 1995

Chapter 10

Approaches to the Synthesis of Haptens for Immunoassay of Organophosphate and Synthetic Pyrethroid Insecticides

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The major groups of insecticides that have been used in agriculture over the last two decades are the organophosphorus insecticides and synthetic pyrethroids. Immunoassays provide a simple means for analysis of large numbers of samples for selected target compounds. The design and development of haptens for immunoassays of the target compounds together with the performance characteristics of the resulting assays are reviewed.

Although several papers in the initial years of the evolution of agrochemical immunoassay concerned the development of antisera to insecticides such as parathion, malathion and DDT (*1*), more recently, the application of immunoassays to insecticides has lagged somewhat behind that of herbicides. One factor has been the greater difficulty of analysis of food matrices that are the most important with insecticides, compared with the water matrices that are typically the targets of many herbicide assays. However, it is possible that the greater chemical lability of many insecticide groups, compared with that of other agrochemicals has been a major factor, too. The move away from organochlorine insecticides since the 1960s has led to greater use of organophosphates and synthetic pyrethroids, because of the lower persistence of many of the parent compounds and their metabolites in soil, water and food samples (*2*). The increased lability of the newer pesticides, which is "designed into" the molecules, brings with it instability in either acid and/or base, and has presented a number of challenges in hapten design. In this review, the focus will be on comparing the chemical routes used to develop the haptens (full synthetic schemes and analyses are usually provided in the original citations) and the properties of the antibodies and assays obtained, namely detection limits, specificity and sensitivity to small increments in target pesticide concentration.

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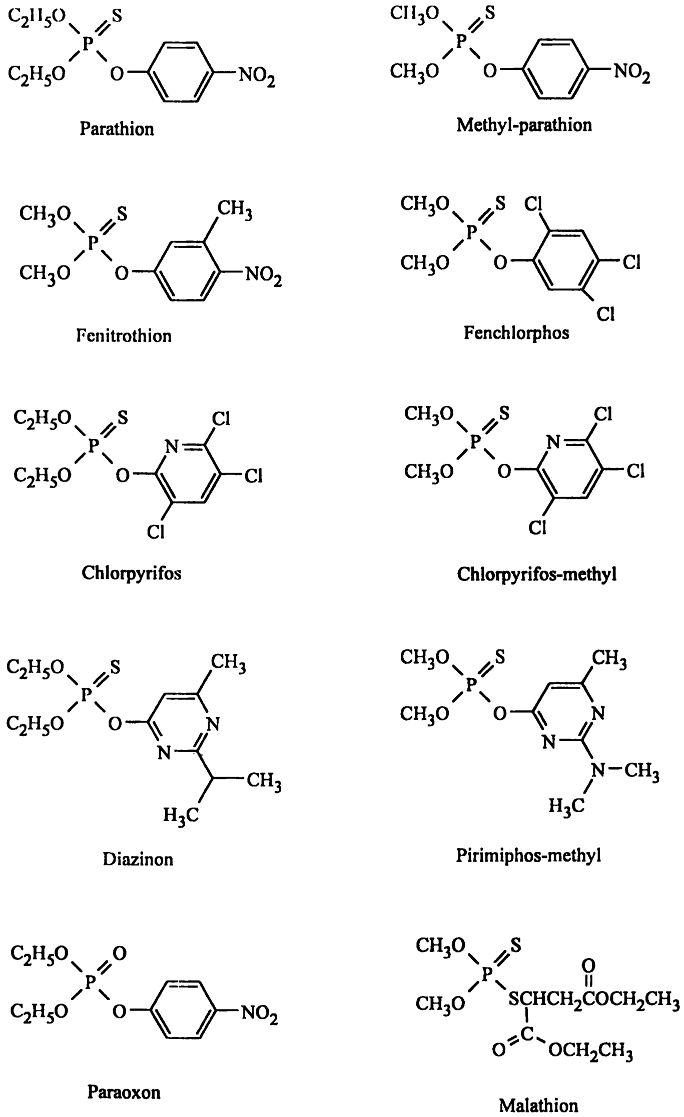
Organophosphate Haptens

The organophosphorus insecticides have a wide spectrum of insecticidal activities and physico-chemical properties, such as solubilities and vapor pressures, and since the 1970's have become the major insecticides used in horticultural crops and on stored commodities such as cereals. There also are several other uses, such as aerial application in cotton and rice cultures, domestic and industrial use as termiticides, and in animal health applications. They can be divided into six main chemical groups, depending on the number and type of appendages on oxygen and sulfur atoms and the leaving group around the phosphorus atom (*2*). The four main variants range from having four oxygen atoms bonded to the phosphorus atom (orthophosphates); three oxygen atoms with a P-S double bond (phosphorothionates); three oxygen atoms, with the S in the bond between P and the leaving group (phosphorothiolates); and phosphorothiolothionates which have sulfur in both the P-S double bond and in the bond between the P atom and the leaving group. The two minor groups are phosphonates and pyrophosphoramides.

The two most important classes in agriculture are the phosphorothionates (or thionphosphates, including parathion, chlorpyrifos, diazinon, fenitrothion and pirimiphos) and the phosphorothiolothionates (also known as dithiophosphates and which include malathion, azinphos-methyl and dimethoate). These two groups have also been the subject of the most intensive development of immunoassays. The structures of some key organophosphorus insecticides are shown in Figure 1. Immunoassays also have been developed for some other cholinesterase-inhibiting compounds, including carbamate insecticides such as carbaryl (*3,4*), aldicarb (*5,6*) and methomyl (*7*), and the potential chemical warfare agent, soman (*8,9*). Soman (methylphosphonofluoridic acid, 1,2,2-trimethylpropyl ester) does not fit into one of the six classes shown above as it has only two P-O bonds, with the P atom also being bonded to a methyl group and a fluorine atom.

Phosphorothionates. Although other organophosphates (with the exception of limited work on malathion) were ignored, a substantial proportion of the earlier pesticide immunoassay papers concerned themselves with development of haptens for the analysis of parathion and its active metabolite, paraoxon (Figure 2). All of the initial studies utilized derivatives of the diethyl ester (parathion), although we have more recently developed a pair of haptens based on parathion and methyl parathion using similar chemical routes and points of coupling. While parathion is primarily used in broadacre agriculture and is the target of analysis because of high human toxicity (*10*), methyl-parathion is much less toxic and is applied directly to stored products as a protectant treatment in countries such as India.

Parathion Haptens Coupled Through the Aromatic Nitro-Group. The simplest approach to the development of pesticide antibodies is to derivatize the pesticide itself to create a means of coupling it to protein. With aromatic nitro-compounds such as parathion or fenitrothion, this can involve reduction to a substituted aniline and either diazotization (Figure 2 (*1*)), or coupling through a spacer arm attached to the aromatic amine via amide bond formation (Figure 2 (*2*)). The initial



Parathion

Methyl-parathion

Fenitrothion

Fenchlorphos

Chlorpyrifos

Chlorpyrifos-methyl

Diazinon

Pirimiphos-methyl

Paraoxon

Malathion

Figure 1. Structures of selected organophosphorus insecticides.

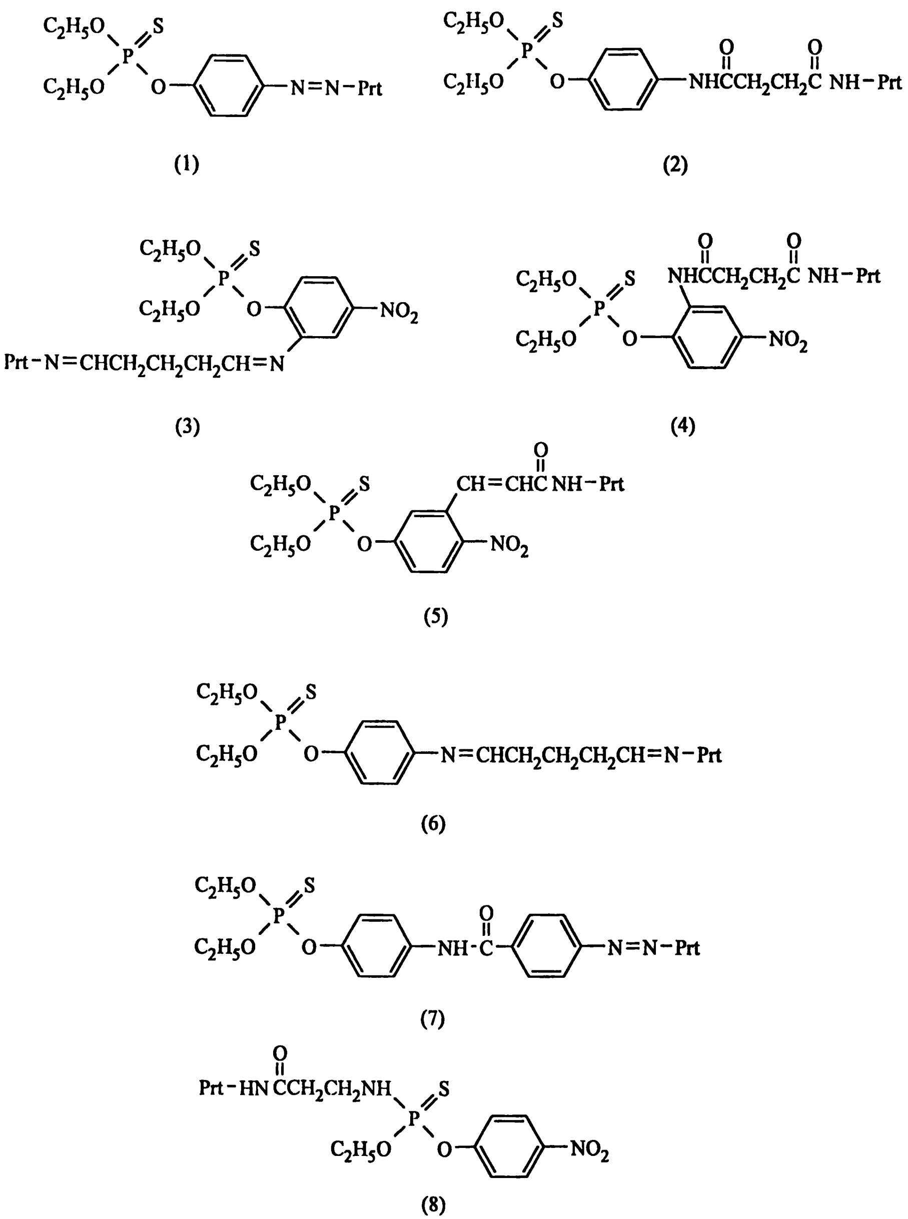


Figure 2. Structures of different parathion haptens. References for their syntheses as follows: Hapten 1 (*11,12*), hapten 2 (*16*), haptens 3–7 (*19*) and hapten 8 (*25*).

approach taken by Al-Rubae (*11*) was to couple the diazotized derivative directly to protein for antibody production. Although the antibody so obtained was relatively low in titer (used at a dilution of 1/1000), it provided moderate sensitivity (lower limit of detection (LLD) of 5 ppb). Surprisingly, cross-reaction with methyl-parathion was not detected. The assay, described in 1978, was one of the first pesticide immunoassays to be applied to food matrices, and good spike/recovery correlations were reported for several fruit and vegetable matrices, without the need for sample cleanup. They later used an antibody derivative to develop a radioimmunoassay, and applied it (*12*) to a similar diazotized parathion derivative and applied it to food analysis. More recently, an antibody prepared using the same approach has been applied to a fiber-optic immunosensor capable of detecting nanomolar levels of parathion (*13*). Others have attempted to develop monoclonal antibodies to either diazotized parathion amine (*14*) or diazotized paraoxon amine (*15*). The assay using the parathion hapten had a reasonable low LLD (1 ppb), but a very flat standard curve, providing 50% inhibition of antibody binding in indirect ELISA of only 5 ppm (*14*). It cross-reacted similarly with methyl-parathion and parathion but did not detect paraoxon. The assay developed using the paraoxon hapten did detect parathion (*15*), but its LLD also was very high (1 ppm) for both compounds.

Diazotization also has been used for a fenitrothion immunogen (Figure 1), the 3-methyl analog of methyl-parathion (Figure 1), and utilized extensively as a direct treament for grain and other stored products (*16*). Initial attempts using diazo-derivatives of either fenitrothion (*17*) or fenitrooxon (*18*) provided antibodies with relatively high detection limits. The standard curves were rather flat (sometimes termed "low sensitivity"), making the assay of little use for quantitative analyses. The first to introduce a separate spacer group coupled through a reduced nitro-group was Lober *et al.* (*19*), who coupled a paraoxon derivative through a succinyl moiety (shown for a parathion derivative in Figure 2 (*2*)). They compared an O,O-diethyl-O-(*p*-aminophenyl) phosphate hapten with the hemisuccinate derivative of the same compound. Using a radioimmunoassay, the latter hapten gave superior results and an apparent affinity constant of 10^6 to 10^7 liters per mole for paraoxon. The assay showed a 10% cross-reaction with parathion but little cross-reaction with methyl-parathion. We also have developed a derivative of fenitrothion, based on reduction of the nitro-substituent to an amine followed by reaction with ethyl adipoylchloride to produce a six-carbon atom spacer arm (*17*). The resultant antisera had a somewhat superior LLD to those developed using a diazo-coupled hapten that lacked a spacer arm.

Other Positions of Derivatization. The position of substitution on parathion haptens was more systematically studied by Vallejo *et al.* (*20*, Figure 2 (3–7)), although they had only modest success in developing a more sensitive immunoassay. Two different strategies were developed for coupling through the 4-amino group produced by reduction of the 4-nitro substituent. These involved reaction of amino-parathion (O,O-diethyl-O-(*p*-aminophenyl)-thiophosphate) with glutaric dialdehyde as well as development of a similar derivative with a benzene ring in the spacer arm (based on diazo coupling of 4-amino-4'-(O,O-diethylphosphorothionyl)-benzanilide). Haptens also were developed by coupling at the 2 and 5 positions on the ring. To synthesize the 2-substituted derivatives, the 2-amino derivative of parathion was first synthesized by reaction of 2-amino-4-nitrophenol and diethylthiophosphoryl chloride. Two spacer arms were developed, one using glutaric dialdehyde (Schiff base derivative) and the other, containing amide bonds, involved an initial reaction with succinic anhydride. The 5-substituted hapten required the initial synthesis of 2'-nitro-5'-hydroxycinnamic acid, which was converted into the parathion analog by reaction with diethylphosphoryl chloride before coupling to amine groups in the carrier protein.

More recently a simple strategy has been developed, also based on ring substitutions (*21*) for the preparation of haptens for chlorpyrifos (O,O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate. These authors carried out a one-step nucleophilic substitution of the chlorine atom (next to the pyridine nitrogen (in position 6)), using 3-mercaptopropionic acid. Attempts to produce a similar hapten based on the benzene analog, fenchlorphos-ethyl, using the same procedure were not successful, since the chlorine substituents were far less activated to nucleophilic substitution reactions. Instead, a ring-derivatized fenchlorphos analog was prepared by reacting 3,5,6-trichlorosalicylic acid with the protected amino acid, *tert*-butyl-3-aminopropanoate, followed by reaction with diethyl chlorothiophosphate. The heterologous immunoassay, with monoclonal antibodies to the first hapten and the fenclorphos derivative immobilized onto the microwell, gave an assay that was reasonably specific for chlorpyrifos and chlorpyrifos-methyl with moderate sensitivity [IC_{50} of 33 nM (about 10 ppb) for chlorpyrifos]. This detection limit was higher than that of the assay developed by our group (*22*) using polyclonal rabbit antisera to a chlorpyrifos derivative coupled through the phosphate ester (see below), but it is similar to that obtained by us using a monoclonal antibody (*23*). Typically, hapten immunoassays based on monoclonal antibodies have higher limits of detection than those based on polyclonal antisera, although exceptions have been found (*24*).

An obvious limitation of many of the methods used to develop haptens for the immunoassay of parathion and fenitrothion, is that they are limited to compounds which contain an aromatic nitro-group that can be reduced to an amino-functionality. Similarly, the approach used by Manclus *et al.* (*21*) for chlorpyrifos is based on the special reactivity of the replaceable halogen next to the pyridine nitrogen.

Coupling Through the Phosphate Ester. Heldman *et al.* (*25*) were the first to develop paraoxon haptens and conjugates that linked the pesticide and carrier protein through the phosphate ester. Their first conjugate directly coupled the pesticide phosphorus atom to amino groups on the protein by reaction of the carrier protein with O-(4-nitrophenyl)-O-ethyl phosphorochloridate (produced by reaction of 4-nitrophenyl phosphorochloridate with ethanol). A conjugate with a four-carbon spacer arm was developed by reacting this compound with a protected (trimethylsilyl) derivative of β-alanine, in the presence of trimethylamine. In a competitive radioimmunoassay, 50% displacement of tracer binding was obtained with 10 pmol paraoxon. The rabbit antisera to these conjugates confered *in vivo* protection against paraoxon poisioning.

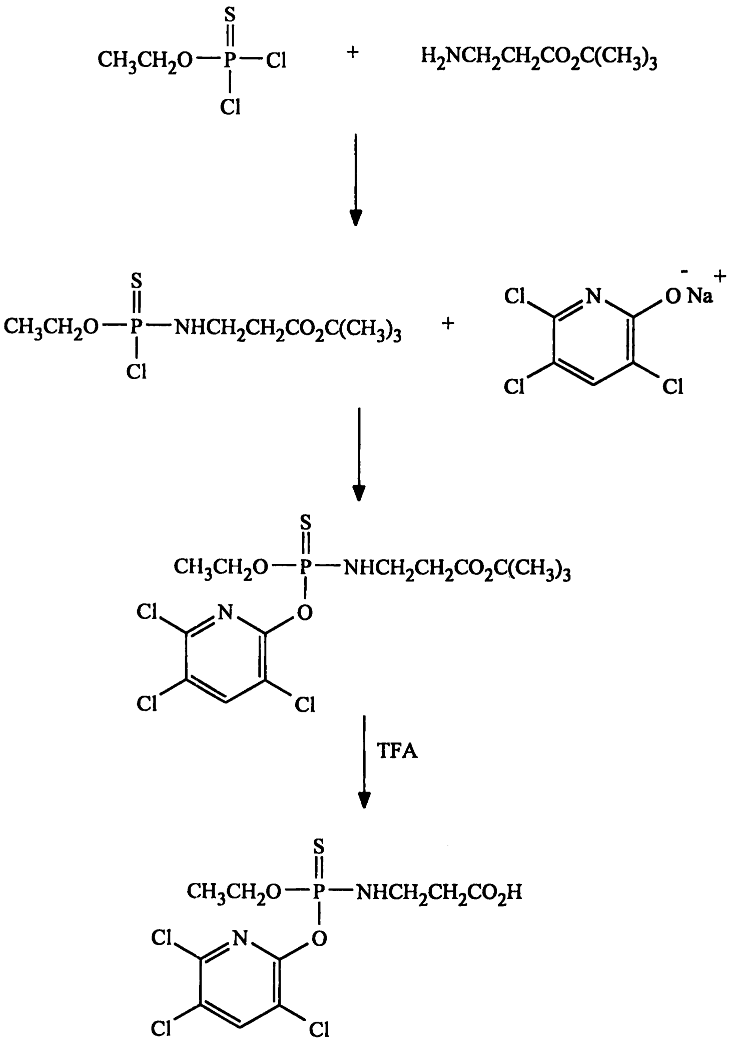
Since this work showed that antibodies raised to a phosphoramide bound with high affinity to the corresponding organophosphates, we developed a generic method for the production of antibodies to organothiophosphates (*16*, Figure 2 (8), Figure 3). This required development of a stable thiophosphoramide bifunctional reagent, *tert*-butyl-3-[chloro(methoxy)-phosphorthioylamino]-propanoate. It was prepared by first synthesizing *tert*-butyl-3-aminopropanoate (*26*) and reacting it with either O-methyl dichlorothiophosphate or its ethyl analog. The bifunctional reagent was stable for extended periods and produced the desired haptens by reaction with the sodium salt of the hydrolysis product of the target organophosphate by refluxing in acetonitrile. The *tert*-butyl protecting group was removed using trifluoroacetic acid, and the resulting carboxylic acid hapten was coupled to protein after conversion to the reactive *N*-hydroxysuccinimide ester.

Several polyclonal and monoclonal antibodies have been produced using haptens developed according to this protocol, including fenitrothion (*27*), parathion and methyl-parathion (*28,29*), chlorpyrifos (*22*), chlorpyrifos-methyl (*23,30*) and pirimiphos-methyl (*30*). The limits of detection achieved with rabbit polyclonal antibodies fall into the range 0.01–1 ppb, even though in general we have not employed hapten heterology. In other words, we have used the same hapten and spacer arm for coupling to the carrier protein for antibody production as we have for the peroxidase conjugate. In most cases, the antibodies were quite specific for the pesticide that was used to develop the corresponding immunogen, although the chlorpyrifos assay detected chlorpyrifos-methyl, the pirimiphos-methyl assay detected pirimiphos-ethyl and the parathion assay detected methyl-parathion. The latter assays also detected fenitrothion, which differs from methyl-parathion only by the presence of a methyl group at the 3-position. Immunoassays based on most of these haptens have been commercialized in microwell and tube formats (*31,32*), and application of the assays were made to several matrices, including grain products, irrigation drainage water, and fruit juices.

Diazinon is an important organophosphate insecticide of relatively high persistence. It is widely used in horticulture, turfgrass management and animal health applications, and is one of the most widely used organophosphorus insecticides in the US. Although it is a close analog of pirimiphos-ethyl (the difference being that the side chain on the pyrimidine ring in pirimiphos-ethyl is $-N(CH_3)_2$ rather than $-CH(CH_3)_2$), this difference made diazinon unstable in both acid and base, whereas most other organophosphates are quite stable under acidic conditions. Under both the conditions used for deprotection of the *tert*-butyl ester used with the β-alanine spacer arm (brief treatment with trifluoroacetic acid) and in a more gentle deprotection of a trimethylsilyl group used as an alternative, the zwitterion intermediate that formed spontaneously hydrolyzed with formation of the pyrimidone. This problem was solved by the use of a neutral spacer arm (3-aminopropanol) rather than the amino acid, β-alanine. The activated form of the diazinon hapten could be synthesized in three steps: 1) Ethyl dichlorothiophosphate was reacted with the sodium salt of the pyrimidone formed by alkaline hydrolysis of diazinon; 2) The chlorine atom bound to the phosphorus atom was then displaced by the amino substituent of 3-aminopropanol; 3) The hydroxy-group on this hapten was then activated for coupling to protein by reaction with carbonyldiimidazole.

These haptens provided a highly sensitive, homologous assay for diazinon using an immobilized antibody (Skerritt and Beasley, CSIRO Division of Plant Industry at

In Immunoassays for Residue Analysis; Beier, R., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.



CH3CH2O—P(=S)(Cl)—Cl
+
H2NCH2CH2CO2C(CH3)3
CH3CH2O—P(=S)(Cl)—NHCH2CH2CO2C(CH3)3
+
Cl
N
O⁻ Na⁺
Cl
Cl
CH3CH2O—P(=S)(O)—NHCH2CH2CO2C(CH3)3
TFA
CH3CH2O—P(=S)(O)—NHCH2CH2CO2H

Figure 3. The synthetic scheme for the chlorpyrifos hapten developed by McAdam and Skerritt (*25*).

Canberra and North Ryde, Australia, unpublished) and a diazinon-peroxidase conjugate. The assay could detect at least 0.02 ppb diazinon in water and exhibited minimal cross-reaction with structurally-related compounds such as pirimiphos. It has been applied to analysis of diazinon residues in various matrices, including lanolin (*33*) and fruit juices (*34*).

Phosphorothiolothionates. Malathion (Figure 1) was one of the first agrochemicals to be studied with respect to immunochemical responses. Conjugates of the metabolites produced by partial hydrolysis of the ethyl-ester, either as a monoacid or as the anhydride derivative of the diacid derived from [O,O-dimethyl-S-(1,2-biscarboxyethyl) phosphorodithioate], were coupled to a carrier protein and shown to produce precipitating and hemagglutinating antibodies in rabbits (*35,36*), and an IgE-mediated allergic response in mice and rats also has been elicited following immunization with protein conjugates of the anhydride derivative (*37,38*). Although malathion has retained considerable importance as an agrochemical, these early haptens have not to date been used in a published, sensitive enzyme-immunoassay. The alternative approaches for "generic hapten synthesis" proposed by groups in New Zealand and the UK (*39–41*, see below) could provide potential routes to hapten synthesis for malathion immunoassays, but such immunoassays have not yet been described.

Recently, a new method for synthesis of organophosphate haptens has been described by New Zealand scientists (Figure 4). It also provides a means for coupling through the phosphate ester, but preserves a dialkyl ester functionality. Thus, instead of one of the phosphorus-ethoxy/methoxy bonds being replaced by a P-N-spacer arm linkage, the P-(O-CH_2)$_2$- motif is retained by incorporation into a six-membered ring that contains one phosphorus, two oxygen and three carbon atoms. The carbon atom opposite the phosphorus atom is connected to a -CH_2OCH_2COOH spacer arm which is used for coupling to carrier proteins. Thus, a rigid ring is formed which is quite disimilar to the other organophosphate haptens, in which the methoxy or ethoxy groups are freely rotating. The advantage of this method, which was initially used to develop monoclonal antibodies to azinphos-methyl (*39*), is that it also can be adapted to a range of organophosphate insecticides, although some modifications as to how the leaving group is presented must be made for each assay. The monoclonal antibodies to azinphos-methyl were highly sensitive, detecting down to 0.05 ppb, and cross-reacting only with azinphos-ethyl. The method has also been used to develop antibodies to several phosphorothionates, including parathion, paraoxon, chlorpyrifos and demeton (*40*). Significant (30–80%) cross-reaction of the parathion antibodies with fenitrothion was noted.

Attempts to Develop Broad-Specificity Organophosphate Assays. Although many of the initial derivatives of parathion (O,O-diethyl-O-4-nitrophenyl phosphate) were coupled distally to the phosphate ester, in most cases the resultant antibodies were quite specific for parathion and did not detect other organophosphates of the structure: $(EtO)_2$-P(S)-O-Ar. Initial attempts to develop broad-specificity antibodies using a simpler hapten design were made by Sudi and Heeschen (*42*). In this work, three phosphates, 4-[(diethoxyphosphinyl)oxy]benzynepropanoic acid

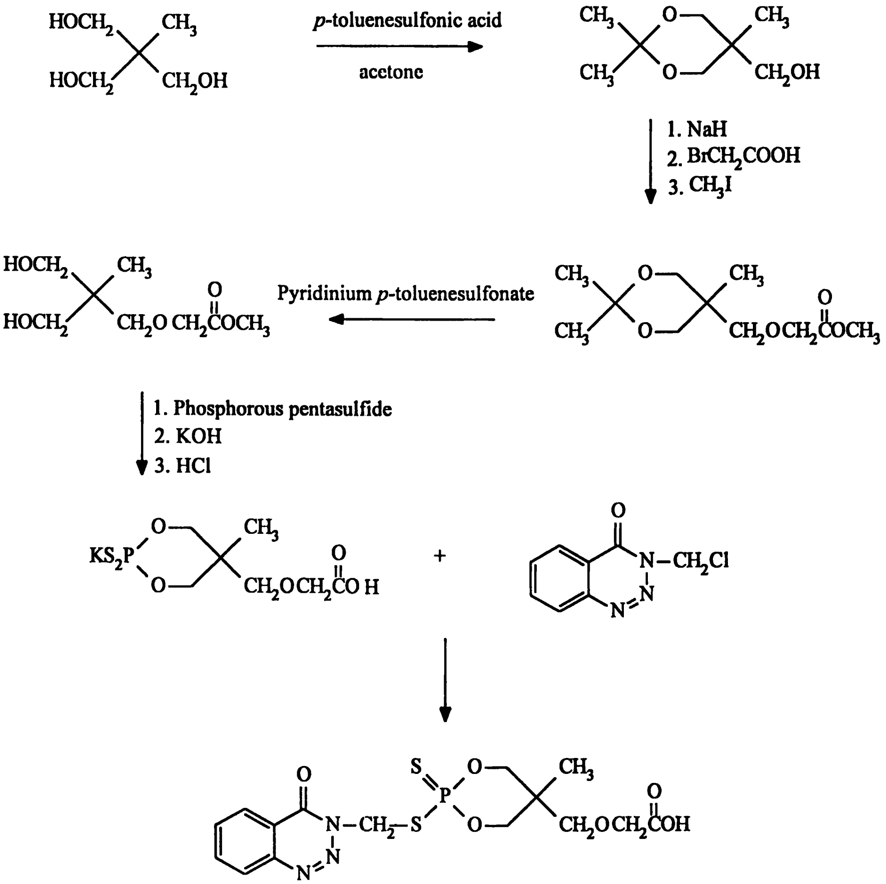

p-toluenesulfonic acid
acetone
1. NaH
2. BrCH2COOH
3. CH3I
Pyridinium p-toluenesulfonate
1. Phosphorous pentasulfide
2. KOH
3. HCl
+

Figure 4. The synthetic scheme for azinphos haptens developed by Jones (*38–39*).

(which is similar to parathion but contains a -CH_2-CH_2-COOH group instead of the nitro-group, and P(O) rather than P(S), 4-(diethoxyphosphinyl)-2-butenoic acid and phosphorochloridic acid diethyl ester were reacted with poly-L-lysine and bovine serum albumin carriers. A series of indirect enzyme-immunoassays using various combinations of immunogen and coating antigen was developed. The aim of developing systems that detect a wide range of organophosphate insecticides was achieved, although the LLD was rather poor, with 50% inhibition for most diethyl thiophosphates (which are included under phosphorothionates) and diethyl dithiophosphates (refered to as phosphorothiolothionates above), in the range of 100 ppb to 1 ppm. Nonetheless, the approach may have considerable value, especially if an alternative format, based on solid-phase antibody, provided enhanced sensitivity utilizing an immobilized antibody (*17,43*).

Recently, workers at the UK Ministry of Fisheries and Food have submitted a patent application claiming the use of a single antibody reagent to screen for several members of a group of phosphorothionates and phosphorothiolothionates (*41*). The type of haptens claimed in the application include compounds of the type $(RO)_2$-P(S)-Z-Y-B-D, where R = Ethyl, Methyl or other lower alkyl; Z = O, S or NH, Y = "a spacer", B = CO- or O-CO- and D = an activating group. The main difference between these studies and the earlier work of Sudi and Heeschen (*41*) is that the hapten may have P-S bonds as well as P-O bonds. It will be interesting to compare the utility of such antisera with broad-specificity assays based on cholinesterase inhibition. The advantage of broad-specificity assays is their utility in screening for "any" target organophosphate in a mixture; the disadvantage is that different compounds almost invariably cross-react to different extents, thus it is quite possible that one compound at a level well below the legal maximum residue limit will provide a similar response to another at a violating level.

Use of Stable Analogs of Organophosphates in Immunoassay. The greater lability of organophosphorus insecticides, compared with the organochlorines that they largely replaced, has been mentioned above (*2*). The least stable component in any competitive immunoassay using immobilized antibody is typically the enzyme-pesticide conjugate. Instability can arise from loss of enzyme activity and/or hydrolysis of the spacer arm or hydrolysis of the coupled pesticide. This factor is especially important in the development of field test kits, where reagents should ideally be stable for repeated periods of several hours away from refrigeration and be able to be used without need for reagent reconstitution or need for further dilution. Duquette *et al.* (*44*), in developing a fieldable kit for paraoxon, decided to use an analog for conjugate development, namely diethyl 4-aminobenzylphosphorate. This compound differs from aminoparaoxon in that it has a methylene group between the phosphorus atom and the aromatic ring; this makes it somewhat less prone to hydrolysis than the ester. We have used a different approach in developing a more stable conjugate. The inductive effect of the pyridine nitrogen atom in chlorpyrifos contributes to its tendency to hydrolyze. The benzene analog of chlorpyrifos-methyl is a known pesticide (fenchlorphos), and we used the trichlorophenol derived from this compound to develop haptens for immunoassay of chlorpyrifos-methyl. As predicted, the conjugates so derived had enhanced stability on long-term storage.

Synthetic Pyrethroids

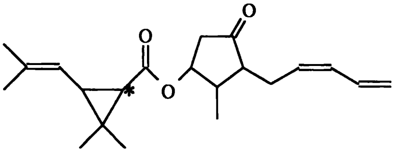
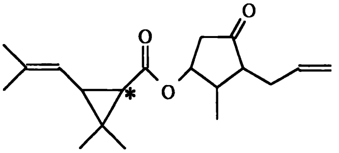
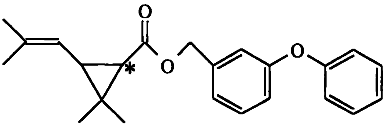
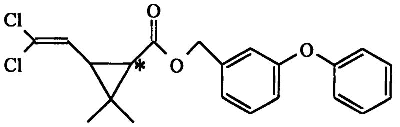
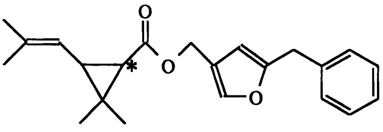
While the initial group of insecticidal compounds with chrysanthemic or pyrethric acid moieties were naturally occurring pyrethrins from the flower of *Chrysanthemum cinerariaefolium*, there are no documented attempts to develop immunoassays specifically for these compounds. The focus on both insecticide development and immunoassay has been on synthetic pyrethroids, which have greater photostability, hydrolytic stability and thus persistence than many of their natural counterparts (*45*). The cross-reaction of some of the pyrethrins has, however, been studied with antibodies to the synthetic analogs (see below). The synthetic pyrethroids can notionally be divided into two groups of compounds on the basis of chemical structure and putative mechanism of action at insect target sites. The "type I" compounds (Table I) are simple esters of 2,2-dimethyl-3-(2-methyl-1-propenyl) cyclopropanecarboxylic acid with another cyclic group, and include the natural pyrethrins as well as some synthetic pyrethroids, and "type II" compounds contain an ester of an arylcyanohydrin (*46*; Table II). These compounds have different toxicological effects and may well have slightly different mechanisms of action at the insect neuron. The carbon-cyano bond alpha to the ester also engenders the type II compounds with greater stability to photolysis. The development of immunoassays for synthetic pyrethroids has mirrored their order of discovery, with initial work carried out on bioallethrin, then permethrin, phenothrin and bioresmethrin, and more recently the type II compounds. Initial research was directed towards development of immunoassays for the type I compounds.

Type I Pyrethroids.

Bioallethrin. The first immunoassay developed for pyrethroid insecticides was for bioallethrin (1*R*,3*R*,4'*S*-isomer of allethrin) (*47*). The hapten was prepared by introducing a hemisuccinate linker at the side chain of the cyclic moiety of *S*-bioallethrin via a hydroboration-oxidation reaction (Figure 5). The resulting anti-Markovnikov alcohol still retained the stereochemistry of *S*-bioallethrin and thus the antibodies produced from the hapten were specific for *S*-bioallethrin. The antibody did not cross react with (-)-*cis-(R)*-allethrin, 1*S*,3*R*-chrysanthemate and 4'*R*-allethrin, suggesting that the configurational or geometrical specificity of the antibody was important in developing an *S*-bioallethrin-specific antibody (*48*). Initial work demonstrated that both free hapten and bioallethrin at low ppm concentrations inhibited an antigen-specific precipitin reaction; the corresponding radioimmunoassay was somewhat more sensitive (*48*).

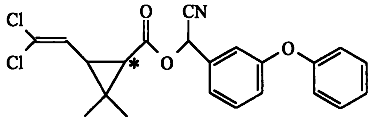
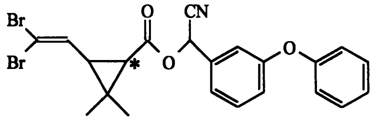
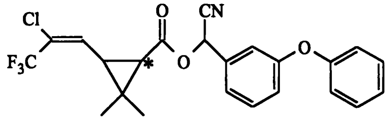
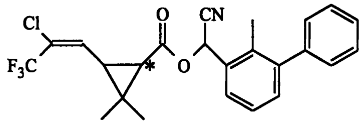
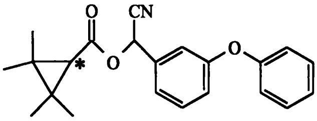
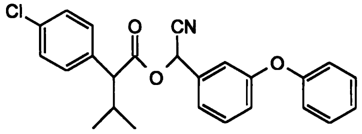
Permethrin/Phenothrin. A different approach was used for preparation of the hapten for permethrin and phenothrin immunoassay (*49*). Ozonolysis of the vinylic side chain of phenothrin was performed to produce a carboxylic acid, followed by the direct coupling of the acid to a carrier protein using the mixed anhydride reaction. The antibody produced from this hapten was selective for permethrin and phenothrin, with 1–10% cross-reaction with some type II compounds. A more sensitive assay was developed using a coating antigen based on phenoxybenzoic acid coupled to

Table I. Type I pyrethroids

Formula	Common Name	Stereochemistry	No. of Isomers
	Pyrethrin I	1R *trans*; S	1
	Bioallethrin	1R *trans*; S	2
	Phenothrin	(±) *cis/trans*	4
	Permethrin	(±) *cis/trans*	4
	Bioresmethrin	1R *trans*	1

* indicates carbon at position 1

Table II. Type II pyrethroids

Formula	Common Name / Stereochemistry / No. of Isomers
	Cypermethrin (±) *cis/trans* 8
	Deltamethrin 1R *cis* α S 1
	Lambda cyhalothrin (±) *cis*, Z 4
	Bifenthrin (±) *cis*, Z 2
	Fenpropanate, Fenpropathrin (±) 2
	Fenvalerate (±) 4

* indicates carbon at position 1.

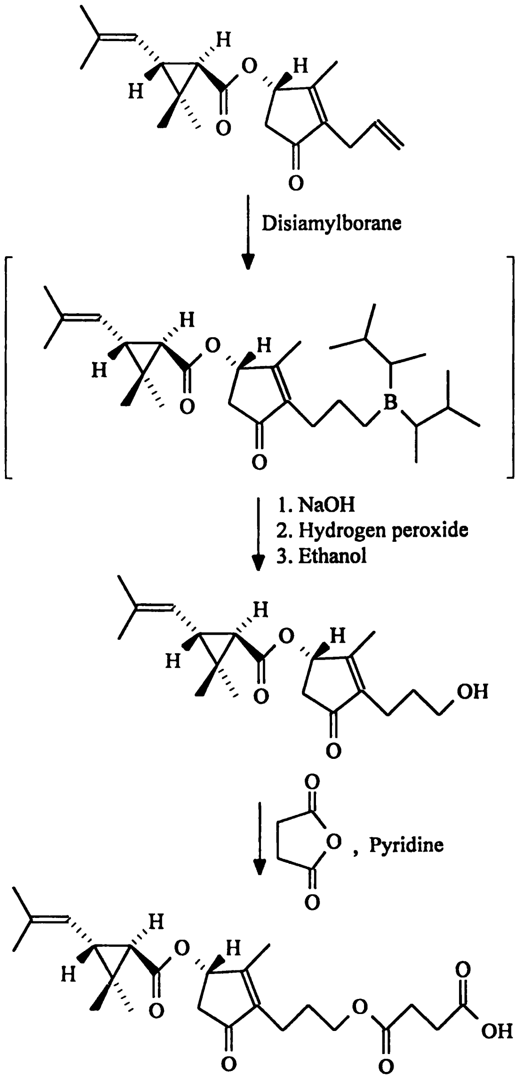

H
O
H
O
O
Disiamylborane
B
1. NaOH
2. Hydrogen peroxide
3. Ethanol
OH
O
O
O
, Pyridine
OH

Figure 5. The synthetic scheme for a bioallethrin hapten developed by Wing and Hammock (*46,47*).

serum albumin; a further improvement of the sensitivity was achieved when phenoxybenzoic acid was coupled to the enzyme via the formation of an amide using a β-alanine spacer arm and incorporated in a direct enzyme-immunoassay format (*49*). The most sensitive assay format detected 1.5 ppb of permethrin. The first assay was used for permethrin detection in meat (*49*) and more recently for flow injection immunoanalysis (*51*). The latter was applied to analysis of residues of permethrin and phenothrin in wheat grain and milling fractions (*50*).

Bioresmethrin. A different synthetic route was attempted for synthesis of the hapten for bioresmethrin, a non-cyano pyrethroid with a benzyl furan rather than a phenoxybenzyl moiety (Figure 6). In this case, the spacer arm also was introduced on the vinylic side chain of bioresmethrin, but a different strategy was required, since the benzylfuran moiety was destroyed upon ozonolysis (*52*). The spacer arm had either a 2 or 6 carbon chain length, and a trimethylsilyl protecting group was introduced into the 1*R*-*trans*-chrysanthemic acid before the oxidative cleavage of the vinyl side chain with $KIO_3/KMnO_4$ to enable formation of an acid. The esterification of the above acid with phenoxymethyl furyl alcohol was followed by the deprotection and formation of an *N*-hydroxysuccimidyl active ester for conjugation. A bulky spacer arm, containing an aromatic functional group, was used to reduce the relative affinity of the antibodies for the conjugate compared with free bioresmethrin in standards and test samples. This resulted in a sensitive immunoassay for bioresmethrin, with the LLD of 2 ppb in buffer. Fifty percent cross-reaction with resmethrin (the (+)-*cis/trans*-form) suggested that the antibody had selectivity for the (+)-*trans*-isomer. There was little reaction with other pyrethroids. This assay was applied to various grain matrices for the quantitative analysis of bioresmethrin residues.

Type II Pyrethroids.

Coupling at the Aromatic Moiety. Demoute *et al.* (*53*) were the first to attempt hapten synthesis of a cyano-pyrethroid, namely deltamethrin. The approach was to introduce a linker on the 3-phenoxybenzyl end of the molecule. The synthesis involved six steps (Figure 7): 1) formation of dioxolanyl phenoxybenzaldehyde; 2) converted to 2-[3-(4-(1,1-dimethylethoxy)-1-oxopropenyl)-phenoxy]-phenyl-1,3-dioxolane using O,O-diethyl-tert-butoxycarbonylmethyl phosphate in a Wittig-Horner reaction; 3) simultaneous reductions of the alkene accompanied by an exchange of the aldehyde protecting group from a dioxolane to the dimethylacetal (shown) plus the reduced acetal (a methyl ether) as a consequence of using methanol as the solvent for the reaction; 4) formation of an aromatic aldehyde; 5) acylation of the cyanohydrin with deltamethric acid chloride; and 6) removal of the *tert*-butyl protecting group. This hapten was labeled with iodine and used in a radioimmunoassay. In developing the immunoassay for the human metabolites of fenvalerate and fenpropathrin, Wengatz *et al.* (*54*) also synthesized the pyrethroid analogs with amine substitution on the aromatic group. The limits of detection of the assays developed from these haptens varied from 2 ppb to 13 ppb for the phenoxybenzyl metabolite and 4 ppb for fenvalerate metabolite.

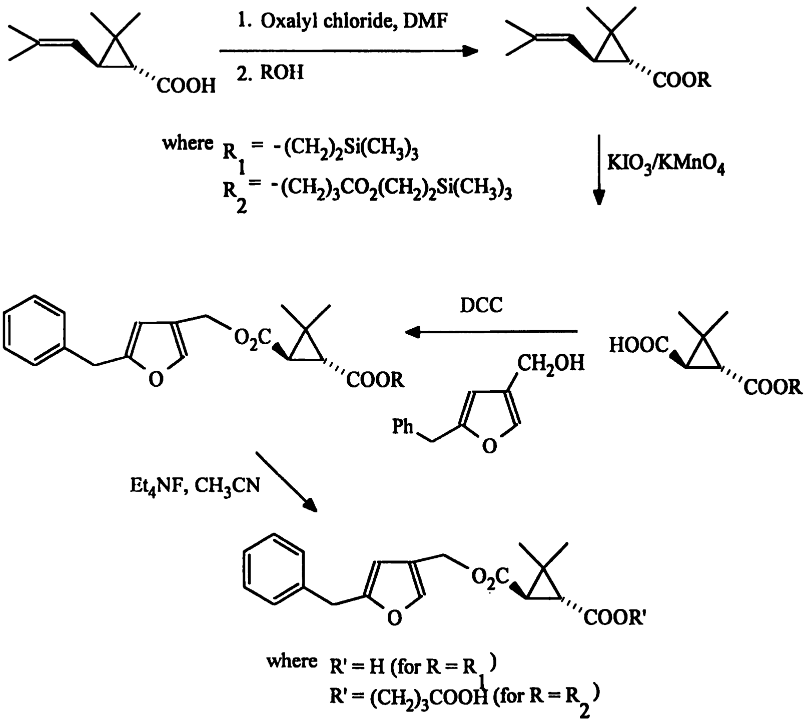

COOH
1. Oxalyl chloride, DMF
2. ROH
COOR
where R_1 = $-(CH_2)_2Si(CH_3)_3$
R_2 = $-(CH_2)_3CO_2(CH_2)_2Si(CH_3)_3$
$KIO_3/KMnO_4$
HOOC
COOR
DCC
CH_2OH
Ph
O
O_2C
COOR
O
Et_4NF, CH_3CN
O_2C
COOR'
O
where R' = H (for R = R_1)
R' = $(CH_2)_3COOH$ (for R = R_2)

Figure 6. The synthetic scheme for bioresmethrin haptens (*51*).

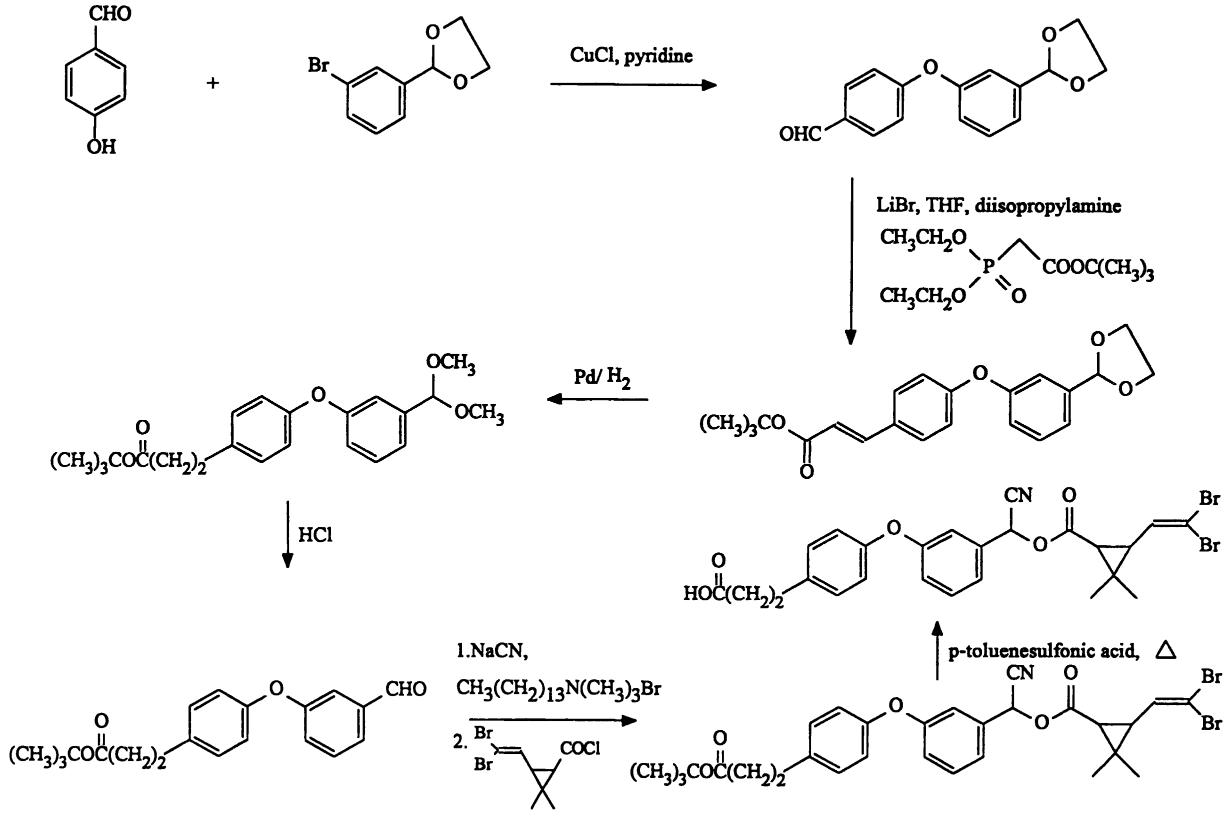

CHO
OH
+
Br
CuCl, pyridine
OHC
LiBr, THF, diisopropylamine
CH3CH2O
P
COOC(CH3)3
CH3CH2O
O
(CH3)3CO
O
Pd/ H2
OCH3
OCH3
O
(CH3)3COC(CH2)2
HCl
CHO
1.NaCN,
CH3(CH2)13N(CH3)3Br
2. Br
Br
COCl
CN
Br
Br
HOC(CH2)2
p-toluenesulfonic acid, △

Figure 7. The synthetic scheme for a deltamethrin hapten developed by Demoute *et al.* (*52*).

A simple two-step reaction has been used in our laboratory to introduce a spacer on the aromatic moiety. Deltamethrin hemisuccinate was prepared by the esterification of deltamethrin alcohol with succinic acid. The deltamethrin alcohols (at the 2- and 4-positions) were prepared using an organometallic reagent, lead tetratrifluoroacetic acid (LTTFA, *55*, Figure 8). As noted by the original authors, the yield of the alcohols produced in this manner was very poor, but sufficient to develop haptens for immunization and enzyme conjugation. Although the antibodies prepared using this strategy provided relatively low sensitivity assays, the enzyme conjugates so derived were useful in combination with other antibodies.

The tendency of deltamethrin to isomerize by chemical or photochemical means has been noted by several groups (*56,57*). The isomerization of deltamethrin in solvents such as methanol, acetone and acetonitrile is due to proton exchange with the solvent, and this reaction only occurs with compounds having a cyano group such as cyfluthrin, cypermethrin and lambda cyhalothrin. The isomerization can be hastened in the presence of base. The most sensitive assay using the enzyme conjugate prepared from the hapten prepared in Figure 8 (together with an antibody prepared from a hapten coupled at the opposite end of the molecule, see below), provided a LLD of about 20 ppb with freshly-prepared deltamethrin, but 0.4 ppb after a brief isomerization treament.

Conjugation Through the Middle of the Molecule. In designing different hapten structures, the linker also was placed in the middle of the pyrethroid (Figure 9, Tables IA and IB). Esterification of 3-phenoxymandelic acid with pyrethric acid chloride (racemic mixture) produced a pyrethroid analog containing a carboxylic acid instead of a cyano-substituent, enabling the formation of an amide derivative with *tert*-butyl-β-alanine (Figure 9). Three different pyrethric acids, deltamethric acid, permethric acid and cyhalothric acid, were employed here to obtain structurally different haptens on the cyclopropane moiety. Cross-reaction studies suggest that these antibodies show preference for the cyclopropane end of the molecule. Several of these assays detect non-isomerized deltamethrin at about 5 ppb, however, somewhat greater sensitivity was observed with isomerized compounds, especially deltamethrin. The most sensitive assay based on these hapten structures detected 0.2 ppb of isomerized deltamethrin. In most cases the portion of the molecule that is distal to the point of attachment is best recognized by the antibody. Yet the antibodies raised against the full pyrethroid structure have preference for the more polar vinyl cyclopropane moiety over the aromatic moiety.

Synthesis of Metabolite Analogs. Attempts to synthesize haptens using the pyrethroid metabolites was first reported by Wraith *et al.* (*58*). The antibody was produced to a protein conjugate prepared via the three-carbon chain length spacer arm with 3-phenoxybenzoic acid and dichlorovinyldimethylcyclopropane carboxylic acid. The antibody recognized 3-phenoxybenzoic acid and cypermethrin, but not dichlorovinylcyclopropane carboxylic acid. The assay was applied to soil and water even though the LLD was very high, with an IC_{50} of 1 ppm. Direct coupling of 1*R*-*cis*-permethric acid, 1*R*-*trans*-permethric acid and 3-phenoxybenzoic acid to the carrier proteins via carbodiimide activation of the carboxylic acids was used by Pullen

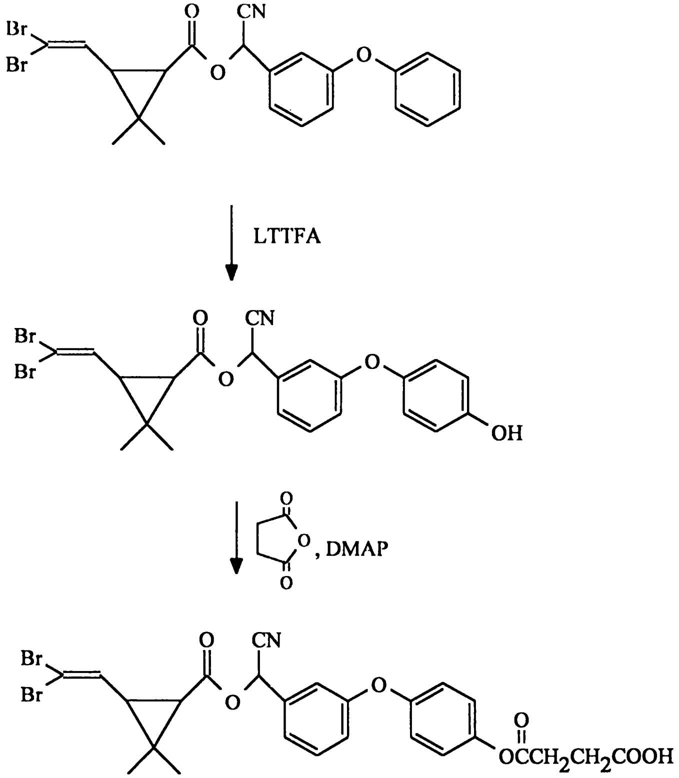

Br
Br
O
CN
O
O
LTTFA
OH
O
O
O
, DMAP
OCCH2CH2COOH

Figure 8. The synthetic scheme for deltamethrin hapten developed by Lee and Skerritt (unpublished). DMAP = *N,N'*-dimethylaminopyridine; LTTFA = lead tetra trifluoroacetate.

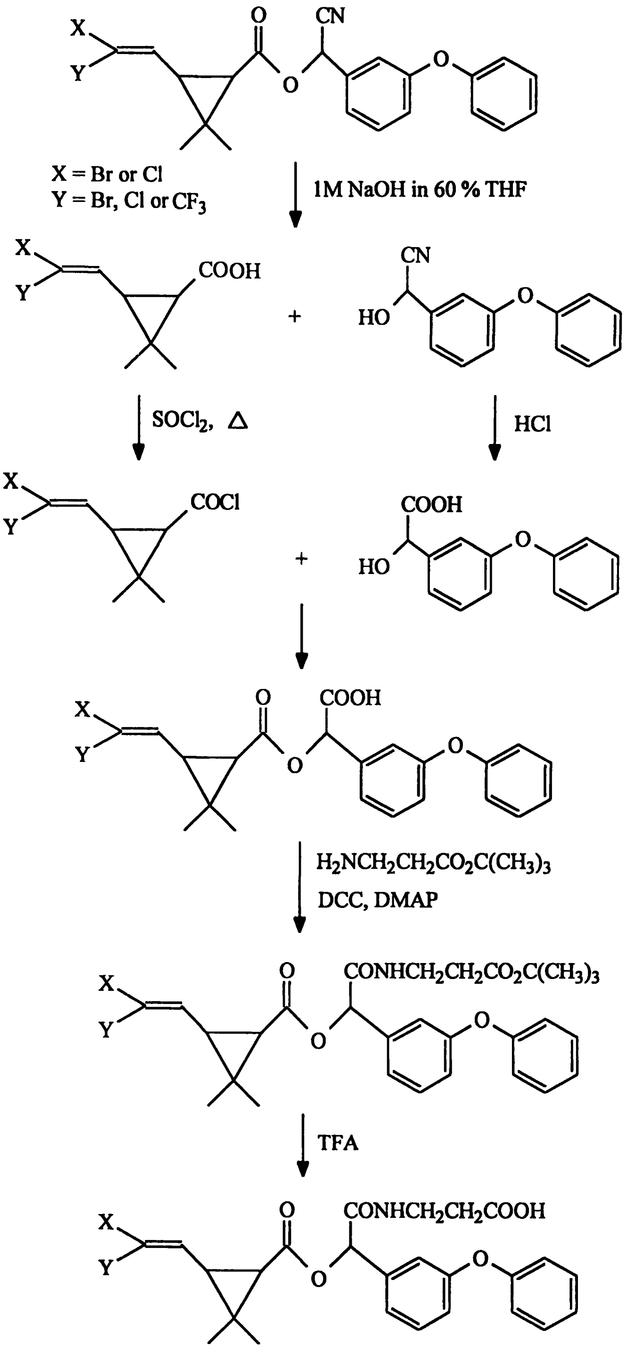

X = Br or Cl
Y = Br, Cl or CF_3
1M NaOH in 60 % THF
$SOCl_2$, Δ
HCl
$H_2NCH_2CH_2CO_2C(CH_3)_3$
DCC, DMAP
TFA
COOH
CN
HO
COCl
$CONHCH_2CH_2CO_2C(CH_3)_3$
$CONHCH_2CH_2COOH$

Figure 9. The synthetic scheme for pyrethroid haptens, based upon coupling through the middle of the compound (Lee and Skerritt, unpublished). DCC = dicyclohexylcarbodiimide. DMAP = dimethylaminopyridine.

and Hock (*59,60*). Both polyclonal and monoclonal antibodies were generated, showing different cross reactions. The monoclonal antibody (*59*) detected allethrin, bioallethrin, *S*-bioallethrin, permethrin, bioresmethrin and pyrethrin, but allethrin was detected with the most sensitivity with a LLD of 1 ppb. The polyclonal antibody (*60*) detected permethrin, cyfluthrin, cypermethrin, *S*-bioallethrin and *trans*-permethric acid with a detection limit of about 0.4 ppb. Good correlations were obtained between levels of permethrin spiked and recovered in buffer. Bonwick *et al.* (*61*) have also reported the synethesis of haptens based on permethrin hydrolysis products, but in this case using either 6-aminohexanoic acid or 4-aminobutanoic acid spacer arms to link the permethric and phenoxybenzoic acids to protein carriers. The best assay, using the latter hapten, was not particularly sensitive (IC_{50} of 1 ppm, LLD of 2 ppb), but a good correlation was obtained between the levels of permethrin measured in sediment samples using immunoassay and GC/MS.

We also have synthesized haptens using the pyrethric acids of deltamethrin, cypermethrin and lambda-cyhalothrin, and the phenoxybenzyl moieties, namely 3-phenoxybenzoic acid and 3-phenoxybenzaldehyde cyanohydrin. Each hapten was prepared via a 3-carbon chain length linker. The antibody produced from 3-phenoxybenzoic acid, in general, detected compounds with the phenoxybenzyl moiety such as cypermethrin, deltamethrin, lambda cyhalothrin, permethrin, esfenvalerate, fluvalinate and phenothrin. In some cases, greater sensitivity was achieved with the isomerized forms of the above compounds. Although the sensitivities of some of these assays were rather low for the un-isomerized forms, the assay could be made generic for phenoxybenzyl compounds. For deltamethrin, the most sensitive assay had a LLD of 0.6 ppb.

For antibodies generated from pyrethric acids, preference for compounds with the vinyl cyclopropane moiety was observed, and the detection of halovinyl cyclopropane compounds was predominant in one case. Several assays using these antibodies also could detect non-isomerized deltamethrin at about 6 ppb. The assay with the lowest limit of detection for deltamethrin used antibodies raised against the deltamethric acid analog; the detection limit for this assay was 0.15 ppb of isomerized deltamethrin. Three enzyme conjugates based upon three different haptens coupled via a spacer arm containing an aromatic group were synthesized: a "bulky" spacer arm was placed on: 1) a hapten based on cypermethrin and conjugated through the middle of the molecule, 2) a hapten based on deltamethric acid, and 3) a hapten based on the phenoxybenzyl moiety. Only the third enzyme conjugate exhibited high sensitivity, with an LLD of 0.4 ppb of isomerized deltamethrin.

Coupling Only the Cyclopropane Moiety. Coupling via the cyclopropane moiety was achieved by oxidation of the dimethylvinyl group. The overall synthetic scheme is very much like that for bioresmethrin, except for the protecting group (*tert*-butyl ester); in addition a racemic mixture of chrysanthemic acid was used. The most sensitive assay gave a limit of detection of 0.4 ppb for deltamethrin.

Conclusions

While there have been over thirty papers on the development of immunoassays for organophosphates and synthetic pyrethroids, using a wide range of innovative hapten synthesis strategies, the production of assays for some of the most important compounds is by no means complete. For example, as of early 1995 there are no commercially available assays for either malathion or the type II pyrethroids, despite their importance in agriculture and residue analysis. This contrasts with the situation for key herbicides such as atrazine, for which there are about half a dozen separate kits now commercially available. Regarding the organophosphates, the relatively simple strategy of diazotization for hapten synthesis has met with rather limited success. The routes of greatest value or potential value would appear to be by derivatization through the phosphate ester (this exposes the leaving group for production of specific antibodies) and development of methods to produce broad specificity antibodies for "generic" organophosphate immunoassays.

Several strategies have lead successful development of immunoassays for synthetic pyrethroids, although the published studies to date pertain largely to the type I compounds. The success of immunoassay procedures is very much dependent on the stereochemistry of the hapten, since the synthetic pyrethroids have several stereoisomers and often only some of these are insecticidally active. This might not matter if the insecticidal form were metabolized under different conditions to the same products, but this may not be the case. One of the difficulties in developing immunoassays for pyrethroids is to maintain the stereochemistry of the target isomer(s) during hapten synthesis. The stereochemistry of type I haptens is more readily maintained by an appropriate selection of reaction conditions and by starting the synthesis with the appropriate isomer.

The development of an immunoassay for type II compounds has been more challenging. Several strategies for spacer arm attachment have been attempted to raise different specific antibodies: 1) at the aromatic moiety; 2) in the middle of the compound; 3) at the vinylcyclopropane moiety (in many cases using half molecule derivatives for either the immunogen or conjugate synthesis). We found that a more sensitive assay was obtained when the enzyme conjugate used in the assay was coupled to a different hapten (i.e., hapten heterology); this contrasts with our experience in the development of organophosphate immunoassays. Generally, the serum titer obtained was lower for full molecule haptens than the half molecule haptens, probably due to the hydrolysis of the hapten before it can be recognized by the immune system of the animal. *In vivo* hydrolysis may account for the observation that despite the efforts of several independent research groups, the immunoassays for pyrethroids developed thus far have lower sensitivities than immunoassays for other agrochemicals, including organophosphates, triazines and sulfonylureas. Other factors, such as isomerization, the lack of nitrogen atoms and the significant hydrophobicity of the pyrethroids also may be important.

Acknowledgments. The authors are grateful to several colleagues and collaborators for advice and assistance, including M. Daunt, R. J. Haseler, D. P. McAdam and A. J. Rigg (CSIRO), J. Elix, M. Rasmussen and M. G. O'Shea (Australian National

University), B. Hock and S. Pullen (Technical University of Munchen, Germany), B. S. Ferguson (ImmunoSystems Inc., ME, USA) and B. D. Hammock, I. Wengatz and S. Gee (University of California, Davis, CA, USA).

Literature Cited

1. Gee, S.; Hammock, B. D.; Skerritt, J. H. In *New Diagnostics for Crop Sciences*; Skerritt, J. H.; Appels, R. A., eds.; CAB Press: Wallingford, UK, 1995; pp 243–276.
2. Hassall, K. A. *The Biochemistry and Uses of Pesticides*; VCH Verlagsgesellschaft: Weinheim, Germany, 1990, 2nd Edn.; pp 81–207.
3. Marco, M.-P.; Gee, S. J.; Cheng, H. M.; Liang, Z. Y.; Hammock, B. D. *J. Agric. Food Chem.* **1993**, *41*, 423–430.
4. Abad, A.; Montoya, A. *J. Agric. Food Chem.* **1994**, *42*, 1818–1823.
5. Brady, J. F.; Fleeker, J. R.; Wilson, R. A.; Mumma, R. O. In *Biological Monitoring for Pesticide Exposure*; Wang, R. G. M.; Franklin, C. A.; Honeycutt, R. C.; Reinert, R. C., eds.; American Chemical Society: Washington, DC, 1989, ACS Symposium Series No. 382; pp 262–284.
6. Itak, J. A.; Selisker, M. Y.; Herzog, D. P. *Chemosphere* **1992**, *24*, 11–21.
7. Charlton, R. R.; Majaran, W. R.; Carlson, C. W.; Carski, T. H.; Liberatore, F. A.; Kenyon, L. H.; Allison, D. A.; Steele, W. J.; Fraser, A. *Environmental Monitoring of Crop Protection Chemicals by Immunoassay*; Proc. 105th AOAC meeting, AOAC International: Phoenix, AZ, 1991, Abstract No. 326.
8. Hunter, K. W.; Lenz, D. E.; Brimfield, A. A.; Naylor, J. A. *FEBS Lett.* **1982**, *149*, 147–151.
9. Buenafe, A. C.; Rittenburg, M. B. *Immunology* **1991**, *73*, 398–406.
10. Worthing, C. R.; Walker, S. B. *The Pesticide Manual—A World Compendium*; British Crop Protection Council: Thornton Heath, UK, 1987, Eighth Edn.
11. Al-Rubae, A. Y. *The ELISA: A New Method For the Analysis of Pesticide Residues*; PhD Thesis, Pennsylvania State University, *Dissertation Abstracts International* **1978**, *39*, 4723–4724.
12. Ercegovich, C. D.; Vallejo, R. P.; Gettig, R. P.; Woods, L.; Bogus, E. R.; Mumma, R. O. *J. Agric. Food Chem.* **1981**, *29*, 559–568.
13. Anis, N. A.; Wright, J.; Rogers, K. R.; Thompson, R. G.; Valdes, J. J.; Eldefrawi, M. E. *Anal. Lett.* **1992**, *25*, 627–635.
14. Ibrahim, A. M. A.; Morsy, M. A.; Hewedi, M. M.; Smith, C. *J. Food Agric. Immunol.* **1994**, *6*, 23–30.
15. Brimfield, A. A.; Lenz, D. E.; Graham, C.; Hunter, K. W. *J. Agric. Food Chem.* **1985**, *33*, 1237–1242.
16. Snelson, J. T. *Grain Protectants*; Australian Centre for International Agricultural Research: Canberra, Australia, 1987, Monograph No. 3.
17. McAdam, D. P.; Hill, A. S.; Beasley, H. L.; Skerritt, J. H. *J. Agric. Food Chem.* **1992**, *40*, 1466–1470.
18. Padki, M. M.; Bhide, M. B. *Indian J. Med. Res.* **1984**, *79*, 137–141.
19. Lober, M.; Krantz, S.; Hermann, I. *Acta. Biol. Med. Ger.* **1982**, *41*, 487–496.

20. Vallejo, R. P.; Bogus, E. R.; Mumma, R. O. *J. Agric. Food Chem.* **1989**, *30*, 572–580.
21. Manclus, J. J.; Primo, J.; Montoya, A. *J. Agric. Food Chem.* **1994**, *42*, 1257–1260.
22. Hill, A. S.; Skerritt, J. H.; Bushway, R. J.; Perkins, L. B.; Larkin, K.; Thomas, M.; Korth, W.; Bowmer, K. H. *J. Agric. Food Chem.* **1994**, *42*, 2051–2058.
23. Edward, S. L.; Skerritt, J. H.; Hill, A. S.; McAdam, D. P. *Food Agric. Immunol.* **1993**, *5*, 129–144.
24. Skerritt, J. H. In *New Frontiers in Agrochemical Immunoassay;* Kurtz, D. A.; Skerritt, J. H.; Stanker, L., eds.; AOAC International: Arlington, VA, 1995; pp 1–16.
25. Heldman, E.; Balan, A.; Horowitz, O.; Ben-Zion, S.; Torten, M. *FEBS Lett.* **1985**, *180*, 243–248.
26. McAdam, D. P.; Skerritt, J. H. *Aust. J. Chem.* **1993**, *46*, 959–967.
27. Hill, A. S.; Beasley, H. L.; McAdam, D. P.; Skerritt, J. H. *J. Agric. Food Chem.* **1992**, *40*, 1471–1474.
28. Ferguson, B. S.; Larkin, K. A.; Skerritt, J. H. *Monitoring Parathion and Parathion-Methyl in Ground and Surface Water with a Rapid and Sensitive Immunoassay*; Poster, 8th IUPAC Congr. Pesticide Chem.: Washington, DC, July 1994, Abstract No. 43.
29. Ferguson, B. S.; Larkin, K. A.; Bushway, R. J.; Perkins, L. B.; Skerritt, J. H.; Hill, A. S. *Low Cost, Sensitive Screening for Organophosphate Pesticides on Rice by Enzyme Immunoassay*; 209th ACS National Meeting, Anaheim, CA, April, 1995, Abstract No. BTEC-049.
30. Skerritt, J. H.; Hill, A. S.; Thwaites, H. L.; Edward, S. L.; McAdam, D. P. *J. AOAC Int.* **1992**, *73*, 519–528.
31. Beasley, H. L.; Skerritt, J. H.; Hill, A. S.; Desmarchelier, J. M. *J. Stored Prod. Res.* **1993**, *29*, 357–369.
32. Edward, S. L.; Hill, A. S.; Ashworth, P.; Matt, J. L.; Skerritt, J. H. *Cereal Chem.* **1993**, *70*, 748–752.
33. Ferguson, B. S.; Larkin, K. A.; Skerritt, J. H.; Beasley, H. L. Association of Official Analytical Chemists International: Washington, DC, 1993, Abstract No. 241.
34. Larkin, K. A.; Ferguson, B. S.; Bushway, R. J.; Perkins, L. B.; Skerritt, J. H.; Beasley, H. L. *Detection of Diazinon in Water and Fruit Juice by Enzyme-Immunoassay;* Proc. 6th Ann. California Pesticide Workshop, Sacramento, CA, 1994.
35. Centeno, E. R.; Johnson, W. J.; Sehon, A. H. *Int. Arch. Allergy* **1970**, *37*, 1–13.
36. Haas, G. J.; Guardia, E. J. *Proc. Soc. Exp. Biol. Med.* **1968**, *129*, 546–551.
37. Vijay, H. M.; Mendoza, C. E.; Lavergne, G. *Toxicol. Appl. Pharamacol.* **1978**, *44*, 137–142.
38. Cushman, J. R.; Street, J. C. *Toxicol. Appl. Pharmacol.* **1983**, *70*, 29–42.
39. Jones, W. T.; Harvey, D.; Jones, S. D.; Ryan, G. B.; Wynberg, H.; Ten Hoeve, W.; Reynolds, P. H. S. *Food Agric. Immunol.* **1995**, *7*, 9–19.
40. Jones, W. T.; Wynberg, H.; Ten Hoeve, W. *Immunological Detection of*

Organophosphates; World Patent Application PCT WO 93/17030, September 2, 1993.

41. Banks, J. N.; Chaudhry, M. Q.; Matthews, W. A. *Novel Hapten-Protein Conjugates Useful for Generating Antisera—for Detection of Organophosphorus Pesticides, e.g., Methacrifos*; World Patent Application 9427149, filed November 24, 1994.
42. Sudi, J.; Heeschen, W. *Kiel. Milchwirtsch. Forschungsber* **1988**, *40*, 179–203.
43. Hill, A. S.; Mei, J. V.; Yin, C.-M.; Ferguson, B. S.; Skerritt, J. H. *J. Agric. Food Chem.* **1991**, *39*, 1882–1886.
44. Duquette, P. H.; Guire, P. E.; Swanson, M. J. In *Field Screening Methods for Hazardous Waste Site Investigations*; First International Symposium, US Environmental Protection Agency: Las Vegas, NV, October 11–13, 1988; pp 239–242.
45. Elliott, M.; Janes, N. F. *Chem. Soc. Rev.* **1978**, *7*, 473–505.
46. Gammon, D. W.; Brown, M. A.; Casida, J. E. *Pestic. Biochem. Physiol.* **1981**, *15*, 181–189.
47. Wing, K. D.; Hammock, B. D.; Wunster, D. A. *J. Agric. Food Chem.* **1978**, *26*, 1328–1333.
48. Wing, K. D.; Hammock, B. D. *Experientia* **1989**, *35*, 1619–1620.
49. Stanker, L. H.; Bigbee, C.; Van Emon, J.; Watkins, B.; Jensen, R. H.; Morris, C.; Vanderlaan, M. *J. Agric. Food Chem.* **1989**, *37*, 834–839.
50. Skerritt, J. H.; Hill, A. S.; McAdam, D. P.; Stanker, L. H. *J. Agric. Food Chem.* **1992**, *40*, 1287–1292.
51. Wengatz, I.; Plant, A. L.; Stanker, L. H.; Schmid, R. D. In *Strategies for Food Quality Control and Analytical Methods in Europe*; Proc. 6th European Conf. on Food Chemistry, Hamburg, Lebensmittelchemie Gesellschaft: Frankfurt, Germany, 1993, Vol. 2; pp 770–773.
52. Hill, A. S.; Mc Adam, D. P.; Edward, S. L.; Skerritt, J. H. *J. Agric. Food Chem.* **1993**, *41*, 2011–2018.
53. Demoute, J. P.; Touer, G.; Mouren, M. *Preparation of Pyrethroidal Radioactive Iodine-Labelled Amino Acid Derivatives for Radioimmunoassay of Deltamethrin*; French Patent, 1986, No. 2593503.
54. Wengatz, I.; Stoutamire, D.; Gee, S. J.; Hammock, B. D. *Immunoassays for Fenvalerate and Fenpropathrin-Based Human Metabolites*; Proc. 8th IUPAC Congr. Pesticide Chem., Washington, DC, July 1994, Abstract No. 64.
55. Zhang, M.; Scott, J. G. *J. Agric. Food Chem.* **1994**, *42*, 1779–1782.
56. Maguire, R. J. *J. Agric. Food Chem.* **1990**, *38*, 1613–1617.
57. Perschke, H.; Hussain, M. *J. Agric. Food Chem.* **1992**, *40*, 686–690.
58. Wraith, M. J.; Hitchings, E. J.; Cole, E. R.; Cole, D.; Woodbridge, A. P.; Roberts, T. R. *Development of Immunoassay Methods for Pyrethroid Insecticides*; Sixth IUPAC Congr. on Pesticide Chemistry, IUPAC, Ottawa, Canada, 1986, Abstract No. 5C-10.
59. Pullen, S.; Hock, B. *Anal. Lett.* **1995**, *28*, 765–779.
60. Pullen, S.; Hock, B. *Anal. Lett.* **1995**, *28*, 781–795.
61. Bonwick, G. A.; Abdul-Latif, P.; Sun, C.; Baugh, P. J.; Smith, C. J.; Armitage, R.; Davies, D. H. *Food Agric. Immunol.* **1994**, *6*, 341–356.

RECEIVED November 3, 1995

Chapter 11

Analysis of Benomyl Residues in Commodities by Enzyme Immunoassay

Extraction, Conversion, and High-Performance Liquid Chromatography Validations

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Use of a simple acetone extraction scheme afforded complete conversion of benomyl residues in food commodities for immunoassay analysis. Preliminary investigation of various extraction/hydrolysis schemes revealed this to be the most advantageous, and it was optimized for sensitivity, speed and simplicity. Residues were assayed using Benomyl/Carbendazim Kits and the RaPID Assay System (Ohmicron Corp., Newtown, PA). Ohmicron's Food Prep Kits (for sample extract cleanup) were also evaluated. The optimized extraction scheme, with or without the Food Prep Kit, generated results comparable to those by HPLC analysis. For five crop matrices — mustard greens, Italian dandelions, parsley, apples and strawberries — with real field residues ranging from 0.062 to 1.2 ppm, the correlation of immunoassay versus HPLC, across the range of all measurable samples in the study, was r = 0.963, n = 18. Overall performance data demonstrated the ability of this immunoassay system to adequately extract and quantitate bound residues in field-treated commodities.

Cornell Analytical Laboratories, in co-operation with Ohmicron Corporation, studied the analysis of benomyl residues in and on plant tissue by competitive enzyme-linked immunosorbent assay. Sample analyses were performed using the RaPID Assay system (Ohmicron Corp., Newtown, PA) and Ohmicron's 100-test Benomyl/Carbendazim Kits. The primary objective of this study was twofold: 1) to investigate and assess the efficiencies of various extraction/hydrolysis schemes leading to extracts compatible with Enzyme ImmunoAssay (EIA); and 2) to obtain more meaningful immunoassay data for the systemic fungicides benomyl and its major degradation product carbendazim (MBC). Most important in the second was analyzing field-treated, environmentally-realistic samples. We approached this first by spiking and determining recoveries with both benomyl and MBC. This also served to further characterize the performance profile of the Benomyl/Carbendazim Kits by RaPID

0097–6156/96/0621–0150$15.00/0

Assay, as previously published data had been generated by spiking "clean" samples with MBC shortly before extraction and EIA analyses.

Additional consideration was given to optimizing the selected, most advantageous immunoassay scheme for sensitivity, speed and simplicity. Close attention also was given to evaluating Ohmicron's Food Prep Kits, containing a proprietary reagent for sample extract cleanup. High-Performance Liquid Chromatography (HPLC) was used for immunoassay method validation.

Materials and Reagents

Materials employed but not included in the RaPID Assay kits were: Eppendorf 12.5-mL reservoir repeater pipette (Brinkmann Instruments, Inc., Westbury, NY); Eppendorf tri-volume pipette (100, 200 and 250 µL); variable-volume micropipettes capable of delivering 20 and 50 µL (VWR Scientific, Philadelphia, PA); 10.0-mL polystyrene and 5.0-mL glass serological pipettes (Corning Glass Works, Corning, NY); Class A grade glass volumetric pipettes and flasks (Corning Glass Works, Corning, NY); 15-mL polypropylene centrifuge tubes with screw caps and 250-mL polypropylene centrifuge bottles (VWR Scientific); magnetic separation unit (Ohmicron Corp.); Maxi Mix II variable speed vortex mixer (Barnstead/Thermolyne Corp., Dubuque, IA); Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The spectrophotometer utilized was an Ohmicron RPA-I RaPID Photometric Analyzer.

The analytical reference standards, benomyl (methyl 1-[butylcarbamoyl]-2-benzimidazolecarbamate) and carbendazim (methyl 2-benzimidazolecarbamate), were obtained from DuPont Agricultural Products, Wilmington, DE. HPLC-grade acetonitrile and ethyl acetate and certified A.C.S. grade sodium chloride were purchased from Fisher Scientific, Fair Lawn, NJ. Commercial grade acetone (Potter Paint Company, Cortland, NY) was redistilled in-house, and HPLC/nanopure-grade water was obtained from an in-house Barnstead water purification system.

Commodity Field Samples

Sample Information. Three of the five crop materials analyzed in this project (mustard greens, Italian dandelions and fresh parsley) were from the IR-4 Minor Use Pesticide Registration program. Treated samples were sprayed with Benlate 50% WP (E.I. DuPont, 50% active ingredient, wettable powder). Application rates were 0.25 or 0.50 # a.i./A (pounds of active ingredient per acre). The pre-harvest intervals (time period between last benomyl application and sampling) for these three commodities were 14, 7, and 19 days, respectively. The seventeen IR-4 samples were received frozen and stored at -20 °C. The fourth crop material was *Red Delicious* apples harvested the first week of October 1993. The apples were stored at 0 °C before and after benomyl treatment for storage preservation on November 18, 1993; one lb. Benlate 50% WP per 200 gallons of water was applied to the apples in a hydrocooler tank with water circulating at 100 gallons per min at 50 °F through 144 – 3/8" holes/20-bushel bin. The last crop included in this project was strawberries. The variety of treated samples was *Seneca*, supplied by a commercial fruit/vegetable farm. Benlate 50% WP had been applied at a rate of 5 oz a.i./A. Two days after spraying, the strawberries were hand-picked by random sampling from all rows throughout the plot, the non-edible tops were discarded, and the berries were frozen at -20 °C. As a control sample for this variety was not available, a combination of organically grown *Honeoye* and *All Star* strawberries was used.

Sample Preparation. Vegetables and fruits were ground in a Hobart Food Chopper to obtain a homogeneous mixture. Mustard greens, Italian dandelions, fresh parsley and strawberry samples were ground partially frozen. Refrigerated apples were processed into three factions before grinding — flesh plus peel, flesh, and peel. The non-edible portions of the apples were discarded before grinding and freezing. Ground samples were divided into two sets, one each for immunoassay and HPLC analyses, and stored frozen at -20 °C.

Stock Standards and Spiking Solutions

Benomyl and carbendazim stock standards were prepared from analytical reference standards in accordance with the HPLC method (*1*). A 100 µg/mL stock standard of benomyl was prepared by dissolving 20 mg of benomyl (98.9% purity) in ethyl acetate in a 200-mL volumetric flask, followed by sonication. An equal volume and concentration of carbendazim stock standard was prepared with carbendazim (99.6% purity) and 0.1 N H_3PO_4. Both stock standards were divided for use between immunoassay and HPLC analyses.

Spiking solutions for immunoassay were prepared at 10 µg/mL from the above stock standards. Acetonitrile was the solvent used in preparing the spiking solution for benomyl; water was used to prepare the carbendazim spiking solution. Stock standards and spiking solutions for benomyl were stored at -20 °C, whereas those for carbendazim were refrigerated (2–8 °C).

Immunoassay Protocol

All samples were analyzed for benomyl/carbendazim residues by immunoassay using the RaPID Assay system. 200 µL of sample or calibrator, 250 µL of enzyme conjugate, and 500 µL of antibody-coupled magnetic particle suspension were first added to a disposable polystyrene test tube. After incubation at room temperature for 20 min, the mixtures were magnetically separated with a magnetic rack and the supernatant decanted. The magnetic particles were washed twice with 1 mL of washing buffer (0.1% Tween in tris-buffered saline). To each tube, 500 µL of a 1:1 mixture of substrate (0.02% hydrogen peroxide in a citric acid buffer) and chromogen solution (0.4 g/L 3,3',5,5'-tetramethylbenzidine in an organic base) were added. Color was allowed to develop for 20 min, then stopped and stabilized with a 500 µL addition of 2 M sulfuric acid.

The color formation in each sample or calibrator was analyzed spectrophotometrically using the RPA-I Photometric Analyzer set at 450 nm. A pre-programmed cartridge (Ohmicron Corp.) was used, and the instrument processed the data input by linear regression data reduction; the calibration curve is generated into a straight line by the least squares regression method and analyte concentration is determined from this equation. The data was transformed by L_n/LogitB (LogitB = log

$(A/A_0)/(1\text{-}A/A_0)$, where A = absorbance response of sample or calibrator and A_0 = absorbance response at zero dose concentration). Standard curves were constructed from the $\%B/B_0$ ($\%B/B_0 = (A/A_0) \times 100$) of the calibrators. At the beginning of each run, the calibrators were assayed in duplicate in ascending order (0.0, 0.25, 1.0, and 5.0 ppb). Each run also included a 2.5 ppb control, assayed in singlicate. A run was deemed valid if: 1) the correlation (r) of the calibration curve was 0.990 or greater; and 2) the EIA result for the 2.5 ppb control was within ±0.6 ppb. The Least Detectable Dose (LDD) for carbendazim, estimated at 90% B/B_0, is 0.10 ppb in water. The LDD for unconverted benomyl is 0.38 ppb in water (28.5% cross-reactivity). Benomyl residues are analyzed as carbendazim after conversion.

A Non-Specific Binding (NSB) tube was included with each set of assayed samples; this tube contained 50 times the pesticide concentration of the highest calibrator (250 ppb) in water and was used as the reagent blank in the RPA-1 Analyzer. Absorbance from the NSB tube was automatically subtracted from the standard calibrators and samples by the RPA-1 after programming. This served to delete positive bias resulting from the low level of direct binding of enzyme-antigen conjugate to a solid support surface found typically in heterogeneous immunoassays.

Preliminary Experimental Test Procedures

Due to conflicting reports in the scientific literature as to the rate of, and completeness of, benomyl conversion to MBC in water, acids and organic solvents (*2–8*), preliminary tests were designed to address this issue. Four extraction/hydrolysis schemes were chosen for consideration — acidic-aqueous, aqueous-organic, organic-acidic, and aqueous-heat. Additionally, the results of these tests would provide information relative to each scheme's compatibility with the RaPID Assay system and determine if further, more comprehensive, testing of a particular scheme was desirable.

Acidic-Aqueous Scheme. There did seem to be common agreement on one point when published procedural steps were outlined to achieve total benomyl conversion to MBC — some form of acid was necessary, either with or without heat. The cited methods varied in degree of complexity, ranging from a simple addition of acid to the extraction solvent to a more elaborate procedure involving an extended heating interval (*9,10*).

Taking this information into account, a preliminary examination of an acidic-aqueous scheme was conducted using two test acids — 0.2 M HCl and 0.2 M H_3PO_4, with and without applied heat. Water only was initially employed to evaluate results free from plant matrix interference. Six 50-mL aliquots of water (in 250-mL centrifuge bottles) were spiked, each at 200 ppb benomyl. Two mL of each acid were added to three of the benomyl-spiked water samples. To each centrifuge bottle, 100 mL of HPLC-grade water were added to simulate the volume of solvent added to a sample, and the bottle was covered. A hot water bath (Isotherm, Fisher Scientific, Springfield, NJ) at 90 °C was then used as a heat source for four of the spiked water aliquots. Thirty- and sixty-minute heating intervals were allotted for acid hydrolysis of benomyl to MBC, then cooled. Aliquots (with and without heat treatment) were diluted with Benomyl Sample Diluent (sodium acetate buffer, pH 4.0, with 1 mM ethylenediaminetetraacetic acid [EDTA] and 0.1% gelatin, Ohmicron Corp.) into the linear range of the calibrators. Immunoassay was performed on these acid-treated spiked waters in duplicate. Concurrently, acid-treated control waters were assayed by EIA.

Aqueous-Heat Scheme. Examination of an aqueous-heat scheme was undertaken, as benomyl is heat labile and carbendazim is very water soluble (*11*). The possibility of eliminating the need for an organic solvent was attractive. Three 50-g subsamples of chopped mustard greens (benomyl-free control sample) were weighed in tared 250-mL centrifuge bottles. Two of the subsamples were spiked, one at 200 ppb MBC and the other at 500 ppb benomyl. HPLC/nanopure water (100 mL) was added to each spiked subsample and homogenized with a Polytron for 15 min. A hot water bath at 90 °C was then employed for 60 min. The mixtures were cooled, reblended, then 50-mL aliquots were gently centrifuged for 10 min. The extract supernatants were assayed by EIA in duplicate, both with and without the Food Prep Kit.

Aqueous-Organic Scheme. Aqueous-organic schemes were evaluated by comparing acetonitrile as an extracting solvent to Ohmicron's recommended extractant — acetone. Two extraction procedures recommended by Ohmicron were employed. In the first, Ohmicron recommended the following for the immunoassay determination of carbendazim in vegetables or fruits:

> Add 100 mL of acetone to 50 g of chopped sample and blend thoroughly. Transfer approximately 50 mL of the blended mixture to a 50 mL conical tube and allow the solids to settle (5 min). Transfer 4 mL of the upper extract layer to a 15 mL centrifuge tube and add approximately 2.0 g of sodium chloride. Cap the tube and vortex to mix thoroughly. Allow the aqueous and acetone phases to separate (5 min). Transfer 100 μL of the acetone phase (top layer) to a glass tube and evaporate to dryness under nitrogen. Redissolve the residue in exactly 5.0 mL of the Benomyl Sample Diluent (*12,13*).

The second extraction procedure employed the use of Ohmicron's Food Prep Kits; each kit consists of test tubes containing 0.5 g of a powdered polymer mixture for sample cleanup of food extracts and a bottle of sodium chloride. The cleanup reagent serves to reduce interferences with the EIA test due to phenolic substances, such as flavenoids (*14*). The procedure is the same as that cited above with one additional step — prior to the salt partitioning step, 4 mL of extract supernatant were added to a tube containing the proprietary cleanup reagent and vortexed for 15 s.

The two extraction procedures above served as the general framework upon which acetonitrile was tested as the extracting solvent and also formed the standard against which procedural modifications were tested and evaluated.

Acetonitrile was selected as a test solvent due to the RaPID Assay antibody's higher tolerance for acetonitrile in water (10%) compared with 3% for acetone. Because of this higher tolerance, the use of acetonitrile had the potential for eliminating the nitrogen evaporation step recommended by Ohmicron to eliminate any solvent effect. In addition, it was necessary that the solvent-of-choice effect a clean phase separation in the salt partitioning step. Historically, the Mills method, a widely-used multiresidue procedure in traditional chemical methodology, utilizes acetonitrile as the extraction solvent and also includes a saline partitioning step (*15*).

Six 50-g subsamples of chopped mustard greens (benomyl-free control sample) were weighed in tared 250-mL centrifuge bottles. Four subsamples were spiked with benomyl or MBC, two each at 400 ppb. The remaining two subsamples were used as control samples and extracted, one each, with acetonitrile or acetone. Using the above-cited procedures (with and without the Food Prep Kit), the benomyl- and MBC-spiked samples were extracted with 100 mL each of acetonitrile or acetone. Immunoassay was then performed on the sample extracts in duplicate. Similarly, 50-g subsamples of a field-treated sample (0.25 # a.i./A Benlate 50% WP) of mustard greens were extracted with both organic solvents, and the results were compared.

Immunoassay Procedure for the Benomyl Residue Analysis of Commodity Field Samples

Results from the above extraction schemes were evaluated and one scheme was selected, optimized and used for the benomyl residue analysis of the 23 field samples. Control samples for each commodity were spiked with benomyl and carbendazim as separate fortifications. When fortifying samples, time was allotted (approximately 20 min) for spiking solutions to stand in contact with the plant material before the addition of extracting solvent. Recovery spikes were prepared at concentrations commensurate with the residue levels found in the field-treated samples. As the tissue matrix is different for every crop (and sometimes variety), each commodity should be validated by a traditional chemical method before treated field samples are committed.

HPLC Apparatus, Operating Conditions and Procedures

The HPLC system consisted of a Tracor 951 LC pump and a Rheodyne Model 7125 valve loop injector (100 µL). The system was equipped with a 757 Absorbance Detector (Applied Biosystems, Foster City, CA) set at 280 nm. The mobile phase was 0.025 N tetramethylammonium nitrate in 0.025 N nitric acid at a flow-rate of 0.5 mL/min. A Zipax SCX strong cation 1.0 m x 2.1 mm i.d. exchange column (E.I. Du Pont de Nemours & Co., Inc., Wilmington, DE) was maintained at 60 °C. Chart speed was 0.25 cm/min, and the retention time for carbendazim was approximately 18 min.

Control and field-treated crop samples were analyzed by HPLC for benomyl residues, determined as MBC, in accordance with the method as described by Spittler *et al*. (*1*). Method modifications were as follows: 1) ethyl acetate was used as the extracting solvent; and 2) prior to analyses, samples were filtered through 0.45 µm Gelman PTFE Acrodisc filters.

Spiking solutions of benomyl in ethyl acetate were used to fortify control samples for each commodity. Recoveries were benomyl-spiked at concentrations commensurate with the levels found in the field samples. The system detection limit was defined as the lowest spike concentration analyzed by HPLC for a given crop material. An estimated detection limit was extrapolated to visualize low residue trends. Results for controls, samples, and spikes were obtained from calibration curves generated from carbendazim standards at appropriate levels.

Results

Extraction/Hydrolysis. Initial examination of an acidic-aqueous scheme revealed complete hydrolysis of benomyl to carbendazim for both test acids — HCl and H_3PO_4, with and without applied heat. With HCl, 200 ppb benomyl spikes generated comparable results when employing either 30- or 60-min heating intervals; recoveries were 117 and 97%, respectively. With H_3PO_4, 101 and 100% recoveries were achieved for the same respective time intervals. Without heat, recoveries were comparable to those with heat treatment for both test acids. The control samples were "clean," with no false-positives. All intra-assay Coefficients of Variation (CVs) were less than 7%. These data indicated acceptable method accuracy and precision, along with EIA compatibility.

An aqueous-heat scheme, utilizing mustard greens, generated the following results. Spike recoveries were low, with 67% recovery of the 200 ppb MBC spike and 62% recovery of the 200 ppb benomyl spike. Even though recoveries were poor, precision was acceptable with intra-assay CVs for the assayed spike extracts of benomyl and MBC of 6.2 and 12%, respectively. When the Food Prep Kit was employed, recovery of the MBC spike improved to 81%, indicating a minimization of matrix effect. The control sample result was "clean," showing no false-positives. Precision was acceptable with an intra-assay CV of 15%.

Investigation of aqueous-organic schemes produced the following results. Without the Food Prep Kit and employing acetonitrile as the extracting solvent, 400 ppb benomyl- and MBC-spiked fortifications of mustard greens had recoveries of less than 100%, but were within 9%. Precision was good with intra-assay CVs of less than 7%. However, acetonitrile did not perform well with the Food Prep procedure. Recovery of MBC was low (63%), although precision was still excellent with an intra-assay CV of 5.4%. When water was used as the final extract diluent, recovery improved to 79% with an intra-assay CV of 0.5%.

Without the Food Prep Kit and employing acetone as the extracting solvent, spiked mustard greens at 400 ppb benomyl and MBC gave acceptable recoveries of 106 and 114%, respectively; intra-assay CVs were good — less than 9% for both assayed spikes. With the Food Prep Kit, the same fortifications had recoveries of 105% with intra-assay CVs of less than 13%. When a control sample of mustard greens was extracted with either acetone or acetonitrile, negative results were obtained (below the 25 ppb detection limit of the EIA system), both with and without the Food Prep Kit.

Field-Treated Samples versus Spikes. For the field-treated sample (0.25 # a.i./A Benlate 50% WP), without the Food Prep Kit, the result was 26% less with the acetonitrile extraction than with the acetone extraction. Because of the low result obtained with the acetonitrile extraction, the remaining extraction mixtures were allowed to stand at room temperature for two days. After being shaken, particles were allowed to settle and extracts were prepared for immunoassay and reanalyzed.

For the acetone extraction, the result was comparable to that obtained two days previously (differing by only +6.2%) and indicated that the first analysis reflected essentially complete conversion of benomyl to MBC and complete extraction. With the acetonitrile extraction, the analyte residue two days later was 21% higher. However, this result was still 12% lower than the acetone-extracted sample at day two. This observation agrees with work cited by Chiba *et al.* (*11*), but disagrees with that reported by Zweig *et al.* (*16*), who claimed complete conversion of benomyl to MBC in acetonitrile within 3 h.

The field-treated crop samples revealed acetonitrile to be an insufficient extraction/conversion solvent, in contrast to the good results obtained using spikes only. The higher residue levels in the field-treated sample after an additional two days of conversion time serves to highlight the matrix differences, and therefore the need for immunoassay methods developed by analyzing field-treated samples in addition to recovery spikes.

Of the extraction/hydrolysis schemes investigated, the results indicated the acetone extraction to be the most advantageous. The data showed that use of this solvent afforded complete conversion of benomyl to MBC in plant tissue within the normal time frame of the immunoassay procedure (sample extraction and assay). This appeared to conflict with the observation by Peterson *et al.* (*8*), who cited benomyl as being relatively stable in acetone. Benomyl residues were also quantitative both with and without use of the Food Prep Kit. Once this was manifest, the major focus was then shifted toward optimization of the acetone extraction scheme.

Procedural Modifications of the Acetone Extraction Scheme.

Direct Dilution versus Evaporation/Redissolution. EIA results obtained by direct dilution of the final extract supernatant were compared to those obtained with the evaporation/redissolution step, i.e. 50 μL of sample extract were directly diluted with Sample Diluent (1:100 dilution) versus 100 μL of sample extract being evaporated and then redissolved in 5.0 mL of Benomyl Sample Diluent (1:50 dilution). Comparable or improved results were achieved with the former. When 400 ppb MBC-spiked homogenates of mustard greens were extracted, results were comparable, both with and without use of the Food Prep Kit. (The difference between the two alternative steps was 3.6% without the Food Prep Kit and 0.96% using the Food Prep procedure.) When 50 ppb MBC-spiked homogenates of apple flesh were tested, improved results were obtained by direct dilution. Without the Food Prep Kit, recovery improved from 138% (69 ppb) to 116% recovery (58 ppb), and with the Food Prep Kit, recovery improved from 76% (38 ppb) to 96% recovery (48 ppb).

In addition to comparable or improved recoveries, eliminating the evaporation step along with the associated nitrogen evaporation apparatus, including a dedicated exhaust hood, would enhance the method for several reasons — less laboratory equipment, space, and labor, plus, it would increase the options of where one could perform the test. Therefore, the evaporation step was eliminated and a direct 1:100 dilution of the final sample extract was adopted. This did alter the detection limit of the EIA system, from 25 to 50 ppb, but it was still well below benomyl tolerance levels. In exchange for the comparable or improved results seen and the above advantages, this slight decrease in sensitivity seemed worthwhile.

HPLC-grade Water versus Benomyl Sample Diluent. HPLC-grade water was tested as the final extract diluting medium to see if water was a viable alternative to the proprietary Benomyl Sample Diluent for the crop matrices under study. Ohmicron recommends that Sample Diluent be used for all sample dilutions, as it contains buffer and chelating agents to neutralize sample extracts, but water has the advantages of availability and ease-of-use (the Sample Diluent does not pipet as readily).

Using spiked and field-treated homogenates of mustard greens, comparable results were obtained with HPLC-grade water as the final sample diluent, both with and without the Food Prep Kit. Without the Food Prep Kit, MBC-spiked homogenates of mustard greens (400 ppb) were recovered at 105%, a difference of -1% when compared to the result obtained with Sample Diluent. With the Food Prep Kit, MBC recovery was 97%, differing -8% from that obtained with Sample Diluent. The corresponding intra-assay CVs employing water as the extract diluent for both procedures were less than 6% and less than 10%, respectively. Similar comparisons were achieved with field-treated benomyl samples (1X and 2X application treatments) of mustard greens. With the Food Prep procedure, EIA results for the two crop samples using HPLC water as the final extract diluent varied by +1.5 and -1.1% to that with Sample Diluent. When extracted with the Food Prep Kit, results varied by -12 and -5.7%. The above results indicated water to be an acceptable final extract diluent. Therefore, HPLC water was adopted as the final extract diluent and for all samples requiring additional dilution into the range of the calibrators. However, because the Sample Diluent generated slightly better results when using the Food Prep Kit, it was maintained as the final extract diluent for this purpose only.

One last modification was the elimination of the first decantation; this step involved the decanting of a 50-mL aliquot of sample/extractant mixture into a 50-mL centrifuge tube, followed by settling and the removal of a 4-mL aliquot. This proved to be unnecessary, with a 4-mL aliquot being removed directly from the settled, blended mixture.

Future Consideration. Using the optimized acetone extraction scheme, sample size reduction was tested. For each of the five commodities used in this project, a 10-gram field-treated subsample was extracted with 20 mL of acetone and assayed by EIA in duplicate. These results were then compared with their corresponding 50-gram sample results. Results were comparable; CVs between the two sample sizes were less than 9% (average CV, 4.9%). Within-assay CVs for the 10-gram samples were 9% or less (average CV, 4.8%). This indicated that use of a 10-gram sample size was representative with this immunoassay scheme for the types of crop matrices tested and agreed with a study by Bourke *et al.* (*17*).

Immunoassay Analyses of Commodity Field Samples employing the Optimized Acetone Extraction Scheme. EIA recovery and precision studies and field sample analyses for benomyl residues were performed using the optimized acetone extraction scheme. The extraction procedure without the Food Prep Kit will be hereon referred to as Method I and the procedure with the Food Prep Kit will be referred to as Method II.

Recovery Fortifications. Control samples for each commodity were spiked with benomyl and carbendazim at analyte concentrations commensurate with the levels found in the samples (Table I). Spike concentrations for the five crops varied from

Table I. EIA Recovery Data for Benomyl- and MBC-Spiked Control Samples.

CROP SAMPLE Spike	Method I (ppm)	% Recovery	Method II (ppm)	% Recovery
MUSTARD GREENS				
0.20 ppm MBC	0.21	105	0.21	105
0.40 ppm MBC	0.42	105	0.35	88
0.80 ppm MBC	0.81	101	0.71	89
0.40 ppm Benomyl[a]	0.28	106	0.26	99
ITALIAN DANDELIONS				
0.20 ppm MBC	0.22	111	0.20	99
1.0 ppm MBC	1.0	104	0.87	86
1.0 ppm Benomyl[a]	0.67	102	0.61	94
FRESH PARSLEY				
0.20 ppm MBC	0.22	110	0.22	112
2.0 ppm MBC	2.0	100	1.7	86
0.60 ppm Benomyl[a]	0.40	102	0.40	102
APPLES				
0.050 ppm MBC (Flesh)	0.058	116	0.050	100
1.2 ppm MBC (Peel)	1.2	99	1.1	88
0.60 ppm Benomyl[a] (Flesh and Peel)	0.46	117	0.37	93
STRAWBERRIES				
0.050 ppm MBC	0.17[b]	340[b]	0.052	104
0.10 ppm Benomyl[a]	0.19[b]	288[b]	0.068	104

Method I, optimized acetone extraction procedure; Method II, Method I plus Food Prep Kit.

[a] Added as benomyl, measured and calculated as carbendazim.

[b] False-positive.

0.050 to 2.0 ppm for MBC, and 0.10 to 1.0 ppm for benomyl. MBC recoveries ranged from 99 to 116% (mean recovery, 106%) for Method I, and from 86 to 112% (mean recovery, 96%) for Method II. Benomyl recoveries ranged from 102 to 117% (mean recovery, 107%) using Method I, and 93 to 104% (mean recovery, 98%) using Method II. Results for strawberries prepared by Method I were not included in the above calculations, as a false positive was observed with Method I; it was adequately minimized using Method II. All control sample results were "clean," that is below the detection limit of the immunoassay system, except, of course, for strawberries extracted without the Food Prep Kit.

Interassay Precision Studies Performed with Spiked and Field-Treated Crop Samples. For the 5 commodities tested, between-assay CVs, day-to-day variation and within-sample or sample-to-sample variation demonstrated excellent reproducibility (Tables II, III). Using Method I, CVs generated from repeat analyses of sample extracts assayed on different days were all below 7%, averaging 3.5%, and using Method II, between-assay CVs for two sample extracts were 2.8 and 12%. When duplicate extractions of several field-treated crop samples were assayed, the percent CVs between first and second extractions for Method I were less than 6% (average CV, 2.7%), and for Method II, 12% and less (average CV, 5.5%). The data by both Methods I and II demonstrated good precision and reproducibility.

Field Sample Analyses. Using Methods I and II, the twenty-three crop samples were analyzed for benomyl as carbendazim (Table IV). As was seen in the spike recoveries, strawberries exhibited a false-positive result using Method I, but matrix interference was adequately minimized using Method II. The levels of residue found in the field-treated samples ranged from a low of 0.062 ppm in strawberries to a high of 1.8 ppm in fresh parsley. Within-assay (intra-assay) CVs between duplicate analyses of sample extracts were less than 19% (average CV, 7.4%) using Method I. Similarly, with Method II, within-assay CVs were less than 20% (average CV, 6.1%). All control sample results were negative, below the detection limit of the EIA system, except as noted for strawberries.

Comparison of results between Methods I and II were favorable, with an average CV of 7.4%. Configured in graphic form, this comparison is illustrated in Figure 1 (r = 0.985, n = 17). Strawberries were excluded due to the false-positive response with Method I.

Calculations for Immunoassay Analysis. Final pesticide concentration in each sample was obtained by multiplying the assay result (ppb) by the appropriate dilution factor consisting of two parts — one to correct for the initial 2 to 1 extracting solvent to crop sample ratio, and the second to correct for the 1:100 final dilution of sample extract. This equates to a 200X correction factor if no additional sample dilution is required. Therefore, ng of MBC per g of sample (ppb) would equal:

$$\text{EIA result (ppb)} \times [\text{vol. of extraction solvent (mL)} \div \text{wt. of sample (g)}] \times [\text{vol. extract (mL)} + \text{vol. diluent (mL)}] \div \text{vol. extract (mL)} \qquad (1)$$

Table II. Comparison of EIA Results for Sample Extracts Assayed on Different Days.

CROP SAMPLE Field-Treated or Spike	Day 1–Day 2 Method I (MBC ppm)[a]	Interassay % CV[b]	Day 1–Day 2 Method II (MBC ppm)[a]	Interassay % CV[b]
Mustard Greens TRT 1X	0.45–0.48	4.5	0.39–0.37	2.8
Mustard Greens TRT 2X	0.78–0.79	0.36	–	–
Mustard Greens MBC Spike (0.40 ppm)	0.42–0.46	6.6	–	–
Fresh Parsley TRT 1X	0.16–0.17	3.4	0.15–0.18	12
Fresh Parsley TRT 2X	1.1–1.2	3.0	–	–
Fresh Parsley MBC Spike (0.20 ppm)	0.21–0.22	2.0	–	–
Ap–ple Peel MBC Spike (1.2 ppm)	1.3–1.2	4.6	–	–

Method I, optimized acetone extraction; Method II, Method I plus Food Prep Kit.

[a] Each EIA result is the average of duplicate assays of a single extract.

[b] CV = Coefficient of Variation, calculation based on three significant figures.

Table III. Comparison of MBC Immunoassay Results for Duplicate Extractions of Field-Treated Samples.

COMMODITY	1st Extraction–2nd Extraction Method I (ppm)[a]	% CV[b]	1st Extraction–2nd Extraction Method II (ppm)[a]	% CV[b]
MUSTARD GREENS	0.45–0.42	5.5	0.46–0.39	12
FRESH PARSLEY	0.29–0.29	2.0	0.27–0.26	3.2
ITALIAN DANDELIONS	1.1–1.1	1.6	0.96–0.90	4.6
APPLES (Flesh & Peel)	0.31–0.32	1.8	0.26–0.27	2.1

Method I, optimized acetone extraction; Method II, Method I plus Food Prep Kit.

[a] Each EIA result is the average of duplicate assays of a single extract.

[b] CV = Coefficient of Variation, calculation based on three significant figures.

Table IV. Benomyl Residues Determined as MBC by Immunoassay and High-Performance Liquid Chromatography (HPLC) in Commodity Field Samples.

COMMODITY FIELD SAMPLE	Method I ppm[a]	Method II ppm[a]	CV[b] EIA I & II	HPLC ppm[c]	CV[b] EIA[d] & HPLC
MUSTARD GREENS					
Control	<0.050	<0.050	–	<0.10	–
TRT 1X,reps 1 & 2	0.45	0.46	0.62%	0.48	4.1%
TRT 1X,reps 3 & 4	0.21	0.26	13%	0.16	19%
TRT 2X,reps 1 & 2	0.79	0.72	5.6%	0.69	9.3%
TRT 2X,reps 3 & 4	0.18	0.20	9.4%	0.19	6.2%
ITALIAN DANDELIONS					
Control	<0.050	<0.050	–	<0.40	–
TRT 1X, rep 1	1.1	0.96	9.9%	0.89	15%
TRT 1X, rep 2	0.73	0.68	4.2%	0.82	8.3%
TRT 1X, rep 3	1.5	1.5	0.23%	1.3	9.1%
TRT 1X, rep 4	0.54	0.56	1.9%	0.64	12%
FRESH PARSLEY					
Control	<0.050	<0.050	–	<0.10	–
TRT 1X, rep 1	0.29	0.27	6.0%	0.22	21%
TRT 1X, rep 2	0.16	0.15	2.7%	0.15	1.3%
TRT 1X, rep 3	0.31	0.26	14%	0.31	1.6%
TRT 2X, rep 1	1.2	0.92	18%	1.3	5.2%
TRT 2X, rep 2	1.8	1.7	4.1%	1.7	2.2%
TRT 2X, rep 3	1.8	1.6	5.4%	1.5	13%
APPLES					
Flesh&Peel(Control)	<0.050	<0.050	–	<0.10	–
Flesh&Peel (TRT)	0.31	0.26	12%	0.25	15%
Flesh (TRT)	0.078	0.070	7.6%	0.078	0.0%
Peel (TRT)	1.2	0.99	11%	1.1	1.9%
STRAWBERRIES					
Control	0.082[e]	<0.050	–	<0.10	–
Treated	0.16[e]	0.062	62%	0.063	1.1%

Method I, optimized acetone extraction; Method II, Method I plus Food Prep Kit.
System Detection Limit: EIA-0.050 ppm; HPLC-ppm of lowest spike for each crop.
Estimated Detection Limit: HPLC-0.050 ppm for apples, 0.025 ppm for strawberries.
TRT 1X, 0.25 # a.i./A Benlate 50% WP; TRT 2X, 0.50 # a.i./A Benlate 50% WP.

[a] Each EIA result is the average value of a single extract assayed in duplicate.

[b] CV = Coefficient of Variation, calculation based on three significant figures.

[c] Each HPLC residue value was corrected for recovery.

[d] Method I results used, except for treated strawberries, where Method II was used.

[e] False-positive.

Using the lowest calibrator in the Carbendazim RaPID Assay (0.25 ppb) as the LDD (Least Detectable Dose) and the 200X correction factor, the detection limit of the system equates to 50 ppb.

In the recovery studies, percentage recoveries for benomyl were calculated by first converting added amounts of benomyl to carbendazim concentrations; the concentration of added benomyl (ppb) was divided by the molecular weight conversion factor of 1.53 to give the concentration of MBC if all benomyl underwent conversion. Therefore, % recovery would equal:

$$\text{EIA result (ppb MBC)} \div [\text{concentration of added benomyl (ppb)} \div 1.53] \times 100 \qquad (2)$$

HPLC Analyses of Commodity Field Samples and Recovery Fortifications. The twenty-three field samples (control and benomyl-treated) analyzed by immunoassay also were analyzed for benomyl residues, as carbendazim, by HPLC. These results are given in Table IV. For each sample, the residue value was corrected for recovery. Control samples were spiked with benomyl, measured and calculated as carbendazim, at levels found in the field-treated samples (Table V).

Comparison of Field Sample Results by Immunoassay and HPLC. Comparison of immunoassay and HPLC results and their corresponding coefficients of variation are shown in Table IV. Method I results were used in all CV calculations except for strawberries, where Method II was used. CVs ranged from 0.0 to 21% with an average of 7.9%. Graphing this comparison (Figure 2) depicts the excellent correlation between the results determined by immunoassay, using Method I or II, and those determined by HPLC across the range of all measurable samples in the study ($r = 0.963$, $n = 18$).

Conclusions

Use of a simple acetone extraction scheme afforded complete conversion of benomyl to carbendazim in plant tissue for immunoassay analysis. Results of this study indicate that added acid is not always needed nor is an additional hydrolysis step necessarily required.

Streamlining the acetone extraction scheme enhanced the advantages typically cited as characteristic of an immunochemical method compared to traditional chemical methods — less capital equipment, laboratory materials, cost, time and labor, ease-of-use, etc. The favorable result from the brief examination of sample size reduction, obtained for each of the five commodities, indicated that a 10-gram sample size is representative. Sample size reduction would result in even further savings.

By employing environmentally-realistic samples — field-treated commodities — for test and analytical purposes and by spiking with both benomyl and carbendazim, more meaningful immunoassay data were obtained to further characterize the performance profile of the Benomyl/Carbendazim RaPID Assay and Food Prep Kits. Residue levels determined in the field-treated samples ranged from 0.062 to 1.8 ppm. This provided a good overview of method and EIA performance over a range of ppb analyte concentrations at practical agricultural levels. Overall performance data demonstrated the ability of this immunoassay system to quantitate trace residues of

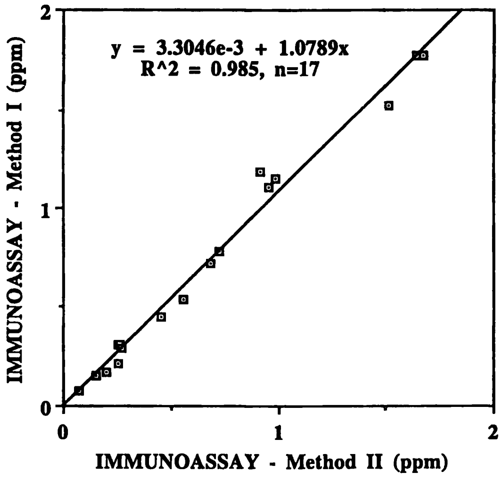

Figure 1. Comparison of Benomyl Residues as MBC in Field-Treated Crop Samples by Immunoassay Methods I and II.

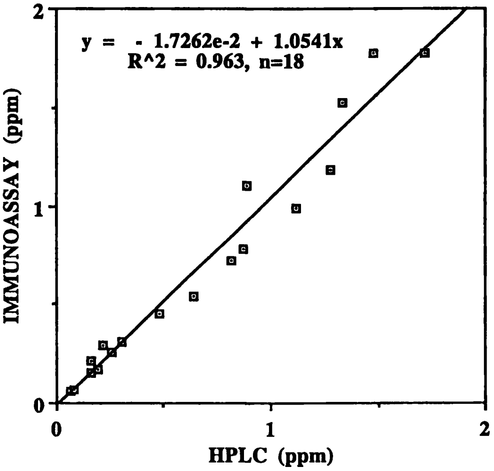

Figure 2. Comparison of Benomyl Residues as MBC in Field-Treated Crop Samples by Immunoassay, using Methods I or II, and HPLC Analyses.

Table V. HPLC Recovery Data for Benomyl-Spiked[a] Control Samples.

CROP SAMPLE Spike	HPLC ppm	Recovery
MUSTARD GREENS		
0.40 ppm	0.16	62%
ITALIAN DANDELIONS		
0.40 ppm	0.20	75%
1.0 ppm	0.57	88%
FRESH PARSLEY		
0.10 ppm	0.040	61%
2.0 ppm	0.76	58%
APPLES		
0.10 ppm (Flesh)	0.048	74%
1.0 ppm (Peel)	0.41	62%
0.10 ppm (Flesh and Peel)	0.036	55%
STRAWBERRIES		
0.10 ppm	0.039	59%

[a] Added as benomyl, measured and calculated as carbendazim.

benomyl as MBC in various plant matrices with accuracy, precision, and thoroughness at concentrations below tolerance. This is particularly valuable for benomyl, as analysis of this material is not readily incorporated into standard multiresidue schemes. Since a separate method will almost always be required, development of a simple and efficient one becomes of utmost importance.

This study further demonstrates enzyme immunoassay technology as a valuable and viable means to meet the heavy demands of increased testing that are being required for proper health risk assessment and increased regulatory compliance.

Acknowledgments

The authors wish to give special acknowledgment to the sponsors of this project — Ohmicron Corporation and the National Agricultural Pesticide Impact Assessment Program. Any opinions, findings, conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of Ohmicron Corporation or the National Agricultural Pesticide Impact Assessment Program.

Literature Cited

1. Spittler, T. D.; Marafioti, R. A.; Lahr, L. M. *J. Chromatogr.* **1984**, *315*, 527–531.
2. Singh, R. P.; Brindle, I. D.; Hall, C. D.; Chiba, M. *J. Agric. Food Chem.* **1990**, *38*(8), 1758–1762.
3. Singh, R. P.; Marvin, C. H.; Brindle, I. D.; Hall, C. D.; Chiba, M. *J. Agric. Food Chem.* **1992**, *40*(8), 1303–1305.
4. Calmon, J. P.; Sayag, D. R. *J. Agric. Food Chem.* **1976**, *24*(2), 311–314.
5. Calmon, J. P.; Sayag, D. R. *J. Agric. Food Chem.* **1976**, *24*(2), 426–428.
6. Clemens, G. P.; Sisler, H. D. *Phytopathology* **1969**, *59*, 705–706.
7. Chiba, M.; Cherniak, E. A. *J. Agric. Food Chem.* **1978**, *26*(3), 573–576.
8. Peterson, C. A.; Edgington, L. V. *J. Agric. Food Chem.* **1969**, *17*, 898.
9. Bushway, R. J.; Kugabalasooriar, J.; Perkins, L. B.; Harrison, R. O.; Young, B. E. S.; Ferguson, B. S. *J. AOAC Int.* **1992**, *75*(2), 323–327.
10. Report #440/1–83/079C; U. S. Environmental Protection Agency: Washington, DC, 1983.
11. Chiba, M.; Doornhos, F. *Bull. Environ. Contam. Toxicol.* **1974**, *11*(3), 273–274.
12. RaPID Assays Application Procedures, No. T00035 and T00036, Ohmicron Corp.: Newtown, PA.
13. RaPID Assays Application Procedures, No. T00048, T00050, T00057, Ohmicron Corp.: Newtown, PA.
14. RaPID Prep Application Procedures, No. A00149, Ohmicron Corp.: Newtown, PA.
15. Mills, P. A.; Onley, J. H.; Gaither, R. A. *J. Assoc. Off. Anal. Chem.* **1963**, *46*, 186.
16. Zweig, G.; Gao, R. *Anal. Chem.* **1983**, *55*, 1448–1451.
17. Bourke, J. B.; Spittler, T. D.; Young, S. J. In *Principles of Environmental Sampling*; Keith, L. H., ed.; American Chemical Society: 1988; pp 355–361.

RECEIVED October 3, 1995

Chapter 12

Detection of Pesticides in Human Milk Samples Collected in Egypt by Enzyme-Linked Immunosorbent Assay

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Human milk samples (80) collected from 10 different cities in Egypt were tested for ethyl parathion, diflubenzuron and dieldrin using an indirect enzyme linked immunosorbent assay (ELISA). Ethyl parathion was detected in all samples (100%) at levels ranging from 0.0065 to 20 μg/mL, but dieldrin was detected in 73 samples (91.25%). On the other hand, diflubenzuron also was detected in 48 samples (60%). These results present evidence for the persistence (or continued use) of these pesticides in Egyptian agriculture and their transmission through the food chain.

Insecticides have been used for many years in plant protection and public health programs; however, they are now being shown to cause serious environmental problems. The problems are such that certain pesticides have been classed as priority pollutants. These priority pollutants, concentrated along the food chain, reach the human body in the daily diet and are deposited and accumulated in adipose tissues (*1,2*). The persistence of such pesticides within the environment leads to significant contamination of foods, mainly milk and its products, which in Egypt are the main source of food for pregnant and lactating women (*3*).

Egypt used 617,507 metric tons of pesticides in the period between 1952 and 1984 (*4*). Nearly 50% of this quantity found its way into the soil and is a permanent source of persistent pesticides, affecting plants, soil fertility, air, water and useful fauna (*5,6*).

Pesticides have played an important part in the dramatic increases in agricultural productivity which have been achieved in the developed world over the last few decades. However, public concern about the indiscriminate use of pesticides, specifically the organochlorine insecticides, such as dichloro-diphenol tetrachloroethan (DDT), a-BHC, dieldrin and aldrin (highly stable, lipophilic, and metabolize slowly) (*7,8*) has increased (*9–11*). The toxic effects of organochlorine compounds differ in detail, but all are neurotoxic substances. One major difference is

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that DDT initially affects the peripheral nervous system; whereas, g-BHC and aldrin appear to attack the central nervous system. At the physiological level, only the effect of DDT on nerve-muscle systems has been investigated intensively. Despite some differences, all organochlorine compounds destabilize neural activity, manifested by a hyper-excitability of nerves and muscles (*8,11,12*). Organophosphorus compounds came into general use as insecticides during the late 1950s and early 1960s. The growth in the use of these compounds resulted in part from the resistance which certain insects were developing toward chlorinated hydrocarbons (*13*). The site of action of organophosphorus compounds is the synaptic gap between nerve cells. The phosphorus-containing pesticides deactivate the enzyme acetylcholinesterase (ACHE) and prevents it from destroying acetylcholine. Normally, ACHE breaks down the chemical acetylcholine after it has carried an impulse from one nerve fiber to another (across the synaptic gap). The result of interfering with normal activity is a build-up of acetylcholine and a barrage of extraneous nerve impulses which disrupt normal function; tremors, convulsion, paralysis and death follow (*8,11,12*). On the other hand, the growth regulator diflubenzuron is a potent insecticide by virtue of its ability to inhibit synthesis of cuticle chitin, thus disrupting normal insect growth and development (*13–16*). Its specificity for those species whose structural integrity depends upon chitin (the arthropod phylum) makes diflubenzuron more selective than the broad spectrum pesticides (*17*). However, its effects on nontarget arthropods (*18*), its persistence in the environment (*19–25*), and the carcinogenic activity of 4-chloroaniline (a potential diflubenzuron metabolite) (*26,27*), serves to retain interest in the short- and long-term actions of diflubenzuron in the environment. Chromatography methods have been widely used to monitor levels of pesticides in foodstuffs such as milk and dairy products (*28–34*). However, these techniques are time consuming and labor intensive. A robust, yet simple system is required for routine analysis.

This paper reports the use of an ELISA to determine the levels of ethyl parathion, dieldrin and diflubenzuron in human milk samples (80) collected form 10 different cities in Egypt. These results present evidence for the persistence (or continued use) of theses pesticides in Egyptian agriculture and their possible transmission through the food chain.

Materials and Methods

Preparation of Antigens.

Ethyl parathion. The preparation of antigen was carried out according to the method of Ercegovich *et al.* (*35*).

Reduced Parathion. Ethyl parathion (3.44 mmol) (British Greyhound and Allied Chemicals, Birkenhead, Merseyside, UK) was dissolved in 10 mL of diethyl ether and extracted with cold 1% Na_2CO_3 to remove impurities. To the phenol-free ether solution was added 10 mL of 9:1 acetic acid: concentrated HCl and zinc powder (2 g). The yellow reaction mixture was stirred under reflux for 45 min, after which time the solids were filtered from the colorless solution and subsequently washed with distilled water and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure (Figure 1).

BSA-Reduced Parathion. Reduced parathion (105 mg, 0.4 mmol) was dissolved in 50 mL of water containing 2 mmol of HCl. The solution was cooled in an ice bath, and chilled 0.1 N $NaNO_2$ solution was added dropwise until a positive starch-iodide paper test was obtained. The mixture was stirred for 30 min and the excess nitrous acid was decomposed with urea. The reaction mixture was added to 500 mg of bovine serum albumin (BSA) and dissolved in 100 mL of borate buffer (pH 9), and the mixture was stirred in an ice bath for 2 h. The bright orange chloride reaction mixture was dialyzed using two changes of distilled water (4 L) daily for 5 d at 4 °C. The pH of the dialysis water was adjusted to neutrality to avoid precipitation of the conjugate. The dialyzed product was lyophilized and the conjugate was stored at -20 °C until required (Figure 1).

Dieldrin. 6,7-dihydro-6-carboxyaldrin was prepared as described previously. This material was then conjugated to bovine serum albumin.

Diflubenzuron. Preparation of the conjugate was carried out according to the method of Wie and Hammock (*36*). Diflubenzuron (0.052 mmol) was dissolved in 500 µL of dry dioxane. To this solution was added tri-*N*-butylamine (0.062 mmol) and then isobutyl chloroformate (0.062 mmol). The resulting mixture was stirred at room temperature for 30 min and then added dropwise to a solution of ovalbumin (150 mg) in 15 mL of 0.2 M borate buffer, pH 8.7, and stirred at room temperature overnight. The conjugate was then dialyzed extensively in 7 nM phosphate buffer containing 0.15 M NaCl and 0.02% NaN_3. This procedure resulted in the covalent binding of 2.5 mol of hapten/50 kilodaltons of protein (Figure 2).

Antibody Production. New Zealand white rabbits (20 weeks old) were immunized with a solution of conjugate (prepared in water) mixed with an equal volume of Freund's complete adjuvant and maintained by booster injections of antigen in Freund's incomplete adjuvant at three month intervals. Each rabbit received 2 mg of immunogen injected subcutaneously into the neck flap at five sites. Blood samples were collected from the lateral ear vein at monthly intervals.

ELISA Methods.

Microtitre Plate Sensitization. Microtitre plates 96-well (Dynatech Immunol II) were coated with the conjugate (5 µg/mL in 0.1 M carbonate/bicarbonate buffer, pH 9.6, 100 µL per well). After overnight incubation at 4 °C the plates were washed three times with coating buffer, emptied and dried at 37 °C. Finally the plates were sealed with tape and stored at 4 °C.

Non-Competitive Indirect ELISA for the Detection of Antibody. Serum samples were tested for antibody by means of a non-competitive ELISA. Serial dilutions of the serum in the range of 1:10 to 1:10^5 were prepared in wash buffer (0.01 M

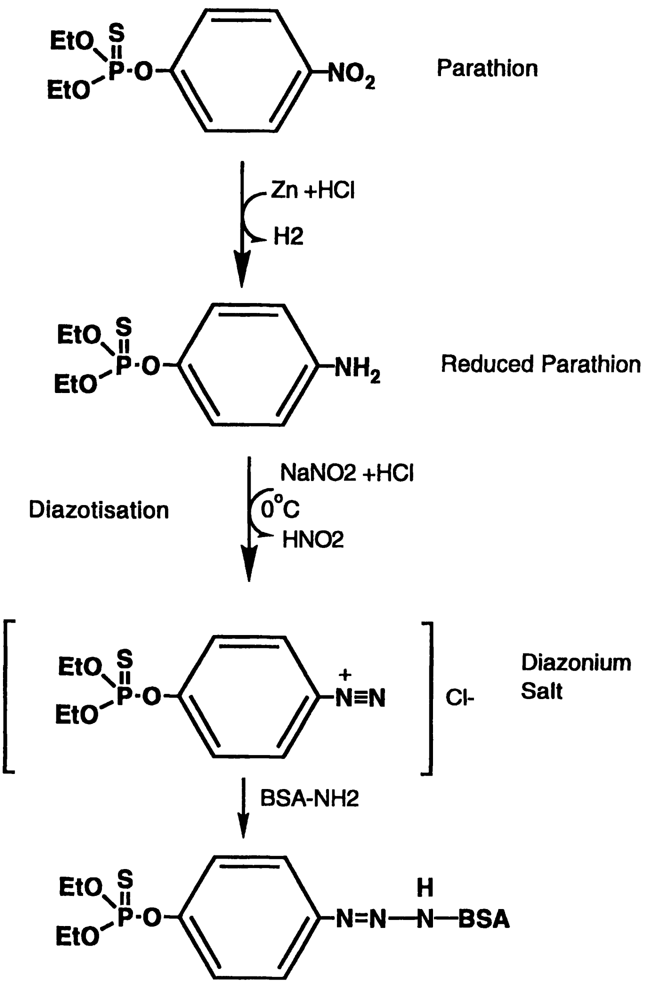
EtO
S
P-O
EtO
NO2
Parathion
Zn +HCl
H2
NH2
Reduced Parathion
Diazotisation
NaNO2 +HCl
0°C
HNO2
+
N≡N
Cl-
Diazonium Salt
BSA-NH2
H
N=N—N—BSA

Figure 1. Preparation of parathion-BSA conjugate.

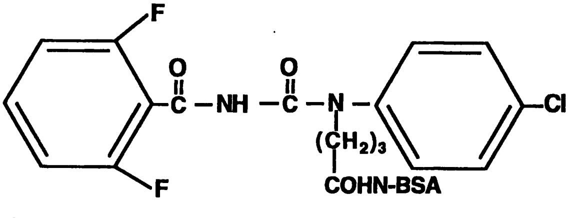
F
O
C
NH
O
C
N
(CH2)3
COHN-BSA
Cl
F

Figure 2. Preparation of diflubenzuron-BSA conjugate.

phosphate containing 0.5 M NaCl, 0.5% Tween 80, 0.01% Thimerosal, pH 7.8) containing 1% bovine serum albumin to reduce non-specific reactions. Samples of each dilution (0.1 mL) were added in duplicate to wells of a sensitized microtitre plate. The negative control was dilution buffer. The plate was then incubated for 1 h at room temperature with constant shaking, emptied, washed 5 times with wash buffer, and blot dried. Goat anti-rabbit IgG peroxidase conjugate (0.1 mL) (Sigma Chemical Co., St. Louis, MO), diluted 1:3000 in wash buffer, was added to each well. The plate was then incubated 1 h, washed 5 times, and blot dried as before. The substrate was prepared from a stock solution of 2,2'-azino bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) 20 mg/mL in water. Substrate solution [0.15 mL ABTS stock solution + 4.85 mL citrate buffer 0.1 M citrate monohydrate, pH 4.0 with 0.05 mL of H_2O_2 (30%)] 0.1 mL was added to each well. Plates were incubated with shaking at room temperature. The absorbance was measured at 405 nm on a Titertek Multiscan (Flow Laboratories, Ltd., Rickmansworth, Hertfordshire, UK).

Samples were recovered following adsorption of BSA, and the reactive fractions were tested in order to determine their optimum working dilution prior to use in the competitive ELISA.

Indirect Competitive ELISA. Analysis of control pesticide samples was by competitive ELISA. Dieldrin (Fluorochem Ltd., Derbyshire, UK) was dissolved in methanol while ethyl parathion and diflubenzuron were dissolved in propanol, and then different concentrations were prepared by dilution in human milk containing 1% BSA (to reduce non-specific reactions). Dilutions were prepared such that the final concentration of methanol was always less than 1%. Prior analysis indicated that methanol and propanol up to 10% did not interfere with binding of the antibody to the coated wells. Control samples and human milk samples to be tested for pesticides (50 µL) were added to wells of the sensitized plates. Purified antibody (50 µL) was added to each test and control well, milk and buffer samples without pesticides (a positive control, maximum antibody binding). Plates were incubated for 1 h at room temperature with constant shaking. The subsequent steps were the same as for the antibody detection ELISA above.

Results and Discussion

Human milk samples were collected from 10 different cities in Egypt and tested for the presence of pesticides using an indirect ELISA. The results showed that the samples contained a considerable amount of pesticides (Table I, II and III). The results showed that all the samples contained ethyl parathion from 0.006 to 20.0 ppm. Although, there are restrictions on the use of parathion before the harvesting of sprayed crops, farmers do not always follow these regulations, so we believe that this is the main reason for detecting parathion in the samples. Diflubenzuron and dieldrin were detected in 48 (60%) and 73 (91.25%) out of the total samples ranging from 0.005 to 5 ppm, and 0.006 to 28 ppm, respectively. The highest values of ethyl parathion, diflubenzuron and dieldrin were 20, 5 and 28 ppm, and were detected in El-Mahala, Kafer El-Zaiyat and Shibin El-Kom, respectively. The values from each city varied considerably with no apparent relationship between location and pesticide

Table I. Parathion levels in human milk samples (ppm).

Sample No.	1	2	3	4	5	6	7	8	Average
Cairo	0.120	0.090	0.100	0.100	0.100	0.110	0.110	0.130	0.082
Giza	10.000	0.080	0.100	0.070	0.070	0.110	0.007	0.125	1.319
Benha	0.130	0.135	0.090	0.125	0.100	0.135	0.090	0.120	0.115
Tanta	0.070	0.070	0.040	0.006	0.120	0.060	0.260	0.100	0.090
K. El-Zaiyat	4.000	10.000	10.000	0.520	0.140	10.000	0.700	9.000	5.545
Mahala	0.650	0.680	20.000	4.000	0.500	0.009	0.140	0.130	3.263
Zagazig	10.000	0.140	0.650	10.000	0.700	0.600	0.500	10.000	4.073
Shibin El-Kom	0.080	0.070	0.070	0.700	0.110	0.100	0.120	0.135	0.164
Fayoum	0.110	0.100	0.130	0.100	0.110	0.110	0.075	–[a]	0.175
Beni Suef	0.140	0.100	0.140	0.500	0.140	0.135	0.140	0.130	0.160

[a] level of parathion was less than 0.005 ppm.

Table II. Diflubenzuron levels in human milk samples (ppm).

Sample No.	1	2	3	4	5	6	7	8	Average
Cairo	0.025	0.025	0.007	–[a]	4.000	0.041	0.005	–[a]	0.512
Giza	0.041	0.015	–[a]	0.250	0.020	0.250	0.220	–[a]	0.098
Benha	–[a]	0.020	–[a]	1.000	–[a]	–[a]	0.040	1.000	0.257
Tanta	0.040	0.005	–[a]	0.025	–[a]	0.006	–[a]	–[a]	0.009
K. El-Zaiyat	–[a]	0.010	0.250	0.010	–[a]	–[a]	–[a]	5.000	0.658
Mahala	–[a]	0.025	–[a]	1.000	–[a]	1.000	0.250	–[a]	0.248
Zagazig	–[a]	–[a]	0.040	0.006	0.020	–[a]	0.020	–[a]	0.010
Shibin El-Kom	–[a]	0.220	–[a]	0.006	–[a]	–[a]	1.000	–[a]	0.153
Fayoum	0.010	0.010	–[a]	0.045	0.250	–[a]	0.220	–[a]	0.660
Beni Suef	0.010	0.005	0.250	0.006	0.040	0.006	8.000	0.250	0.071

[a] level of diflubenzuron was less than 0.005 ppm.

Table III. Dieldrin levels in human milk samples (ppm).

Sample No.	1	2	3	4	5	6	7	8	Average
Cairo	0.830	3.000	0.083	0.830	1.000	0.670	0.670	3.000	1.260
Giza	0.200	1.050	2.000	0.390	0.350	8.750	7.500	3.000	2.905
Benha	2.500	3.000	2.000	3.000	1.000	15.000	7.000	5.000	4.812
Tanta	0.320	10.000	7.000	0.830	0.200	0.010	0.200	2.500	2.632
K. El-Zaiyat	0.070	0.080	–[a]	0.350	0.280	–[a]	5.000	0.050	0.971
Mahala	0.090	0.280	0.040	0.040	5.500	3.000	8.750	0.350	2.256
Zagazig	0.390	–[a]	0.030	0.050	–[a]	–[a]	0.006	–[a]	0.119
Shibin El-Kom	–[a]	0.520	5.000	15.000	28.000	0.830	5.000	0.670	7.860
Fayoum	0.020	2.000	0.350	0.030	2.000	2.500	7.000	2.500	2.050
Beni Suef	0.010	0.480	1.000	0.200	0.350	0.520	3.000	20.000	3.195

[a] level of dieldrin was less than 0.005 ppm.

concentrations. However, there are no formal statements indicating that these pesticides are allowed to be used; similarly, no clinical, age or social data was available on the mothers supplying the samples, and thus there may be some relationship between the accumulated level of pesticide and age. This study demonstrates the usefulness of the ELISA for the detection of pesticides and its applicability in studing human breast milk. The technique is sensitive, detecting pesticides in the range of 0.005 to 40.0 ppm. It is simple, robust and could provide a means of screening large numbers of samples, and it also could be used in assessing the risk to infants from maternal breast milk. The results also indicate the need to monitor these levels routinely and to collect biographical data of the donors so that correlation with age, employment and geographic location can be determined.

Literature Cited

1. Morita, M.; Mimura, S.; Yagyu, G. OHI. *Environ. Poll.* **1975**, 175–179.
2. Mori, Y.; Masanori, K.; Ercuko, O.; Toshhihiro, O. *Bull. Environ. Contam. Toxicol.* **1983**, *30*, 74–79.
3. Abdel-Gawaad, A. A.; Shams El-Dien, A. *J. Egypt. Soc. Toxicol.* **1989**, *5*, 49–52.
4. Abdel-Gawaad, A. A. *Survey of pesticides used in Egypt*; 2nd International Congress for Soil Pollution and Protection from Pesticide Residues: Cairo, Egypt, 1985, Vol. II; pp 33–86.
5. Abdel-Gawaad, A. A.; EL-Khishin, M.; Hefney, M. S. H. *Soil pollution by pesticide residues: side effect on germination, growth and crop yield;* 1st International Congress for Soil Pollution and Protection from Pesticide Residues: Cairo, Egypt, 1981a, part II; pp 189–326.
6. Abdel-Gawaad, A. A.; Ahmed, S. M.; Hosieny, T.; Khaled, O. M. *Soil pollution by pesticide residues: 2-side effect on soil fertility*; 1st International Congress for Soil Pollution and Protection from Pesticide Residues: Cairo, Egypt, 1981b, Part II; pp 196–219.
7. *Pesticides, Chemicals and Health;* British Medical Association, 1990.
8. Hassall, K. A. *The Chemistry of Pesticides;* Macmilan Education, Ltd.: London, UK, 1982.
9. Carson, R. *The Silent Spring;* Penguin: London, UK, 1962.
10. Gibbons, J. H. *Pesticide Residues in Food*; Technomic Publishing Co., Inc.: USA., 1988.
11. Fletcher, W. W. *The Best War*; Basil Blackwell: Oxford, 1974.
12. Bockris, J. O. M. *Environmental Chemistry*; Plenum Press: New York, NY, 1977.
13. Hajjar, N. P.; Casida, J. E. *Science* **1978**, *200*, 1499–1500.
14. Mulder, R.; Gijswjt, M. *J. Pestic. Sci.* **1973**, *4*, 737.
15. Post, L. C.; deJong, B. J.; Vincent, W. R. *Pestic. Biochem. Physiol.* **1974**, *4*, 473.
16. Verloop, A.; Ferrell, C. D. *Pesticide Chemistry in the 20th Century*; Plimmer, J., ed.; American Chemical Society: Washington, DC, 1977, ACS Symposium Series No. 37; p 237.

17. Marx, J. L. *Science* **1977**, *197*, 1170–1172.
18. Muzzarelli, R. *CRC Crit. Rev. Environ. Control* **1986**, *16*, 141–146.
19. Ivie, G. W.; Bull. D.; Veech, J. A. *J. Agric. Food Chem.* **1980**, *28*, 330–337.
20. Mansager, E. R.; Still, G. G.; Frear, D. S. *Pestic. Biochem. Physiol.* **1979**, *21*, 172–182.
21. Martinant, P. J.; Christman, V.; Cooper, R. J.; Dodge, K. M.; Whitmore, R. C.; Booth, G.; Seidel, G. *Bull. Environ. Contam. Toxicol.* **1987**, *39*, 142–149.
22. Mutanen, R. M.; Siltanen, H. T.; Kuukka, V. P. *J. Pestic. Sci.* **1988**, *23*, 131–140.
23. Nigg, H. N.; Cannizaro, R. D.; Stamper, J. H. *Bull. Environ. Contam. Toxicol.* **1986**, *36*, 833–838.
24. Schaefer, C. H.; Dupras, E. F. *J. Agric. Food Chem.* **1967**, *24*, 733–739.
25. Van den Berg. *Internal Report No.* 56637/47/1986; Duphar BV: Weesp, Holland, 1986.
26. Seuferer, S. L.; Breymer, H. D.; Dunn, J. *J. Pestic. Biochem. Physiol.* **1979**, *10*, 174–180.
27. Chhabra, R. S. *Toxicology and Carcinogenesis Studies of para-Chloroaniline Hydrochloride*; U.S. Department of Health and Human Services: Washington, DC, 1989, National Toxicology Program Technical Report No. 351.
28. Downey, W. K.; FlynnL, M. P.; Aherne, S. A. *J. Dairy Res.* **1975**, *42*, 21–29.
29. Bauza, C. A. *Arch. Pediatr. Urug.* **1975**, *46*, 139–148.
30. Tyllinen, H.; Karppanen, E.; Nurmi, E. *J. Sci. Agric. Soc. Finl.* **1975**, *47*, 533–540.
31. Pompa, G.; Fiordelisi, R.; Ostinelli, G.; Zecconi, A. *Arch. Vet. Ital.* **1984**, *35*, 74–81.
32. Ezz, A. E.; Seleim, Z. M.; Abdel-Gawaad, A. A. *J. Egypt Soc. Toxicol.* **1991**, *7*, 125–134.
33. Wimmer, M. J.; Smith, R. R. *J. Agric. Food Chem.* **1991**, *39*, 280–286.
34. Association of Official Agricultural Chemists. *Official Methods of Analysis*; AOAC: Washington, DC, 1980, 13th ed.
35. Ercegovich, C. D.; Vallejo, R. P.; Getting, R. R.; Woods, L.; Bogus, E. R.; Mumma, R. O. *J. Agric. Food Chem.* **1981**, *29*, 559–583.
36. Wie, S. L.; Hammock, B. D. *J. Agric. Food Chem.* **1982**, *30*, 949–957.

RECEIVED October 11, 1995

Chapter 13

Detection of Dieldrin by Enzyme-Linked Immunosorbent Assay in Some Dairy Products

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An enzyme-linked immunosorbent assay (ELISA) was used to monitor the distribution of dieldrin in Domiati cheese, Cheddar cheese and Yogurt. The effect of the salt concentration (0–10% w/v) on the distribution of aldrin/dieldrin between curd and whey in Domiati cheese was studied. There was a positive correlation between the amount of salt added to the milk and the amount of aldrin/dieldrin detected in the whey. On the other hand, in cheddar and yogurt filtrate (acid whey), aldrin/dieldrin were generally not detected.

The use of pesticides has contributed in a large part to the high production levels which modern agriculture in developed countries have been able to achieve. Egypt used 61,705 metric tons of pesticides in the period between 1952 and 1984 (*1*), nearly 50% of this quantity found its way into the soil and is a permanent source of persistent pesticides (*2,3*). Measurable amounts of pesticide residues in our food including dairy products present a variety of problems. For example organochlorine insecticides which have been in use for more than four decades in agriculture and public health programs all over the world, have caused one of the most serious environmental problems and are commonly detected in air, soil, aquatic and terrestrial wild life, and in different food items (*4–7*). The organochlorine insecticides are characterized by their capability to travel great distances from application sites, and they are sufficiently stable and retain their identity for years, they tend to concentrate in fatty tissues of higher members of the food chain. Several organochlorine residues including DDT and dieldrin, were detected in the liver and adipose tissues of humans at higher levels than other pesticide groups. Food contaminated with pesticide residues are reported to be the major source of human's daily intake. Milk, milk based formula, and its by-products contain a profound amount of pesticide residues (*6*). Animal feed, feed mixtures and fodder grasses have been shown to be the major source of contamination of animal tissues and milk and milk products (*8–11*). Studies have clearly demonstrated the transfer of pesticide residues from animal food

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stuffs into the tissues of farm animals (*12*). Further, organochlorine insecticides have been the pesticides found most frequently in milk and dairy products (*13–20*).

Chromatographic methods have commonly been used to analyze pesticide residues in food stuffs (*17,18,21–23*). These methods are time consuming and laborious, tedious, require personal skills and are expensive. The existence of immunoassays for analysis of pesticide residues such as aldrin/dieldrin provides a relatively cheap, sensitive, reliable and rapid assay which can be used to monitor food samples routinely. The present paper was developed to examine the distribution of pesticides, parathion, diflubenzuron and dieldrin spiked into milk and processed into different dairy products.

Materials and Methods

Control Samples of Dairy Products. Control samples were made from cow's milk as follows:

Domiati cheese.

Pasteurized milk, 37 °C
↓
Salting (0, 2.5, 5, 7.5, 10%, w/v)
↓
Coagulation (Renneting), 37 °C
↓
Draining of whey (whey was collected)
↓
Packaging

Cheddar cheese.

Pasteurized milk, 30 °C
↓
Addition of starter 1.5% (30 min/30 °C)
↓
Coagulation (Renneting), 30 °C
↓
Cutting of curd (whey acidity 0.15%)
↓
Gently stirring (10 min) and scalding of curd
(up to 40 °C in 60 min and whey acidity 0.18%)
↓
Draining of whey (at acidity 0.19%) and whey was collected
↓
Cheddaring (curd acidity 0.85% in 3 h)
↓
Milling
↓
Packaging and pressing

Yogurt.

Milk
↓
Heat treatment (90 °C/15 min)
↓
Cooling to 40 °C
↓
Inoculation of the starter (3%)
↓
Incubation (40 °C/3 h)
↓
Cooling of yogurt (4 °C)
↓
Filtration of yogurt, Yogurt filtrate (acid whey)

Spiking of the Milk Samples. Dieldrin was dissolved in methanol and added to the milk to reach a concentration of 5 μg/mL.

Preparation of Cheese Extract. Freshly prepared cheese curd was mixed with distilled water (1:2) in a blender for 5 min at room temperature. The homogenate was centrifuged at 3000 rpm and the supernatant was used as the cheese extract.

Preparation of Whey from Yogurt. Yogurt curd was carefully stirred using a spatula and filtered using filter paper (Whatman No. 1), and the filtrate was collected.

Preparation of Immunogen (Figure 1)

Functionalized Hapten. 6,7-Dihydro-6-carboxyaldrin was prepared as described by Langone and Van Vunakis (*24*), by heating a mixture of hexachlorocyclopentadiene (10 g) (Aldrich Chemical Co., Milwaukee, WI) and bicyclo-(2.2.1)-5-heptene-2-carboxylic acid (5.0 g; 36 mmol) (Lancaster Synthesis Inc., Windham, NH) at 90 °C for 48 h. The black semi-solid was dissolved in hot ethanol (150 mL) and an insoluble solid was separated by filtration. The filtrate was concentrated to dryness in vacuo and the residue was triturated with petroleum ether to give approximately 4.0 g of tan solid. Two recrystallizations from chloroform-petroleum ether gave a white powder. The melting point of the product was 216 °C.

Preparation of Conjugates for Immunization. To 100 mg (0.24 mmol of the hapten derivative and 70 mg (0.61 mmol) *N*-hydroxysuccinimide dissolved in 1.0 mL dimethylformamide (DMF) was added 60 mg (0.29 mmol) of *N*,*N*′-dicyclohexycarbamide (DCC). After 30 min, precipitated *N*,*N*′-dicyclohexylurea was separated by centrifugation. A 0.01 mL aliquot of the supernatant liquid in 1.0 mL dioxane was added to 60 mg of bovine serum albumin (BSA) dissolved in 3.0 mL, water pH 7.2. After standing at 4 °C overnight, the reaction mixture was dialyzed

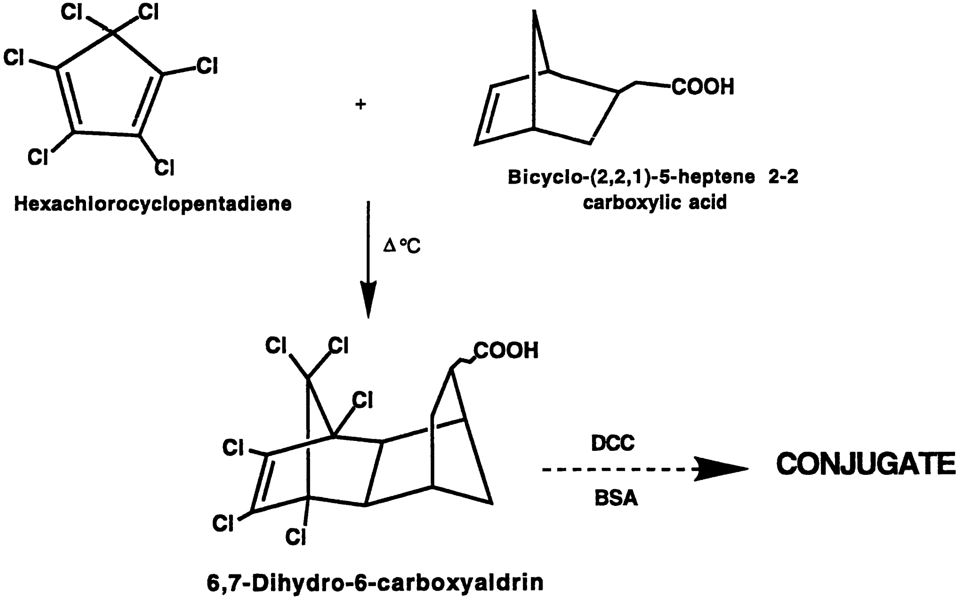
Cl
Cl
Cl
Cl
Cl
Cl
Hexachlorocyclopentadiene
+
COOH
Bicyclo-(2,2,1)-5-heptene 2-2
carboxylic acid
Δ °C
Cl
Cl
Cl
Cl
Cl
Cl
COOH
6,7-Dihydro-6-carboxyaldrin
DCC
BSA
CONJUGATE

Figure 1. Preparation of dieldrin-BSA conjugate.

exhaustively against 50% dioxane-water, then against 0.005 M phosphate, 0.15 M sodium chloride, pH 7.2.

Antibody Production and ELISA Assay. New Zealand white rabbits (20 weeks old) were immunized with a solution of conjugate (prepared in water) mixed with an equal volume of Freund's complete adjuvant and maintained by booster injections of antigen in Freund's incomplete adjuvant at three months intervals. Each rabbit received a total of 2 mg per 1.0 mL of immunogen injected subcutaneously into the neck flap at five sites (200 µL per site). Blood samples were collected from the lateral ear vein at monthly intervals and tested for antibody production using ELISA.

Purification of Antibody. Positive serum samples were treated by affinity chromatography to remove bovine serum albumin antibodies. CNBr-sepharose 4B (Pharmacia, Piscataway, NJ) was linked to BSA as per manufacturers handbook. Serum samples were diluted 1:1 in loading buffer (0.1 M phosphate, 0.5 M NaCl, pH 7.2) and aliquots (5 mL) loaded onto a 10 cm column filled with swollen gel. The unbound fraction containing anti-pesticide antibodies was eluted with loading buffer. The BSA reactive fraction was subsequently recovered by eluting the column with Glycine-HCl (0.1 M) pH 4.0.

Microtitre Plate Sensitization. Dynatech Immunol II 96-well microtitre plates (Dynatech Laboratories Ltd., Billingshurst, Sussex, UK) were coated with the conjugate (5 µg/mL in 0.1 M carbonate/bicarbonate buffer, pH 9.6, 0.1 mL per well). After overnight incubation at 4 °C the plates were washed three times with coating buffer, emptied and dried at 37 °C. Finally the plates were sealed with tape and stored at 4 °C.

Non-Competitive Indirect ELISA for the Detection of Antibody. Serum samples were tested for antibody by means of a non-competitive ELISA. Serial dilutions of the antibody, in the range of 1:10 to 1:10^5 were prepared in wash buffer containing 1% bovine serum albumin (0.01 M phosphate containing 0.5 M NaCl, 0.5% Tween 80, 0.01% Thimerosal, pH 7.8). Samples of each dilution (0.1 mL) were added in duplicate to wells of a sensitized microtitre plate. The negative control was dilution buffer. The plate was then incubated for 1 h at room temperature with constant shaking, emptied, washed 5 times with wash buffer and blot dried. Goat anti-rabbit IgG peroxidase conjugate (0.1 mL) (Sigma Chemical Co., St. Louis, MO), diluted 1:3000 in wash buffer was added to each well. The plate was then incubated 1 h, washed 5 times and blot dried as before. The substrate was prepared from a stock solution of 2,2'-azino-bis(3-ethylbenzthialozine-6-sulphonic acid (ABTS) 20 mg/mL in water. Substrate solution (0.15 mL ABTS stock solution + 4.85 mL citrate buffer 0.1 M citrate monohydrate, pH 4.0 with 0.05 mL of H_2O_2 (30%)) 0.1 mL was added to each well. Plates were incubated, with shaking at room temperature. The absorbance was measured at 405 nm on a Titertek Multiscan (Flow Laboratories Ltd., Rickmansworth, Hertfordshire, UK). Samples recovered following removal of BSA reactive fractions were tested in the same way in order to determine their optimum working dilution prior to use in the competitive ELISA.

Indirect Competitive ELISA Procedure. Analysis of prepared pesticide samples was conducted by competitive ELISA. Dieldrin (Fluorochem Ltd., Derbyshire, UK) was dissolved in methanol (1 mg/mL) then different concentrations were prepared by dilution in control whey or cheese extracts in Domiati cheese (0, 2.5, 5, 7.5 and 10% salt concentration), cheddar cheese and acid whey containing 1% BSA, to reduce non-specific reactions. Dilutions were prepared such that the final concentration of methanol was always less than 1%. Prior analysis indicated that methanol up to 10% did not interfere with binding of the antibody to the coated wells. Pesticide samples (50 µL) were added to wells of the sensitized plates. Purified antibody (50 µL) was added to each well containing pesticide solution and to each well containing buffer or milk (50 µL) without pesticide which were included to provide a positive control (maximum antibody binding). Plates were incubated for 1 h at room temperature with constant shaking. The subsequent steps were the same as for the antibody detection ELISA described previously. Measurement of the absorbance at 405 nm of the test and control samples indicated the degree of inhibition of antibody binding produced by the test samples.

Results and Discussion

The molecular weight of the aldrin/dieldrin hapten is too low to initiate an immune response (*25*). It was therefore necessary to conjugate the dieldrin hapten to a larger carrier protein. Rabbits were immunized with the conjugate and the antiserum used to develop an ELISA capable of detecting pmol levels of dieldrin. The serum contained antibodies which reacted with either the carrier protein (BSA) or the hapten, therefore affinity chromatography was used to remove the BSA reactive antibodies.

The pesticide used in this study is poorly soluble in water, therefore, methanol was used as the primary solvent. To ensure that the presence of methanol in the assay did not interfere with antibody binding, buffer plus methanol over a range of 0–10% methanol was tested. No effect was detected over this range of methanol concentration. Standard pesticide dilutions were prepared by dissolving the analyte in methanol and diluting the resulting solution with whey containing salt in the range of 0, 2.5, 5, 7.5, 10% (Figure 2), Domiati cheese (0, 2.5, 5, 7.5, 10% salt) extracts (Figure 3), Cheddar cheese extract (Figure 4) and Cheddar cheese whey (Figure 5).

Domiati cheese is commonly used in Egypt. This type of cheese differs from the other types in salting method. The salt is added to the milk (pasteurized or raw) at high levels in the range of 5–10%, while for cheddar cheese, the salt was added to the curd after draining the whey to a level of 2%.

Table I describes the Domiati cheese yield of control and contaminated samples. The cheese yield was increased by salt concentration and addition of pesticide. The curd from the contaminated milk had a softer and weaker texture than the control (*26*).

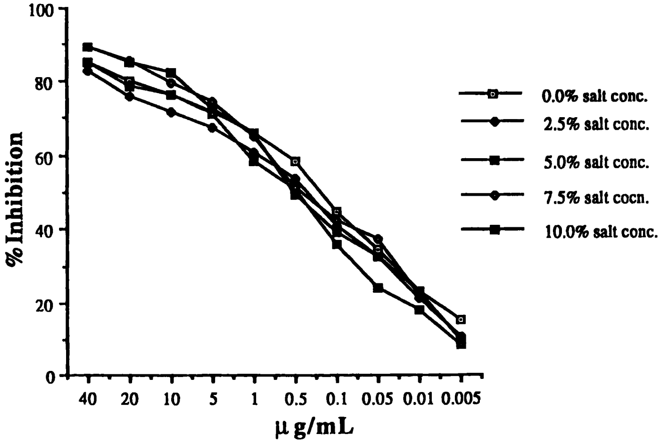

Figure 2. Inhibition curve for dieldrin in 0–10% salting whey.

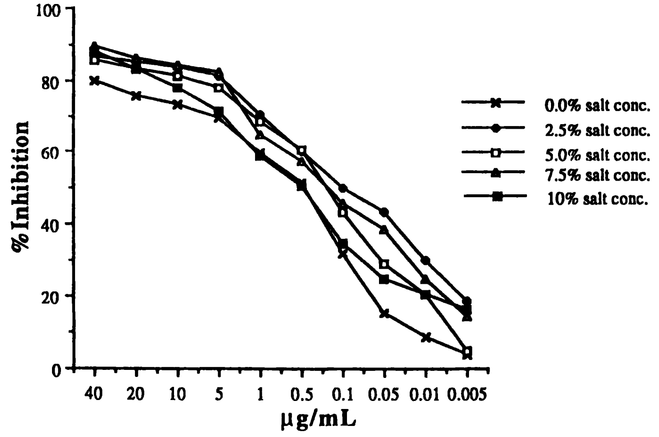

Figure 3. Inhibition curve for dieldrin in 0–10% salting Domiati cheese extract in water (1:2).

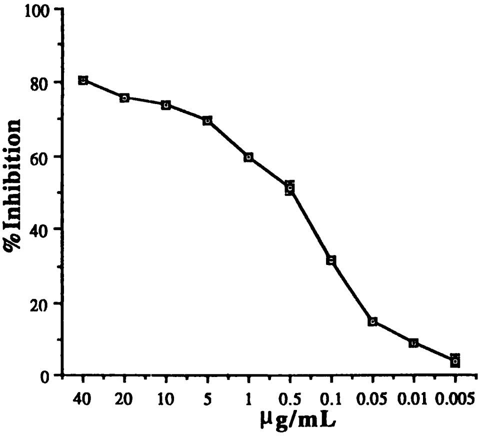

Figure 4. Inhibition curve for dieldrin in cheddar cheese extract in water (1:2).

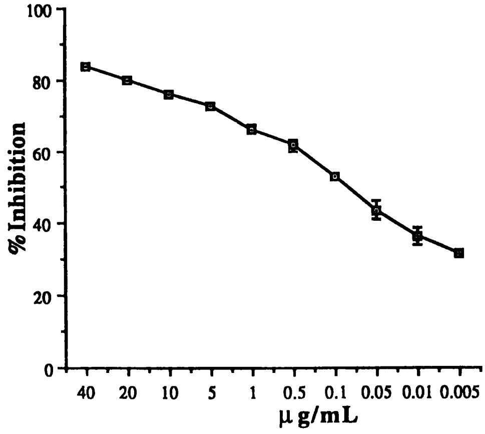

Figure 5. Inhibition curve for dieldrin in cheddar cheese whey.

Table I. Cheese yield of control and contaminated samples at 0–10% salt.

Salt conc. (%)	Cheese yield (%) Control samples	Cheese yield (%) Contaminated Samples
0.0	18.80	20.34
2.5	23.90	28.22
5.0	28.42	33.22
7.5	30.70	33.41
10.0	31.00	37.02

The effect of salt concentration on dieldrin in salted whey is shown in Table II. The results clearly show that the content of dieldrin in whey was increased. The highest value of pesticide at 10% salt concentration was 0.75 ppm while it was 0.1 ppm at 2.5% salt concentration.

Table II. The effect of salt concentration on dieldrin in whey.

Salt conc. (%)	Dieldrin (ppm)
0.0	0.00
2.5	0.10
5.0	0.15
7.5	0.50
10.0	0.75

Table III shows no significant differences in the concentration of dieldrin recovered from cheese extracts. Even with an increase in salt concentrations no dieldrin was detected in the extract. Lack of recovery is probably due to the poor solubility of dieldrin in water (*27,28*).

Table III. Aldrin/dieldrin concentration in domiati cheese extract.

Salt conc. (%)	Dieldrin (ppm.)
0.0	< 0.005
2.5	< 0.005
5.0	< 0.005
7.5	< 0.005
10.0	< 0.005

In the case of cheddar cheese, the level of dieldrin either in whey or in the cheese extract was not detectable (lower than 0.005 ppm, Table IV). The same results were obtained in yogurt and acid whey.

Table IV. Dieldrin content in whey and cheese extract of cheddar cheese.

Samples	Dieldrin (ppm)
Whey (control)	0.000
Whey (contaminated)	< 0.005
Cheese extract (control)	0.000
Cheese extract (contaminated)	< 0.005

In this study the results showed that the concentration of salt, method and stage of salting has an effect on the residual pesticides in cheese. This agrees with results which were obtained by Abou Donia *et al.* (*26*). The type of coagulation (acid or rennet) had no effect on the released pesticide in whey.

Literature Cited

1. Abde-Gawaad, A. A. *Survey of pesticides used in Egypt*; 2nd International Congress for Soil Pollution and Protection From Pesticide Residues: Egypt, 1985, Vol. II; pp 33–86.
2. Abdel-Gawaad, A. A.; EL-Khishin, M.; Hefney, M. S. H. *Soil pollution by pesticide residues: side effect on germination, growth and crop yield*; 1st International Congress for Soil Pollution and Protection From Pesticide Residues: Egypt, 1981a, Part II; pp 189–326.
3. Abdel-Gawaad, A. A.; Ahmed, S. M.; Hosieny, T.; Khaled, O. M. *Soil pollution by pesticide residues: 2-side effect on soil fertility*; 1st International Congress for Soil Pollution and Protection From Pesticide Residues: Egypt, 1981b, Part II; pp 196–219.
4. Edwards, C. A. *Environmental Pollution by Pesticides*; Plenum Press: London, UK, 1973.
5. Peric, Z.; Randelovic, B.; Vojinovic V.; Neskovic, H. *Zast. Bilja.* **1981**, *32*, 61–70.
6. Abdel-Gawaad, A. A. *Pesticide residue levels in food stuffs produced from old valley and reclaimed area in Egypt*; 3rd World Conference on Environmental Health Hazards of Pesticides, Cairo, Egypt, 1989.
7. Abde-Gawaad, A. A.; Shams EL-Dien, A. *J. Egypt. Soc. Toxicol.* **1990**, *5*, 49–52.
8. Duggan, R. E. *J. Pestic. Monitor.* **1968**, *2*, 153–162.
9. Kaphlia, B. S.; Seth, T. D. *Environ. Pollut. Ser. B.* **1982**, *3*, 231–237.
10. Pierson, D. A.; Hoffman J. S.; Nord, P. J.; Gebhart, J. E.; Frank, C. W. *J. Agric. Food Chem.* **1982**, *30*, 187–189.

11. Waliszewski, S. M.; Szymczynski; G. A.; Rogowska, Z. *Bull. Environ. Contam. Toxicol.* **1985**, *34*(4), 518–526.
12. Lucas, J. In *Our Polluted Food*; Charles Knight & Company Ltd.: London, UK, 1975.
13. Bacic, B. *Mljekarstvo* **1970**, *20*, 134–136.
14. Tolie, A. B.; Heeschen, W.; Reichumth, J. *XIX Internal. Dairy Cong.* **1974**, *1E*, 461–462.
15. Rodriguez, C. A.; Rodriguez, P. F.; Gonzales, A. P.; Landa, B. O. *Rev. Cubana Cienc. Vet.* **1972**, *3*, 183–186.
16. Luquet, F.; Goursaud, J. G.; Casalis, J. *Ann. Falsif. Expertise Chim.* **1974**, *67*, 217–239.
17. Downey, W. K.; Flynn, M. P.; Aherne, S. A. *J. Dairy Res.* **1975**, *42*, 21–29.
18. Bauza, C. A., II, *Arch. Pediatr. Urug.* **1975**, *46*, 139–148.
19. AlbertL, L. *Bol. Of. Sanit. Panam.* **1981**, *91*, 15–29.
20. Kampe, K. W. *Dtsch. Milchwirtsch.* **1983**, *34*, 871–874.
21. Tyllinen, H.; Karppanen, E.; Nurmi, E. *J. Sci. Agric. Soc. Fin.* **1975**, *47*, 533–540.
22. Pompa, G.; Fiordelisi, R.; Ostinelli, G.; Zecconi, A. *Arch. Vet. Ital.* **1984**, *4*, 74–81.
23. Ezz, A. E.; Seleim, Z. M.; Abdel-Gawaad, A. A. *J. Egypt Soc. Toxicol.* **1991**, *7*, 125–134.
24. Langone, J. J.; Van Vunakis, H. *Res. Commun. Chem. Pathol. Pharmacol.* **1975**, *10*, 63–171.
25. Erlanger; B. F. *Methods Enzymol.* **1980**, *70*, 85.
26. Abou-Donia, S. A.; Abdel-Shahaid, Y.; Shaker, N.; Salam, A.; Ismail, A. *J. Dairy Sci.* **1985**, *68*(suppl. 1), 99.
27. Hassall, K. A. *The Chemistry of Pesticides*; Macmillan Education, Ltd.: London, UK, 1982.
28. Carson, R. *The Silent Spring*; Penguin: London, UK, 1962.

RECEIVED October 18, 1995

Chapter 14

Development of an Enzyme-Linked Immunosorbent Assay for Hexazinone and Its Application to Water

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Polyclonal antibodies to hexazinone were raised in rabbits using a combination of intradermal and subcutaneous injections. An appropriate hexazinone derivative was prepared by refluxing the metabolite 3-(4-hydroxycyclohexyl)-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4-(1H, 3H)-dione and succinic anhydride in pyridine at 100 °C for 6 h. The resulting hexazinone hemisuccinate was converted to an active ester by reacting it with *N*-hydroxysuccinate and 1-ethyl-3-(dimethylaminopropyl)carbodiimide • HCl (EDC) in dimethylformamide (DMF) overnight at 20 °C. The immunogen was prepared by conjugating the active ester to bovine serum albumin (BSA); the enzyme tracer consisted of the derivative conjugated to horse radish peroxidase (HRP). The immunoassay was performed employing two formats, plates and tubes, and both were precoated with rabbit anti-hexazinone. Thirty-two groundwater samples analyzed for hexazinone using a plate enzyme immunoassay (EIA) correlated well with high-performance liquid chromatography (HPLC) (correlation coefficient of 0.9468). A correlation coefficient of 0.9563 was calculated for 30 water samples run by tube EIA vs. HPLC. Of the eight known hexazinone metabolites, seven were tested for cross-reactivity and five were cross-reactive.

Ever since 1979 when aldicarb and its metabolites were found in well water from Long Island (*1*), there has been an increased effort to determine the extent of pesticide contamination in aquifer systems and to evaluate the fate and transport mechanisms associated with pesticide contamination of our water resources (*2–4*). Because groundwater represents a significant water resource and provides

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approximately 52.5% of the domestic water supply in the nation (*5*), preservation and/or remediation of this resource is essential. Recent studies have indicated that the extent of groundwater contamination due to agricultural inputs is significant (*5*), and that there is need to determine the potential for accumulation and chemical transformation or degradation of these contaminants within the ecosystem. One pesticide of particular interest is hexazinone. Hexazinone (trade name–Velpar) is a herbicide that can be used as a foliar spray or applied directly to soils to control perennial grasses and broadleaf weeds in blueberries, sugar cane, pineapple and alfalfa. Recent studies have identified the presence of hexazinone in groundwaters within the blueberry growing areas of eastern, southern, and western Maine (Yarborough, D. E., University of Maine at Orono, unpublished data; Reed, A. W., University of Maine at Orono, unpublished data).

Due to the concern regarding hexazinone contamination of drinking water, over 75 residents in Maine have requested groundwater analyses from our laboratory over the past year. More than half of these samples were positive for hexazinone ranging in concentration from 0.1 ppb to 24 ppb. Unfortunately, research conducted to date has not been extensive enough to ascertain the extent of contamination over the region. While the concentrations of hexazinone determined in these systems were well below recommended drinking water quality criteria, the fate of hexazinone in these environments must be assessed to determine the potential for accumulation and/or transport.

This contamination problem has led to the development of hexazinone antisera and an EIA procedure for the analysis of hexazinone in groundwater which is described in this paper. Present methods employ chromatographic techniques (*6–8*) for analyzing hexazinone, but this EIA technique, like all EIA methods, is more cost-effective and will allow for rapid analysis of numerous hexazinone water samples. Because of these two reasons EIA is gaining in popularity for the analysis of environmental toxins in soil and water (*9*).

Experimental

Hexazinone and Its Metabolites. Hexazinone (3-cyclohexyl-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4-(1H,3H)-dione) and the following 7 metabolites: metabolite A [3-(4-hydroxycyclohexyl)-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4-(1H,3H)-dione]; metabolite B [3-cyclohexyl-6-(methylamino)-1-methyl-1,3,5-triazine-2,4-(1H, 3H)-dione]; metabolite C [3-(4-hydroxycyclohexyl)-6-(methylamino)-1-methyl-1,3,5-triazine-2,4-(1H,3H)-dione]; metabolite D [3-cyclohexyl-1-methyl-1,3,5-triazine-2,4, 6-(1H,3H,5H)-trione]; metabolite E [3-(4-hydroxycyclohexyl)-1-methyl-1,3,5-triazine -2,4,6-(1H,3H,5H)-trione]; metabolite A-1 [3-(trans-2-hydroxycyclohexyl)-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4-(1H,3H)-dione]; and metabolite 1 [3-(4-oxycyclohexyl)-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4-(1H,3H)-dione] were gifts from E. I. DuPont de Nemours & Company, Experimental Station, Wilmington, DE. Structures of hexazinone and its metabolites are shown in Figure 1.

Water Samples. All water samples were collected from private wells located in eastern, western and southern Maine. Samples were collected by home owners and sent to the University of Maine, Department of Food Science in quart canning jars. Samples were kept cold during shipment and analyzed immediately upon arrival.

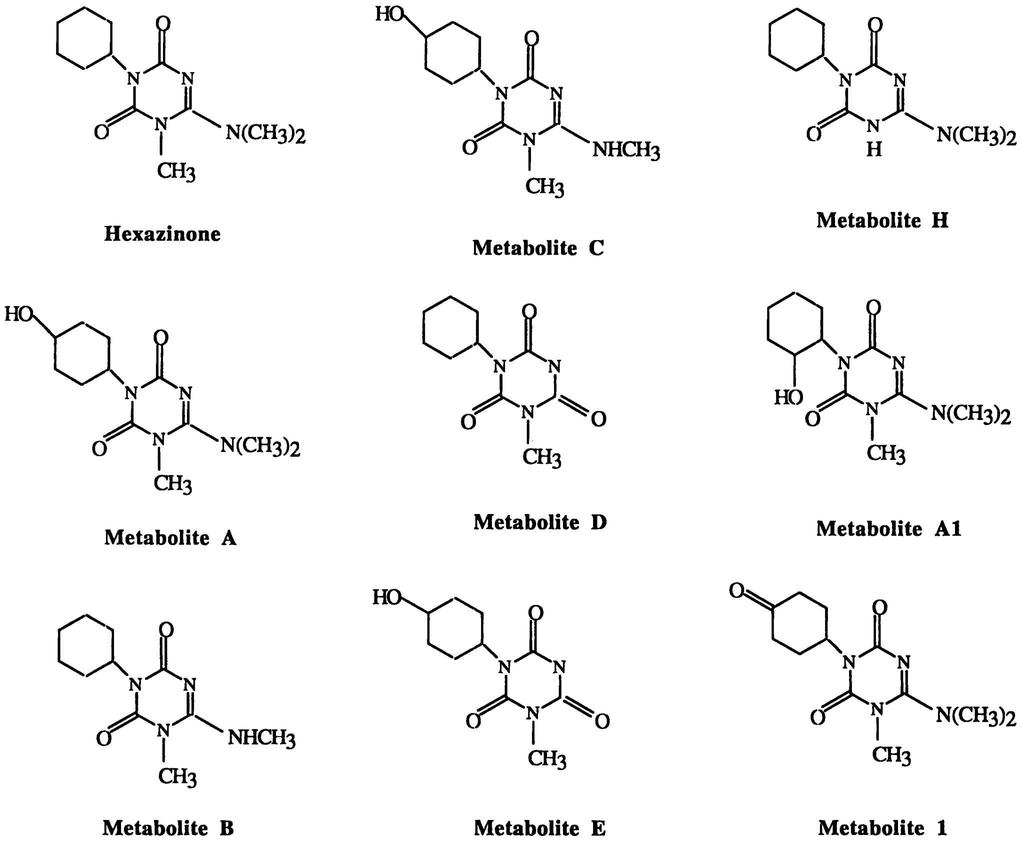
Hexazinone
Metabolite C
Metabolite H
Metabolite A
Metabolite D
Metabolite A1
Metabolite B
Metabolite E
Metabolite 1

Figure 1. Structure of hexazinone and eight metabolites.

Home owners were instructed to use a clean canning jar and to allow the water to run 10 min before taking the sample.

Preparation of Standards for Immunoassay. Stock solutions of hexazinone and its 7 metabolites were prepared by weighing 20 mg of each into a 5 mL volumetric flask and bringing each to volume with HPLC grade methanol. An intermediate standard solution was made by pipetting 50 µL of each stock solution into 50 mL volumetric flasks and bringing them to volume with HPLC grade water. Working standards (0.11, 0.22, 0.44, 1.1, 2.2, 4.4, 8.8, 17.6, 35.2, 70.4, 140.8, 281.6, 583.2 ppb) of each compound were prepared by serially diluting the intermediate standards into HPLC grade water.

Preparation of Hexazinone Standards for HPLC. The same hexazinone stock solution as was used for immunoassay was employed for the HPLC. An intermediate standard was prepared by pipetting 50 µL of the stock standard into a 50 mL volumetric flask and bringing it to volume with methanol-acetonitrile-water (10:40:40, v/v/v). Working standards (12.5, 62.5, 312.5, 1562.5, and 7812.5 ng/5 mL) of hexazinone were prepared by making serial dilutions of the intermediate standard with the methanol-acetonitrile-water solution.

Production of Antisera. Polyclonal antisera to hexazinone was raised in rabbits using a combination of intradermal and subcutaneous injections. A hexazinone derivative was prepared by refluxing metabolite A, 3-(4-hydroxycyclohexyl)-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4-(1H,3H)-dione and succinic anhydride in pyridine at 100 °C for 6 h. The resulting hexazinone hemisuccinate was converted to an active ester by reacting it with *N*-hydroxysuccinamide and EDC in DMF overnight at 20 °C.

Preparation of Immunogen. The immunogen was prepared by conjugating the active ester to BSA; the enzyme tracer consisted of the derivative conjugated to HRP.

EIA Analysis of Hexazinone. The immunoassay was performed using two formats, plates and tubes, and both were precoated with rabbit anti-hexazinone using a proprietary method (ImmunoSystems, Scarborough, ME). The procedures employed for each type of immunoassay kit (EnviroGard from Millipore Corp., Bedford, MA) were as follows: (1) The analysis of hexazinone in water by the tube EIA kit was performed by pipetting 200 µL of standards or water samples to no more than 10 EIA tubes followed by 200 µL of enzyme conjugate. Each tube was mixed briefly by swirling. After 20 min incubation at room temperature, the tubes were rinsed 4 times under tap water and blotted dry before the addition of 500 µL of K-blue® (Elisa Technologies, Lexington, KY) substrate to each tube. A 10 min incubation at room temperature was used. The reaction was then stopped with 350 µL of 1 N HCl which causes a change in color from blue to yellow. Absorbance of each tube was read at 450 nm using an EnviroGard tube reader. (2) The analysis of hexazinone in water by the plate EIA kit was performed by pipetting 100 µL of standards and samples to each microtiter well followed by 100 µL of enzyme conjugate. Wells were incubated at room temperature for 60 min while shaking at 200 rpm. Following this incubation the wells were rinsed 4 times under tap water and blotted dry before 100 µL of substrate was added to each well. A 30 min incubation at room temperature was completed at 200 rpm followed by the addition of 1 N HCl (100 µL) to stop the reaction. Each well was read at 450 nm with a Bio-Tek Model EL 301 microwell strip reader (Burlington, VT).

Quantitation of Hexazinone and its Metabolites by EIA. Control tubes were run with each set of tubes to calculate % B_0 values of standards and samples (absorbance at 450 nm of standard or sample/absorbance at 450 nm of control X 100). Standards were run at the beginning and end of each day with the average of both runs were used to prepare the standard curve which was made by plotting % B_0 versus the log of hexazinone concentration using semi-log graph paper. The amounts of hexazinone in unknown water samples were interpolated from the standard curve.

HPLC Operating Conditions. A Zorbax C_{18} column (stainless steel, 4.6 mm I.D. X 250 mm) (Phenomenex, Torrance, CA) was employed for the separation along with a mobile phase comprised of methanol-acetonitrile-water (20:40:40) at a flow rate of 1 mL/min using a Hewlett-Packard (HP) 1050 pump (Wilmington, DE). Injections (50 µL) were performed by a HP 1050 auto-injector. The samples were detected with a HP 1050 photodiode array detector set at 247 nm while a Vectra HP 486 Chem Station for windows was used to measure peak areas.

Analysis of Hexazinone in Water by HPLC. Water samples (500 mL) were passed through an activated C_{18} Sep-Pak® (Waters Associates, Milford, MA) (activation was done by passing 5 mL of HPLC methanol through a Sep-Pak® followed by 5 mL of HPLC water) at 5 ml/min. After drying the Sep-pak® for 20 min under vacuum, it was eluted with 4 mL of a mixture of ethyl acetate-methyl tertiary butyl ether (20:80). The 4 mL was evaporated to dryness under nitrogen and the residue was dissolved in 1 mL of HPLC mobile phase. A 50 µL aliquot was injected into the HPLC.

Results and Discussion

Hexazinone is not a typical triazine herbicide since it has no chlorine atoms where it could be easily conjugated to BSA. However, there is a hydroxylated metabolite of hexazinone that is appropriate for conjugation to BSA. Metabolite A (Figure 1), after being changed to an active ester, was conjugated to the protein and the resulting immunogen was injected into rabbits. On the 3rd bleed a sufficient titer of hexazinone polyclonal antibodies was obtained. The ester derivative of hexazinone metabolite A also was conjugated to horse radish peroxidase (HRP) to produce the enzyme conjugate. Once the antibody and conjugate were produced, a proprietary method was used to manufacture the plate and tube kits. Both type of kits were employed in developing EIA procedures for the analysis of hexazinone in groundwater.

Typical EIA standard curves for both formats are shown in Figure 2. The curves parameters such as linearity and sensitivity are very similar with the best linear range being between 0.2 to 4.4 ppb; the IC_{50} (concentration of hexazinone at a % B_0

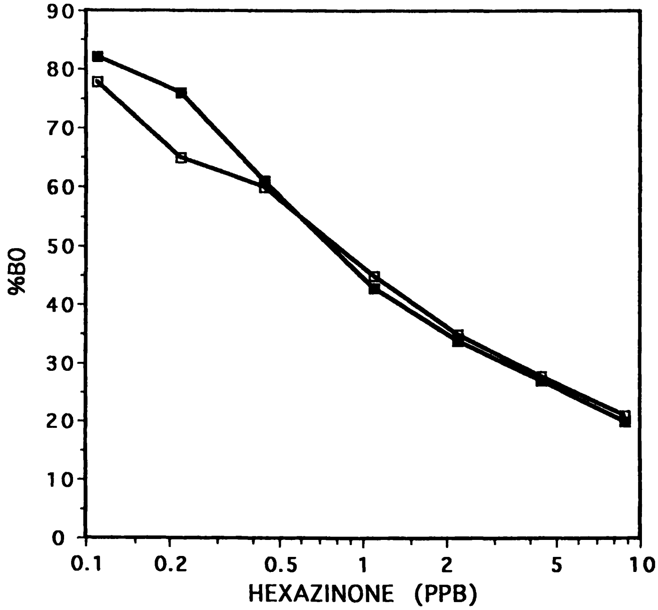

Figure 2. EIA standard curves for the plate kit (■) and tube kit (□).

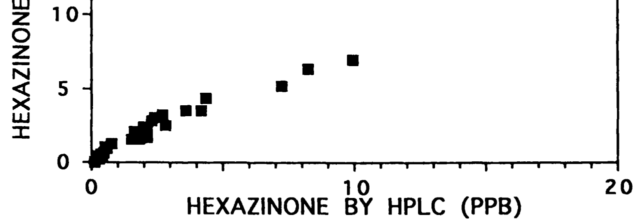

Figure 3. Comparison of the hexazinone concentration of 30 groundwater samples by tube EIA versus HPLC.

value of 50) was 1.0 ppb and the lower limit of detection (LLD) was 0.1 ppb hexazinone. Samples greater than 4.4 ppb indicated by a % B_0 less than 30 were diluted in order to obtain the best quantitative results.

As with any analytical technique, precision within and between days is important. Reproducibility results for hexazinone in water, for tubes and plates with standards and fortified samples are presented in Tables I–IV. For the plate EIA the intra- and interassay CVs ranged from 1.6 to 12% for the standards and 4.1 to 25% for the fortified samples, while the tube EIAs the CVs varied from 4.0 to 13% for the standards and 5.7 to 18% for samples. Although both formats have acceptable intra- and interassy reproducibility, the tube format demonstrates better reproducibility (Tables III and IV) especially with water samples fortified from 0.1 to 25 ppb hexazinone. Most CVs were 15% or less for the tube kit while they were 20% for the plate assay.

The accuracy of both EIA formats was tested using fortified samples spiked at 0.1, 0.5, 1, 2.5, 5, 10 and 25 ppb. Results are shown in Tables V and VI. There were no significant differences between results obtained with the plate or tube kit. Both kits demonstrated similar recoveries and linear regression equations.

A study was performed to determine the cross-reactivity of the hexazinone polyclonal antisera. There are 8 known metabolites of hexazinone (Figure 1), and seven were tested for their ability to cross-react. Cross-reactivity results of these metabolites along with 23 other structurally similar pesticides including several common triazines are given in Table VII. Five metabolites (A, A1, 1, B, and C) were very cross-reactive with sensitivities close to hexazinone. Two metabolites, D and E, demonstrated no cross-reactivity at a level of 1 ppm along with 23 other pesticides. From these cross-reactivity results one can conclude that the dimethylamino group in the 6 position (metabolites A, A1, 1) is the most important for binding followed by the methylamino group in the 6 position (metabolites B and C). When the amino group is completely degraded, as is the case with metabolites D and E, then no cross-reactivity was demonstrated. Based on this observation, the eighth hexazinone metabolite which was not tested for cross-reactivity also should be very reactive.

Although the polyclonal antisera does demonstrate a great deal of cross-reactivity for 5 hexazinone metabolites, this is not detrimental. Many of these metabolites are not found in water, but for the ones that are, it would be imperative to be able to screen for them.

Correlation studies between both EIA formats and HPLC were performed (Figures 3 and 4). Thirty-two groundwater samples were used in the plate kit comparison while 30 groundwaters were employed for the tube study. In both cases the correlation coefficients were almost identical (0.9468 for plate and 0.9550 for tube). The equations were different in that the plate format yielded a regression line of $y = 0.83\ x + 0.58$ which indicates a high bias by EIA, while the equation for the tube format was $y = 1.04\ x - 0.09$, demonstrating no bias.

Tabulated results of the comparison studies are given in Tables VIII and IX. From these data it is easy for one to see the exact values for hexazinone. Groundwater samples ranged from 0.1 ppb to 24 ppb with most samples containing less than 3.0 ppb having excellent agreement with HPLC. Thus the hexazinone EIA formats should prove beneficial as a screening method for groundwater. Of the groundwater

Table I. Reproducibility of the hexazinone plate EIA for standards.

Hexazinone Std. (ppb)	CV[a] intra-assay (%)	CV[b] interassay (%)
0.12	5.3	3.9
0.23	3.1	5.2
0.46	1.7	5.6
1.1	5.6	12.0
2.2	1.6	8.7
4.4	3.6	9.3
8.8	10.0	6.6

[a] Percent coefficients of variation based on 3 determinations in 1 day.
[b] Percent coefficients of variation based on 4 determinations in 4 different days.

Table II. Reproducibility of the hexazinone plate EIA for spiked water samples.

Hexazinone spike (ppb)	CV[a] intra-assay (%)	CV[b] interassay (%)
0.1	15.0	7.1
0.25	–	–
0.50	4.1	25.0
1.0	22.0	15.0
2.5	12.0	7.7
5.0	15.0	19.0
10.0	14.0	18.0
25.0	16.0	13.0

[a] Percent coefficients of variation based on 4 determinations in 1 day except for spike 0.50 which was based on 3 determinations in 1 day.
[b] Percent coefficients of variation based on 3 determinations in 3 different days.

Table III. Reproducibility of the hexazinone tube EIA for standards.

Hexazinone Std. (ppb)	CV[a] intra-assay (%)	CV[b] interassay (%)
0.12	6.4	4.4
0.23	6.1	4.2
0.46	7.7	8.0
1.1	5.9	9.0
2.2	5.6	2.7
4.4	4.0	12.0
8.8	8.7	13.0

[a] Percent coefficients of variation based on 6 determinations in 1 day.
[b] Percent coefficients of variation based on 6 determinations in 6 different days.

Table IV. Reproducibility of the hexazinone tube EIA for spiked water samples.

Hexazinone Spike (ppb)	CV[a] intra-assay (%)	CV[b] interassay (%)
0.1	13.0	12.0
0.25	7.3	10.0
0.50	18.0	7.4
1.0	15.0	6.5
2.5	11.0	14.0
5.0	7.4	9.0
10.0	7.0	5.7
25.0	6.7	46.0

[a] Percent coefficients of variation based on 6 determinations in 1 day.

[b] Percent coefficients of variation based on 5 determinations in 5 days except for the 0.25 ppb spike which was based on 4 determinations in 4 days.

Table V. Accuracy and comparison with HPLC of hexazinone plate EIA for spiked water samples.[a]

Amount hexazinone added (ppb)	Amount hexazinone found (ppb)
0.1	0.11
0.50	0.63
1.0	1.3
2.5	2.5
5.0	4.6
10.0	9.9
25.0	22.0

[a] Correlation coefficient was 0.9987 with an equation of $y = 0.88\,x + 0.33$.

Table VI. Accuracy and comparison with HPLC of hexazinone tube EIA for spiked water samples.[a]

Amount hexazinone added (ppb)	Amount hexazinone found (ppb)
0.1	0.14
0.25	0.25
0.50	0.50
1.0	0.92
2.5	2.1
5.0	4.2
10.0	7.3
25.0	20.0

[a] Correlation coefficient was 0.9992 with an equation of $y = 0.79\,x + 0.047$.

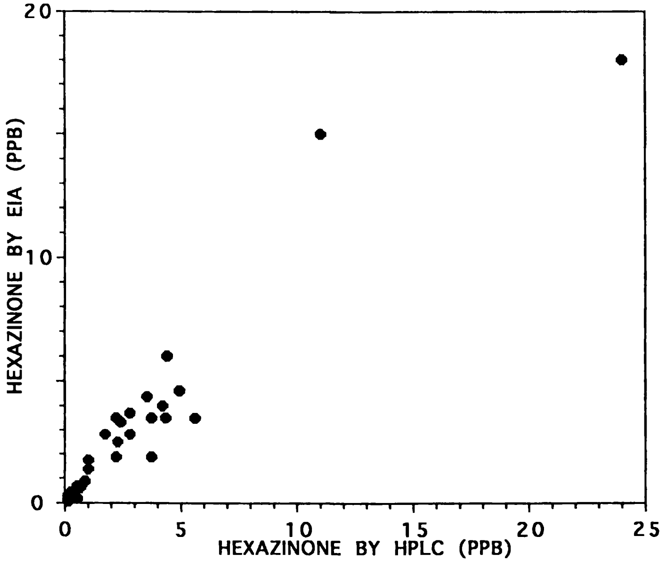

Figure 4. Comparison of the hexazinone concentration of 32 groundwater samples by plate EIA versus HPLC.

Table VII. Cross-reactants in the hexazinone plate and tube EIA.

Compound[a]	Plate EIA IC_{50}[b] (ppb)	Plate EIA LLD[c] (ppb)	Tube EIA IC_{50}[b] (ppb)	Tube EIA LLD[c] (ppb)
Hexazinone	1.0	0.10	1.0	0.10
Metabolite A	2.8	0.22	3.8	0.22
Metabolite A1	7.0	0.44	8.0	0.44
Metabolite 1	2.8	0.22	2.3	0.22
Metabolite B	4.2	1.1	5.4	1.1
Metabolite C	8.0	1.1	11.0	1.1

[a] The following compounds showed no cross-reactivity at 1 ppm: Metabolite D and E, simazine, cyromazine, prometyrn, procyazine, propazine, trietazine, terbutylazine, cyprazine, prometon, cyanazine, atrazine, analazine, amitraz, ametryn, amitrole, dichlone, bromacil, imidan, captan, iprodione, terbacil, lenacil, bentazon, capafol, and maleic hydrazide.

[b] Concentration that causes 50% inhibition.

[c] Lowest limit of detection at % B_0 of less than 90.

Table VIII. Comparison of plate EIA and HPLC for the determination of hexazinone in water.[a]

Water Sample	EIA Hexazinone (ppb)	HPLC Hexazinone (ppb)
1	0.70	0.50
2	1.8	1.0
3	0.46	0.27
4	0.09	0.10
5	0.60	0.57
6	0.33	0.34
7	0.34	0.33
8	0.86	0.90
9	0.36	0.36
10	0.32	0.10
11	0.28	0.13
12	0.18	0.51
13	3.5	3.7
14	1.9	2.2
15	18.0	24.0
16	2.8	2.8
17	3.5	4.3
18	1.9	3.7
19	4.0	4.2
20	3.5	5.6
21	3.3	2.4
22	15.0	11.0
23	3.7	2.8
24	4.6	4.9
25	3.5	2.2
26	2.8	1.7
27	0.14	0.15
28	2.5	2.3
29	6.0	4.4
30	1.4	1.0
31	0.62	0.67
32	4.4	3.5

[a] Correlation coefficient was 0.9468 with an equation of $y = 0.83\,x + 0.58$.

Table IX. Comparison of tube EIA and HPLC for the determination of hexazinone in groundwater.[a]

Water Sample	EIA Hexazinone (ppb)	HPLC Hexazinone (ppb)
1	2.3	2.1
2	6.4	8.3
3	2.1	1.7
4	2.4	2.0
5	1.6	1.8
6	21.0	17.0
7	0.25	0.18
8	2.9	2.3
9	1.2	0.76
10	0.66	0.46
11	0.47	0.35
12	3.0	2.4
13	4.4	4.4
14	0.34	0.34
15	0.41	0.39
16	7.0	10.0
17	0.9	0.58
18	1.7	2.1
19	3.6	4.2
20	2.5	2.8
21	0.4	0.17
22	5.2	7.8
23	1.0	0.53
24	1.6	1.5
25	1.9	1.9
26	0.1	0.14
27	3.2	2.7
28	0.24	0.27
29	0.46	0.28
30	3.6	3.5

[a] Correlation coefficient was 0.9550 with an equation of $y = 1.04x - 0.09$.

samples screened thus far by both EIA and HPLC, there have been no metabolites found by HPLC. Therefore, hexazinone may not be metabolized in Maine. If this is true, then the EIA value will reflect the hexazinone concentration. Furthermore, since the water quality guideline is 210 ppb for hexazinone, it appears that Maine's groundwater may be well below that level. More monitoring needs to be performed before such a statement can be made with certainty. This research shows that the EIA for monitoring hexazinone in groundwater is rapid, accurate and cost-effective.

Acknowledgement

This manuscript is number 1949 of the University of Maine Agricultural and Forestry Experiment Station.

Literature Cited

1. Zaki, M. H.; Moran, D.; Harris, D. *Am J. Pub. Health.* **1982**, *72*, 1392–1395.
2. Krawchuk, B. P.; Webster, G. R. B. *Water Pollut. Res. J. Can.* **1987**, *22*, 129–146.
3. Roy, D. N.; Konar, S. K.; Charles, D. A.; Feng, J. C.; Prasad, R.; Campbell, R. A. *J. Agric. Food Chem.* **1989**, *37*, 443–461.
4. Drinking Water Health Advisory: *Pesticides*; Cook, M. B., ed.; U. S. Environmental Protection Agency, Office of Drinking Water Health Advisories; Lewis Publishers: Chelsea, MI, 1989; pp 471–486.
5. Bedient, B. P.; Rifai, H. S.; Newell, C. J. *Ground Water Contamination Transport and Remediation*; Prentice-Hall Publishers: Englewood Cliffs, NJ, 1994; pp 20–90.
6. Neary, D. G. *South. J. Appl. For.* **1983**, *10*, 217–223.
7. Thompson, D. G.; MacDonald, L. M.; Staznik, B. *J. Agric. Food Chem.* **1992**, *40*, 1444–1449.
8. Lavy, T. J.; Mattice, J. D.; Kochenderfer, J. N. *Environ. Qual.* **1989**, *18*, 507–514.
9. Van Emon, J. M.; Lopez-Avila, V. *Anal. Chem.* **1992**, *64*, 879–882.

RECEIVED October 11, 1995

Chapter 15

Immunochemical Approaches to Research on Natural Toxicants and Phytoprotectants in Food

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Immunochemical research on low molecular weight, biologically-active non-nutrient components of food materials has concentrated on contaminants such as mycotoxins and pesticides. Inherent components have attracted considerably less attention, despite evidence of considerably greater potential for *in vivo* bioactivity in man. Such activity might be deleterious (as is possible for the potato glycoalkaloids and the psoralens) or beneficial (such as the proposed protection against certain cancers provided by phytoestrogens like the isoflavones). Development and application of immunoassay methods of analysis offers advantages of specificity, a high rate of sample through-put and comparatively low cost. Particularly important is the potential high sensitivity. Application of immunoassays to human serum and tissues, where sample volumes are necessarily small, allows study of absorption and metabolism, and can result in improved assessments of implications for health. Examples of immunoassay approaches to the determination of the potato glycoalkaloids, isoflavones and psoralens will be presented.

Immunoassay methods of analysis are now in wide use for detection and quantitative determination of low molecular weight contaminants of agri-food material. Indeed, a dedicated immunodiagnostics industry now provides all manner of antibody-based kits for a user community including the food producers, processors, retailers and consumers, as well as regulators and all those with environmental interests. Most of the attention has been directed towards contaminants such as the mycotoxins (including the aflatoxins, trichothecenes and fumonisins) and pesticides, in contrast, the natural bioactive, non-nutrient constituents of plant foods have received little attention. Plants are able to produce an extraordinarily diverse range of compounds through their pathways of secondary metabolism, many of them highly toxic to insects, fungii and other 'predators'. Indeed, the concept of 'natural pesticides' has been used to explain their activity and, possibly, their role in the plant. So poor is our basic knowledge of how the non-nutrient

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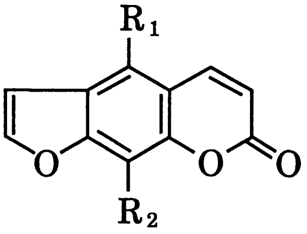
components of plant foods are absorbed and metabolized following ingestion, and what the effects are — if any — on *in vivo* metabolism and health, that we can make few authoritative recommendations to consumers or plant breeders. This comment applies both to potentially deleterious and potentially beneficial plant non-nutrients. Food plants are rich sources of toxicants of wide-ranging activities, including many carcinogens. Despite this, virtually all available evidence points to the beneficial effects of consuming more fruit and vegetables, in terms of 'protective' action against cancers and coronary heart disease. Such effects are related to the fibre and vitamin components, but there is a growing acceptance of the importance of the non-nutrient fraction (*1*).

Immunoassay methods can readily provide information on dietary intake, absorption and metabolism. High levels of specificity and sensitivity coupled with high rates of sample through-put can be achieved. Specificity is needed because of the number of closely related compounds that might be present. Sensitivity is essential for several reasons; because of the low levels, because of the small sample volumes normally encountered in human studies in particular, and because sample work-up can be greatly simplified (eliminating purification steps) when it is possible to dilute the sample prior to analysis. The latter possibility also removes potentially interfering matrix effects.

However, surprisingly few immunoassays have been developed for low molecular weight, non-nutrient components of plant foods (*2*). By far the most research has been devoted to the potato glycoalkaloids, toxicant steroidal alkaloids, and this work will be summarized in this and other chapters (See Sporns *et al.* and Stanker *et al.*, this volume). Of other toxicant plant secondary metabolites, the psoralens are, perhaps, of greatest interest because of their potent bioactivity, their high levels in certain plants, and the small safety margin that has been estimated between levels normally consumed and levels at which deleterious actions have been observed in humans. In this chapter we will further delineate reasons for conducting research involving immunoassay development for these compounds. As has been described previously, some of the non-nutrients are believed to provide beneficial health effects. Immunoassay can help elucidate the mechanisms involved, and lead to improved dietary advice. In this chapter we describe some of the immunochemical research carried out on the isoflavones, much of it related to deleterious effects in farm animals, but there is an increasing awareness because of the 'protective' effects that these compounds are thought to provide against certain human cancers.

Psoralens. Psoralens (or linear furanocoumarins) are found in many commonly consumed vegetables (*3*) and fruits, including the plant families Rutaceae (citrus fruits), Umbelliferae (parsley, parsnip, celery, celeriac, fennel) and Moraceae (fig), and are thought to play an important role in protecting against attack by herbivores, insects and pathogens (*4–9*). A large number of psoralens have been isolated from plants, the most common are shown in Figure 1. Psoralen is the biosynthetic precursor (*9*) for the other psoralens and is elaborated by hydoxylation and O-alkylation. The levels of psoralens found in commonly consumed vegetables (Table I) (*10*) are variable and can be considerably higher in stressed plants (*11,12*). On the surface of spoiled parsnips, levels of xanthotoxin, psoralen and bergapten of 110.9 mg/100 g, 53.7 mg/100 g and 9.0 mg/100 g, respectively, have been found and these levels persisted in apparently healthy tissue below the diseased surface (*13*).



Common name	**R_1**	**R_2**
Psoralen	H	H
Xanthotoxin	H	OMe
Bergapten	OMe	H
Isopimpinellin	OMe	OMe
Isoimperatorin	$OCH_2CH{=}C(CH_3)_2$	H
Oxypeucedanin	$OCH_2\underset{O}{CH{-}C}(CH_3)_2$	H
Heraclenin	H	$OCH_2\underset{O}{CH{-}C}(CH_3)_2$
Oxypeucedanin hydrate	$OCH_2CHOHCOH(CH_3)_2$	H

Figure 1. Structures of commonly occurring plant psoralens.

Table I. Levels of psoralens in healthy vegetables (mg/100 g).

Vegetable	*Bergapten*	*Isopimpinellin*	*Psoralen*	*Xanthotoxin*
Celeriac	0 – 3.15	0 – 1.26	0 – 1.03	0.12 – 2.16
Celery	0 – 1.85	0 – 2.05	0 – 1.24	0 – 0.82
Parsley	0.10 – 1.82	0 – 0.22	0 – 0.31	0 – 0.45
Parsnip	0.22 – 0.80	----------	0.10 – 1.36	0.26 – 5.45

Levels of psoralens vary considerable from one part of the plant to another, in general the concentrations are highest in the outer leaves (*14*), and peel. When the peel is used, as in the production of essential oils from citrus fruit, the levels of psoralens can be very high (*15*), for example bergamot oil can contain up to 0.8% bergapten (*16*).

Pharmacology. In combination with UV light, many psoralens are potent photosentisizers, and the consumption of psoralens has resulted in phototoxic reactions in man and animals (*17*). The psoralens are known to cause mutagenic and lethal effects *in vitro* and *in vivo* (*18*). Understanding of the biological actions of psoralens has come primarily through interest in their pharmacological properties. The therapeutic benefits of psoralens and light have been known from ancient times (*19*). Psoralens, commonly bergapten, xanthotoxin, and 4,5,8-trimethylpsoralen in combination with UVA light (320–400 nm), are used clinically in the treatment of a range of skin disorders including psoriasis (*20*), vitiligo (*21*), mycosis fungoides (*22*), and leukaemic cutaneous T-cell lymphomas (*23*). Psoralens have been used in a number of sun-tan preparations as a stimulus to the production of melanin (*18*). In addition, psoralens show anti-depressant activity (*24*) (possibly via the stimulation of the production of melatonin), are effective potassium channel blockers with a possible role in treating demyelinating diseases like multiple sclerosis (*25*), and show cytostatic activity (*26*).

Psoralens also have a number of overtly deleterious actions (*27*); they interact with DNA, are activated by cytochrome *P*-450 enzymes, and cause photosentization reactions.

DNA Adduct Formation. Psoralens intercalate into the minor groove of DNA duplexes, with the 5′-TA sequence as the preferred site (*28*), and in combination with UV light, undergo a [2+2] cycloaddition (*29*) reaction with the pyrimidine bases (primarily thymine) forming both monofunctional and bifunctional (cross linking) adducts (*27*). *In vitro* and *in vivo* studies have shown these photoadducts to be mutagenic and carcinogenic. In addition, psoralens have been shown to be mutagenic in the absence of light (*30*). Studies in humans suggest that patients who have received psoralen treatment are at a ten times greater risk of developing cutaneous squamous-cell carcinoma (*31,32*). The World Health Organization has cited psoralen phototherapy as a cause of human skin cancer (*33*).

Interaction with Cytochrome *P*-450. Psoralens are potent modulators of cytochrome *P*-450 (*34,35,36*). Xanthotoxin has been shown to be biotransformed *in vitro* to reactive intermediates (possible intermediates are a furan epoxide and an unsaturated dicarbonyl compound) that bind covalently to liver microsomes. These activated species react with a wide range of nucleophilic groups including DNA, RNA and proteins. It should be noted that aflatoxin B_1 is activated in a similar manner, to give a 2,3-epoxy-aflatoxin, and is known to be a potent carcinogen causing cancer of the liver, colon, and kidney in some animals (*37*). Psoralens also deactivate certain Cytochrome *P*-450 enzymes affecting the metabolism of xenobiotics. The mechanism of inactivation of cytochrome *P*-450 is not clear, but might result from covalent binding of activated xanthotoxin intermediates to specific target cytochrome *P*-450 (*35*).

Photosentisation. The precise mechanism by which psoralens cause skin photosensitization reactions is not known (*38*). Some of the biological effects of psoralens in the skin have been attributed to the formation of adducts with DNA. However an alternative mechanism has been proposed involving the binding of psoralens to specific receptors in cell membranes and cytoplasm that are important in the regulation of growth factor-induced signal transduction pathways. Following UV irradiation the receptor is activated by photoalkylation, leading to a cascade of biochemical changes that result in alterations in cell growth and differentiation.

Human Exposure. Humans are exposed to psoralens through handling or ingesting psoralen containing fruits and vegetables. High levels have been associated with outbreaks of photodermatitis in workers handling produce, especially parsnips, celery (*14,39*), citrus fruit (*40*), and the use of fragrances. A number of cases have been documented where severe phytophotodermatitis has occurred following consumption of limes (*40*), and celery (*41,42,43*). In addition, cataract (pigmented spots) formation is a possible risk associated with the consumption of psoralens, and has been noted in farm animals (*17*), rats (*44*), and humans (*45*).

Studies in humans show that the phototoxic threshold of an ingested dose of a mixture of bergapten and xanthotoxin is about 10 mg xanthotoxin and 10 mg of bergapten, and for xanthotoxin alone about 15 mg. This dose is not normally reached by the consumption of healthy vegetables under normal dietary habits, however the estimated safety factor is relatively small, about 2–10 µg/g (*46*). Concentrations of psoralens of 8–10 µg/g celery were found to cause phytodermatitis in grocery store workers (*40*).

Metabolism of Bergapten and Xanthotoxin in Man. Bergapten and xanthotoxin are extensively metabolized in man (*47–49*), and are rapidly cleared, with serum half-lives of about 1 h (*50*). The major metabolites of bergapten and xanthotoxin are glucuronic acid conjugates, mainly of arylacetic acid and arylalcohols resulting from oxidation of the furan ring, possible via an intermediate 2′,3′-epoxide. Reduction of the 3,4-double bond in the lactone ring of bergapten, followed by hydrolysis to give an arylpropionic acid derivative, is thought to result from metabolism by gastrointestinal microflora. Metabolism of the lactone ring of xanthotoxin occurs to a minor extent yielding an arylpropenoic acid derivative.

Development of ELISA Methods for Psoralens. No work has been reported in the literature concerning the development of immunoassays for the analysis of free psoralens of food origin. However, ELISA methods have been developed for DNA-psoralen adducts (*31,51,52*), and applied to the quantification of DNA adducts in plasma from patients undergoing psoralen treatment, and to human keratinocytes and animal skin samples. No work was done on the quantification of adducts arising from the exposure to, or consumption of psoralens in the diet; such work might give an indication as to what the long term risks are.

The four most commonly found psoralens (psoralen, bergapten, xanthotoxin, and isopimpinellin) in fruits and vegetables are currently the subject of immunoassay development in this laboratory. The aim of this work will be to explore the dietary absorption and bioavailability of these compounds with a view to further elucidate the real risks (if any) of dietary psoralen consumption. Antisera against xanthotoxol and xanthotoxin have been produced using xanthotoxin immunogens shown in Figures 2 and 3 (Creeke, Lee, Wilkinson and Morgan, Institute of Food Research at Norwich, UK, unpublished data) and are currently being assessed for suitability for plant and human studies. It is hoped that further understanding of the role of psoralens in human health will be forthcoming.

Isoflavones. Isoflavones are heterocyclic diphenols. More than 200 types have been described principally in plants of the Leguminosae sub-family (*53*) some of which are plants regularly consumed by humans such as soya (*Glycine max*) and chickpea (*Cicer arietinum*) (*54*). Of the 200 different types, a small number have been shown to be estrogenic in animals.

Interest in the estrogenic activity of plant isoflavones stems from the 1940s when they were shown to be responsible for 'clover disease', an infertility syndrome in sheep that devastated the Western Australian sheep breeding industry (*55*). The affected sheep had fed on subterranean clover (*Trifolium subterraneum*) which had levels of up to 5% dry matter (*56*) of isoflavones consisting of formononetin, biochanin A, genistein and daidzein. The latter pair occurring as 7-O-glycosides (genistin and daidzin; Figure 4). Subsequently, isoflavones have been shown to be estrogenic in a number of animal species such as cattle (*57*), rats (*58*), mice (56), Californian quails (*60*) and cheetahs (*61*). The relative estrogenic activity of individual isoflavones varies with animal species and also route of administration (*62,63*). The most estrogenic all have a spatial arrangement of functional groups, particularly phenolic groups, similar to natural and synthetic estrogens (*64*) (Figure 4).

Isoflavones and Human Health. The estrogenicity of isoflavones is weak in comparison with natural estrogens such as estradiol (Table II) and as a result they were not considered to be a health problem for humans. However, over the last 10 years increasing interest has been shown in dietary isoflavones as a growing body of evidence suggests that they may be beneficial to human health.

Epidemiological data shows that in Asian countries there is a much lower incidence of certain cancers such as breast and prostate, than in western countries. This difference has been attributed to the much greater use of soya in Asian diets (*64,66–72*). Soya foods can contain up to 3% by weight of isoflavones, mainly the glycosides daidzin and

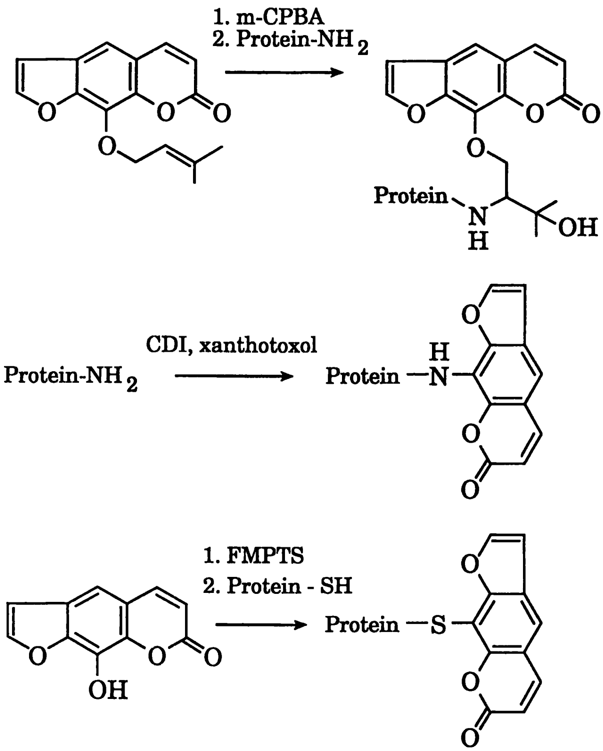

1. m-CPBA
2. Protein-NH$_2$
O
O
O
O
O
O
O
Protein
N
H
OH
Protein-NH$_2$
CDI, xanthotoxol
Protein
H
N
O
O
O
1. FMPTS
2. Protein - SH
O
O
O
OH
Protein
S
O
O
O

Figure 2. Synthesis of xanthotoxol-protein conjugates. (*m*-CPBA = 3-chloroperoxybenzoic acid; FMPTS = 2-fluoro-1-methyl-pyridinium *p*-toluenesulfonate; CDI = 1,1'-carbonyl-diimidazole).

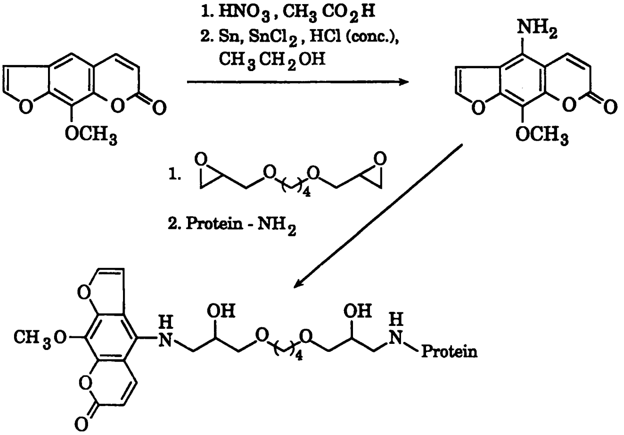

1. HNO$_3$, CH$_3$CO$_2$H
2. Sn, SnCl$_2$, HCl (conc.), CH$_3$CH$_2$OH
O
O
O
OCH$_3$
NH$_2$
O
O
O
OCH$_3$
1.
O
O
O
4
O
2. Protein - NH$_2$
O
CH$_3$O
O
O
H
N
OH
O
O
4
OH
H
N
Protein

Figure 3. Synthesis of xanthotoxin-protein conjugate.

genistin (*73*). Endogenous and synthetic hormones are important in the development of a number of hormone dependent cancers, including cancer of the breast and prostate. It is thought that because of their low estrogenicity, isoflavones can antagonize hormone-dependent cancer development through binding estrogen receptors, or by altering sex hormone metabolism, in a manner that reduces the total effective life-time exposure to endogenous estrogens (*64,70,72*). In addition, isoflavones have the potential to protect against cancer in a non-steroidal manner because they are antioxidants, and can inhibit cell proliferation, tumour induction and angiogenesis (*64,70*).

Table II. Relative estrogenicity of phytoestrogens to estradiol upon a human endometrial cell line.

Compound	*Relative Estrogenicity*
Estradiol	100
Genistein	0.08
Equol	0.06
Daidzein	0.01
Biochanin A	< 0.01
Formononetin	< 0.01

Adapted from ref. *65*

Isoflavone Metabolism. Metabolism of isoflavones in humans is not well understood. But following ingestion of soya rich in isoflavones, the following isoflavonoids have been reported in human urine; daidzein and genistein, the daidzein metabolites equol and O-desmethylangolensin (both formed by intestinal microbes) and compounds intermediate in the metabolism of both equol and O-desmethylangolensin (*54,66,68,74–79,80*). These compounds also have been found in plasma (*79*) and feces (*79,81*).

In plasma and urine the isoflavonoids occur mainly as glucuronides and sulfoglucuronides, but also as the 'bioactive' free and sulphated conjugates. Both the free and conjugated isoflavonoids can undergo entereohepatic circulation, in a manner similar to the natural steroidal estrogens (*80*). Fecal excretion of isoflavones has been reported to be 1–2% of the amounts ingested (*79*), of which greater than 90% of the metabolites are excreted unconjugated (*81*).

The profile of the measured metabolites varies from one individual to another, particularly in regard to equol formation where approximately one third of the population excrete low levels, but this seems to be correlated with higher levels of O-desmethylangolensin suggesting differentially active metabolic pathways (*14,24*).

In the above studies the analytical techniques used were HPLC (*26,27*) or GC/MS isotope dilution spectrometry (*60,68,76,77,80–84*). A profile of the metabolites and isoflavonoid conjugates can be attained using these methods, however the complexity of sample preparation necessary for both techniques limits the number of samples that can be analyzed. To address this problem and to provide further data on the bioavailability of isoflavones and the putative health protective properties, we are developing enzyme-linked immunoassays to measure isoflavones and their metabolites in human body fluids and tissues, as well as foods.

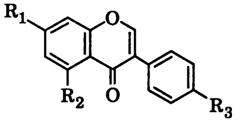
Isoflavone Immunoassays. Previously, polyclonal antisera was raised against genistein (*85,86*), daidzein (*86*), formononetin (*86,88*) and equol (*87*). Cox *et al.* (*86*) produced immunogens by synthesis of 2-carboxylic acid derivatives of the plant derived isoflavones and a carbocyclic analogue of equol. Using the 2-carboxylic acid derivative of genistein, Bauminger *et al.* (*85*) developed a radioimmunoassay that specifically recognized genistein with an assay detection limit of 43 µg.

Wang *et al.* (*88*) raised anti-formononetin antisera in rabbits by immunization with a 7-O-(carboxymethyl) ether derivative of formononetin conjugated to bovine serum albumin. The formononetin specific radioimmunoassay developed had a detection limit of 200 pg and has been applied to measure formononetin in sheep plasma and ruminial fluid (85) as well as in mouse plasma and mammary tissue homogenates (*89*).

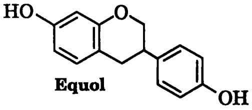
Antisera specific for daidzein have been obtained (Wilkinson, Lee, Creeke, Price, Rhodes and Morgan, Institute of Food Research at Norwich, UK, unpublished data) using an immunogen derived from a puerarin-protein conjugate made after sodium periodate clevage of the 8-C-sugar residue. The antibodies will be used to further elucidate the absorption and metabolism of the phytoestrogens in man.

Potato Glycoalkaloids. The potato glycoalkaloids have been the most extensively studied by immunochemical means of all the non-nutrients. These compounds (Figure 5) are generally regarded as being toxic in nature, and normally occur at levels between 1 and 15 mg/100 g potato tissue in tubers destined for human consumption (*90,91*). Processing and cooking do not significantly affect the levels (*92*). Glycoalkaloids are found in particularly high concentrations in the skin of the tuber and in the leaves and fruit (*93,94*). The glycoalkaloids have anti-insect and anti-fungal activities, which might be an explanation as to why they are present in the potato (*95*). Levels are affected by genetic (*96*) and environmental factors (*97*). Of the latter, conditions during growth, and post-harvest treatment are particularly important. Stress, such as occurring in damaged tubers will increase levels significantly. The predominant members of the group are α-chaconine and α-solanine, both glycosidic derivatives of solanidine (*98*). It has normally been the custom to analyze the group as a whole.

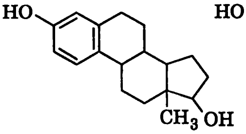
In vitro and *in vivo*, the glycoalkaloids inhibit cholinesterase activity (*99*) and membrane permeability effects (*100*). Reported symptoms of poisoning include gastrointestinal and neurological disturbances. It is generally accepted that glycoalkaloid levels in retail tubers should not exceed 20 mg/100 g tissue. At these levels, in any case, the tubers taste extremely bitter to most consumers. Prior to the development of immunochemical and HPLC procedures, time-consuming and low through-put chemical analyses were performed, often using noxious reagents (*101*). The development of immunoassays has increased the rate of analysis considerably, and probably as a result of their simplicity, improved the quality of the analytical data (*102–107*). Normally, only selection of the potato sample and a solubilization step are required prior to analysis. Results correlate extremely well with other procedures, including HPLC, even when the ELISAs are used independently of the developers (*108*)! Immunoassays do not require



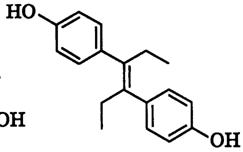
Compound	R_1	R_2	R_3
Daidzin	Glucose	H	OH
Daidzein	OH	H	OH
Formononetin	OH	H	OCH_3
Genistin	Glucose	OH	OH
Genistein	OH	OH	OH
Biochanin A	OH	OH	OCH_3



Equol

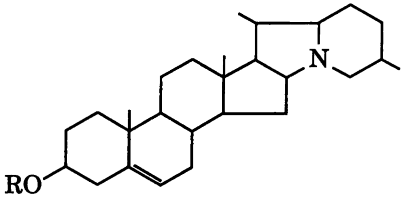


Estradiol



Diethylstilbestrol

Figure 4. Comparison of isoflavonoid structures with natural and synthetic estrogens.



R = H Solanidine

R = Gal – Glu Solanine
|
Rham

R = Glu – Rham Chaconine
|
Rham

Figure 5. Structures of potato glycoalkaloids.
(Gal = galactose; Glu = glucose; Rham = rhamnose).

expensive equipment, and have the considerable potential to be applied away from the specialized laboratory. In this chapter it is not proposed to detail each of the immunoassays for glycoalkaloids that have been described. Further details can be found in other chapters in this volume and also in review articles (*109–111*). Rather, it is proposed to outline application of the immunochemical methodology to studies of absorption and metabolism in man. Such studies would have been considerably more difficult and expensive to carry out using alternative procedures.

Human Studies Using Immunoassay. The polyclonal antibodies used in the following studies were raised using an immunogen synthesized by sodium periodate cleavage of the sugar residues of α-chaconine to generate dialdehydes which were coupled to protein via a Schiffs base linkage. The antisera raised by this route recognized the potato glycoalkaloid group but do not cross-react with compounds in which there are changes in the steroid or alkaloid moieties (*102*). Further differentiation can be achieved by using chloroform to preferentially extract solanidine, the aglycone, at the expense of the more water-soluble glycoalkaloids (*111*).

A number of studies have shown the presence of glycoalkaloids or solanidine (*111–113*) in all serum and saliva samples examined. Levels could be related to a crude measure of potato glycoalkaloid intake determined for individuals by questionnaire (*113*). A time-course study in the same report showed a long half-life for solanidine (relative to frequency of ingestion) of several days. Thus, although dietary absorption is relatively poor — given the quantities consumed — there is potential for deposition within the body, probably within the liver (*114*). Solanidine was shown to be present in postmortem human liver samples, suggesting that solanidine is stored in the body (*115*).

What is the evidence for regarding potato glycoalkaloids as potential toxicants in man? The evidence is suprisingly scarce, and is complicated by the presence of many other possible candidates for deleterious effects, especially those occuring in stressed potatoes. In one of the best documented cases, an outbreak of poisoning in a school in the UK (*116*), the cause of the poisoning was clearly traced by demonstrable *in vitro* activities, to a batch of potatoes that had begun to sprout. Unfortunately, it was not possible to quantify the glycoalkaloids directly, but it was estimated that levels might have been 20–30 mg/100 g — high, but not as high as might have been assumed given the rarity of poisoning events associated with potatoes. The authors postulated the involvement of another factor(s) that could have modified the normal poor absorption of the glycoalkaloids themselves.

More recently, the membrane permeability effects of the glycoalkaloids have received attention, particularly as might occur *in vivo* at the gut wall. Such an activity might not be important in itself — there are many other important dietary components that would have similar effects, including the saponins. Indeed, it is suspected that the 'normal' gut wall is in a permanent state of damage and repair. However, increased consumption of fruit and vegetables would be expected to lead to increased consumption of membrane permeabilizing agents. Associated with this is evidence that gut wall damage can allow access to the circulation of biopolymers such as proteins (*100,117*).

Structural Studies. The potato glycoalkaloid fraction has usually always been quantified as one, and not separated into components. The broad specificity of the anti-glycoalkaloid antisera has been, in consequence, ideal. Recent research linking molecular structure to bioactivity might lead to the necessity for a new approach in the future. Structures in which the sugar residues have adopted an extended conformation could clearly be differentiated in membrane permeabilizing activity from more folded structures (*118*). The approach of Stanker and colleagues in selecting monoclonal antibodies with unique specificities (*106*) and in exploring a recombinant antibody approach (See Kamps-Holtzapple and Stanker, this volume) might be extremely important to a change in strategy.

Future Directions. Immunoassays have many advantages, not least that they can be applied in difficult circumstances to provide new information. As we seek to elucidate further the link between diet and health, antibody-based methods will be an important tool in providing the understanding. For the plant food bioactive non-nutrients, it is important to understand the levels in the foods being consumed and what affects the levels. Genetic, environmental and processing/cooking factors all need to be considered. The relevant *in vivo* bioactivity (and directly relevant *in vitro* tests) need to be understood and accepted. Absorption and bioavailability studies, using biomarkers as necessary, can then be performed to provide informed risk/benefit assessments.

Immunoassays have one further advantage in this area of research — once the antibody has been produced and the assay established, it can be applied at any point in the long chain between plant breeding, through agricultural production and processing to human metabolic studies. Antibody tests can be applied to single seeds whilst retaining viability. This could save considerable time in conventional plant breeding programs, which normally take several years to reach a conclusion, aimed at reducing levels of toxicants or increasing levels of beneficial 'protective' factors. Further, recombinant technology offers the prospect in the coming decade of not only speeding up the process of new variety production but of offering totally new options. An example might be the transference of pathways leading to biosynthesis of beneficial constituents into food plants in which they were not previously present, or the removal of deleterious pathways. The former offers exciting possibilities for improving the positive side of the diet and health balance; the latter is more problematic since the removal of, for example, 'natural pesticides' will have direct consequences for the health of the growing plant, and indirect problems if the secondary metabolite pathways are disturbed. Immunoassays will be useful in providing analytical data quickly with high sample through-put, at low cost and with high precision.

The approach described will require a much more holistic attitude than has prevailed up to now. Analytical methodology should be applied at all points of the agri-food chain. There is little evidence that this is currently the case. Thus plant breeders need to be sharing the concerns of the producers, the regulators and the consumers. Are, for example, plant breeders concerned about glycoalkaloid levels in potatoes until a very late stage of their programs? This is despite the availability of simple and cheap immunoassay methods, and the considerable expense caused when a problem occurs (which is, of course, greater as the program proceeds). Are farmers aware of the effects of their treatment of the growing potatoes, of the critical role of harvesting and storage practice on glycoalkaloid levels? Are retailers aware that if they purchase a known variety grown in an unusual geographical location then the glycoalkaloid levels can be abnormally high? Are retailers fully aware of the link between storage conditions for potatoes and the glycoalkaloid levels? A more coordinated analytical approach across the range of analytes of interest will require development of more novel immunoassay formats, truly applicable away from the specialized laboratory. This means more than simple manipulations; it means greater robustness, built-in control procedures, and clear interpretation of results and action. The latter point, in particular, may need far more sophistication on the part of the immunochemists in choosing their analytes than previously has been necessary.

Literature Cited

1. Johnson, I. T.; Williamson, G.; Musk, S. R. R. *Nutr. Res. Rev.* **1994**, *7*, 175–204.
2. Morgan, M. R. A. In *New Diagnostics in Crop Sciences*; Skerritt, J. H., ed.; CAB International: Oxford, UK, in press.
3. Ramaswamy S. *Indian Food Pack.* **1975**, *29*(4), 37–46.
4. Ivie, G. W.; Bull, D. L.; Beier, R. C.; Pryor N. W. *Allelochemicals: Role in Agriculture and Forestry*; Waller G. R., ed.; American Chemical Society: Washington, DC, 1987, ACS Symposium Series No. 330; pp 455–462.
5. Neal, J. J. *J. Chem. Ecol.* **1989**, *15*(1), 309–315.
6. Afek, U.; Carmeli, S.; Aharoni, N.; Roizer, L. *Acta Hort.* **1993**, *343*, 357–360.
7. Zangerl, A. R. *Ecology* **1990**, *71*(5), 1926–1932.
8. Ivie, G. W. *Rev. Latinoamer. Quim.* **1987**, *18*(1), 1–6.
9. Brown, S. A.; El-Dakhakhny, M.; Steck, W. *Can. J. Biochem.* **1970**, *48*, 863–871.
10. NOTIS Database; Institute of Food Research: Norwich Research Park, Colney, Norwich NR4 7UA.
11. Chaudhary, S. K.; Ceska, O.; Warrington, P. J.; Ashwood-Smith, M. J. *J. Agric. Food Chem.* **1985**, *33*, 1153–1157.
12. Trumble, J. T.; Millar, J. G., Ott, D. E.; Carson, W. C. *J. Agric. Food Chem.* **1992**, *40*, 1501–1506.
13. Ceska, O.; Chaudhary, S.; Warrington, P.; Poulton, G.; Ashwood-Smith, M. *Experimentia*, **1986**, *42*(11/12), 1302–1304.
14. Diawara, M. M.; Trumble, J. T.; Quiros, C. F.; Hansen R. *J. Agric. Food Chem.* **1995**, *43*, 723–727.
15. McHale, D.; Sheridan, J. B. *J. Ess. Oil Res.* **1989**, *1*, 139–149.
16. Mondello, L.; Stagno d'Alcontres, I.; Duce, R. D. *Flavour Fragr. J.* **1993**, *8*, 17–24.
17. Ivie, G. W. *J. Agric. Food Chem.* **1978**, *26*(6), 1394–1403.
18. Ashwood-Smith, M. J.; Poulton, G. A.; Barker, M.; Mildenberger, M. *Nature* **1980**, *285*, 407–409.
19. Scott, B. R.; Pathak, M. A.; Mohn, G. R. *Mutat. Res.* **1976**, *39*, 29–74.
20. Parrish, J. A.; Fitzpatrick, T. B.; Tanenbaum, L.; Pathak, M. A. *New Engl. J. Med.* **1974**, *291*(23), 1207–1211.
21. Parrish, J. A.; Fitzpatrick, T. B.; Shea, C.; Madhu, A.; Pathak, M. A. *Arch. Dermatol.* **1976**, *112*, 1531–1534
22. Lowe, N. J.; Cripps, D. J.; Dufton, P. A.; Vickers, C. F. *Arch. Dermatol.* **1979**, *115*, 50–53.

23. Edelson, R.; Berger, C.; Gasparro, F.; Jegasothy, B.; Heald, P.; Wintroub, B.; Vonderheid, E.; Knobler, R.; Wolff, K.; Plewig, G.; McKiernan, G.; Christiansen, I.; Oster, M.; Honigsmann, H.; Wilford, H.; Kokoschka, E.; Rehle, T.; Perez, M.; Stingl, G.; Laroche, L. *New Engl. J. Med.* **1987**, *316*(6), 297–303.
24. Souetre, E.; Salvati, E.; Belugou, J. L.; Krebs, B.; Darcourt, G. *J. Clinical Endocr. Metab.* **1990**, *71*(3), 670–674.
25. Bohuslavizki, K. H.; Hansel, W.; Kneip, A.; Koppenhofer, E.; Reimers, A.; Sanmann, K. *Gen. Physiol. Biophys.* **1993**, *12*, 293–301.
26. Gawron, A.; Glowniak, K. *Planta Med.* **1987**, *53*(6), 526–529.
27. Anderson, T. F.; Voorhees, J. J. *Ann. Rev. Pharmacol. Toxicol.* **1980**, *20*, 235–257.
28. Hwang, G-S.; Choi, B.-S. *Bull. Korean Chem. Soc.* **1994**, *15*(1), 6–8.
29. Semin, D. J.; Winkler, P. C.; Rowlen, K. L. *Photochem. Photobiol.* **1994**, *60*(3), 185–195.
30. Wagner, S. J.; White, R.; Wolf, L.; Chapman, J.; Robinette, D.; Lawlor, T. E.; Dodd, R. Y. *Photochem. Photobiol.* **1993**, *57*(5), 819–824.
31. Santella, R. M.; Yang, X. Y.; DeLeo, V. A.; Gasparro, F. P. *IARC Sci. Publ.* **1988(89)**, 333–340.
32. Yang, X. Y.; Delohery, T.; Santella, R. M. *Cancer Res.* **1988**, *48*, 7013–7017.
33. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Chemicals, Industrial Processes and Industries Associated with Cancer in Humans. Supplement 4. Methoxsalen with Ultra-violet A Therapy (PUVA) (group 1).* International Agency for Research on Cancer: Lyon 1982; pp 158–160.
34. Cai, Y.; Bennett, D.; Nair, R. V.; Ceska, O.; Ashwood-Smith, M. J.; DiGiovanni, J. *Chem. Res. Toxicol.* **1993**, *6*, 872–879.
35. Mays, D. C.; Hilliard, J. B.; Wong, D. D.; Gerber, N. *Biochem. Pharm.* **1989**, *38*(10), 1647–1655.
36. Neal, J. J.; Wu, D. *Pestic. Biochem. Physiol.* **1994**, *50*, 43–50.
37. Ellis, W. O.; Smith, J. P.; Simpson, B. K.; Oldham, J. H. *Crit. Rev. Food Sci. Nutr.* **1991**, *30*(3), 403–439.
38. Laskin, J. D. *Food. Chem. Toxicol.* **1994**, *32*(2), 119–127.
39. Austad, J.; Kavli, G. *Contact Dermatitis* **1983**, *9*, 448–451.
40. Nigg, H. N.; Nordby, H. E.; Beier, R. C., Dillman, A.; Macias, C.; Hansen, R. C. *Food Chem. Toxicol.* **1993**, *31*(5), 331–335.
41. Ljunggren B. *Arch. Dermatol.* **1990**, 126, 1334–1336.
42. Seligman, P. J.; Mathias, C. G. T.; O' Malley, M. A.; Beier, R. C.; Fehrs, L. J.; Serrill, W. S.; Halperin, W. E. *Arch. Dermatol.* **1987**, *123*, 1478–1482.
43. Berkley, S. F.; Hightower, A. W.; Beier, R. C.; Fleming, D. W.; Brokopp, C. D.; Ivie, G. W.; Broome, C. V. *Ann. Intern. Med.* **1986**, *105*, 351–355.
44. Wulf, H. C.; Andreasen, M. P. *Invest. Ophthalmol. & Visual Sci.* **1982**, *22*(1), 32–36.
45. Lerman, S.; Kuck, J. F., Jr.; Borkman, R. F.; Saker, E. *Ophthalmic Res.* **1976**, *8*, 213–226.
46. Schlatter J.; Zimmerli B.; Dick R.; Panizzon R.; Schlatter C. H. *Food. Chem. Toxicol.* **1991**, *29*(8), 523–530.
47. John, B. A.; Chasseaud, L. F.; Wood, S. G.; Forlot, P. *Xenobiotica* **1992**, *22*(11), 1339–1351.

48. Busch, U.; Schmid, J.; Koss, F. W.; Zipp, H.; Zimmer, A. *Arch. Dermatol. Res.* **1978**, *262*, 255–265.
49. Schmid, J.; Prox, A.; Reuter, A.; Zipp, H.; Koss, F. W. *Eur. J. Drug Metab. Pharmacokinet.* **1980**, *5*(2), 81–92.
50. de Wolff, F. A.; Thomas, T. V. *Clin. Pharmacokinet.* **1986**, *11*, 62–75.
51. Santella, R. M.; Dharmaraja, N.; Gasparro, F. P.; Edelson, R. L. *Nucleic Acids Res.* **1985**, *13*(7), 2533–2544.
52. Yang, X. Y.; DeLeo, V.; Santella, R. M. *Cancer Res.* **1987**, *47*, 2451–2455.
53. Dewick, P. M. In *The Flavonoids: Advances in Research since 1980*; Harborne, J. B., ed.; Chapman and Hall: London, UK, **1988**; pp 552–570.
54. Price, K. R.; Fenwick, G. R. *Food Addit. Contam.* **1985**, *2*, 73–106.
55. Bennetts, H. W.; Underwood, E. J.; Shier, F. L. *Aust. Vet.* **1946**, *22*, 2–12.
56. Beck, A. B. *Aust. J. Agric. Res.* **1964**, *15*, 223–230.
57. Kallela, K.; Heinonen, K.; Saloniemi, H. *Nord. Veterinaermed.* **1984**, 36, 124–128.
58. Leavitt, W. W.; Meismer, D. M. *Nature* **1969**, *218*, 181–182.
59. Burroughs, C. D.; Mills, K. T.; Bern, H. A. *Reprod. Toxicol.* **1990**, *4*, 127–135.
60. Leopold, A. S.; Erwin, M.; Oh, J.; Browning, B. *Science* **1976**, *191*, 98–99.
61. Setchell, K. D. R.; Gosselin, S. J.; Welsh, M. B.; Johnston, J. O.; Balisteri, W. F. *Gastroenterology* **1987**, *93*, 225–233.
62. Lundh, T. J.-O.; Pettersson, H.; Kiessling, K.-H. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 938–941.
63. Stob, M. In *CRC Handbook of Naturally Occuring Food Toxicants*; Recheigh, M., Jr., ed.; CRC Press: Boca Raton, Florida, 1983; pp 81–100.
64. Messina, M. J.; Persky, V.; Setchell, K. D. R.; Barnes, S. *Nutr. Cancer.* **1994**, *21*, 113–131.
65. Markiewicz, L., Garey, J.; Adlercreutz, H.; Gurpide, E. *J. Steroid Biochem. Mol. Biol.* **1993**, *45*, 399–405.
66. Kelly, G. E.; Nelson, C.; Waring, M. A.; Joannou, G. E.; Reeder, A. Y. *Clin. Chim. Acta.* **1993**, *223*, 9–22.
67. Setchell, K. D. R.; Borriello, S. P.; Hulme, P.; Kirk, D. N.; Axelson, M. *Am. J. Clin. Nutr.* **1984**, *40*, 569–578.
68. Adlercreutz, H.; Honjo, H.; Higashi, A.; Fotsis, T.; Hämäläinen, E.; Hasegawa, T.; Okada, H. *Am. J. Clin. Nutr.* **1991**, *54*, 1093–1100.
69. Tomatis, L. In *Cancer, Causes, Occurences and Control*; Tomatis, L., ed.; IARC: Lyon, 1990, Sci. Pub. 100.
70. Barnes, S.; Peterson, G.; Grubbs, C.; Setchell, K. In *Diet and Cancer: Markers, Prevention and Treatment*; Jacobs, M. M., ed., Plenum Press: New York, NY, 1994; pp 135–147.
71. Lee, H. P.; Gourley, S. W.; Duffy, J.; Esteve, J.; Lee, J.; Day, N. E. *Lancet* **1991**, *337*, 1197–1200.
72. Cassidy, A.; Bingham, S.; Setchell, K. D. R. *Am. J. Clin. Nutr.* **1994**, *60*, 333–340.
73. Franke, A. A.; Custer, L. J.; Cerna, C. M.; Narala, K. K. *J. Agric. Food Chem.* **1994**, *42*, 1905–1913.

74. Adlercreutz, H.; Höckerstedt, K.; Bannwart, C.; Bloigu, S.; Hämäläinen, E.; Fotsis, T.; Ollus, A. *J. Steroid Biochem.* **1987**, *27*, 1135–1144.
75. Setchell, K. D. R.; Adlercreutz, H. In *Role of the Gut Flora in Toxicity and Cancer;* Rowland, I. R., ed.; Academic Press: New York, NY, 1988; pp 315–345.
76. Kelly, G. E.; Joannou, G. E.; Reeder, A.Y.; Nelson, C.; Waring, M. A. *Proc. Soc. Exp. Biol. Med.* **1995**, *208*, 40–43.
77. Adlercreutz, H.; Fotsis, T.; Bannwart, C.; Wähälä, K.; Brunow, G.; Hase, T. *Clin. Chim. Acta* **1991**, *199*, 263–278.
78. Franke, A. A.; Custer, L. J. *J. Chromatog. B.* **1994**, *662*, 47–60.
79. Xu, X.; Wang, H.-J.; Murphy, P. A.; Cook, L.; Hendrich, S. *J. Nutr.* **1994**, *124*, 825–832.
80. Adlercreutz, H.; van der Wildt, J.; Kinzel, J.; Attalla, H.; Wähälä, K.; Mäkelä, T.; Hase, T.; Fotsis, T. *J. Steroid Biochem. Mol. Biol.* **1995**, *52*, 97–103.
81. Adlercreutz, H.; Fotsis, T.; Kurzer, M. S., Wähälä, K.; Mäkelä, T.; Hase, T. *Anal. Biochem.* **1995**, *225*, 101–108.
82. Adlercreutz , H.; Markkanen, H.; Watanabe, S. *Lancet* **1993**, *342*, 1209–1210.
83. Adlercreutz, H.; Fotsis, T.; Lampe, J.; Wähälä, K.; Mäkelä, T.; Brunow, G; Hase, T. *Scand. J. Clin. Lab. Invest.* **1993**, *53*, 5–18.
84. Morton, M. S.; Wilcox, G.; Wahlqvist, M. L.; Griffiths, K. *J. Endocrin.* **1994**, *142*, 251–259.
85. Bauminger, S.; Lindner, H. R.; Perel, E.; Arnon, R. *J. Endocrin.* **1969**, *44*, 567–578.
86. Cox, R. I.; Wong, M. S. F.; Braden, A. W.; Trikojus, V. M.; Linder, H. R. *J. Reprod. Fertil.* **1972**, *28*, 157–158.
87. Cox, R. I.; Wilson, P. A.; Mattner, P. E. *Toxicon* **1983**, *3*, 85–88.
88. Wang, W.; Tanaka, Y.; Han, Z.; Cheng, J. *J. Agric. Food Chem.* **1994**, *42*, 1584–1587.
89. Wang, W.; Tanaka, Y.; Han, Z.; Higuchi, C. M. *Nutr. Cancer* **1995**, *23*, 131–140.
90. Sinden, S. L.; Webb, R. E. *Am. Potato. J.* **1972**, *49*, 334–338.
91. Davies, A. M. C.; Blincow, P. *J. Sci. Food Agric.* **1984**, *35*, 553–557.
92. Bushway, R. J.; Ponnampalam, R. *J. Agric. Food Chem.* **1981**, *29*, 814–817.
93. Wolf, M. J.; Duggar, B. M. *Am. J. Bot.* **1940**, *27*, 20s.
94. Lampitt, L. H.; Bushill, J. H.; Rooke, H. S.; Jackson, E. M. *J. Soc. Chem. Ind.* **1943**, *62*, 48–51.
95. Tingey, W. M. *Am. Potato J.* **1984**, *61*, 157–167.
96. Sanford, L. L.; Sinden, S. L. *Am. Potato J.* **1972**, *49*, 209–217.
97. Jadhav, S. J.; Sharma, R. P.; Salunkhe, D. K. *CRC Crit. Rev. Toxicol.* **1981**, 21–104.
98. Paseshnichenko, V.; Guseva, A. R. *Biochem. (USSR).* **1956**, *21*, 606–611.
99. Patil, B. C.; Sharma, R. P.; Salunkhe, D. K.; Salunkhe, K. *Food Cosmet. Toxicol.* **1972**, *10*, 395–398.
100. Gee, J. M.; Price, K. R.; Ridout, C. L.; Johnson, I. T.; Fenwick, G. R. *Toxicol. in Vitro* **1994**, *3*, 85–90.
101. Coxon, D. T. *Am. Potato J.* **1994**, *51*, 169–183.
102. Morgan, M. R. A.; McNerney, R.; Matthew, J. A.; Coxon, D. T.; Chan, H. W.-S. *J. Sci. Food Agric.* **1994**, *34*, 593–598.

103. Morgan, M. R. A.; Coxon, D. T.; Bramham, S.; Chan, H. W.-S.; van Gelder, W. M. H.; Allison, M. J. *J. Sci. Food Agric.* **1985**, *36*, 282–288.
104. Plhak, L. C.; Sporns, P. *J. Agric. Food Chem.* **1992**, *40*, 2533–2540.
105. Plhak, L. C.; Sporns, P. *Am. Potato J.* **1994**, *71*, 297–313.
106. Stanker, L. H.; Kamps-Holtzapple, C.; Friedman, M. *J. Agric. Food Chem.* **1994**, *42*, 2360–2366.
107. Thomson, C. A.; Sporns, P. *J. Agric. Food Chem.* **1995**, *43*, 254–260.
108. Hellenas, K.-E. *J. Sci. Food Agric.* **1994**, *37*, 776–782.
109. Morgan, M. R. A.; Coxon, D. T. In *Natural Toxicants in Food: Progress and Prospects;* Watson, D. H. ed.; Ellis Horwood: London, UK, 1987; pp 221–230.
110. Morgan, M. R. A.; Lee, H. A. In *Development and Application of Immunoassays for Food Analysis*; Rittenburg, J. H.; ed.; Elsevier Applied Science: London, UK, 1990; pp 143–170.
111. Matthew, J. A.; Morgan, M. R. A.; McNerney, R.; Chan, H. W.-S.; Coxon, D. T. *Food Chem. Toxicol.* **1983**, *21*, 637–640.
112. Harvey, M. H.; McMillan, M.; Morgan, M. R. A.; Chan, H. W.-S. *Hum. Toxicol.* **1985**, *4*, 187–194.
113. Harvey, M. H.; Morris, B. A.; McMillan, M.; Marks, V. *Hum. Toxicol.* **1985**, *4*, 503–512.
114 Claringbold, W. D. B.; Few, J. D.; Brace, C. J.; Renwick, J. H. *J. Steroid Biochem.* **1980**, *13*, 889–895.
115. Claringbold, W. D. B.; Few, J. D.; Renwick, J. H. *Xenobiotica* **1982**, *12*, 293–302.
116. McMillan, M.; Thompson, J. C. *Q. J. Med.* **1979**, *48*, 227–243.
117. Gee, J. M.; Johnson, I. T.; Wal, J. M.; Atkinson, H. A. C.; Miller, K. *Hum. & Exp. Toxicol.* **1994**, *13*, 619.
118. Price, K. R.; Gee, J. M.; Ng, K.; Wortley, G. M.; Johnson, I. T.; Rhodes, M. J. C. *Nat. Toxins*, in press.

RECEIVED October 11, 1995

Chapter 16

Analysis of Proteinaceous Antinutritional Compounds in Soya Oilseed Products

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In the food and feed industry large amounts of grain legumes are used for the formulation of endproducts meant for human and animal nutrition. In particular the use of soybean (*Glycin max*) products as milk replacers in the animal diet and the problems related to antinutritional compounds present in soya are well described. Soya contains compounds like trypsin inhibitors, lectins, nonstarch polysaccharides, phytic acid, anti- and/or allergenic proteins. A special need exists for the identification and characterization of these factors. In this study, procedures have been developed for the detection of soya trypsin inhibitors, using both immunochemical and trypsin inhibitory activity methods, for the detection of soya lectins by both sandwich ezyme-linked immunosorbent assay (ELISA) and functional lectin immuno assay (FLIA), and for the detection of the apparent anti- and/or allergenicity by immunochemical methods such as ELISA and Westernblotting using anti-soya calf and human sera, mouse anti-allergen soya P34, and rabbit anti-soya glycinin and β-conglycinin antibodies.

During the last few decades, nutritionists have made it clear that among the factors interfering with the availability of nutrients to animals feeding on legume seeds, protease and amylase inhibitors, lectins as well as anti- and/or allergenic proteins are of great importance (*1*). Plant protease and amylase inhibitors and also lectins may have an important role as defense agents against insect attack (*2*) or micro-organism invasion. The best known protease inhibitors found in seeds are those which are reactive with serine proteases (EC 3.4.21.-), like trypsin and chymotrypsin. However, inhibitors of sulphydryl and metalloproteases also are known. In legume seeds the more widely distributed protease inhibitors belong to the Bowman-Birk (BBI), Kunitz (KTI) and Potato Inhibitor 1 (PI-1) families. The proteins of the BBI family have molecular weights (MWs) around 8–9 kDa and contain a high amount of cysteines

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involved in 7 disulphide bridges. The BBI's are double-headed inhibitors that able to bind different proteases, especially trypsin and chymotrypsin, at different reactive sites. The BBI's generally consist of 2 domains, each containing a distinct reactive site. The sequence alignment of these domains shows a large homology, and it is generally assumed that these double-headed inhibitors have evolved by gene duplication (*3–5*). Odani and Ikenaka (*6*) have separated the 2 internal homologous regions and have shown that both reactive sites are each individually reactive with proteases. The three-dimensional structures for the BBI's of peanut (*7*) and soybean (*8*) have been established and confirmed. These proteins exist as two domain structures, and in addition, the proteinase inhibiting sites are located in exposed external loops. Each inhibitory domain is held in place by the disulphide bridges within a domain, and by cross-links of a domain to an intervening sequence. This may explain their fairly rigid structure. In the soybean BBI the reactive sites have been identified at LYS (16)–SER (17) and at LEU (42)–SER (43). The Kunitz trypsin inhibitors (KTI) are characterized by a MW of about 21 kDa and generally contain four cysteines forming two disulphide bonds (*9*). The soybean KTI is a single-headed inhibitor of trypsin, and also was shown to be weakly reactive against chymotrypsin at two reactive sites, one of them overlapping with the trypsin reactive site. The reactive site in the soybean KTI has been localized at the ARG (63)–ILE (64) bond and the three dimensional structure of this inhibitor has been determined (*10*). Inhibitors homologous to the soybean KTI have been found in many plant seeds. The existence of many isoforms has been described for the protease inhibitors present in various seeds: three trypsin/chymotrypsin inhibitors in red kidney bean (*Phaseolus vulgaris*) (*11*), four in lentils (*Lens culinaris*) (*12*), ten BB trypsin iso-inhibitors (*13*) and three genetically different variants of KTIs in soybean seeds (*Glycin max*).

According to the antinutritional effects of the protease inhibitors, their stability against heat treatment and pepsin also has been studied. It is well known that protease inhibitors are relatively heat stable, due to their rigid structure and high content of disulphide bridges. Only high temperatures are able to inactivate these components. Among the antinutrients that are inactivated by cooking are the lectins or phytohemagglutinins (PHA). The term 'lectin', derived from the Latin 'legere' to choose, was proposed already in 1949 to indicate the selectivity of their interaction with specific blood cells. Lectins possess specific affinity for certain carbohydrate residues, and since sugars are normal structural features of most animal cell membranes, lectins may attach to these sugars and specific receptors. The receptor sites must exist in an exposed position in order to react with a specific lectin. As the 'old' name indicates, hemagglutinins can be characterized and detected by their activities on red blood cells, while other cells can be agglutinated as well. In many cases, binding of lectins may occur without visible clumping. The hemagglutination process is only one of the numerous detectable consequences of the lectin-receptor interaction and plays a key role in the control of various normal and pathological processes in living organisms (*14,15*).

The structure of lectins in relation to their binding characteristics has been studied in detail. Most lectins are glycoproteins that contain different *N*-linked glycosyl residues. The glycosylation is likely not essential for sugar binding. Calcium or manganese ions are often found as ligands in specific sites and removal of these bivalent ions abolishes the lectin activity. Lectins form a rather homologous family of proteins, that on the other hand, are rather heterogeneous with respect to subunit composition and structure (*16*). The existence of isolectins in plant tissues may be explained from the fact that lectins are commonly found as oligomeric structures of 2 or 4 similar or different subunits. The isolectins or individual subunits possess varying degrees of toxicity. As each subunit has one carbohydrate-binding site, monomeric or hemilectins are nonagglutinating molecules, but still have the ability to be toxic. In order to exert an antinutritional action, lectins from legume seeds must resist digestion, in cooked foods or feeds be thermo-stable, and interact with the brush-border membrane (BBM) of the intestinal mucosa. In the case of raw beans or winged bean agglutinin given to experimental animals, the hemagglutinating activity and the specific precipitation of thyroglobulin can easily be detected in extracts of feces, thus indicating that at least part of the ingested lectins have resisted digestion (*17,18*). Soybean lectins, on the other hand, seemed to be digested by gastrointestinal enzymes (*19*). Recent new insights appear to indicate that the role of lectins as being an antinutrient is debatable apart from a possible role as a growth factor; also, unknown effects on the gut flora may have beneficial effects.

Apart from the various antinutrients, in the feed industry it is generally accepted that the overall antigenicity of (particular) soya proteins contributes to the problem of maldigestion and gastrointestinal disturbances. Antigenic (or better immunogenic) proteins may well be defined as proteins able to activate the immune system and consequently induce the generation of various types of antibodies. This phenomenon of immunogenicity of particular proteins in the animal diet appears to be related with a suboptimal digestion, enhanced maintainance requirements, and loss of endogenous protein, but it is essentially not understood. Under common conditions, the intestinal tract provides an effective barrier against the excessive absorption of microorganisms and feed antigens that normally exist in the intestinal lumen. However, during gastrointestinal infection, and also in newborn infants and animals, the mucosal barrier appears to be less effective than in adults and the uptake of feed or food antigens may be increased. This will lead to the generation of antibodies against the absorbed antigens (*20,21*), and may under particular circumstances favor the development of an allergy due to the failure to develop an oral tolerance. Gastrointestinal adverse responses in preruminant calves, which were fed with soya (*22,23*), were assumed to be induced by the soya proteins and especially glycinin and β-conglycinin (*24,25*). Using calf anti-soya protein sera obtained from orally sensitized animals, Hessing *et al.* (*26,27*) observed that the calf antibody responses were mainly directed against β-conglycinin and an unknown low MW antigenic P22–25 soya protein.

In clinical studies using sera from soya allergenic human individuals, various IgE-binding soya proteins have been reported (*28–32*). More recently, Ogawa *et al.* (*33*) demonstrated the occurence of the major allergen in soya beans, named *Gly m* Bd 30 K, that was most strongly and frequently bound by the IgE antibodies of the sera of soya allergenic human Japanese individuals. In 1993, this soya bean allergenic protein, *Gly m* Bd 30 K, was further characterized (*34*) and identified as the soya bean seed 34-kDa oil-body-associated protein (*35*). The 34-kDa oil-body-associated protein or P34 showed considerable sequence similarity to the thiol proteinases of the papain superfamily and demonstrated a remarkable homology of about 30% with the house dust mite allergenic thiol proteinase *Der p* I, from *Dermatophagoides pteronyssinus*. It was recommended that *Gly m* Bd 30K (or P34) should be named as *Gly m* I as the first characterized allergenic protein in soya beans (*34*). Recently, we have compared the previously recognized unknown low MW antigenic P22–25 soya protein (*26,27*) with the *Gly m* I (or P34) soya bean allergen, and observed that those soya proteins are clearly different (Hessing *et al.*, submitted for publication in *Food Agric. Immunol.*).

Materials and Methods

Materials. Soya products as raw beans, meal and concentrate were obtained from different origins. Bovine serum albumin (BSA), dithiothreitol (DTT) and *N*-acetyl-D-galactosamine were obtained from Sigma Chemical Co. (St. Louis, MO). 1,2-Phenylendiamine-dihydrochloride and glycerol was obtained from Fluka (Buchs, Switzerland). Microtiter plates were from Costar (Cambridge, MA). (Gal NAc-β-O-CETE)$_n$ BSA was obtained from Janssen, Biochimica (Brussel, Belgium).

Preparation and Extraction of Samples. Each soya sample was either supplied as flour or milled prior to analysis to a particle size of ≤0.5 mm using a Laboratory Mill (Retsch, 0.5 mm). Samples were milled, but not defatted. For immunochemical analysis, 1 g of soya flour was extracted by the addition of 20 mL 50 mM Tris-HCl, pH 8.2. The pH was adjusted to 8.2 if required and samples were thoroughly mixed on a suitable magnetic stirrer for 60 min at room temperature. Subsequently, 2 mL of the extract was centrifuged for 15 min at 7500 x g and supernatants were either immediately analyzed or frozen at -20 °C.

Trypsin Inhibitors. Kunitz and Bowman Birk trypsin inhibitor were purchased from Sigma (St. Louis, MO). In addition, a BBI preparation was obtained from Nestle (Switzerland) for comparison. For the inactivation of the KTI either *N*-acetylcysteine or ethanol was used.

Lectins. Soya lectins were purified from defatted soybean meal by a two-step procedure including affinity chromatography on *N*-acetyl-D-galactosamine-Sepharose CL-4B and gel filtration on Sephadex G-25 using Pharmacia-LKB BioPilot equipment. Finally, the purified lectins were lyophilized and frozen at -20 °C. Sodium dodecylsulfate polyacryamide gel electrophoresis (SDS-PAGE) analysis of the isolated preparations under reducing conditions revealed the characteristic 30 kDa lectin subunits. IEF-analysis of the purified lectin preparation revealed 2 major bands at IEP 5.2 and 5.65 and *N*-terminal sequencing analysis confirmed the identity of the purified soya lectin.

Glycinin, β-conglycinin and *Gly m* I. The soya storage proteins glycinin and β-conglycinin were purified as is described (Hessing *et al.*, submitted for publication in *J. Agric. Food Chem.*). *Gly m* I (or P34) was purified essentially as described (*34*) and was generously provided by Dr. Ogawa (University of Tokushima, Japan). The purified P34 preparations were lyophilized and frozen at -20 °C.

Antibodies. Monoclonal antibodies (Mabs) against the soya KTI, BBI and soya allergen *Gly m* I (P34) were obtained as described elsewhere (*33, 36–38*). Rabbit anti-soya lectin specific rabbit anti-lectin antibodies were prepared according to standard procedures and the immunoglobulins were obtained via protein A affinity chromatography according to the instructions of the manufacturer (Pharmacia-LKB, Uppsala, Sweden).

SDS PAGE and Western Blotting Assays. SDS PAGE and Western blotting were performed under reducing conditions, using a modification of the method of Laemmli (*39*). The extraction buffer was 0.063 M Tris (hydroxymethyl)aminomethane pH 6.8, 2% sodium dodecylsulfate (SDS), 20% glycerol, 0.01% bromphenol blue. The reducing agent was 1% dithiothreitol. Samples were heated for 15 min at 100 °C and centrifuged at 10,000 rpm. After electrophoresis the protein bands were stained with coommassie brilliant blue. Another gel was blotted after electrophoresis according to the Western blot method (*40*).

Results and Discussion

Various methods were developed for the measurements of antinutritional compounds as trypsin inhibitors and lectins. In addition, methods for the detection and quantification of immunogenic proteins and the problem of allergy have been studied.

Measurement of the Trypsin Inhibitory Activity. The most commonly used methods for measuring the trypsin inhibitory activity (TIA) in legume seeds are based on the original method of Kakade *et al.* (*41*). In the original Kakade method, TIA was measured by the inhibition of trypsin, using the chromogenic substrate benzoyl-DL-arginine-*p*-nitroanilide-hydrochloride (DL-BAPA). The Kakade method has been published as an AOCS official method (Ba12–75, 1983), and is widely used as a quality parameter for soybean products. However, recently at several laboratories a number of modified TIA-procedures are under investigation that are either adapted for reasons of optimal performance, or for TIA measurement in other legume seeds. For peas (*Pisum sativum*) and faba beans (*Vicia faba*), Valdebouze *et al.* (*42*) have developed modified methods for TIA measurement. The most important modification involved the extraction procedure; i.e., the extraction is performed under acidic instead of alkaline conditions. It was speculated that under acidic conditions any putative interference due to lipoxygenase activity and phenolic compounds was abolished. Since 1988, we have been developing another modified TIA procedure for the analyses of soybean products, which has been evaluated by a collaborative study (*43*), and also is based on the original AOCS Kakade method. The modifications of this method are both present in the sample extraction and assay performance procedures and are briefly summarized in Table I and II. More recently, the Kakade-like methods have been adapted to a microtitre plate procedure (*44*), which allows the TIA analyses of a large number of samples in breeding programs or for feed control (Hessing *et al.*, TNO Nutrition and Food Research Institute at The Netherlands, unpublished results).

Table I. Comparison of the extraction procedure of the Kakade- and TNO TIA methods.

Steps:	Original Kakade method:	TNO modified TIA method:
1.	Extraction buffer: 0.01 N NaOH, pH 8.4–10.0	pH constant at pH 9.5
2.	Shaking 1 h at room temperature	Settle down during 15–24 h at 4 °C
3.	Centrifugation (10,000 x g)	
4.	Preparation of dilutions	Preparation of dilutions
5.	Incubation	Incubation
6.		Centrifugation (10,000 x g)

Table II. Comparison of the incubation procedure of the Kakade- and TNO TIA methods.

Steps:	Original Kakade method:	TNO modified TIA method:
	Incubation procedure for samples:	
1.	Sample extract + trypsin	Sample extract + L-BAPA
2.	Addition of DL-BAPA 10 min incubation at 37 °C	Addition of trypsin 10 min incubation at 37 °C
3.	Inhibition reaction with acetic acid	Inhibition reaction with acetic acid
	Incubation procedure for controls:	
4.	Sample extract + DL-BAPA	Sample extract + L-BAPA + acetic acid
5.	Incubate 10 min at 37 °C	
6.	Addition of acetic acid + trypsin	Addition of trypsin and incubation for 10 min at 37 °C

Immunochemical Analysis of the Legume Seed Trypsin Inhibitors. Alternatively, immunochemical methods for the measurement of the trypsin inhibitors can be applied for soybeans (*36,37,45*), peas (*46*) and chick peas (*47*). The presently used immunochemical methods, however, have a very high specificity, and as a consequence may not detect all relevant inhibitory activity of the various kinds of inhibitors. In addition, the used antibodies should ideally be able to discriminate between native; i.e., (re)active, complexed and denatured inhibitors. On the other hand, all inhibitors having enzyme inhibitory ability present in the seed need to be determined. We have developed both competitive ELISA methods for both the measurement of the native forms of the soya KT and BB Inhibitors. For this, microtiter plates were coated with goat anti-mouse antibodies and subsequently monoclonal anti-KTI and BBI antibodies were bound. In the third step of the assay, free KTI, BBI and biotinylated KTI and BBI were applied, and during a 1 h incubation these proteins were allowed to compete for recognition by the immobilized anti-KTI and anti-BBI Mabs. For obtaining a standard curve, the free KTI and BBI preps were added to the wells in a serial dilution, while the concentration of the biotinylated inhibitors was kept constant. Following this incubation, unbound trypsin inhibitor molecules were removed by washing and bound biotinylated inhibitors were measured by peroxidase conjugated avidin and tetramethylbenzidin staining. Both the competitive KTI and BBI ELISAs demonstrated a lower detection limit of about 5 ng/mL and could be applied for the analysis of a large number of soybean materials as raw beans, soybean meals and concentrates (Table III).

Table III. Immunochemical Analysis of antinutritional soya compounds (mg/g).

Soybean product	TIA	KTI	BBI	SBL ELISA	SBL FLIA	GLYC	CON
Soybean	18.9	8.0	7.9	5.8	5.4	233	68
SBM	2.0	0.8	n.d.	0.34	0.37	50	20
SPC	2.5	0.27	n.d.	< 0.01	< 0.01	< 0.001	< 0.001

SBL, Soybean lectin; GLYC, glycinin; CON, conglycinin; SBM, Soybean meal; SPC, Soyprotein concentrate; n.d., not done.

Analytical and Functional Methods for Lectins. The detection of legume seed lectins has in the past, as well as today, most commonly been performed by hemagglutination assays. In for example, microtiter plates, constant amounts of normal or protease-treated red blood cells (RBC) are added, and clumping due to the addition of serial dilutions of lectin-containing samples, is visually determined. The acceptance of this method is widespread, among other considerations, because of the small amounts of lectin samples that are required. In principle, screening of single seeds is feasible with this method. The origin of RBC and the individual quality of these cells is essential for a good performance of the assay because RBC from different species are susceptible in different degrees to various lectins (*48*). The semiquantitative nature of the serial dilution test has been improved by using either a photometric measurement of the RBC that are left unagglutinated by lectin (*49*), or by using an electronic particle counter to count the nonagglutinated cells in microtiter wells (*50*).

In general, the haemagglutination assays are hampered by both a lack in specificity and sensitivity. In addition, some lectins are not detected by haemagglutination. The screening of the presence of lectins in foods has also been realized by a combination of immunoelectrophoresis and a hemadorption lectin test (*51*). Using polyclonal antibodies against isolated bean lectins, microgram quantities of lectin also could be measured by rocket immunoelectrophoresis (*52*). Nanogram quantities of castor bean lectins were detected by a technique that was based on the interaction of the lectin with sugar immobilized to lysozyme, and the measurement of the remamnent lytic effect of *Micrococcus luteus* cells (*53*).

In 1989, we have begun to exploit the functional property of *Phaseolus vulgaris* lectins to bind to specific sugar groups for the development of a so called Functional Immuno Assay (FLIA), which was compared by also measuring the total amounts of those lectins present in the sample by an ELISA procedure that was based on the use of a sandwich of polyclonal anti *Phaseolus vulgaris* lectin antibodies (*54*). The difference between the FLIA and ELISA method lies in the fact that the FLIA uses a carbohydrate-containing agent, instead of a polyclonal antibody, to form a sandwiched enzyme-immunosorbent assay. The FLIA method is based on the concept that functional lectins are defined as lectins capable of binding to a specific suitable sugar-containing substrate. Since then, we also have developed FLIA and ELISA methods for the measurement of lectin contents in soya beans, peas and *Vicia faba*. For each type of legume lectin specie, both specific antibodies and specific carbohydrate-containing substrates have been developed. For soya lectin, either rabbit polyclonal anti-soya lectin antibodies (ELISA) or (Gal NAc-β-O-CETE)$_n$ BSA (FLIA) were coated on microtiter plates. Subsequently, plates were blocked and incubated with purified lectin as reference and the soya samples diluted at appropriate concentrations. The plates were washed and peroxidase-conjugated anti-lectin antibodies were applied and bound conjugated antibody was developed for peroxidase activity using 1,2-phenylenediamine. The soybean lectin ELISA and FLIA methods demonstrated a lower detection limit of about 0.01 mg/g and could be applied for the analysis of a large number of soybean materials like raw beans, soybean meals, and concentrates (Table III).

Analysis of Soya Proteins in Relation with Allergy, Antigenicity or Hypersensitivity Phenomena.

Food allergy and hypersensitivity can occur in humans and also in animals, and result in an immunological reaction to an ingested food or feed protein. It is important to differentiate between a true allergy and an intolerance. Allergy is mediated via an immunological mechanism, usually accompanied by a specific IgE synthesis that induces the release of mediators from mast cells and basophils. These mediators are responsible for the tissue reactions occurring in allergic diseases (*55*). Intolerance is an inability to consume a diet containing a particular antigen without distress and does not imply a specific immunological mechanism. It may be due to digestive enzyme deficiencies, fat intolerance, physiological causes or an undetermined ethiology (*55*). The development of a food allergy depends on several factors like heredity, intestinal permeability, immune responsiveness and exposure to food.

Laboratory assays have mostly proven to be of minor value for the diagnosis of food/feed allergies. Exceptions are the radio-allergosorbent test (RAST) and the crossed radioimmunoelectrophoresis (CRIE) for IgE antibodies in blood and serum. Recently, Gjesing and Ipsen (*56*) compared different assay methods (RAST, CRIE, dot blotting, immunoblotting) for the estimation of specific IgE antibodies against food proteins using wheat flour and soya as antigens. All four methods failed to identify specific IgE reactions to these antigens. However, although the four methods showed good correlations when statistically compared, misleading diagnosis was possible when only one technique was used. Using the ELISA and immunoblotting techniques (*30–34*), a number of groups have studied IgE and IgG antibodies reacting with fractionated soya proteins and it may be concluded that a multiplicity of soya allergens exists that are able to induce allergy related IgE formation. Whereas, the production of IgE towards food is a disadvantageous reaction, the production of IgG antibodies towards some dietary antigens is an apparent normal reaction. Laboratory tests can be useful for predicting the value of soya based products for young animals and the use of ELISA and haemagglutination methods for the examination of possible relationships between various soya products and the performance of these products in the animals.

ELISA for Soya Glycinin and β-Conglycinin Immunoreactivity Levels. As the underlying factors behind the immunogenicity and allergenicity problems associated with soya proteins are difficult to indicate from presently existing knowledge, in collaboration with a number of soya producers it was decided in 1991 at TNO to develop a collaborative ELISA method to measure the soya storage proteins, glycinin and β-conglycinin, immunoreactivity levels using rabbit antibodies (for reasons of convenience), and to aim at introducing a general accepted quality control method for the determination of an antigenicity parameter for soya products. A direct double antibody sandwich enzyme-linked immunosorbent assay (ELISA) method using specific polyclonal rabbit antibodies against soya glycinin and β-conglycinin, respectively, was developed as is described elsewhere (Hessing *et al.*, submitted for publication in *J. Agric. Food Chem.*). Polyclonal rabbit anti-glycinin and anti-β-conglycinin antibodies were coated on microtiter plates with the respective antibodies, and subsequently, plates were blocked and incubated with purified glycinin, and β-conglycinin, and the soya samples were diluted to the appropriate concentration. The plates were washed and peroxidase-conjugated anti-glycinin and anti-β-conglycinin antibodies were applied and finally the plates were washed and bound conjugated antibody was developed for peroxidase activity using 1,2-phenylenediamine. The glycinin and β-conglycinin ELISA methods demonstrated a lower detection limit of about 0.001 mg/g and could be used for the analysis of a large number of soybean materials like raw beans, soybean meals, and concentrates (Table III).

ELISA for Total Soya Antigenicity Content. In 1988, Ventling and Hurley (*57*) applied the immunoblotting procedure for determination of soya proteins in food and feed products. However, these authors used rabbit antisera against all soya proteins, which may not be completely representative for the allergenicity of those soya proteins. In 1991, van Oort *et al.* (*58*) showed that the use of antisera from sensitized calves in similar experiments did result in different reactivities of the various soya proteins compared with rabbit antisera that were obtained after parenteral immunization. Therefore, an ELISA procedure was developed using the calf antibodies (*26*) to obtain a more realistic product parameter that allowed for a proper prediction of the antigenicity of a soya product. For the determination of the so called total soya antigenicity, the same procedure (sandwich ELISA) was performed as described above, with the exception that now calf-antibodies obtained through oral sensitization were used (*26,27*). Presently, the application of this method for various soya products is being evaluated and a comparison with broiler, piglet and human anti-soya protein sera is being made. This study may allow for the development of methods that enable a better *in vitro* product evaluation. Also, the first identified soya allergen *Gly m* I can now be measured by a recently developed *Gly m* Bd 30 K sandwich ELISA (*59*).

Concluding Remarks. In this study, an overview of the state-of-the-art on analytical methods for the measurements of proteinaceous antinutritional compounds is presented. Also, some newly developed immunochemical procedures for the detection of soya proteins as lectins, KTI and BBIs and antigenic and/or allergenic proteins (glycinin, β-conglycinin and *Gly m* I allergen (P34)) are discussed. The assays can be used to evaluate the characteristics of food and feed products during processing and in the final products.

Literature Cited

1. Gueguen, J.; Van Oort, M. G.; Quillien, L.; Hessing, M. *2nd International Workshop on Antinutritional Factors in Legume Seeds*; Wageningen, The Netherlands, 1993; pp 9–30.
2. Shade, R. E.; Schroeder, H. E.; Pueyo, J. J.; Tabe, L. M.; Murdock, L. L.; Higgins, T. J. V.; Chrispeels, M. *J. Biotechnology* **1994**, *12*, 793–795.
3. Tan, C. G. L.; Stevens, F. C. *Eur. J. Biochem.* **1971**, *18*, 515–523.
4. Shimokawa, Y.; Kuromizu, K.; Araki, T.; Ohata, J.; Abe, O. *Eur. J. Biochem.* **1984**, *143*, 677–684.
5. Birk, Y. *Int. J. Peptide Protein Res.* **1985**, *25*, 113–131.
6. Odani, S.; Ikenaka, T. *J. Biochem. (Tokyo)* **1977**, *82*, 1513–1522.
7. Susuki, A.; Tanaka, I.; Yamane, T.; Kikkawa, J.; Ashida, T.; Noriaka, S.; Hara, S.; Ikenaka, T. *J. Biochem. (Tokyo)* **1987**, *101*, 267–274.
8. Werner, M. H.; Wemmer, D. E. *Biochem.* **1991**, *30*, 3356–3364.
9. Richardson, M. *Methods Plant Biochem.* **1991**, *5*, 259–305.
10. Sweet, R. M.; Wright, H. J.; Janin, J.; Chothia, C. H.; Blow, D. *Biochem.* **1974**, *13*, 4212–4228.

11. Wu, C.; Whitaker, R. *J. Agric. Food Chem.* **1991**, *39*, 1583–1589.
12. Weder, J. K. P.; Mueller, R. *J. Food Biochem.* **1989**, *13*, 81–103.
13. Tan-Wilson, A. L.; Chen, J. C.; Duggan, M. C.; Chapman, C.; Obach, R. S.; Wilson, K. A. *J. Agric. Food Chem.* **1987**, *35*, 974–981.
14. Grant, G. *Prog. Food & Nutr. Sci.* **1989**, *13*, 317–348.
15. Grant, G.; van Driessche, E. *2nd International Workshop on Antinutritional Factors in Legume Seeds*; Wageningen, The Netherlands, 1993; pp 45–60.
16. Sharon, N.; Lis, H. *FASEB J.* **1990**, *4*, 3198–3208.
17. Jaffe, W. G.; Vega-Lette, C. L. *J. Nutr.* **1968**, *94*, 203–210.
18. Higuchi, M.; Kawada, T.; Iwai, K. *J. Nutr.* **1989**, *119*, 490–495.
19. Liener, I. E. *J. Biol. Chem.* **1976**, *233*, 401–405.
20. Kilshaw, P. J. *Curr. Top. Vet. Med. Anim. Sci.* **1981**, *12*, 203–223.
21. Taylor, S. L.; Lemanske, R. F., Jr.; Bush, R. K. *Comments Agric. Food Chem.* **1987**, *1*, 51–70.
22. Kilshaw, P. J.; Sissons, J. W. *Res. Vet. Sci.* **1979a**, *27*, 361–365.
23. Kilshaw, P. J.; Sissons, J. W. *Res. Vet. Sci.* **1979b**, *27*, 366–371.
24. Sissons, J.; Smith, R. H.; Hewitt, D.; Nyrup, A. *Br. J. Nutr.* **1982**, *47*, 311–318.
25. Sissons, J. W.; Thurston, S. M. *Res. Vet. Sci.* **1984**, *37*, 242–246.
26. Hessing, M.; Van de Hoef, R.; Van Biert, M.; Vlooswijk, R. A. A.; Van Oort, M. G.; Hamer, R. J. *Recent Advances of Research in Antinutritional Factors in Legume Seeds*; Wageningen, The Netherlands, 1993; pp 55–59.
27. Hessing, M.; Bleeker, H.; Van Biert, R.; Vlooswijk, R. A. A.; Van Oort, M. G. *Food Agric. Immunol.* **1994**, *6*, 315–320.
28. Moroz, L. A.; Yung, W. H. *N. Engl. J. Med.* **1980**, *302*, 1126–1128.
29. Shibasaki, M.; Suzuki, S.; Tajima, S.; Emoto, H.; Kuroume, T. *Int. Arch. Allergy Appl. Immunol.* **1980**, *61*, 441–448.
30. Burks, A. W.; Williams, L. W.; Helm, R. M.; Connaughton, C.; Cockrell, G.; O'Brien, T. *J. Allergy Clin. Immunol.* **1991**, *88*, 172–179.
31. Burks, A. W., Jr.; Brooks, J. R.; Sampson, H. A. *J. Allergy Clin. Immunol.* **1988**, *81*, 1135–1142.
32. Herian, A. M.; Taylor, S. L.; Bush, R. K. *Int. Arch. Allergy Appl. Immunol.* **1990**, *92*, 193–198.
33. Ogawa, T.; Bando, N.; Tsuji, H.; Okajima, H.; Nishikawa, K.; Sasaoka, K. *J. Nutr. Sci. Vitaminol.* **1991**, *37*, 555–565.
34. Ogawa, T.; Tsuji, H.; Bando, N.; Kitamura, K.; Zhu, Y. L.; Hirano, H.; Nishikawa, K. *Biosci. Biotechnol. Biochem.* **1993**, *57*, 1030–1033.
35. Kalinski, A.; Melroy, D. L.; Shyam Dwivedi, R.; Herman, E. M. *J. Biol. Chem.* **1992**, *267*, 12068–12076.
36. Brandon, D. L.; Bates, A. H.; Friedman, M. *J. Agric. Food Sci.* **1989**, *37*, 1192–1196.
37. Van Duijn, G.; Heijstek, F.; Van Oort, M. *Recent Advances of Research in Antinutritional Factors in Legume Seeds*; Wageningen, The Netherlands, 1993; pp 51–54.
38. Tsuji, H.; Bando, N.; Kimoto, M.; Okada, N.; Ogawa, T. *J. Nutr. Sci. Vitaminol.* **1993**, *39*, 389–397.

39. Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
40. Towbin, H. J.; Staehelin, Th.; Gordon, J. *Proc. Natl. Acad. Sci. USA.* **1979**, *76*, 4350–4354.
41. Kakade, M. L.; Rackis, J. J.; Mcghee, J. E.; Puski, G. *Cereal Chem.* **1974**, *51*, 376–382.
42. Valdebouze, P.; Bergeron, P.; Gaborit, T., Delort-Laval, J. *Can. J. Plant Sci.* **1980**, *60*, 695–701.
43. Dukel, F.; Van Westerop, H. J. M.; Hessing, M.; Bokhorst, H. J.; Daalhuisen, H. J.; Van Schalm, K. J.; Sittrop, N.; Verwijs, T. *J. Sci. Food Agric.* **1995**, in press.
44. Gaborit, T.; Quillien, L.; Gueguen, J. *2nd International Workshop on Antinutritional Factors in Legume Seeds*; Wageningen, The Netherlands, 1993; pp 27–33.
45. Brandon, D. L.; Bates, A. H.; Friedman, M. *J. Food Sci.* **1988**, *53*, 102–107.
46. Frokiaer, H.; Horlyck, L.; Sorensen, H.; Sorensen, S. *Recent Advances in Antinutritional Factors in Legume Seeds*; European Association for Grain Legume Research, Copenhagen-Denmark, ed., Wageningen, The Netherlands, 1993, pp 75–78.
47. Henmar, H.; Frokiaer, H.; Savage, G.; Sorensen, S. *Improving Production and Utilization of Grain Legumes*; 2nd European Conference on Grain Legumes; 1995; p 412.
48. Burger, M. M. *Methods Enzymol.* **1974**, *32*, 615–621.
49. Turner, R. H.; Liener, I. E. *Anal. Biochem.* **1975**, *68*, 651–653.
50. Coffey, D. G.; Uebersax, M. A.; Hosfield, E. L.; Brunner, J. R. *J. Food Sci.* **1985**, *50*, 78–87.
51. Andersen, M. M; Ebbesen, K. *Biol. Biochem. Clin. Biochem.* **1986**, *5*, 95–108.
52. Osborn, T. C.; Bliss, F. A. *J. Am. Soc. Hort. Sci.* **1985**, *110*, 484–488.
53. Ghosh, M.; Bachhawat, B. K.; Suriola, A. *Biochem. J.* **1979**, *183*, 185–188.
54. Hamer, R. J.; Koninkx, J.; Van Oort, M. G.; Mouwen, J.; Huisman, J. *Recent Advances of Research in Antinutritional Factors in Legume Seeds*; van der Poel, A. F. B.; Huisman, J.; Saini, H. S., eds.; Pudoc, Wageningen, The Netherlands, 1989; p 30.
55. Wilson, N. W.; Hamburger, R. N. *Ann. Allergy* **1988**, *61*, 323–328.
56. Gjesing, B.; Ipsen, H. H. *Ann. Allergy* **1987**, *59*, 77–81.
57. Ventling, B. L.; Hurley, W. L. *J. Food Sci.* **1988**, *54*, 766–767.
58. Van Oort, M. G.; Hamer, R. J.; Tolman, G. H. *Proceedings of the International Symposium on Veal Calf Production, March 1990*; EAAP Publication: Wageningen, The Netherlands, 1991, Vol. 52; pp 247–252.
59. Tsuji, H.; Okada, N.; Yamanishi, R.; Bando, N.; Kimoto, M.; Ogawa, T. *Biosci. Biotech. Biochem.* **1995**, *59*, 150–151.

RECEIVED October 11, 1995

Chapter 17

Detecting Ergot Alkaloids by Immunoassay

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The ergot alkaloids are seldom a problem in grain products destined for human consumption, due to the relative ease of removing sclerotia of *Claviceps* from most cereal grains which are hosts for this genus of toxigenic fungus. Animal feeds, on the other hand are often contaminated with ergot alkaloids from *Claviceps*, as well as from fungi of the genus *Acremonium*, which are endophytic in many pasture grasses worldwide. We have employed two strategies in the development of polyclonal and monoclonal antibodies against these alkaloids. Depending on the point of conjugation with the carrier protein, antibodies can be produced which are specific for a narrow range of target moieties, or antibodies which are more general and react with a broad range of ergot alkaloids are produced. These antibodies are mainly used in detection in common ELISA formats.

The ergot alkaloids differ from other natural food toxicants in several important respects. This mycotoxin has probably been responsible for cases of human toxicosis since prehistoric times, and is certainly one of the few for which mass human poisonings can be traced to a known specific class of mycotoxin from the historic record. "Holy fire" or "St. Anthony's fire" was the name given to the gangrenous ergotism which plagued medieval Europe until consumption of ergot sclerotia was recognized as the cause of the illness *(1)*. The production of sclerotia from infected seed of the host grain is another distinctive feature of this fungal plant pathogen. Unlike other mycotoxigenic fungi, the genus *Claviceps* typically produces enlarged sclerotia in place of the grain, which are dark in color, and can be easily separated from the healthy grain. At least 10 species of *Claviceps* are recognized on 50 genera of grass hosts (Table I). Finally, the ergot alkaloids exhibit a broad range of pharmacologic effects. Rather than being acutely toxic or carcinogenic, as many mycotoxins apparently are, these compounds can actually be beneficial pharmaceutical

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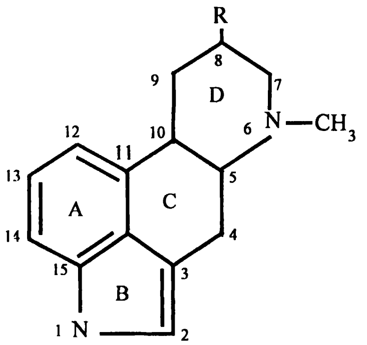
compounds when administered properly. For this reason the ergot drugs are still produced by growing the pathogen on rye, harvesting sclerotia, and extracting the crude ergot compounds. These compounds can be purified and then modified to produce other semisynthetic forms.

Table I. Fungi Known to Produce Ergot Alkaloids

Pathogen	Host	Reference
Acremonium coenophialum	*Festuca arundinacea*	*(2)*
Acremonium 3 spp.	5 species in 4 genera	*(3)*
Epichloe typhina	8 spp. in 4 genera	*(3)*
Claviceps 10 spp.	200 spp, 50 genera (all *Poaceae*)	*(4)*
Aspergillus *Penicillium* *Balansia* *Sphacelia* *Hypomyces* *Phycomyces* *Rhizopus*	Various	*(1)*

Like many other mycotoxins, the ergot alkaloids exist as a large family of compounds, the various forms of which differ widely in toxicologic or pharmacologic effect, depending upon the substitutions on the D ring of the 5-membered ergoline ring structure (Figure 1). There are now about 100 members of this family, including semisynthetic drugs. They range in pharmacologic activity from the semisynthetic *N,N*-diethyl-*D*-Lysergamide (LSD-25), one of the most powerful psychomimetics ever produced, to ergotamine, used to treat migraine headache. The range of pharmacologic activity produced by modifications of this simple ergoline ring structure may still produce fascinating insights into basic endocrinology and neurochemistry. An exhaustive review of ergot chemistry can be found in Berde and Schild *(5)*.

As mycotoxins, this group of compounds rarely poses a threat to human health, owing to current sanitation procedures in grain processing. The ergot alkaloids are common in grain products, particularly rye *(6)*, but at levels which probably have minimal negative health potential. They do, however, pose a significant health risk to animals. Grazing livestock can frequently ingest *Claviceps* sclerotia in the infected grain directly from the field on any of the 50-odd genera of grasses which are potential hosts for this fungus. Grain which has been refused for human consumption could potentially be used as livestock feed, or when used directly on the farm where



COMPOUND	R	DOUBLE BOND
ERGONOVINE	CONHC (CH$_3$) CH$_2$OH	9 - 10
LYSERGIC ACID	COOH	9 - 10
ERGOSINE	CYCLOL ALA-PRO-LEU	9 - 10
ERGOVALINE	CYCLOL ALA-PRO-VAL	9 - 10
ERGOTAMINE	CYCLOL ALA-PRO-PHE	9 - 10
FESTUCLAVINE	β- CH$_3$	8 - 9
ELYMOCLAVINE	CH$_2$ OH	8 - 9

Figure 1. Ergot alkaloids generalized structure.

it is produced without appropriate testing. Another common form of ergotism in livestock arises from ingestion of pasture grasses infected with endophytic fungi, predominantly *Acremonium coenophialum* in tall fescue, and *Acremonium lolii* in perennial ryegrass. The symptoms produced in animals ingesting ergot alkaloids typically include poor weight gain, poor milk production in lactating animals, various reproductive disorders, and in extreme cases, the classical gangrenous loss of extremities such as feet, tails, and ears. In the cases of animal intoxication, like those of ergotism in humans, the effect is produced only when there is constant dietary intake of the contaminated food source as is the case when animals are confined to an infected paddock or pasture.

Methods of Detection

Several methods are available for detecting ergot alkaloids. Perhaps the oldest is the colorimetric assay based on *p*-dimethylaminobenzaldehyde *(7)*. This chromagen, also, can be used as a spray reagent with thin-layer chromatography (TLC), where the color of the spot developed is characteristic of the alkaloid *(8)*. The ergot compounds that have a double bond in the 9,10 position are strongly fluorescent (Figure 1), making detection in solution or on a TLC plate relatively simple. Fluorescence detection is also nondestructive, making it possible to harvest pure alkaloid from the TLC plate or liquid chromatography outflow. The ergot alkaloids not having the 9,10 double bond, usually, can be detected by ultraviolet absorbance of the ergoline ring structure. Fluorescence and U.V. absorbance facilitate high-performance liquid chromatography (HPLC) *(9–11)*. Tandem mass spectrometry (MS/MS) also has been used to detect and identify ergot alkaloids in fescue *(2)*.

In a diagnostic laboratory or field situation, the need often arises to survey large numbers of samples. In these situations, it is more important that samples contaminated with trace amounts of the target compound be identified, rather than quantitative accuracy. It is this situation for which immunoassay techniques are ideally suited. We typically have available more than one method to detect a target compound. This enables us to select a method which is most economical and efficient, as well as providing a methods check whereby analysis by one method can be compared with that of a second or third method. In the case of ergot alkaloids, for example, we can use TLC, HPLC, and immunoassay.

Each method yields a different type of data, useful in its own way. HPLC data is typically the most quantitatively accurate, since each moiety in a complex mixture is separated, detected, integrated by computer, and compared with analytical standards. The nature of the procedure, however, demands a high degree of training of personnel, expensive equipment, and utmost attention to detail. In practical terms, without an automated HPLC, only about 15 samples per day can be analyzed at a cost of about $25.00 per sample. The immunoassay format requires less investment in equipment, training, and time per sample. We can analyze hundreds of samples per day at a fraction of the cost per sample. The trade-off comes in the sacrifice of quantitative accuracy of the data. There are inherent sources of error in all immunoassay methods, particularly when the target compound is in a complex background of related molecular structures.

Early Immunoassays

The ergot alkaloids were one of the first mycotoxin groups for which immunoassay methods were developed. The early interest in radioimmunoassay (RIA) methods stems from the importance of ergot alkaloids as drugs *(12–16)*. These methods were developed for use in pharmacokinetic studies where picogram amounts of ergot drugs were detected in plasma and other biological fluids. These early immunoassays pointed out the effectiveness of the competitive immunoassay in adding quantitative precision to the assay. Another important prior discovery was the observation by Arens and Zenk *(16)* that the point of conjugation on the ergot alkaloid molecule results in antibodies with dramatically different specificities. These early successes at producing antibodies against ergot alkaloids suggested that it would be practical to develop our own antibodies and immunoassays to detect ergot compounds in agricultural commodities. They also suggested conjugation strategies which would result in immunogens that should produce antibodies of different affinities and specificities.

Ergovaline and Endophytes

Our laboratory serves primarily a diagnostic function to assist livestock owners, veterinarians, and the seed industry in identifying fields which may have endophyte toxicosis problems. This has been done mainly by micropscopic examination of plant and seed material for evidence of fungal hyphae *(17)*. With the discovery of ergovaline in endophyte-infected tall fescue *(2)* another potential diagnostic tool became available. If ergovaline proved to be the "endophyte toxin", then levels of this compound should be a good indicator of toxic potential in the pasture. A reliable HPLC method for analysis of ergovaline in fescue was available, and this quickly became a standard procedure in our lab, but the previously discussed limitations of HPLC make immunoassay for this mycotoxin an attractive alternative. Research samples submitted to the lab sometimes consist of large numbers of individual samples, sometimes less than 1 g dry weight. Plant breeders, for example, might wish to determine the toxic potential of large numbers of individual plants from a greenhouse experiment, where the plants tend to be smaller.

Our goal was to develop an immunoassay which would enable us to identify toxic plant material in samples collected by researchers and the general public. The development of specific anti-ergot alkaloid antibodies was proven technology, but could be improved upon by the use of enzymes rather than radioisotope labels in the assay. This would avoid the inherent problem of isotopes being a hazardous waste, and result in an assay using a chromagen which could be interpreted visually. The enzyme-linked immunosorbent assay (ELISA) has essentially replaced the RIA for these reasons. The technology of monoclonal antibodies also would adds specificity and reproducibility to an ELISA.

A problem associated with production of antibodies to ergovaline was the very limited amount of pure ergovaline available. Milligram quantities were obtained from Sandoz (Basel, Switzerland) but it was practical to use this only for standards in chromatography. This meant that our initial attempts to produce antibodies had to be with structurally related compounds. Because of the pharmacological importance of ergot drugs, several other similar compounds are readily available. We chose ergotamine because of its structural similarity to ergovaline.

Ergotamine Polyclonal Antibody

Our first attempt to produce anti-ergot antibodies were essentially a duplication of the work of several predecessors *(18)*. To make the immunogen, ergotamine was linked to the carrier protein bovine serum albumin (BSA) at the indole nitrogen (Figure 1) by the Mannich reaction using glutaraldehyde as the linking agent (Figure 2). A second conjugate was made utilizing ovalbumin (OVA) as the carrier for subsequent use in coating polystyrene microtiter plates in the ELISA. Estimation of conjugation efficiency or molar substitution ratios can be made by measuring the amount of unbound hapten after completion of the reaction. This is removed by dialysis before use. A second relatively simple way to estimate conjugation efficiency is to run the conjugate in the TLC system for these alkaloids, and view the plate in U.V. light or spray with Erlich's reagent. Unbound ergotamine will move to the usual position on the plate as indicated by standards, while the conjugate will remain at the origin of the plate. Relative size of the spots is an indication of conjugation efficiency. We estimated the molar substitution ratios to be 7.7 for BSA and 1.3 for OVA. This reflects the relative abundance of lysine residues to which the aldehyde links the hapten in the two proteins. We measured the protein in the conjugate by colorimetry and adjusted the dosage to approximately 1 mg per injection.

Rabbits were immunized following the general protocols found in Harlow and Lane *(19)*. Titers were measured by competitive inhibition (CI) ELISA (Table II). Serum titers were acceptable after the second boost, and a single lot of serum was chosen for purification and testing. Using the competitive inhibition protocol, with a second antibody conjugate (Goat-antimouse-peroxidase) makes purification of the antiserum unnecessary. We have found that simple ammonium sulfate precipitation is sufficient. Using commercially available second-antibody enzyme conjugates assures a reproducible enzyme reaction if purchased from a reliable vendor with adequate quality control.

As expected, this antibody showed the greatest affinity for the target alkaloid, ergotamine, and ergot alkaloids with related structures (Table III). It appears that the 2' position in the molecule is generally not involved in recognition (Figure 1). However, it is readily apparent that the cyclol-peptide portion of the molecule is the site mainly involved in recognition. A rule of immunology was borne out: the region of the hapten involved in recognition is distal to the point of conjugation. Fortunately, many of these alkaloids are produced by *Claviceps (20)* and the antibody could be used in this assay. Unfortunately, the antibody had little or no affinity for ergovaline, and was unsuitable for analysis of endophyte-infected fescue.

Ergonovine Monoclonal Antibody

As mentioned above, several previous researchers noted that using other reactive sites on the ergot alkaloid molecule, resulted in differing reactivity spectra of the resultant antibodies. Arens and Zenk *(16)* found that using lysergic acid as the hapten, linking BSA at the carboxyllic acid moiety at the C8 position resulted in an antibody which would react with simple lysergic acid derivatives, clavines, and peptide alkaloids. We

Table II. CI-ELISA Generalized Protocol

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1. ELISA plates = Dynatech Immulon 4.

2. Coat with hapten-carrier conjugate 1/1000 in 9.6 carbonate buffer, 60 min 30 °C.

3. Wash 5x phosphate buffered saline + 0.05% tween 20 (PBST), no wait.

4. Sample preparation. Dilute standards from 1mg/mL stocks in PBST. Start with 1000 ppb, or dilute standards in sample extract of plants or seeds. Prepare samples by grinding to pass a 2 mm screen. Weigh out 1 g in disposable plastic cup. Add 10 mL PBST. Allow to sit at room temperature 30–60 min while plate is coating or overnight at 5 °C. Pipette supernatant into ELISA well (50 microliters).

5. Add antibody (50 μL) diluted in PBST. Optimum dilution varies and must be determine for each Ab lot. Sit 15 min at room temperature.
6. Wash 5x.

7. Add goat anti-mouse peroxidase conjugate 1/1000 in PBST at room temperature and incubate for 15 min.

8. Wash 5x.

9. Chromagen = orthophenylinediamine dihydrochloride (OPD) 1 mg/mL in urea peroxide buffer at room temperature for 15 min is average. Stop reaction with 3 M sulfuric acid.

10. Read on an ELISA plate reader at 490 nm.

11. Calculate regression equation for standard curve. Solve unknowns for ppb.

Table III. Cross-reactivity of Poly- and Monoclonal Antibody

	50% inhibition (ng/mL)[a]	
Compound	Polyclonal	Monoclonal
Ergotamine	0.99	129.0
Ergotaminine	3.5	1129.0
Ergostine	3.7	3361.0
Ergocristine	4.4	545.0
Ergostinine	12.0	3031.0
Ergosinine	38.3	216.0
Ergosine	50.6	66.0
Ergoptine	238.0	191.0
α-ergokryptine	345.0	1863.0
Ergovaline	1533.0	71.0
Ergonine	1937.0	533.0
Festuclavine	2995.0	181.0
Setoclavine	3121.0	72.0
Ergonovine	3720.0	0.05
Ergocornine	3775.0	446.0
Elymoclavine	4361.0	59.0
Rugulovasine	7338.0	>10,000.0
Pyroclavine	17151.0	560.0
Agroclavine	-	40.0
LSD	-	167.0
Lysergic Acid	-	337.0
Costaclavine	-	3879.0
L-tryptophan	-	>10,000.0

[a]Concentration of compound causing a 50% reduction in ELISA values when compared to buffer-only controls.

used ergonovine by first making the hemisuccinate following the procedure of Stason, et al. *(21)* (Figure 3). We also decided to attempt a monoclonal antibody, since that capability had recently been made available to us. An additional change was to make a second coating conjugate, using poly-l-lysine as the carrier. This resulted in a much higher substitution ratio than could be obtained with natural proteins.

The production of the monoclonal antibody proceeded along the same general protocol used above, with some exceptions. Mice were tested for polyclonal titers using the same CI-ELISA as before, and the mouse with the highest titer was chosen for boosting and fusion using a standard protocol *(23)*. Next comes the arduous task of selecting the best antibody-producing clones. Important criteria to consider are: 1) monoclonality of the hybridoma, 2) vigorous growth of the hybridoma, 3) high rate of antibody production, and 4) specificity of the antibody for the target. Of secondary consideration is the globulin subclass produced by the cell line. The above criteria are assured by scrupulous attention to detail, including keeping accurate records of cell lineage when replating cells. If one is not especially ruthless in discarding inferior cell lines, the exponential progression of growing cells can quickly be overwhelming. Before proceeding with a fusion, it is imperative that the screening protocol be thoroughly tested to ensure that the test (in our case, CI-ELISA) will correctly identify targe-specific clones. Using the polyclonal sera from tail bleedings of the mice accomplishes this.

We selected the best clone from several promising hybridoma lines, based on the criteria mentioned above. The resulting antibody works well in the CI-ELISA when the source is unpurified hybridoma supernatant solutions. Appropriate concentration of hybridoma supernatant solutions must be determined empirically for each hybridoma lot derived from 24-well plates or from bulk production. Maximum antibody production is obtained from ascites fluid.

The selected clone, EN9F10, demonstrateed a wide spectrum of anti-ergot alkaloid activity (Table III) which makes it suitable for testing not only *Claviceps* infected material, but also *Acremonium* infected fescue as well. The enhanced sensitivity of this assay when compared to the polyclonal assay derives from several factors. The cross-reactivity of the antibody makes more target available to which the antibody can bind. This is due to the fact that both *Claviceps* and endophyte infections result in more than one member of the ergot alkaloid family being produced. In the case of *Claviceps*, at least 6 different alkaloids and their epimers are being produced *(6)* and this would not include clavines and nonfluorescent alkaloids. Another reason for enhanced sensitivity is probably the higher substitution ratio of the PLL-conjugate. This makes for a greater affinity of the antibody for the polystyrene plate, resulting ultimately in a faster, darker chromagen reaction. We calculated that, with this antibody in the CI-ELISA, we could detect one *Claviceps* sclerotium in 20 kg of grain.

Practical Considerations

Several factors contribute to quantitative inaccuracy in this assay. The same may also apply to other types of immunoasssays. First, quantitative inaccuracy resulting from the fact that the target alkaloids are a family or population of cross-reactive antigen targets which are never present alone in naturally occurring sources. Since each antigenic target has a slightly different coefficient of cross-reactivity (50% inhibition

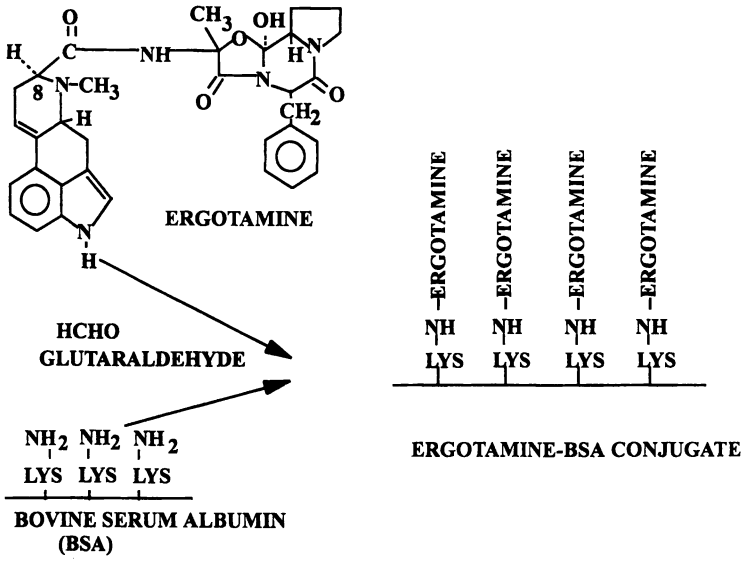
ERGOTAMINE
HCHO
GLUTARALDEHYDE
NH2 NH2 NH2
LYS LYS LYS
BOVINE SERUM ALBUMIN (BSA)
NH -ERGOTAMINE
LYS
ERGOTAMINE-BSA CONJUGATE

Figure 2. Cross linking of Ergotamine to BSA via glutaraldehyde.

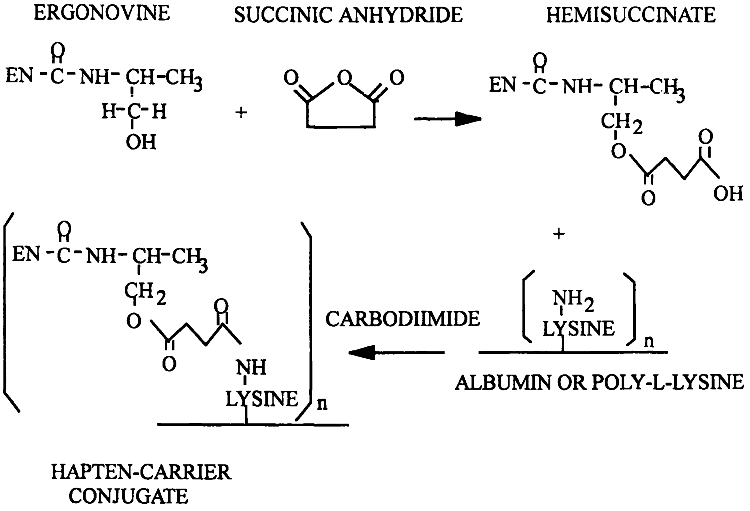
ERGONOVINE
SUCCINIC ANHYDRIDE
HEMISUCCINATE
EN-C-NH-CH-CH3
H-C-H
OH
CARBODIIMIDE
NH2
LYSINE
n
ALBUMIN OR POLY-L-LYSINE
NH
HAPTEN-CARRIER CONJUGATE

Figure 3. Conjugation of Ergonovine and PLL.

ratio), this makes quantitative analysis difficult. Competitive inhibition in an ELISA will be the sum of each individual reactant and the standard curves generated from a single antigen will not apply to the mixture. Since the ratios of alkaloids in the mixture cannot be predicted, a standard curve produced by an artificial mixture of alkaloid cannot be generated. The best approximation would be to select a standard antigen target and express the data in units of that antigen. For example, we use ergonovine, and express the results as parts per billion (ppb) ergonovine in solution, understanding, of course, that this is erroneous. All antibodies, even monoclonal antibodies, may be somewhat cross-reactive, so all ELISA tests of a naturally occurring compound in a natural substrate will suffer to some extent from this error. Mycotoxins, being at the end of a biosynthetic pathway, and suffering from bio- and other degradation, are especially liable to this error.

The other flaw contributing to quantitative inaccuracy is the problem of insolubility. Since antibodies have clear optima of ionic strength, pH, temperature and other parameters, they function optimally in an aqueous solution of phosphate buffered saline. Any deviation from these optima will result in reduced affinity of the antibody and reduced accuracy of the immunoassay. Many of the targets of immunoassays are poorly soluble in aqueous solutions, so other solvents like methanol or acetonitrile must be used. The ergopeptides fall into this category. While some of the clavines and simple lysergic acid derivatives are more hydrophilic, most organic solvents will denature the antibody. ELISA tests for hydrophobic compounds must use dilute mixtures of denaturing solvents. Barna-Vetro et al. (*24*) suggest that alcohol or acetonitrile concentrations should not exceed 10% in the antibody reaction mixture. We have observed the same in our laboratory.

The quantitative inaccuracy of the CI-ELISA using EN9F10 is acceptable if the test is used for screening purposes to eliminate samples which are not contaminated, and require no further testing. Those samples indicated as positive in the assay can be further scrutinized by more accurate methods, such as HPLC.

Acknowledgments

The author wishes to thank Dr. Virginia Kelley for her invaluable assistance and immunological expertise in the development of the antibodies described herein.

Literature Cited

1. Rehacek, Z.; Sajdl, P. *Ergot Alkaloids;* Academia Press: Prague, Czech Republic, 1990.
2. Yates, S. G.; Plattner, R. D.; Garner, G. B. J. Agric. Food Chem. **1985**, *33*, 719–722.
3. Siegel, M. R.; Latch, G. C. M.; Bush, L. P.; Fannin, F. F.; Rowan, D. D.; Tapper, B. A.; Bacon, C. W.; Johnson, M. C. J. Chem Ecol. **1990**, *16*, 3301–3315.
4. Farr, D. F.; Bills, G. F.; Chamuris, G. P.; Roseman, A. Y. *Fungi on Plants and Plant Products in the United States;* APS Press: St. Paul, MN, 1989; p 632.
5. *Ergot Alkaloids and Related Commpounds;* Berde, B.; Schild, H. O., eds.; Handbook of Experimental Pharmacology; Springer Verlag: Berlin, Germany, 1978, Vol 49.

6. Scott, P. M.; Lombart, G. A.; Pellaers, P.; Bacler, S.; Lappi, J. *J. AOAC.* **1992,** *75*, 773–779.
7. Allport, N. L.; Cocking, T. T. *Yearbook Pharm.* **1932,** *5*, 341–46.
8. Svendsen, A. B.; Verpoorte, R. *Chromatography of Alkaloids.* Chromatography Library 23A; Elsevier: Amsterdam, 1983; pp 388–389.
9. Heacock, R. A.; Langille, K. R.; MacNeal, J. D.; Frei, R. W. *J. Chromatogr.* **1973,** *77*, 425–430.
10. Scott, P. M.; Lawrence, G. A. *J. Agric. Food Chem.* **1980,** *28*, 1258–1261.
11. Yates, S. G.; Powell, R. G. *J. Agric. Food Chem.* **1988,** *36*, 337–340.
12. Van Vunakis, H.; Farrow, J. T.; Gjika, H. B.; Levine, L. *Proc. Natl. Acad. Sci. U.S.A.* **1971,** *68*, 1483–1487.
13. Taunton-Rigby, A.; Sher, S. E.; Kelley, P. R. *Science* **1973,** *181*, 165–166.
14. Rosenthaler, J.; Munzer, H. *Experentia.* **1975,** *32*, 234–235.
15. Schran, H. F.; Schwartz, H. J.; Talbot, K. C.; Loeftler, L. *J. Clin. Chem.* **1979,** *25*, 1928–1933.
16. Arens, H.; Zenk, M. H. *Planta Medica.* **1980,** *39*, 336–347.
17. Shelby, R. A.; Dalrymple, L. W. *Plant Disease.* **1987,** *71*, 783–786.
18. Shelby, R. A.; Kelley, V. K. *J. Agric. Food Chem.* **1980,** *38*, 1130–1134.
19. Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual;* Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1988; pp 92–120.
20. Porter, J. K.; Bacon, C. W.; Plattner, R. D.; Arrendale, R. F. *J. Agric Food Chem.* **1987,** *35*, 359–361.
21. Stason, W. B.; Vallotton, M.; Haber, E. *Biochim. Biophys. Acta.* **1966,** *133*, 582–584.
22. Shelby, R. A.; Kelley, V. K. *J. Agric. Food Chem.* **1992,** *40*, 1090–1092.
23. Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual;* Cold Spring Harbor Laboratory; Cold Spring Harbor, NY, 1988; pp 139–243.
24. Barna-Vetro, I.; Gyongyosi, A.; Solti, L. *Appl. Environ Microbiol.* **1994,** *60*, 729–731.

RECEIVED November 16, 1995

Chapter 18

Detection and Quantification of Glycoalkaloids

Comparison of Enzyme-Linked Immunosorbent Assay and High-Performance Liquid Chromatography Methods

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Glycoalkaloids (GA's) represent an important group of naturally occurring plant toxins. They are found in a wide variety of plants including potato and tomato. Analysis of GA's is complicated, particularly in tomato because these compounds do not readily absorb in the UV. Thus, extensive sample cleanup is usually required followed by HPLC. In this chapter we summarize our efforts to develop simple, rapid immunoassays for the major GA's found in potato and in tomato. The assay we developed is a monoclonal antibody (Mab) based competition enzyme-linked immunosorbent assay (cELISA). Hapten synthesis and antibody production is discussed. GA levels measured in identical samples using an HPLC method and the cELISA showed a high degree of correlation (correlation coefficient = 0.998). The cELISA clearly has widespread application and is able to rapidly and accurately measure GA levels.

Glycoalkaloids (GA's) are naturally occurring, potentially toxic, nitrogen-containing secondary plant metabolites that are found in a number of agriculturally important species including potatoes, tomatoes, and eggplant (*1*). In commercial potatoes (*Solanum tuberosum*) there are two major glycoalkaloids, α-chaconine and α-solanine, both glycosylated forms (trisaccharides) of the aglycon solanidine (Figure 1). They are thought to function as a defense against insects and other pests (*2*). These two GA's, along with a number of other natural chemicals in potatoes, are phytoalexins (*3*). Phytoalexins are low-molecular-weight antimicrobial compounds that are both synthesized by and accumulated in plants as a result of exposure to microorganisms (*4*). Recent studies suggest that the potato GA's provide protection to the Colorado potato beetle and to the potato leafhopper (*2, 5–7*).

Wild potatoes (*Solanum chacoense*) and eggplants (*Solanum melongena*) contain the glycoalkaloid solasonine (Figure 1). Because wild potatoes often contain higher

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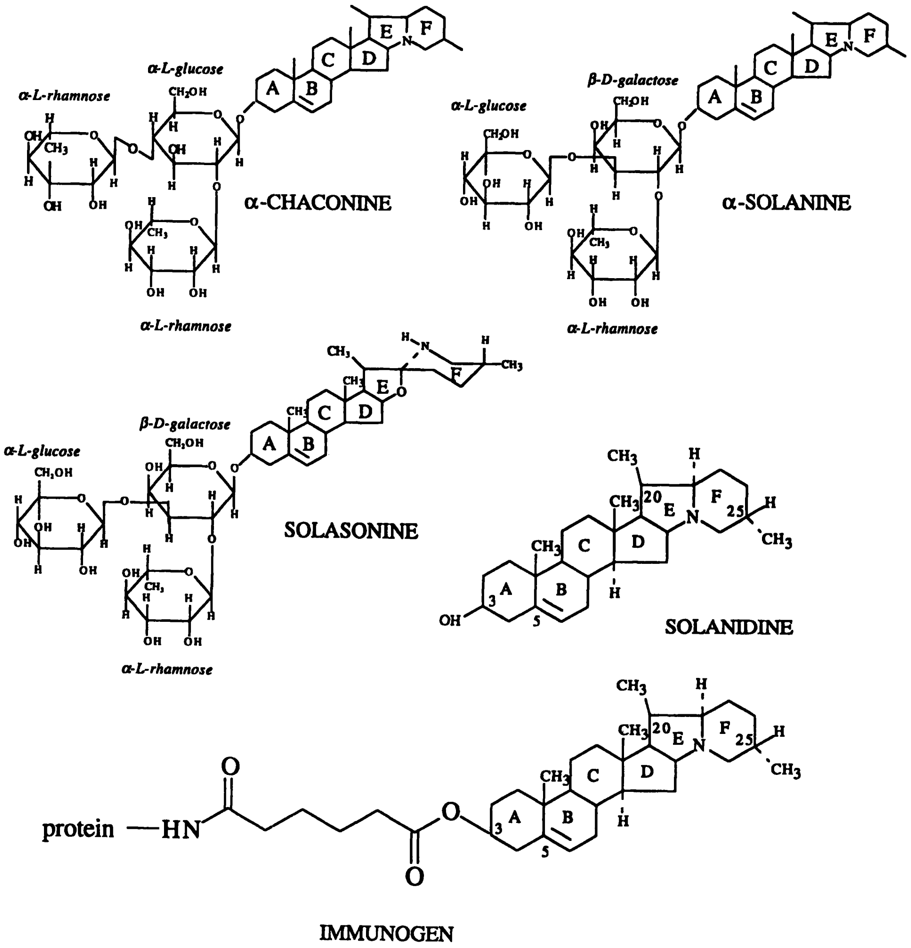
α-L-rhamnose
α-L-glucose
α-L-rhamnose
α-CHACONINE
β-D-galactose
α-L-glucose
α-L-rhamnose
α-SOLANINE
β-D-galactose
α-L-glucose
α-L-rhamnose
SOLASONINE
SOLANIDINE
protein
IMMUNOGEN

Figure 1. Chemical structure of the potato glycoalkaloids, α-chaconine, α-solanine, solasonine, the aglycon solanidine, and the immunogen used to produce monoclonal anti-GA antibodies.

GA levels than commercial varieties, they have been used by plant breeders who are attempting to generate improved cultivars. Such cultivars, however, can have GA levels above 20 mg/100 g of tuber, the generally accepted cutoff level between safe and unsafe potatoes (*8,9*). This guideline limiting the glycoalkaloid content of new potato cultivars has been recommended (*10,11*) because of the potential human toxicity of these compounds, including reported deaths.

One mode of GA toxicity might be their ability to inhibit cholinesterases (*12–14*). Three cholinesterases can be differentiated which are inhibited by α-solanine (*12*), and α-chaconine is a stronger inhibitor than α-solanine (14). Teratogenic effects were produced in mice by α-chaconine (*15*). Neural-tube defects were reported in Syrian hamsters by α-chaconine and α-solanine (*16,17*). Keeler *et al.* (*18*) reported that the new sprouts of the seven potato varieties they tested were teratogenic in hamsters. Likewise, sprouts of the British potato, Arran Pilot, were reported by Renwick *et al.* (*17*) to cause cranial bleb, encephaly, exencephaly, and spina bifida in Syrian hamsters. α-Chaconine and α-solanine have been shown to be embryotoxic in a frog embryo assay (*19,20*). This effect may be due to the ability of the steroidal glycoalkaloids to alter ion fluxes across cell membranes (*21,22*).

In tomatoes (*Lycopersicon esculentum*), the major glycoalkaloid is α-tomatine, which is a glycosylated (tetrasaccharide) derivative of the aglycon tomatidine (Figure 2). As with the *Solanum* glycoalkaloids, α-tomatine is reported to be potentially toxic (*19,23-24*). It has been found to possess antifungal activity (*25*), to inhibit fruitworm and spiny bollworm larval growth (*26,27*), and to interfere with moth eggs (*28*). Figures 1 and 2 illustrate the structures of these potato and tomato glycoalkaloids.

Current methods for analysis of GA's include gas chromatography (GC) (*29–31*), and high-performance liquid chromatography (HPLC) (*11,32–37*). While these methods are accurate and sensitive, they are time consuming, require complex instrumentation and are not easily adapted to rapid screening programs. Part of the difficulty associated with analysis, especially for the tomato GA, α-tomatine, is its lack of absorbency except at low UV wavelengths (e.g., at 205 nm). Thus, extensive cleanup is necessary prior to HPLC in order to remove confounding substances (*38,39*). This has been partially solved by Friedman *et al.* (*37*), who developed an HPLC method for α-tomatine that utilized pulsed amperometric detection. This method was found to be useful for both tomatoes and processed tomato products such as juice, ketchup, sauce and soup (*40*).

The difficulties associated with the analysis of GA's have resulted in the development of alternative immunochemical methods. Enzyme immunoassays, radioimmunoassays, and fluorescence polarization immunoassays (FPA) have been reported for the potato GA's by a number of different groups (*41–46*). Immunoassays for the tomato GA α-tomatine also have been reported (*47*). The above studies all have used the GA itself, or a modified GA as immunogen, linking it to the carrier protein via a modification in the sugar. In contrast, our studies have utilized a protein conjugate of the aglycon of α-solanine as the immunogen. Thus, our immunogen does not contain any structural information in the sugar component of the GA. Using this immunogen, we report here on the ability of one of our monoclonal antibodies, Sol-129, to bind both tomato and potato GA's with a high degree of correlation between the competition ELISA we developed and HPLC methods.

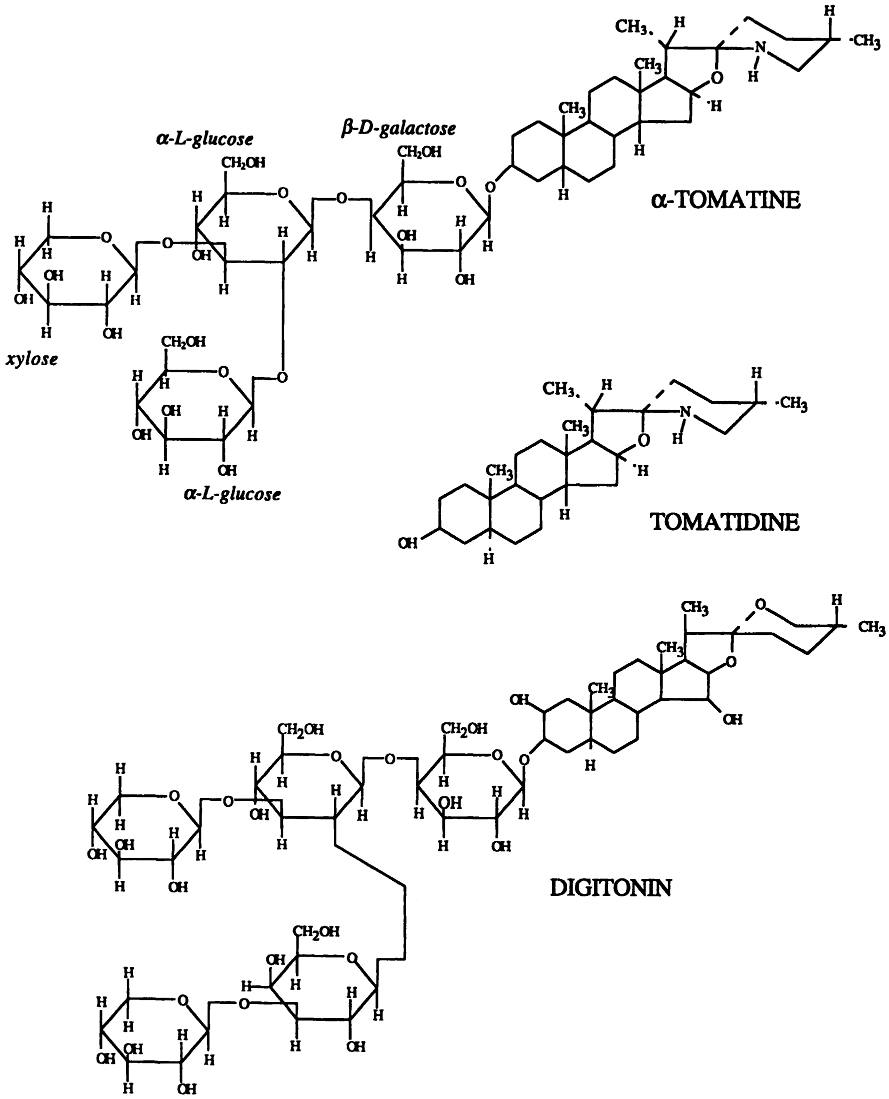
α-L-glucose
β-D-galactose
xylose
α-L-glucose
α-TOMATINE
TOMATIDINE
DIGITONIN

Figure 2. Chemical structure of the tomato glycoalkaloid α-tomatine, its aglycon tomatidine, and the glycoside digitonin.

Materials and Methods

The anti-glycoalkaloid monoclonal antibody Sol-129 and the competition (c)ELISA used in these studies were reported earlier (*48*). Briefly, 96-well microtiter wells were coated with a solanidine-bovine serum albumin (Sol-BSA) conjugate (Figure 1). Standards and/or unknowns were then added, followed by Mab Sol-129. The Mab was then allowed to partition between the GA bound to the bottom of the microtiter well and the GA present in solution. The plates were washed to remove unreacted reagents. The amount of bound Mab was detected by addition of an enzyme-conjugated anti-mouse immunoglobulin sera (Sigma, St. Louis, MO) and substrate. Thus, the color developed in the assay is inversely proportional to the amount of analyte in the sample. The data is expressed as a percent inhibition of control (IC) using the following expression

$$\% \text{IC} = (1 - \text{B/Bo}) \times 100$$

where Bo is the value obtained when no GA is present in the sample (buffer is added instead of sample or standard).

Sample extraction and HPLC analysis was as previously described (*36,37*). Tomato samples were extracted, an aliquot of the extract was then injected for HPLC analysis, and a second aliquot was used in the cELISA. The aliquot used in the cELISA was dried under a stream of nitrogen gas, resuspended in DMF (approximately 1 mL), diluted in assay buffer (usually a 1/1000 dilution was made), and then further diluted in a 1:2 fashion in assay buffer. Unknown concentrations were determined by comparison to a percent B/Bo standard curve near the IC_{50} point (B/Bo between 40–60%).

Results and Discussion

Monoclonal Antibodies. We have previously described a set of eleven Mabs that bound different potato and tomato glycoalkaloids (*48*). The most sensitive of these Mabs, referred to as Sol-129, had 50% of its binding activity inhibited (IC_{50}) when 100 µL of a 2.5 ppb solution of α-solanine was added to the reaction. A typical standard curve for α-tomatine is shown in Figure 3. Extensive cross reactivity studies, using various potato and tomato glycoalkaloids, their aglycons, and nonnitrogen-containing glycosides as competitors have been reported (*48*) for each of the 11 Mabs described. A subset of these data is shown in Table 1. Cross reactivity was calculated by comparison of the IC_{50} values and assigning 100% activity to that for solanidine (the immunizing compound).

These data clearly showed that only one of the 11 Mabs isolated, Sol-129, recognized the tomato glycoalkaloid, α-tomatine, in addition to the potato GA's. Furthermore, of the 11 Mabs isolated, Sol-129 was observed to have the greatest relative affinity for all of the antigens recognized.

A possible interpretation of this data is that a greater number of contacts between residues in the combining site of the antibody and the analyte exists for Mab Sol-129, than there is for the other ten Mabs.

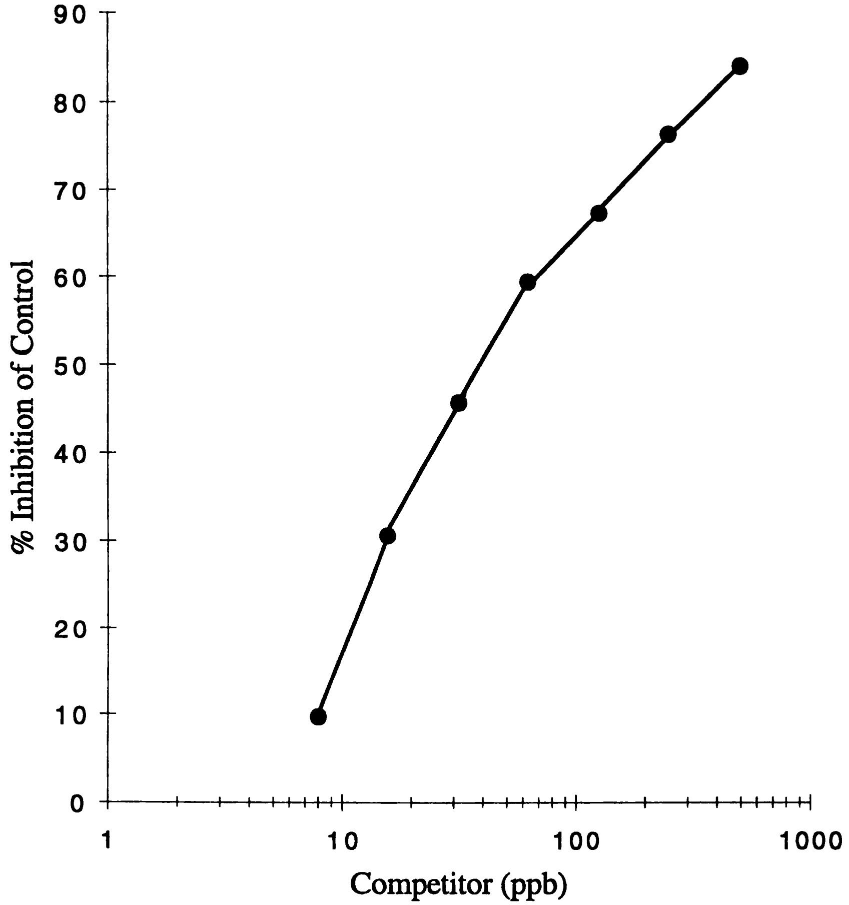

Figure 3. A typical cELISA curve for Mab Sol-129 using α-tomatine as competitor.

The ability of Mab Sol-129 to bind tomato GA's and solasonine in addition to the potato GA's is more difficult to explain since the animals were immunized with a protein conjugate of the aglycon of α-solanine (Figure 1). The orientation of the F-ring of solanidine and the commercial potato glycolalkaloids α-solanine and α-chaconine is fixed (Figure 1). In contrast, the F-ring in solasonine, α-tomatine and tomatidine (Figures 1 and 2) is not fixed and can adopt various conformations. Since conjugation to the carrier protein was achieved through the hydroxyl group on the number 3 carbon of solanidine, conventional wisdom suggests that the resultant antibodies should be most sensitive to that portion of the molecule most distal to the attachment site, which would be the F-ring. This is in fact what we observed (Table 1). Ten of the 11 Mabs bound only α-solanine, α-chaconine or solanidine. Using solanidine as the immunogen, we would not have predicted that an antibody capable of binding the tomato GA's would be isolated.

Table 1. % Cross reactivity[a] observed with different anti-glycoalkaloid Mabs.

Compound Tested	Monoclonal Antibody 8	48	54	55	59	67	68	71	91	106	129
solanidine	100	100	100	100	100	100	100	100	100	100	100
α-solanine	18	4	61	51	46	56	0[b]	0	35	29	96
α-chaconine	16	5	80	29	64	77	0	0	44	26	89
solasonine	0	0	0	0	0	0	0	0	0	0	7
α-tomatine	0	0	0	0	0	0	0	0	0	0	46
tomatidine	0	0	0	0	0	0	0	0	0	0	24

Original data adapted from Stanker *et al.* (*48*)

[a] Cross reactivity was calculated by comparing the IC_{50} value obtained with that from solanidine.

[b] No competition was observed at the highest level of competitor tested.

A number of explanations can be put forth to explain our observations. The Mab Sol-129 may not form any contacts with the F-ring of the immunogen but instead bind exclusively via contacts on the A-E rings; these regions of the potato and tomato aglycons are similar. This interpretation is not favored since binding to nonnitrogen containing GA's such as digitonin (Figure 2) was not observed. Conversely, the antibody may have contacts with the F-ring, but enough contacts with other regions of the aglycon to provide sufficient binding energy to allow binding (albeit with a lower relative affinity) with the tomato GA's. This latter explanation would require that the orientation of the F-ring in the tomato GA's not inhibit binding because of steric or electronic differences. A third possibility is that the higher affinity of Sol-129 forces the F-ring of the tomato GA's into an orientation preferred by the antibody. We have not yet determined which, if any, of these hypotheses most accurately describes the situation with Sol-129 binding. Studies aimed at cloning the genes and expressing functional single-chain variable fragments (scFv) (Kamps-Holtzapple *et al.* this volume) will provide us with the tools (e.g., the amino acid sequences of the antibody heavy- and light-chains) necessary to test these hypotheses.

Regardless of the exact nature of Sol-129 binding, it represents a specific interaction that probably would not be observed in a polyclonal antiserum obtained from animals immunized as above, because most of the resulting B-cell clones appeared to be producing potato-GA-specific antibodies. Barbour et al. (*47*) were able to produce polyclonal antibodies to tomato GA's. Rather than the hapten we used in our studies, they used a BSA conjugate of α-tomatine for immunization. Linkage was achieved via the sugar molecules using the sodium periodate cleavage reaction. Thus their immunogen included the aglycon (tomatidine) as well as sugar (albeit modified by the periodate cleavage reaction). The resulting antisera bound α-tomatine and tomatidine with equal affinity, as well as having a 41% and 24% cross reactivity with α-solanine and demissine, respectively. They also noted that their antisera had a 30% cross reactivity with digitonin. Following an analysis of extracts of tomato foliage, Barbour *et al.* (*47*) conclude that their polyclonal antisera suffered from significant interference from "other compounds" resulting in a "non-linear" and nonproportional response in their ELISA. While monoclonal antibody Sol-129 described here cross-reacted with potato and tomato GA's, we did not observe cross reactivity to digitonin, cholesterol, stigmasterol, or β-sitosterol. The difference in binding specificity is not surprising. All of the antibodies in a given Mab preparation bind the same epitope compared to a collection of antibodies in a sera that may all bind the immunogen but with different affinities and to slightly different epitopes. Thus, Mabs often display a more restricted specificity than observed with antisera. Furthermore, the immunogen we used did not contain any of the sugar component of the alkaloid.

Table 2. Glycoalkaloid level (mg/g) measured in five freeze-dried potato samples by HPLC and cELISA

Sample	HPLC		ELISA
	α-Chaconine	α-Solanine	GA Equivalents
Klamath tuber flesh (no peel)	trace	0	0.004
Russet whole tuber	0.1	0.1	0.13
3194 Whole tuber	0.5	0.3	0.97
Z whole tuber	0.8	0.5	1.63
Lenape tuber peel	2.1	0.8	> 2.5

Data from Stanker *et al.* (*48*).

Analysis of Potato and Tomato Extracts. Monoclonal antibody Sol-129 bound the potato GA's α-solanine and α-chaconine with virtually identical affinity. Therefore, it was used in initial experiments to quantify the total GA level in a series of potato samples. The data from these experiments with potato samples was reported previously and is summarized in Table 2. The potato samples analyzed by HPLC were first extensively extracted using a multistep process previously described (*36*). Briefly, the samples were extracted with a 2% acetic acid solution, basified, reextracted with butanol, concentrated, filtered, and analyzed by HPLC. In contrast, duplicate samples analyzed by the cELISA were simply extracted in an acetic acid solution, neutralized and analyzed. The cELISA results are expressed as glycoalkaloid equivalents since Mab Sol-129 has a comparable binding affinity for α-chaconine and α-solanine, and they are the major GA's in commercial potatoes. These data clearly indicate that the cELISA analysis gave results comparable to that obtained using HPLC. However, because of the simplified sample preparation used with the cELISA, it is a much faster assay and could be used to rapidly screen large numbers of potato samples.

The above data obtained with potato samples encouraged us to apply the immunoassay for measurement of α-tomatine in tomatoes. α-Tomatine is an ideal candidate for immunochemical analysis. It is readily water soluble, and since it contains no chromophore, it is not easily measured by spectrophotometric detection using traditional methods. Furthermore, since there is only one major GA in tomato, α-tomatine, the cELISA results should more closely match the HPLC results than was seen when potatoes were analyzed. A number of samples representing different tomato cultivars and maturation stages, processed tomato products, and tomato plant root samples were analyzed using both the cELISA method and an HPLC method described by Friedman *et al.* (*37*). Specifically, tomatoes were extracted by stirring 1 g in 20 mL of 1% acetic acid for 2 h. The suspension was then centrifuged for 10 min at 13,000 relative centrifugal force, and the supernatant was filtered through a Whatman GF/C filter. The pellet was resuspended in 10 mL of 1% acetic acid, centrifuged, and filtered, and the two extracts were combined. This extract was further purified using solid phase extraction (SPE). A C_{18} SPE tube, equipped with 60 mL reservoir (Supelco) was conditioned with 5 mL of methanol followed by 5 mL of water. The aqueous extract (now about 30 mL) was applied and allowed to gravity drip. When the sample was fully absorbed onto the packing, the tube was washed with about 10 mL of water, followed by 5 mL of 30:70 acetonitrile/1% NH_4OH and then 5 mL of water. The a-tomatine was eluted with 10 mL of 70:30 acetonitrile/pH 3 citric acid/disodium phosphate buffer. The organic solvent was then evaporated off. The aqueous residue was basified with ammonia water and extracted twice into water-saturated butanol, using a separatory funnel. This sample was then dried on a rotovapor. The residue was taken up in 1 mL of 50% methanol/0.1% acetic acid and filtered through a 0.45 μm HV membrane obtained from Millipore (Bedford, MA). This filtrate was ready for HPLC injection. The extracts were then split and analyzed by ELISA and HPLC. We felt it important to demonstrate that regardless of the detection method, HPLC or ELISA, the same GA value was observed if the samples were prepared using the same preparation scheme. (thus eliminating differences of analyte recovery associated with different extraction methods). These data are summarized in Table 3 and Figure 4. The values obtained by the cELISA and HPLC were highly correlated having a correlation coefficient of 0.998 (N = 20). In addition, we observed good correlations regardless of the matrix (Table 3).

Conclusions

The protein conjugate we developed based on the aglycon of α-solanine, solanidine, was a highly effective immunogen. We were able to isolate a large number of monoclonal anti-GA antibodies. Cross reactivity studies (*48*) suggested that this collection of Mabs could be divided into four epitope groups, each group presumably interacting differently with the aglycon. All of the Mabs except one, Sol-129, reacted only with potato GA's, and some of these bound only the aglycon, solanidine. In contrast, Sol-129 was capable of binding the largest number of GA's including the tomato GA α-tomatine and its aglycon. Mab Sol-129 also demonstrated the greatest relative affinity for each of the GA's it bound. We have yet to determine the molecular details of the binding site for Sol-129. However, studies aimed at cloning the heavy– and light–chain genes (Kamps-Holtzapple and Stanker, this volume) will aid in such studies.

The results from analysis of a variety of potato and tomato samples by HPLC methods and using the cELISA method are highly correlated. Little if any matrix effect was observed in the tomato samples. Future studies are aimed at determining whether a more simplified sample extraction and preparation method can be used for analysis of GA's in plant material and body fluids and tissues. In addition, studies are underway to adapt the cELISA method for GA's in potato and tomato leaves. Preliminary studies

Table 3. Analysis of α-Tomatine in Freeze Dried Tomatoes (μg/g)

SAMPLE	HPLC	ELISA
Control Red Tomato	60	67 ±4
Mature Green Control Tomato	122	115 ± 9
Manteca Red Tomato	10	11.3 ± 0.9
Manteca Green Tomato	308	312 ± 31
Precipitated Control Red Tomato	19	15 ± 2
Immature Green Tomato	168	173 ± 13
Immature Green Tomato Replicate	192	204 ± 4.4
Mature Green Tomato	39	39 ± 2.2
Mature Green Tomato Replicate	38	37 ± 2
Breaker Tomato	61	59 ± 1.5
Breaker Tomato Replicate	92	90 ± 1.5
Large Immature Tomato	409	400 ± 27.5
Large Immature Tomato Replicate	385	371 ± 9.6
Tomato Plant Roots	320	308 ± 14.5
Tomato Plant Roots Replicate	353	347 ± 7.8
Tomatillos	6	6.2 ± 0.1
Tomatillos Replicate	6	6.1 ± 0.2
Canned Tomato Sauce	64	57 ± 1.8
Pickled Tomatoes	121	114 ± 2.5
Commercial Mature Green Tomatoes	144	135 ± 2.3

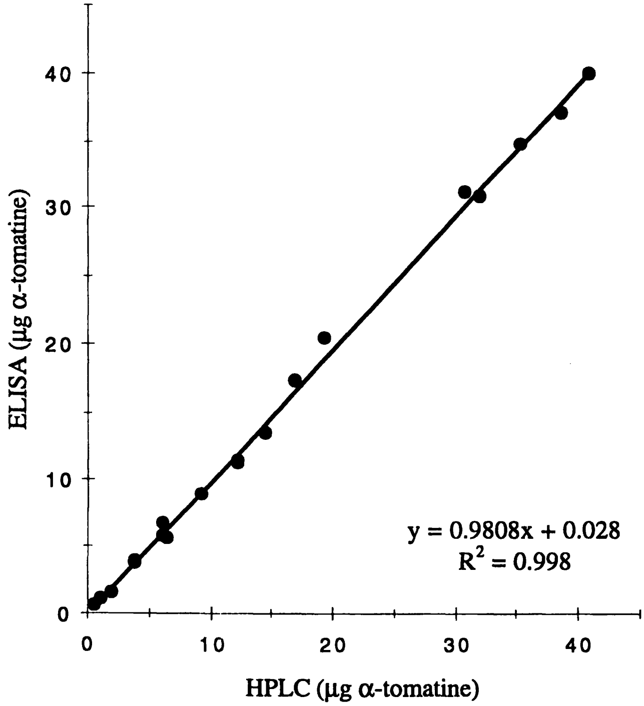

Figure 4. Analysis of α-tomatine in tomatoes using the cELISA and an HPLC method. The line represents the linear regression of this data, with an R^2 of 0.998.

suggest that the cELISA accurately measures α-tomatine in tomato leaves. The ability to measure GA's in individual leaves would be useful for early screening of newly developed cultivars.

Literature Cited

1. Friedman, M. In *Evaluation of Food Safety*: Finley, J.W., Robinson, S.F., Armstrong, A., eds.; ACS Symposium Series 484; American Chemical Society: Washington DC, 1992, pp 429-462.
2. Norris, D. M. In *Chemistry of Plant Protection*; Haug, H., Hoffman, H. eds.; Springer Verlag: Berlin, Germany, 1986; pp 97–146.
3. Beier, R. C.; Nigg, H. N. In *Foodborn Disease Handbook, Diseases Caused by Hazardous Substances*; Hau, Y. H.; Gorham, J. R.; Murrell, K. D.; Oliver, D. O., eds.; Marcel Dekker, Inc.: New York, NY, 1994, Vol. 3; pp 68–70.
5. Flanders, K. L.; Hawkes, J. G.; Radcliffe, E. E.; Florian, I. L. *Euphytica* **1992**, *61*, 83–111.
6. Gaffield, W.; Keeler, R. F.; Baker, D. C. In *Handbook of Natural Toxins, Toxicology of Plant and Fungal Compounds*; Keeler, R. F., Tu, A. T., eds.; Marcel Dekker, Inc.: New York, NY, 1991, Vol. 6; pp 135–158.
7. Roddick, J. G.; Rijnenberg, A. L.; Osman, S. F. *J. Chem Ecol.* **1988**, *14*, 889–902.
8. Slalina, P. *Food Chem.* **1990**, *28*, 759–761.
9. Van Gelder, W. J. M. In *Handbook of Natural Toxins, Toxicology of Plant and Fungal Compounds*; Keeler, R. F.; Tu, A. T., eds.; Marcel Dekker Inc.: New York, NY, 1991, Vol 6; pp 101–134.
10. McMillian M.; Thompson J. C. *Q. J. Med.* **1979**, *48*, 227–243.
11. Morris S. C.; Lee T. H. *Food Technol. Aust.* **1984**, *36*, 118–124.
12. Harris, H.; Whittaker, M. *Ann. Hum. Genet. Lond.* **1962**, *26*, 73–76.
13. Patil, B. C.; Sharma, R. P.; Salunkhe, D. K.; Salunkhe, K. *Food Cosmet. Toxicol.* **1972**, *10*, 395–398.
14. Alozie, S. O.; Sharma, R. P.; Salunkhe, D. K. *J. Food Biochem.* **1979**, *2*, 259–276.
15. Pierro, L. J.; Haines, J. S.; Osman, S. F. *Teratology* **1977**, *15*, 31A.
16. Renwick, J. H. *Lancet* **1982**, *1*, 748.
17. Renwick, J. H.; Claringbold, D. B.; Earthy, M. E.; Few, J. D.; McLean, A. C. S. *Teratology* **1984**, *30*, 371–381.
18. Keeler, R. F.; Young, S.; Brown, D.; Stallknecht, G. F.; Douglas, D. *Teratology* **1978**, *17*, 327–334.
19. Friedman, M.; Rayburn, J. R.; Bantle, J. A. *Food. Chem. Toxicol.* **1991**, *29*, 537–547.
20. Friedman, M.; Rayburn, J. R.; Bantle, J. A. *J. Agric. Food Chem.* **1992**, *40*, 1617–1624.
21. Blankemeyer, J. T.; Stringer, B. K.; Rayburn, J. R.; Bantle, J. A.; Friedman, M. *J. Agric. Food Chem.* **1992**, *40*, 2022–2026.
22. Blankemeyer, J. T.; Atherton, R.; Friedman, M. *J. Agric. Food Chem.* **1995**, *43*, 636–639.

23. Ripperger, H.; Schreiber, K. *In The Alkaloids - Chemistry and Physiology*; Manske, R. H. F.; Rodrigo, R. G. A., eds.; Academic Press: New York, NY, 1981, Vol. XIX; pp 81–192.
24. Roddick, J. G. *Phytochemistry* **1974**, *13*, 9–25.
25. Jiratko, J. *Ochr. Rostl.* **1993**, 29, 93–98 (Czech): *Biol. Abstr.* **1993**, *96*, 140872.
26. Elliger, C. A.; Wong, Y.; Chan, B. G.; Waiss, A. C., Jr. *J. Chem. Ecol.* **1981**, *7*, 753–758.
27. Weissenberg, M.; Klein, J.; Meisner, Ascher, K.R.S. *Entomol. Exp. Appl.* **1986**, *42*, 213–217.
28. Lu, F. M.; Chu, Y. I. *Chin. J. Entomol.* **1992**, *12*, 318–323.
29. Herb, S. F.; Fitzpatrick, T. J.; Osman, S. F. *J. Agric. Food Chem.* **1975**, 23, 520–523.
30. Lawson, D. R.; Erb, W. A.; Miller, A. R. *J. Agric. Food Chem.* **1992**, 40, 2186–2191.
31. Van Gelder, W. M. J.; Tuinstra, L. G. M. Th.; Van Der Greef, J.; Schefter, J.J.C. *J. Chromatogr.* **1989**, *482*, 13–22.
32. Bushway, R. J. *J. Liq. Chromatogr.* **1982**, *5*, 1313–1322.
33. Bushway, R. J.; Barden, E. S.; Bushway, A. W.; Bushway, A. A. *J. Chromatogr.* **1979**, *178*, 533–541.
34. Bushway, R. J. M.; Bureau, J. L.; King, J. *J. Agric. Food Chem.* **1986**, *34*, 277–279.
35. Carman, A. S. Jr.; Kuan, S. S.; Ware, G. M.; Francis, O. J., Jr.; Kershenheuter, G. P. *J. Agric. Food Chem.* **1986**, *34*, 279–282.
36. Friedman, M.; Levin C. E. *J. Agric Food Chem.* **1992**, *40*, 2157–2163.
37. Friedman, M.; Levin, C. E.; McDonald, G. M. *J. Agric. Food Chem.* 1994, *42*, 1959–1964.
38. Bushway, R. J.; Perkins, L. B.; Paradis, L. R.; Vanderpan, S. *J. Agric. Food Chem.* **1994**, *42*, 2824–2829.
39. Keukens, E. A. J.; Hop, M. E. C. M.; Jongen, W. M. F. *J. Agric. Food Chem.* **1994**, *42*, 2475–2477.
40. Freidman, M.; Levin, C. *J. Agric. Food Chem.* **1995**, *43*, 1507–1511.
41. Morgan, M. R. A.; McNerney, R.; Matthew, J. A.; Coxon, D. T.; Chan, H. W-S. *J. Sci. Food Agric.* **1983**, *34*, 593–598.
42. Morgan, M. R. A.; McNerney, R.; Coxon, D. T.; Chan, H. W-S. In *Immunoassays in Food Analysis*; Morris, B. A., Clifford, M. N., eds.; Elsevier Applied Science Publishers: London,UK, 1985; pp 187–195.
43. Plhak, L. C.; Sporns, P. *J. Agric. Food Chem.* **1992**, *40*, 2533–2540.
44. Plhak, L. C.; Sporns, P. *Am. Potato J.* **1994**, *71*, 297–313.
45. Thomson, C. A.; Sporns, P. F *J. Agric. Food Chem.* **1995**, *43*, 254–260.
46. Vallejo, R. P.; Ercegovich, C. D. In *Methods and Standards for Environmental Measurements*; National Bureau of Standards: Washington, DC, 1978; Publication 519; pp 333–340.
47. Barbour, J. D.; Kennedy, G. G.; Roe, R. M. *Rev. Pesticide Toxicol.* **1991**, *1*, 289–303.
48. Stanker, L. H., Kamps-Holtzabble, C., Friedman, M. *J. Agri. Food Chem.* **1994**, *42*, 2360–2366.

RECEIVED October 6, 1995

Chapter 19

Immunoassays for Toxic Potato Glycoalkaloids

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The antibody populations produced in rabbits from two different solanidine glycoalkaloid conjugates (glycosidic-linked and hemisuccinate ester-linked) were very similar in their properties in a competitive indirect immunoassay. Suitable tomatidine glycoalkaloid conjugates could not be prepared using any of the solanidine conjugation methods. A tomatidine conjugate was prepared using selective succinylation of the glycoalkaloid tomatine, followed by separation on an anion exchange column and active ester conjugation to BSA. A comparison of a flourescence polarization immunoassay (FPI) with a solid phase microtiter immunoassay indicated several advantages for FPI including better quantification, less requirement for standards, and easily-modeled kinetics. However, FPI required the use of considerably more concentrated polyclonal serum.

Glycoalkaloids (GAs) are naturally occurring toxic compounds found in plants of the *Solanaceae* family. Important human foods that contain these compounds include potatoes, eggplants, and tomatoes *(1)*. Potatoes are the fourth most important food crop in the world and there have been numerous reported episodes of GA poisoning *(2)*, some resulting in death, associated with consumption of potatoes. It has been suggested that GAs represent one of the most serious toxic components in the human diet *(3)*. GAs are not removed or destroyed by cooking, baking or frying *(4)* and actually add to potato flavour *(7)*. Post-harvest GA synthesis can be initiated by a

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variety of genetic, environmental *(5,6)* and stress conditions including different growing conditions *(7)*, improper storage, disease *(8)*, mechanical *(9)* and insect damage *(10)*.

Given the above concerns it might be expected that potato breeders have concentrated on the elimination of GAs and that analysis for these toxins is a routine procedure in the food industry; however, this is not the case. GAs are important to the plant because they confer pest and disease resistance *(8, 11–14)*. Wild potato varieties which generally contain much higher GA levels and a wider variety of GAs than cultivated potatoes *(18–21)* are often employed in breeding programs to gain better yield, hardiness, disease resistance or some other attribute *(5,13,15–17)*. Due to the inherent toxicity of GAs, there has been a requirement for GA analysis of new potato varieties. Despite this requirement, the food industry relies on a secondary indicator, greening (chlorophyll synthesis), to cull potatoes containing potentially high GA levels. Although chlorophyll production often correlates with GA synthesis, GA levels also increase due to many other factors, as noted above. Even though the identification of green potatoes can result in large losses, this simple method is preferable to the more complex and costly conventional GA quantification methods, such as HPLC *(2)*.

Many GA analysis methods have been developed *(1,20,23,24)*. Earlier gravimetric, colorimetric or titrimetric techniques suffered from incomplete purification and identification or interference from other potato constituents. Most researchers currently prefer chromatographic techniques for GA analysis despite the general requirement of these methods for expensive equipment, time, and technical expertise. Thin-layer chromatography can give qualitative data *(25)* and attempts have been made to render this method quantitative *(26)*. Gas chromatography has the advantage of sensitive detectors but requires derivatization of the GAs or, more often, hydrolysis to obtain the alkaloid *(27,28)*. The most commonly used chromatographic technique is liquid chromatography. Most liquid chromatography methods rely on detection at wavelengths in the 200–208 nm region *(29–36)*, where most compounds have some absorbance, necessitating considerable sample cleanup. Purification of GAs for analysis can be tedious since these soap-like molecules are difficult to effectively remove from potato tissue and require complex extraction solutions *(37)*. Moreover, GAs which lack double bonds absorb weakly in the UV region, precluding effective detection. Acceptable detection has been achieved using pulsed amperometric detection of the sugar moiety of GAs *(38)*.

Among the most exciting new analytical methods for quantification of GAs are immunoassays *(8,39–44)*. The high specificity of antibodies for antigens allows the use of immunoassays to overcome many of the problems associated with extraction and purification of potato samples. Furthermore, immunoassays are rapid, inexpensive procedures which can be developed and permit routine GA analyses in the field by unskilled personnel. The ultimate goal of our research is to develop such assays for all potato GAs.

Experimental

Materials and Methods.

The sources and preparation of most of the immunoassay materials were presented in two recent publications *(41,44)*. Tomatine (80% pure; *36)*, *Limulus polyphemus* hemocyanin (LPH) and bovine serum albumin (BSA, fraction V) were purchased from Sigma Chemical Co., St. Louis, MO. Resin (AG 1-X2, 200–400 mesh, acetate form) was obtained from Bio-Rad Laboratories, Richmond, CA and autoclaved on arrival. Spectrum dialysis tubing was obtained from Fisher Scientific, Edmonton, AB. Thin-layer chromatography (TLC) plates were AL S G/UV from Whatman Ltd., Maidstone, Kent, England. The MALDI-TOF MS was a KOMPACT MALDI I unit operated with KOMPACT 4.0.0 software from Kratos Analytical (Ramsey, NJ) and was used courtesy Dr. Ole Hindsgaul, Department of Chemistry, University of Alberta. Elemental analyses and FAB MS were performed by Spectral Services, Department of Chemistry, University of Alberta. All water was purified using a Milli-Q system (Millipore Corp., Milford, MA). Other chemicals used were reagent grade or better.

Synthesis of Solanidine Hemisuccinate Immunogen. Solanidine hemisuccinate was prepared as described by Plhak and Sporns *(41)*. The hemisuccinate (87.9 mg, 0.182 mmol) was stirred overnight at 4 °C with *N*-hydroxysuccinimide (172.6 mg, 1.5 mmol) and 1,3-dicyclohexylcarbodiimide (167.8 mg, 0.8133 mmol) in 3 mL anhydrous dimethylformamide. The next day the reaction mixture was filtered into 2 mL PBS containing 54.2 mg LPH (0.74 µmol). This reaction mixture was stirred for 23 h at 4 °C. The solution was transferred to 12–14000 MWCO dialysis tubing and the flask rinsed with 2 x 1 mL water. Conjugate was dialyzed against 1 L of 8 M urea for 2 days, 50 mM ammonium bicarbonate (4 L) for 2 days and 4 L of 25 mM ammonium bicarbonate for 2 days. Conjugate was transferred to a vial and lyophilized. Elemental analysis found 13.53% N for the conjugate while LPH alone had 15.47% N; therefore, the conjugate was estimated to contain 27 molecules of alkaloid per mole LPH.

Immunization of Rabbits with Solanidine Hemisuccinate Immunogen. Two rabbits (3-month, female, Flemish Giant x Dutch Lop Ear) were immunized with 1.5 mg of solanidine hemisuccinate in 1.5 mL sterile PBS mixed thoroughly with 1.5 mL Freund's complete adjuvant. Injections were 0.5 mL intramuscular and 0.25 mL to four sites subcutaneous to each rabbit. A boost was made after 4 weeks in a similar manner but using Freund's incomplete adjuvant. Test-bleed samples (2–3 mL each) were taken from the ear artery 10 days after the second boost. Blood samples were allowed to clot at room temperature and centrifuged in Microtainer brand serum separator tubes (Becton Dickinson, Sparks, MD) to remove blood cells. Supernatant sera ("Serum B") were collected and stored at -20 °C.

Improved Competitive Enzyme Immunoassay. Use of 3,3',5,5'-tetramethylbenzidine (TMB) rather than *o*-phenylenediamine for color development with horseradish peroxidase allowed for reduction in the concentration of rabbit serum prepared using solanidine glycosidic-linked immunogen ("Serum A") over the previously published method *(41)*. The solanidine hemisuccinate-BSA conjugate (9 molecules of alkaloid per mole BSA, *41)* was prepared at a concentration of 0.1 µg/mL in PBS and 200 µL added to each well of a microtiter plate. Plates were covered with adhesive acetate sealers and incubated for 18 h at 4 °C. Solutions were removed the next day and replaced with 200 µL/well of 1% BSA in PBS. After 1 h at room temperature this solution was removed and wells were washed with 3 x 200 µL of PBST. Rabbit serum, diluted 1/500,000 with 0.05% BSA in PBST, was added to each well (100 µL/well) followed by 100 µL/well of methanol containing standards or samples. After 2 h of competition at room temperature, solutions were removed and the wells washed as before. Goat anti-rabbit peroxidase conjugate diluted 1/3000 with 0.05% BSA in PBST was added (200 µL/well) and incubated for 2 h at room temperature. After washing as before, 200 µL/well of TMB solution was added to each well for color development. TMB solution was prepared freshly prior to each analysis from 10 mg 3,3',5,5'-tetramethylbenzidine dihydrochloride/mL dimethyl sulfoxide diluted 1:100 with 0.1 M, pH 4.0 citrate buffer containing 1 mg/mL urea peroxide. After 15 min at room temperature, 50 µL of 2 M sulfuric acid was added to each well and the absorbance measured (A_{450nm} - A_{650nm}).

Tomatine Succinylation. Tomatine (201.4 mg, 0.195 mmol) was dissolved in 5 mL dry pyridine (distilled over calcium hydride) and the solution was cooled to 4 °C. Succinic anhydride (78.3 mg, 0.782 mmol) was added and the solution stirred at 4 °C and protected from moisture with a drying tube. The reaction was monitored by TLC using the organic layer of methanol: chloroform: 1% (v/v) ammonia (2:2:1) (v/v/v). Spots were visualized using 5% (v/v) sulfuric acid in ethanol followed by charring. A series of spots developed which corresponded to various succinylation products, with R_f values decreasing with increasing degree of succinylation. After 72 h, 10 mL water were added and the solution stirred for 10 min at room temperature. The mixture was evaporated to dryness. A further 10 mL of water were added to the residue and evaporated to dryness. Residue dissolved in 2 mL water was applied to a 37 x 1.5 cm AG 1-X2 acetate column that had been washed with 150 mL of 1 M ammonium acetate and 100 mL of water at a flow rate of 1 mL/min. The flask was rinsed with an additional 2 mL of water and this rinse was added to the column. After sample application, the column was eluted with 100 mL water followed by 1.6 L of a linear gradient (0.0 M to 0.5 M) ammonium acetate and finally 300 mL of 1.0 M ammonium acetate. Fractions were identified by TLC, as noted above.

The first 20 mL of eluant were free of GA followed by 60 mL of GA-containing Fraction 1. Three later GA fractions contained combinations mainly of di- and trisuccinylated tomatine. Only the first fraction was used for preparation of the immunogen. Fast atom bombardment mass spectroscopic analysis of lyophilized Fraction 1 (47.7 mg) indicated approximately 30% unreacted tomatine and 70% monosuccinylated tomatine as assessed by peak heights.

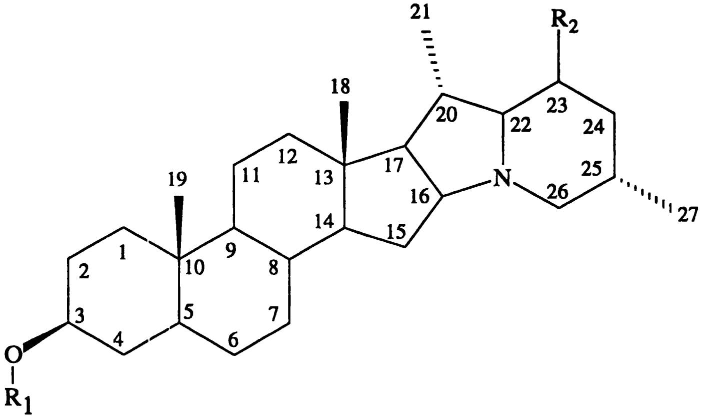
Tomatine Immunogen. After lyophilization of Fraction 1 it was dissolved (10.2 mg, containing approximately 6 μmol monosuccinylated tomatine) along with dicyclocarbodiimide (38.5 mg, 0.187 mmol) and N-hydroxy succinimide (22.2 mg, 0.193 mmol) in 1 mL dimethylformamide and stirred overnight at 4 °C. This solution was filtered through glass wool into a stirring solution of BSA (30.4 mg, 0.46 μmol) in 1 mL PBS and left to stir overnight at 4 °C. The solution was transferred to 12–14000 MWCO dialysis tubing and the flask rinsed with 2 x 1 mL water. Conjugate was dialyzed against 8 M urea, 50 mM ammonium bicarbonate and 25 mM ammonium bicarbonate solutions as noted above for solanidine hemisuccinate. Conjugate was transferred to a vial and lyophilized to give 30.8 mg conjugate. MALDI-TOF mass spectrometry (average mass 71,322) results indicated approximately 5 alkaloid molecules per mole BSA in the conjugate.

Results and Discussion

All commercial potato varieties contain the GAs α-solanine and α-chaconine, which have different trisaccharides attached at R_1 to solanidine (solanidane skeleton with a 5-ene group; Figure 1). Wild potato varieties can contain other solanidane (Figure 1) or spirosolane (Figure 2) alkaloids *(24)* with a variety of attached carbohydrate groups. To develop an immunoassay for GAs in commercial potato varieties, it is logical to develop antibodies against solanidine.

Efficient methods of linking solanidine to proteins must permit proper exposure of solanidine during immunization or competition in an immunoassay. Methods include linking through the glycosidic portion or through the secondary alcohol at position 3 (Figure 3) *(41)*. All solanidine hapten conjugates reported to date have been prepared using either the former *(8)*, latter *(43,44)* or both procedures *(41,42)*. While the molecular weight of solanidine (397.6) might be large enough that the linking method may not be crucial for proper antibody binding *(45)*, evidence indicates that altering the linking method can affect antibody binding to conjugate *(41)*. In addition to the glycosidic immunogen *(42)*, we have prepared solanidine hemisuccinate-linked *Limulus polyphemus* hemocyanin (LPH). The performance of this serum (Serum B) was tested against antibody prepared using the glycosidic immunogen (Serum A) using the same hemisuccinate-linked BSA as competition conjugate in the immunoassay (Table I). Both polyclonal antibody populations gave similar results in this comparison. The requirement for more concentrated Serum B than Serum A was likely due to the reduced number of immunization injections before collection of Serum B. Since the hemisuccinate linking method is easier, faster and proceeds in higher overall yield, this method is recommended for the preparation of immunogens and competition conjugates for solanidine immunoassays.

Considering solanidine GAs are always present in commercial potato cultivars, quantification is important. Conventional solid phase immunoassays on microtiter plates often have considerable well-to-well and especially plate-to-plate variation. Although variability can be reduced by analyzing many replicates of a sample and including standards on every plate, this increases the cost and time required for analysis. To overcome these disadvantages, a fluorescence polarization immunoassay (FPI) *(44)* was developed for solanidine glycoalkaloids. FPIs require attachment of a fluorescent label to antigen. For solanidine, solanidine hemisuccinate was linked directly to a methylaminofluorescein derivative.


21
R_2
18
19
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
20
22
23
24
25
26
27
N
O
R_1

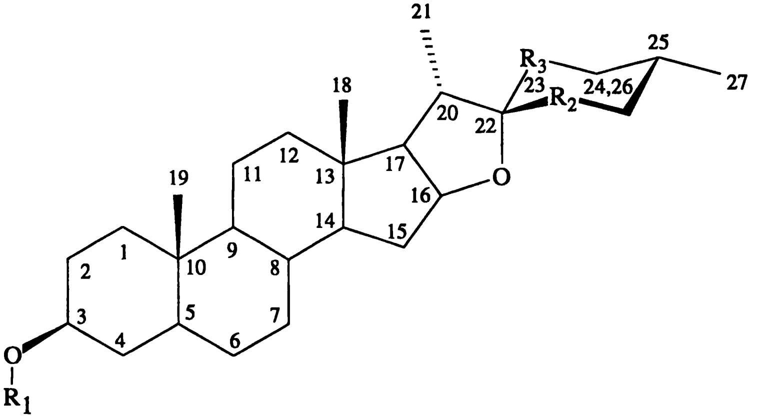
Alkaloid	5-ene	R_1	R_2
solanidine	yes	H	H
demissidine	no	H	H
leptinidine	yes	H	OH
acetylleptinidine	yes	H	OAc

Figure 1. Solanidane Skeleton

Table I. Comparison of Polyclonal Rabbit Serum Produced From Glycosidic or Hemisuccinate Linked Solanidine

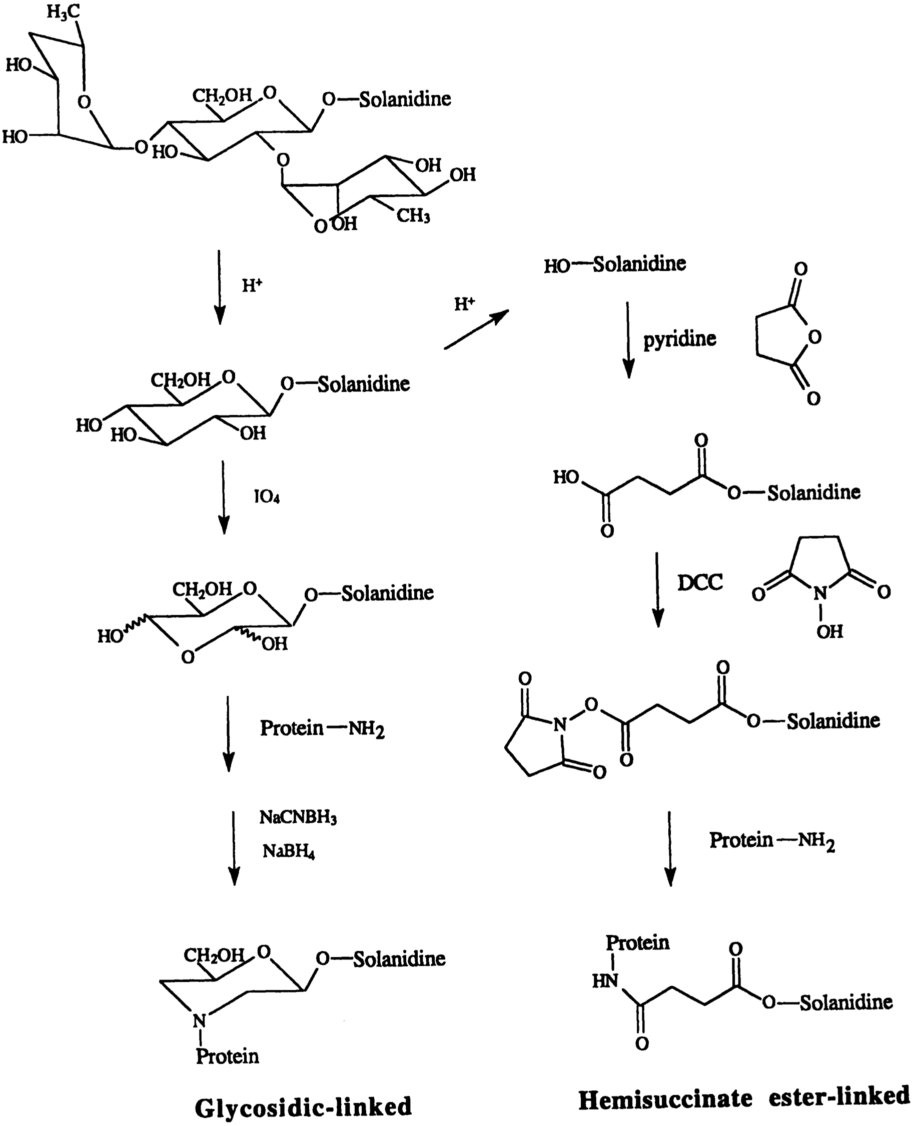
Factors	*Glycosidic Conjugate (Serum A)*	*Hemisuccinate Conjuga (Serum B)*
Number of injections before serum collection	5	2
Serum dilution	1/1,000,000	1/200,000
Maximum absorbance [a] (λ= 450 nm)	0.148	0.208
Concentration of α-chaconine required to reduce the maximum absorbance by 50%	14 nM	14 nM

[a] maximum absorbance with no competing GA added


21
25
27
18
R3
23
R2
24,26
20
22
19
12
11
13
17
16
O
14
15
1
2
10
9
8
3
5
7
4
6
O
R1

Alkaloid	5-ene	R_1	R_2	R_3
tomatidenol	yes	H	NH	CH_2
tomatidine	no	H	NH	CH_2
solasodine	yes	H	CH_2	NH

Figure 2. Spirosolane Skeleton



H_3C
HO
HO
O
O
CH_2OH
O
O—Solanidine
HO
O
OH
OH
O
OH
CH_3
H+
H+
HO—Solanidine
pyridine
O
O
O
CH_2OH
O
O—Solanidine
HO
HO
OH
IO_4
HO
O
O
O—Solanidine
DCC
O
N
O
OH
CH_2OH
O
O—Solanidine
HO
O
OH
Protein—NH_2
O
O
N
O
O
O
O
O—Solanidine
$NaCNBH_3$
$NaBH_4$
Protein—NH_2
CH_2OH
O
O—Solanidine
N
Protein
Protein
HN
O
O
O—Solanidine
Glycosidic-linked
Hemisuccinate ester-linked

Figure 3. Solanidine Conjugates

Competition for binding to the antibody occurred in solution between native GA and fluorescent-labeled solanidine. Fluorescence polarization values are greatest for large, slowly rotating species. Consequently, fluorescence polarization values decrease when fluorescent-labeled solanidine is displaced from the antibody by free (native) GA in samples or standards.

The solid phase microtiter immunoassay and FPI were compared (Table II). Although microtiter immunoassays require considerably more time to complete (several hours versus minutes for FPI), 96 analyses can be performed simultaneously. FPI can be more convenient than solid phase assays, however, because single analyses can be performed rapidly as needed. Furthermore, FPI requires only standardization of reagents and if FPI reagents (antibody and fluorescent-labeled antigen) are stable, standard curves are reproducible. Despite these advantages, FPI requires the use of fairly concentrated antibody solutions. Sensitivity in competitive immunoassays generally increases with lower antibody concentrations. It was surprising, therefore, that FPI was almost as sensitive as the microtiter assay for GAs and was sufficiently sensitive to allow for high dilution of potato extracts. FPIs also display superior reproducibility (lower coefficients of variation) to solid-phase immunoassays and facilitate the determination of antibody binding kinetics since all interactions occur in solution.

Recently there has been increased interest in developing methods of analysis for spirosolane GAs, specifically tomatidine *(34–36,43,46)*. This interest has been sparked in part by the introduction of genetically altered tomatoes and the possibility that these tomatoes could contain high levels of tomatidine. Immunoassays available for tomatidine analysis are limited and include one account of a fortuitously cross-reacting monoclonal antibody *(43)*. A relatively nonspecific immunoassay has also been reported *(40)*. The low specificity of this immunoassay was probably due to opening of the spiroamino ketal moiety during the conjugation procedure (Figure 4); an inefficient immunogen was therefore synthesized *(47)*. Our initial attempts to derivatize tomatine for conjugation to protein also were unsuccessful (Figure 4). Although selective acetylation for spirosolane alkaloids is possible *(48)*, we could not establish conditions to allow for selective succinylation of oxygen without simultaneous succinylation of the secondary nitrogen. Conditions investigated included a variety of different temperatures in pyridine or using acid catalysts such as *p*-toluenesulfonic acid. Attempts to protect the nitrogen group of tomatidine with acid-labile *t*-butylcarbonyl group also failed, probably due to steric hindrance near the secondary nitrogen.

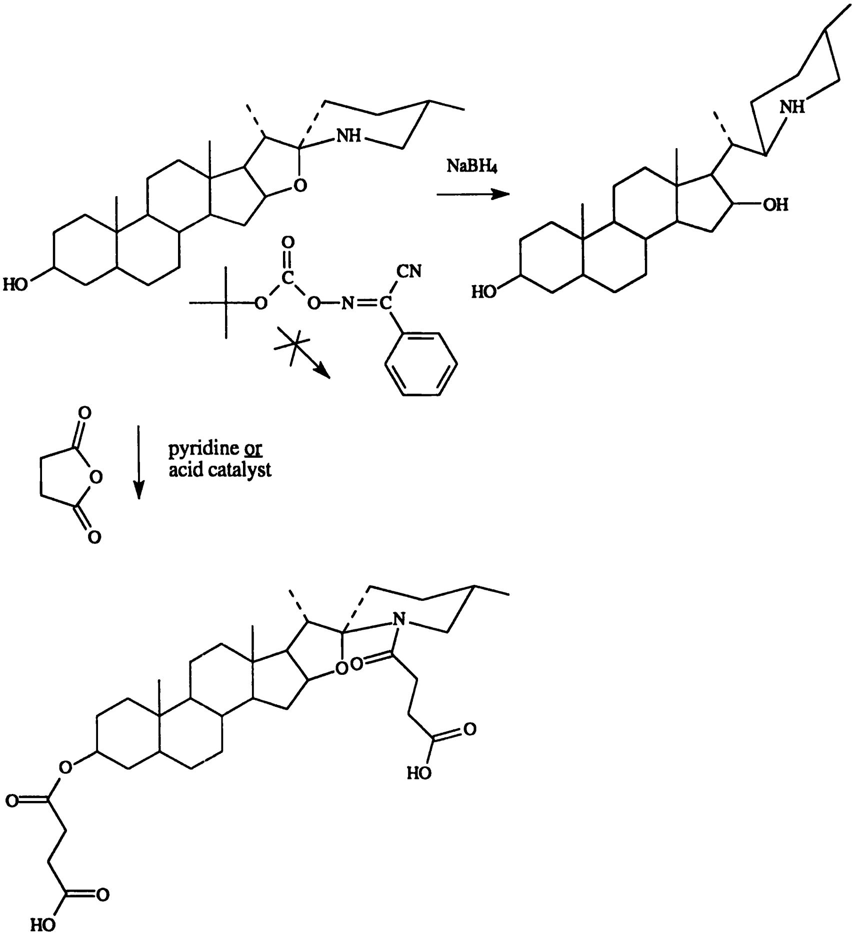

NH
HO
O
NaBH4
NH
OH
HO
O
C
O
O
N=C
CN
O
O
O
pyridine or
acid catalyst
N
O
O
O
HO
O
O
O
HO

Figure 4. Attempts to Prepare Tomatidine Derivatives

Table II. Comparison of Indirect Competitive and Fluorescence Polarization Immunoassays for Quantification of α-Chaconine Using the Same Polyclonal Antibody Serum (Serum A)

Factors	*Indirect Competitive (Microtiter plate)*	*Fluorescence Polarizati (Cuvette)*
Frequency of analysis of standards	with every set of analyses	only on original reagen
Serum dilution	1/1,000,000	1/200
GA concentration for 50% reduction in maximum response	14 nM	20 nM
Coefficient of variation (n=5)	10-20%	~5%
Kinetics	difficult	easy

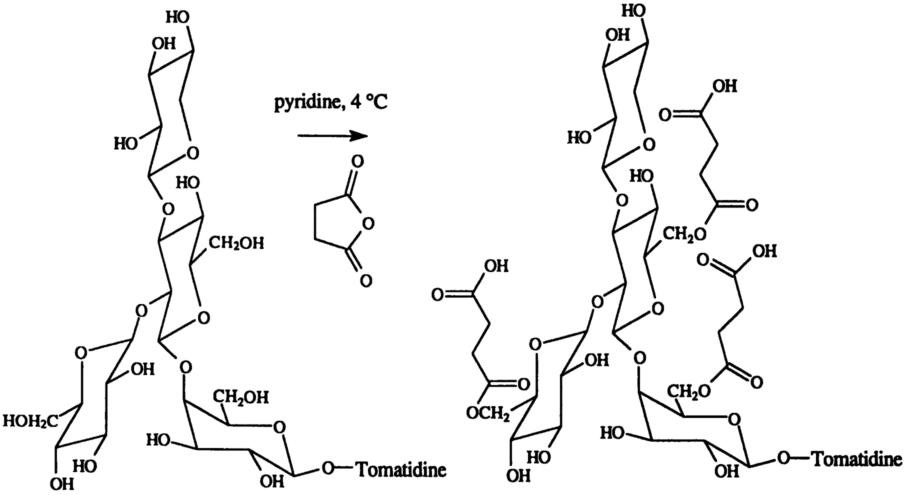


Figure 5. α–Tomatine Succinyl Derivatives

Selective succinylation of the GA tomatine permitted effective conjugate synthesis. Primary hydroxy groups of the sugar were succinylated without altering the alkaloid nitrogen and resulted in a variety of products from mono- to trisuccinylated tomatine (Figure 5). These products could be separated by charge differences using an anion exchange column with an acetate gradient *(49)*. Isolated monosuccinylated tomatine was conjugated to BSA using the active ester method to produce the required immunogen, which is now being evaluated in our laboratory.

In conclusion, we have developed synthetic routes for conjugates in both the solanidane and spirosolane GA series. These conjugates are used both as immunogens and competition conjugates in solid phase immunoassays. We have also shown that fluorescence polarization immunoassays offer several advantages over conventional microtiter-based immunoassays for potato GAs.

Acknowledgments

This work was supported by a Natural Sciences and Engineering Research Council (NSERC) of Canada grant to one of the authors (PS).

References

1. Maga, J. A. *Food Rev. Int.* **1994**, *10*, 385–418.
2. Morris, S. C.; Lee, L. H. *Food Technol. Aust.* **1984**, *36*, 118–124.
3. Hall, R L. *Food Technol.* **1992**, *46*, 109–112.
4. Jadhav, S. J.; Sharma, R. P.; Salunkhe, D. K. *CRC Crit. Rev. Toxicol.* **1981**, *9*, 21–104.
5. Sinden, S. L.; Sanford, L. L.; Webb, R. E. *Am. Potato J.* **1984**, *61*, 141–156.
6. Morris, S. C.; Petermann, J. B. *Food Chem.* **1985**, *18*, 271–282.
7. Roos, H.; Pasemann, P.; Nitzche, W. *Z. Pflanzenzuecht.* **1978**, *80*, 64–79.
8. Morgan, M. R. A.; McNerney, R.; Matthew, J. A., Coxon, D. T. ; Chan, W. S. *J. Sci. Food Agric.* **1983**, *34*, 593–598.
9. Olsson, K. *Potato Res.* **1986**, *29*, 1–12.
10. Hlywka, J. J.; Stephenson, G. R.; Sears, M. K.; Yada, R. Y. *J. Agric. Food Chem.* **1994**, *42*, 2545–2550.
11. Jonasson, T.; Olsson, K. *Potato Res.* **1994**, *37*, 205–216.
12. Fewell, A. M., Roddick, J. G.; Weissenberg, M. *Phytochemistry* **1994**, *37*, 1007–1011.
13. Sanford, L. L.; Deahl, K. L.; Sinden, S. L.; Ladd, T. L., Jr. *Am. Potato J.* **1990**, *67*, 461–466.
14. Tingey, W. M. *Am. Potato J.* **1984**, *61*, 157–167.
15. Deahl, K. L.; Cantelo, W. W.; Sinden, S. L.; Sanford, L. L. *Am. Potato J.* **1991**, *68*, 659–666.
16. Osman, S. F.; Sinden, S. L.; Irwin, P.; Deahl, K.; Tingey, W. M. *Phytochemistry* **1991**, *30*, 3161–3163.
17. Flanders, K. L.; Hawkes, J. G.; Radcliffe, E. B.; Lauer, F. I. *Euphytica* **1992**, *61*, 83–111.
18. Osman, S. *Rec. Adv. Phytochem.* **1980**, *14*, 75–96.
19. Gregory, P.; Sinden, S. L.; Osman, S. F.; Tingey, W. M.; Chessin, D. A. *J. Agric. Food Chem.* **1981**, *29*, 1212–1215.
20. Coxon, D. T. *Am. Potato J.* **1984**, *61*, 169–183.
21. Gregory, P. *Am. Potato J.* **1984**, *61*, 115–122.
22. Zitnak, A.; Johnston, G. R. *Am. Potato J.* **1970**, *47*, 256–260.
23. Morgan, M. R. A.; McNerney, R.; Coxon, D. T.; Chan, H. W. S. In *Immunoassays in Food Analysis*; Morris, B. A.; Clifford, G. D., eds.; Elsevier Applied Science Publishers: London, U.K., 1985; pp 187–195.

24. Van Gelder, W. M. J. In *Handbook of Natural Toxins*; Keeler, R. F. ; Tu, A. T., eds.; Marcel Dekker, Inc.: New York, NY, 1991, Vol. 6; pp 101–134.
25. Svendsen, A. B.; Verpoorte, R. In *Part A. Thin Layer Chromatography.* Elsevier Scientific Publishing Co.: New York, NY, 1983; pp 415–423.
26. Ferreira, F.; Moyna, P.; Soule, S.; Vazquez, A. *J. Chromatogr.* **1993**, *653*, 380–384.
27. Van Gelder, W. M. *J. Chromatogr.* **1985**, *331*, 285–293.
28. Lawson, D. R.; Erb, W. A.; Miller, A. R. *J. Agric. Food Chem.* **1992**, *40*, 2186–2191.
29. Bushway, R. J.; Bureau, J. L.; King, J. *J. Agric. Food Chem.* **1986**, *34*, 277–279.
30. Carman, A. S., Jr.; Kuan, S. S.; Ware, G. S.; Francis, O. J., Jr.; Kirschenheuter, G. P. *J. Agric. Food Chem.* **1986**, *34*, 279–282.
31. Hellenäs, K. A. *J. Sci. Food Agric.* **1986**, *37*, 776–782.
32. Kobayashi, K.; Powell, A. D.; Toyoda, M.; Saito, Y. *J. Chromatogr.* **1989**, *462* 357–364.
33. Friedman, M.; Dao, L. *J. Agric. Food Chem.* **1992**, *40*, 419–423.
34. Kozukue, N.; Kozukue, E., Yamashita, H.; Fujii, S. *J. Food Sci.* **1994**, *59*, 1211–1212.
35. Keukens, E.; Hop, M.; Jongen, W. *J. Agric. Food Chem.* **1994**, *42*, 2475–2477.
36. Bushway, R. J.; Perkins, L. B., Paradis, L. R.; Vanderpan, S. *J. Agric. Food Chem.* **1994**, *42*, 2824–2829.
37. Bushway, R J.; Bureau, J. L.; Stickney, M. R. *J. Agric. Food Chem.* **1985**, *33*, 45–46.
38. Friedman, M., Levin, C. E.; McDonald, G. M. *J. Agric. Food Chem.* **1994**, *42*, 1959–1964.
39. Vallejo, R. P.; Ercegovich, C. D. In *Methods and Standards for Environmental Measurements. Publication 519;* National Bureau of Standards: Washington, D.C., 1979.
40. Barbour, J. D.; Kennedy, G. G.; Roe, R. M. *Rev. Pestic. Toxicol.* **1991**, *1*, 289–303.
41. Plhak, L. C.; Sporns, P. *J. Agric. Food Chem.* **1992**, *40*, 2533–2540.
42. Plhak, L. C.; Sporns, P. *Am. Potato J.* **1994**, *71*, 297–313.
43. Stanker, L. H.; Kamps-Holtzapple, C.; Friedman, M. *J. Agric. Food Chem.* **1994**, *42*, 2360–2366.
44. Thomson, C. A.; Sporns, P. *J. Agric. Food Chem.* **1995**, *42*, 254–260.

45. Chappey, O.; Debray, M.; Niel, E.; Scherrmann, J. M. *J. Immunol. Methods* **1994**, *172*, 219–225.
46. Takagi, K.; Toyoda, M.; Shimizu, M.; Satoh, T.; Saito, Y. *J. Chromatogr.* **1994**, *659*, 127–131.
47. Viloria, E.; Meccia, G.; Usubillaga, A. N. *J. Nat. Prod. (Lloydia)* **1992**, *55*, 1178–1185.
48. Kusano, G.; Aimi, N.; Sato, Y. *J. Org. Chem.* **1970**, *35*, 2624–2626.
49. Saadat, S.; Ballou, C. E. *J. Biol. Chem.* **1983**, *258*, 1813–1818.

RECEIVED August 30, 1995

Chapter 20

Efficacy of a Commercial Enzyme Immunoassay Kit for the Detection of Staphylococcal Enterotoxins in Foods

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Efficacy of an enzyme immunoassay kit, RIDASCREEN (R-Biopharm GmBH, Darmstadt, Germany), has been studied for detection of staphylococcal enterotoxin (SE) in foods. The results show: (a) an excellent sensitivity (0.5–0.75 ng SE/g of food), (b) a high specificity (no false results were observed with any of the foods tested, except for certain foods containing high levels of peroxidase, these false results being eliminated by inactivation of the enzyme with 0.6% NaN_3 prior to analysis, (c) short assay time (a 3-h assay), (d) simplicity (no need for lengthy extraction or concentration), and (e) comparability of repeatability and reproducibility to those of other enzyme immunoassay kit.

Growth of enterotoxigenic strains of *Staphylococcus aureus* to a population of at least 5.0×10^5 cells/g of food is generally considered necessary for production of sufficient amount of enterotoxin to cause food poisoning if the food is consumed (*1, 2*). A number of antigenically different types of staphylococcal enterotoxins (SEs) have been identified: SEA, SEB, SEC_1, SEC_2, SEC_3, SED, and SEE. The enterotoxins SEC_1, SEC_2, and SEC_3 are closely related antigenically and can be identified by their cross-reactions with antibodies prepared against any one of them (*3*). Of the SEs, SEA is the one most commonly involved in staphylococcal food poisoning outbreaks (*4, 5*). Studies indicate that as little as 100 to 200 ng of SEs can produce symptoms of intoxication (*6*). To detect this low level of enterotoxin in 100 g of food (1 to 2 ng of SE/g), several sensitive detection methods are employed: (a) radioimmunoassays (RIA) (*7, 8*); (b) enzyme-linked immunosorbent assays (ELISA) or enzyme immunoassays (EIA) (*9–11*); and (c) reversed passive latex agglutination assays (RPLA) (*12, 13*). Among these assays, some commercial kits are now available. For example, there are five EIA kits, including the Bommeli kit (Swiss Ball kit) made in Switzerland; a visual EIA kit (TECRA) made in Australia; an EIA membrane kit and an EIA tube kit, both made in

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France; and an EIA kit (RIDASCREEN) made in Germany; one RPLA kit is made in Japan. Of these commercial kits, the RIDASCREEN kit is the most recent; therefore, little information on its performance is available.

The purpose of this study was to ascertain whether specificity and sensitivity of the RIDASCREEN kit would meet the criteria for food safety with respect to the detection of staphylococcal enterotoxins in foods, and to evaluate repeatability and reproducibility of the kit.

Materials and Methods

Sources of SE Assay Kits. RIDASCREEN EIA kits produced by R-Biopharm GmbH, Darmstadt, Germany, were obtained from Bioman Products Inc., Mississauga, Ontario, Canada. The kit is a monovalent capture antibody system containing all necessary reagents for the detection of SEA to SEE.

Sources of Standard Enterotoxins. Purified standard SEA and SEE were gifts from M. S. Bergdoll, Food Research Institute, University of Wisconsin, Madison, WI; SEB, SEC, and SED were purchased from Toxin Technology, Inc., Sarasota, FL.

Extraction of SEs from Foods and SE Analyses. SEs were extracted by the procedures described in the previous report (2) and SE assays were carried out according to the manufacturer's instructions unless otherwise stated. The assay procedures involved six steps all of which were carried out at room temperature: (i) binding of toxin and antitoxin by incubation of the microtiter well contents (100 μL food extracts) for 1 h; (ii) removal of unbound materials from the wells by rinsing three times with washing buffer; (iii) formation of sandwich complex (enzyme conjugated antitoxin-toxin-antitoxin) by adding enzyme-labelled conjugate (100 μL) to the toxin-antitoxin complex formed in the wells—this is incubated for 1 h ; (iv) removal of unbound materials by rinsing the wells three times with washing buffer; (v) color development involving 30 min incubation of chromogen with enzyme hydrolyzed substrate and subsequent stopping of the reaction; and (vi) measurement of OD at 450 nm using a microtiter plate reader.

Procedures for Screening and Inactivation of Peroxidase. The presence of peroxidase in food extracts was screened by a spot test mixing equal volumes (50 μL) of substrate and chromogen reagents from the RIDASCREEN kit with sample extract (100 μL). Formation of a dark blue color within 1 min at room temperature indicates a positive test for peroxidase. To determine an optimum concentration of sodium azide for inactivation of peroxidase, each of five different amounts of peroxidase (0.01 to 10.0 purpurogallin units/mL) were treated with six different concentrations of sodium azide (0 to 3.0%), followed by the RIDASCREEN SE-assay procedures. As a positive standard, horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) was used.

Estimation of SE Amounts in Foods. Amounts of SEs in foods were estimated by the RIDASCREEN kit using standard curves. Three standard curves were drawn by using the calibrated ODs of the standard SEs (0, 0.25, 0.50, 0.75, and 1.0 ng/mL) spiked into noodle, ham and cheese extracts representing pasta, meat, and dairy products, respectively. The calibrated OD is obtained by subtraction of the cutoff value from the OD of the sample. The cutoff value is defined as the average OD of two negative controls plus 0.15, which is a constant established by the kit manufacturer.

Results and Discussion

Sensitivity of the RIDASCREEN EIA Kit. Table I shows that the minimum detectable levels of SEs were in the range of 0.10–0.20 ng/mL when SEs were spiked in phosphate buffered saline. The values are similar to those (0.1 ng/mL) claimed by the manufacturer of the kit. With the spiked food extracts, the detectable limit varied depending on the types of food and enterotoxin in question. The ranges of detectable limits with the extracts of beef, chicken, and noodle were between 0.20 and 0.30 ng/mL for all the SEs except SEC; the range of SEC was between 0.30 and 0.35 ng/mL. With cheese extracts, minimum detectable levels of SEs were in the range of 0.30–0.45 ng/mL. The minimum detectable levels of SEA and SEB in chicken and that of SED and SEE in ham were 0.50 ng/g but that of SEC in ham was 0.75 ng/g (Table II). These values are comparable to those in a previous report (*2*). The data of Tables I, II, and III indicate that the sensitivity of the kit fully meets the food safety criteria for detection levels of 1–2 ng/g.

Specificity of the RIDASCREEN EIA Kit. The RIDASCREEN kit did not yield false results with toxin positive and negative food samples when tested by eleven analysts (Table III). It is worthy of note that the kit did not show any false reactions with mussels unlike the false-positive results obtained by other EIA kits (*2, 14*). One should be aware that, since RIDASCREEN kits employ peroxidase labelled anti-SE conjugate, the kits can yield false-positive results with foods containing high levels of peroxidase. If the spot test of the food extract indicates high levels of the enzyme, up to 5.0 purpurogallin units/mL of the enzyme can be inactivated by sodium azide at a concentration of 0.6% (Tables IV and V). This level of sodium azide reduced the serological activity of the SEs by less than 7.0% (data not shown).

Repeatability and Reproducibility of the RIDASCREEN Kit. Standard deviation reflecting repeatability (SD_r) within a laboratory and standard deviation reflecting reproducibility (SD_R) among laboratories are greatly affected by the optical density, i.e. the amounts of toxin present (Table III). With regard to relative standard deviation for repeatability (RSD_r) and that for reproducibility (RSD_R), all toxin-positive samples showed lower values than those of all toxin-negatives (Table III). The overall RSD_R values (24.75–41.60%) were comparable to those (11.74–69.24%) obtained by the EIA kits of the polyvalent capture antibody system (*15*), while RSD_r values (11.93–25.55%) were slightly higher than those (4.88–18.94%) of the latter kits. This is probably due to the fact that the

Table I. Minimum levels of SEs in Buffer and Food Extracts Detectable by the EIA kits (RIDASCREEN).

Sample[a]	SE detection limits (ng/mL)[b]				
	A	B	C	D	E
Buffer	0.15	0.15	0.20	0.15	0.10
Beef	0.20	0.20	0.25	0.20	0.20
Chicken	0.20	0.20	0.25	0.20	0.20
Noodle	0.25	0.25	0.35	0.30	0.25
Cheese	0.35	0.35	0.45	0.35	0.30

[a] The buffer was phosphate-buffered saline (0.05 M phosphate in 0.15 M NaCl containing 0.05% NaN_3, pH 7.6). Food extracts were prepared with SE-free samples by using the phosphate-buffered saline. For SE-positive samples, five levels of different SEs were added to the SE-free samples to arrive at the final concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 ng SE/mL, respectively.

[b] The values are averages for duplicate assays.

Table II. Levels of SEs Detected by the RIDASCREEN Kits in Foods to which Multiple Types of SEs were Added.

Food	SE added	Amounts of each SE			Vol of extracts (mL/g)	Recovery (%)
		Added (ng/g)	Detected[a]			
			ng/mL	ng/g		
Chicken	A and B	0.00	Neg (A) Neg (B)	Neg (A) Neg (B)	1.80	NA[b] (A) NA (B)
		0.25	Neg (A) Neg (B)	Neg (A) Neg (B)	1.79	NA (A) NA (B)
		0.50	0.19 (A) 0.18 (B)	0.34 (A) 0.32 (B)	1.80	68.0 (A) 64.0 (B)
		0.75	0.28 (A) 0.30 (B)	0.50 (A) 0.53 (B)	1.77	66.6 (A) 70.6 (B)
		1.00	0.38 (A) 0.40 (B)	0.68 (A) 0.72 (B)	1.79	68.0 (A) 72.0 (B)
Ham	C, D and E	0.00	Neg (C) Neg (D) Neg (E)	Neg (C) Neg (D) Neg (E)	1.60	NA (C) NA (D) NA (E)
		0.25	Neg (C) Neg (D) Neg (E)	Neg (C) Neg (D) Neg (E)	1.60	NA (C) NA (D) NA (E)
		0.50	Neg (C) 0.19 (D) 0.20 (E)	Neg (C) 0.30 (D) 0.32 (E)	1.58	NA (C) 60.0 (D) 64.0 (E)
		0.75	0.24 (C) 0.30 (D) 0.31 (E)	0.38 (C) 0.48 (D) 0.49 (E)	1.59	50.7 (C) 64.0 (D) 65.3 (E)
		1.00	0.34 (C) 0.42 (D) 0.45 (E)	0.54 (C) 0.67 (D) 0.72 (E)	1.60	54.4 (C) 67.0 (D) 72.0 (E)

[a] The values are averages for duplicate tests. Neg, negative results (no SE detected). The figures for ng/g were calculated by multiplying those (ng/mL) and volume of extracts (mL/g).

[b] NA, not applicable.

Table III. Detection of Staphylococcal Enterotoxins in Foods and the Repeatability and Reproducibility of EIA Kits (RIDASCREEN)

Food	Toxin(s) spiked (ng/g)	Toxin(s) tested	Results[a]					
			OD[b]	R(%)[c]	SD_r[d]	SD_R[e]	RSD_r[f]	RSD_R[g]
Salami	B=1.0	B	0.59	+(100)	0.122	0.161	20.75	27.46
		A, C, D, E	0.14	- (100)	0.034	0.049	23.99	35.16
Chicken	D=2.0	D	0.98	+(100)	0.202	0.242	20.54	24.63
		A,B,C,E	0.15	- (100)	0.041	0.061	25.55	41.60
Cheese	A=1.0	A	0.61	+(100)	0.073	0.114	11.93	18.63
	D=2.0	D	0.85	+(100)	0.141	0.209	16.67	24.75
		B,C,E	0.15	- (100)	0.025	0.038	18.32	26.14
Mussels	None	A,B,C,D, E	0.21	- (100)	0.051	0.086	24.45	41.39

[a] Average of triplicate assays of every food analyzed by eleven analysts.

[b] OD, Optical densisties measured at 450 nm.

[c] R, Results expressed by + and - are positive and negative results, respectively. An OD > cut-off value (CO) is considered positive. CO= mean OD of negative controls plus 0.15.

[d] SD_r, Standard deviation reflecting repeatability.

[e] SD_R, Standard deviation reflecting reproducibility.

[f] RSD_r, Relative standard deviation for repeatability expressed as a percentage.

[g] RSD_R, Relative standard deviation for reproducibility expressed as a percentage.

Table IV. Effect of Sodium Azide on Inactivation of Peroxidase in the EIA system using the RIDASCREEN SE Assay Kits.

Concentration of NaN_3 %	Assay Results[a]				
	Levels of Peroxidase in Food Extract (purpurogallin units/mL)				
	10.0	5.0	1.0	0.1	0.01
0.00	+	+	+	+	-
0.15	+	+	-	-	-
0.30	+	+	-	-	-
0.60	+	-	-	-	-
1.50	+	-	-	-	-
3.00	+	-	-	-	-

[a] + and - are positive and negative results, respectively. The results are averages of triplicate assays.

Table V. Influence of Naturally Occuring Food Peroxidase and NaN_3 Treatment on the SE Assay Results using the EIA Kits (RIDASCREEN)[a]

Food	Peroxidase[b]	Sodium Azide Treatment[c]	
		without	with
Bean Sprouts	+	+	-
Pickel (cucumber)	+	+	-
Salad	+	V	-
Coleslaw	+	V	-
Mussels (raw)	+	-	-
Milk (raw)	V	-	-
Lettuce	+	V	-

[a] The results are averages of triplicate analyses of SE-negative foods.

[b] Presence of Peroxidase was estimated by the spot test described in the text.

[c] Sodium azide treatments were done at a concentration of 0.6% (NaN_3/food extract). +, -, and V designate positive, negative and variable results, respectively.

polyvalent capture antibody system required only one assay per sample without differentiating SE types, while the monovalent EIA kits such as the RIDASCREEN kit required five assays for SEA to SEE per sample.

Acknowledgments

We thank the following participants for analyses of collaborative study samples by the RIDASCREEN kits: D. Warburton, Health Canada, Bureau of Microbial Hazards, Ottawa, ON; K. Catherwood and C. Crawford, Health Canada Western Region, Burnaby, BC; R. Foster, Health Canada Central Region, Winnipeg, MB; C. Fox, Health Canada Atlantic Region, Dartmouth, NS; L. Gour, Health Canada Quebec Region, Longueuil, PQ; K. Jessett, Health Canada Ontario Region, Scarborough, ON; M. Langridge, Ontario Ministry of Health, Etobicoke, ON; E. Mann, Agriculture and Agri-Food Canada, Guelph, ON; A. Morrow, Health Canada Ontario Region, Scarborough, ON; J. Oggel, Agriculture and Agri-Food Canada, Ottawa, ON; U. Purvis and W. Zandstra, Health Canada Ontario Region, Scarborough, ON. We thank G. Sanders for technical assistance in preparation of food samples.

Literature Cited

1. Newsome, R. L. *Food Technol.* **1988**, *42*, 194–195.
2. Park, C. E.; Akhtar, M.; Rayman, M. K. *Appl. Environ. Microbiol.* **1994**, *60*, 677–681.
3. Bergdoll, M. S. *Int. J. Food Microbiol.* **1990**, *10*, 91–100.
4. Bennett, R. W. In *Foodborne Microorganisms and their Toxins: Developing Methodology*; Pierson, M. D.; Stern, N. J., Eds; Marcel Dekkar, Inc.: New York, NY, 1986; pp 345–392.
5. Igarashi, H.; Shingaki, M.; Fujikawa, H.; Ushioda, H.; Terayama, T. *Zentralbl. Bakteriol. Mikrobiol. Hyg. 1 Abt.* **1985**, *14*, 255–257.
6. Evenson, M. L.; Hinds, W. M.; Bernstein, R. S.; Bergdoll, M. S. *Int. J. Food Microbiol.* **1988**, *7*, 311–316.
7. Collins, W. S.; Johnson, A. D.; Metzger, J. F.; Bennett, R. W. *Appl. Microbiol.* **1973**, *25*, 774–777.
8. Johnson, H. M.; Bukovic, J. A.; Kaufman, P. E. *Appl. Microbiol.* **1973**, *26*, 309–313.
9. Ewald, S. *Int. J. Food Microbiol.* **1988**, *6*, 141–153.
10. Fey, H.; Pfister, H.; Rüegg, O. *J. Clin. Microbiol.* **1984**, *19*, 34–38.
11. Freed, R. C.; Evenson, M. L.; Reiser, R. F.; Bergdoll, M. S. *Appl. Environ. Microbiol.* **1982**, *44*, 1349–1355.
12. Park, C. E.; Szabo, R. *Can. J. Microbiol.* **1986**, *32*, 723–727.
13. Bankes, P.; Rose, S. A. *J. Appl. Bacteriol.* **1989**, *67*, 395–399.
14. Park, C. E.; Akhtar, M.; Rayman, M. K. *Appl. Environ. Microbiol.* **1993**, *59*, 2210–2213.
15. Bennett, R. W.; McClure, R. *J. AOAC International*, **1994**, *77*, 357–364.

RECEIVED July 14, 1995

Chapter 21

Immunoassays for Detecting Insect Contamination of Food Products

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Federal standards for regulating the amount of insect contamination in a wide variety of foodstuffs make it imperative that accurate and reliable analytical techniques be available for measuring such contamination. Present analytical methods such as the insect fragment count are time consuming, costly and show wide variability. New analytical techniques for insect detection, based on immunological ELISA assays, have been developed which offer simplicity of use, low cost and excellent accuracy and reliability. One class of ELISA assay, based on the insect muscle protein myosin, is designed to detect a broad range of insect pests in a wide range of stored grains, milled grain products, spices, nuts and dried fruits. This assay is available in several formats, ranging from highly quantitative microwell plate assays for laboratory analysis to simple qualitative dipstick assays for on-site screening. Other insect immunoassays have been developed which are species specific for both beneficial and deleterious insects and also for detecting Tephritid fruitflies, such as the medfly, in import and export fruits. Work is in progress on additional immunoassays for the detection of insect material in cooked processed foods and for rodent contamination of foodstuffs.

Immunoassays have the capability of combining exceptional selectivity with high precision and a relatively low cost in easy to use formats. For this reason they have become a mainstay of clinical laboratory testing. By contrast with their acceptance in the medical fields, immunoassays have been slower to be adopted for use in agriculture, although such testing for pesticide residues and fungal toxins is now becoming more widespread. One area of agriculture which has a potential for introduction of immunoassays is in the testing for insects and insect remains in stored products. The current tests for insect contamination of grain and milled grain products were first introduced many decades ago and have seen few improvements.

0097–6156/96/0621–0281$15.00/0

For whole grains these tests include the insect-damaged kernel (IDK) analysis (*1,2*), and insect exit hole counts and crack and float techniques (*3–5*). For milled grain products, such as wheat flour, the primary method is the insect fragment count (*6*). Each of these methodologies employs visual inspection as part of the procedure and this factor, in addition to other limitations, results in considerable variation between analysts (see for example *7*). X-ray analysis of whole grain kernels (*8*) offers good precision for detecting hidden insects, but the need to provide radiation sources and the considerable training needed to interpret the X-ray films has hampered widespread adoption of this procedure. Other techniques, such as the acoustical detection of live insects (*9,10*) offer considerable potential but are still in the experimental stages.

Immunoassays offer considerable advantages over the present methodologies for detecting insect contamination of food products. First, they have the potential of providing a single type of detection that can be used all the way through the food processing chain, from raw material to processed food. Second, the same basic type of assay can be utilized for analyzing large numbers of samples in a laboratory, or for testing individual samples on site, such as silos, loading docks, or even in the field. Third, simple and rapid immunoassays can be developed which meet the precision and reliability standards required for assessing insect contamination levels of food products. We present here an overview of the immunoassay methodologies that we have developed over the past several years for testing a wide range of commodities including grains, milled grain products, spices, nuts, and dried and fresh fruit.

Materials and Methods

An insect myosin ELISA assay we developed for the detection of insects in grain (*11*) has served as the basis for the development of other methodologies for insect detection. The reliability of this procedure and its application to the detection of different insect life stages is described in Schatzki *et al.* (*12*). The use of a modified version of this assay for detecting larval through adult insects in fruit, as well as an ELISA procedure for detecting insect eggs in fruit is provided in Proske (*13*). Monoclonal antibody based immunoassay procedures for insect species-specific tests are described in Chen (*14*) and Chen and Kitto (*15*). The isolation and characterization of unique Africanized bee proteins and their use in immunoassays for distinguishing between Africanized and European honeybees is given in Davidson *et al.* (*16*) and Verdel (*17*). Immunoassay kits for insect detection, based on this work, are available from Biotect, Inc., Austin, Texas.

Results and Discussion

The Insect Myosin Immunoassays. A prerequisite to developing an immunoassay which would be capable of detecting a variety of insects in a broad array of fruit flies was to select a suitable insect antigen. Ideally such an antigen should be present in large quantities in all the suspected insect pests, and in all major life stages. Additionally, the antigen should differ very little in structure from insect to insect and be readily solubilized from samples of different food products. The insect muscle protein myosin, specifically the "heavy chain," meets these exacting criteria. Myosin is present in relatively large quantities in all insects and in all life stages from larva to adult. Insect myosin also is a slowly evolving protein so that antibodies raised against one insect species should cross-react with a large selection of insects. This proved to be a fortuitous choice of antigen. Rabbit antisera prepared against myosin heavy chain from crickets (*Acheta domesticus*) crossreacts with all of the common stored product insect pests (*11*). Insect myosin is readily extracted from food samples using a high salt buffer. In order to provide an immunoassay that would require minimal setup time, we developed an insect myosin ELISA assay in a sandwich format, which is illustrated schematically in Figure 1. To test a food product for insect contamination, the sample is ground in a common household blender and then extracted in high salt buffer in the same blender. The extraction procedure takes less than 5 min. Duplicate aliquots of the extract are then placed in wells of microtiter plates or strips, along with a range of myosin standards. The wells of microtiter plates or strips are precoated with capture antibody. After a forty-five minute incubation with antigen, the plates are rinsed three times with a wash buffer (0.1 M potassium phosphate, pH 7.0; 0.1% bovine sereum albumin, 0.05% Tween-20). A secondary antibody solution (50 µL) consisting of rabbit antimyosin IgG conjugated to horseradish peroxidase is then added to the wells and allowed to incubate for 30 min. The wells are then emptied by inversion of the plate and rinsed three times, as above. Enzyme substrate (2,2'-azino-di-3-ethyl benzthiazoline sulfonic acid [ABTS]; 100 µL) is added to the wells and color development is allowed to proceed for 25 min in the dark. Color develops in the wells in proportion to the amount of antigen present. After this time, 100 µL of stop solution (0.5 M oxalic acid) is added to each well and the plates are then read at 414 nm in a standard ELISA reader. Up to 24 samples can conveniently be tested in duplicate at one time with this procedure, which takes approximately 2½ h.

The myosin ELISA assay gives an excellent linear response to the amount of myosin present and also correlates extremely well with the number of insects measured in food samples using the X test (Figure 2). This simple assay procedure is highly reproducible both for reassays of a single test sample (Figure 3), and with a variety of industrial wheat mill samples with varying degrees of insect contamination that were assayed 2 or 3 times over a 3 week period, as shown in Figure 4. The myosin ELISA assay detects all of the common stored insect pests and extensive testing has been carried out with all of the major commercial grains including wheat, oats, corn, barley, soybeans, sorghum and rice (*19*). In addition to performing well in in-house and industrial grain mill trials, this procedure has been very favorably tested by the Federal Grain Inspection Service of the U.S. Department of Agriculture (*18*). The myosin assay works well with both whole grain and milled grain products. Collaborative trials with grain mills have established that, for a given mill, the distribution of any insect material in the whole grain is distributed in a consistent fashion into the flour, shorts and bran fractions as illustrated in Figure 5. Thus, ELISA testing of raw material is an excellent indicator of how much insect contamination will turn up in the finished product. In a similar fashion, the myosin immunoassay provides a quantitative tool for the blending of clean and dirty grain to meet regulatory sanitary standards (Figure 6).

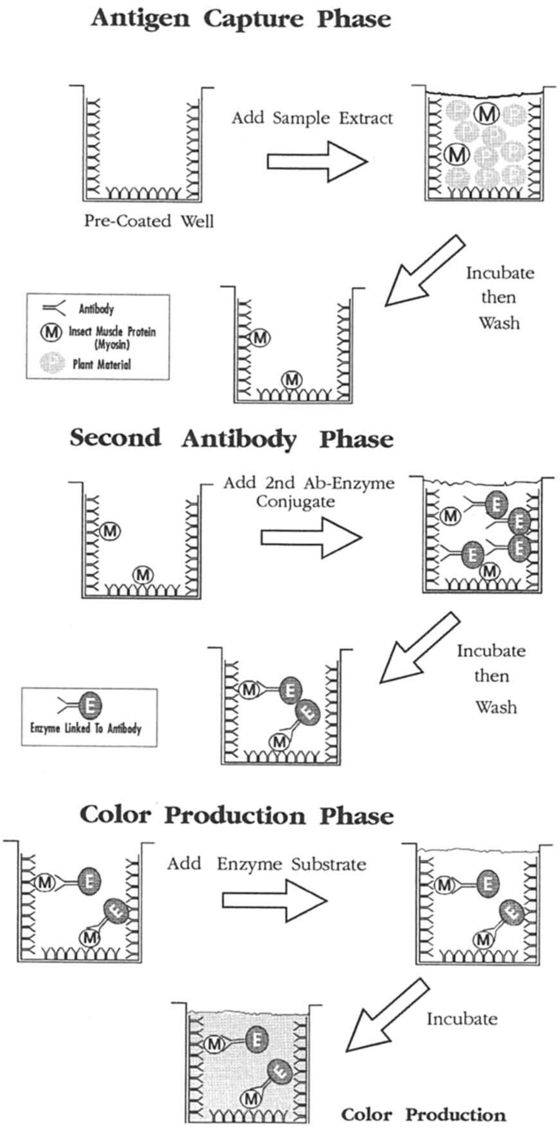
Antigen Capture Phase
Add Sample Extract
Pre-Coated Well
Incubate then Wash
Antibody
M Insect Muscle Protein (Myosin)
Plant Material
Second Antibody Phase
Add 2nd Ab-Enzyme Conjugate
Incubate then Wash
E
Enzyme Linked To Antibody
Color Production Phase
Add Enzyme Substrate
Incubate
Color Production

Figure 1: **A schematic representation of the sandwich ELISA procedure. Reproduced with permission from reference (19), CAB International, 1994.**

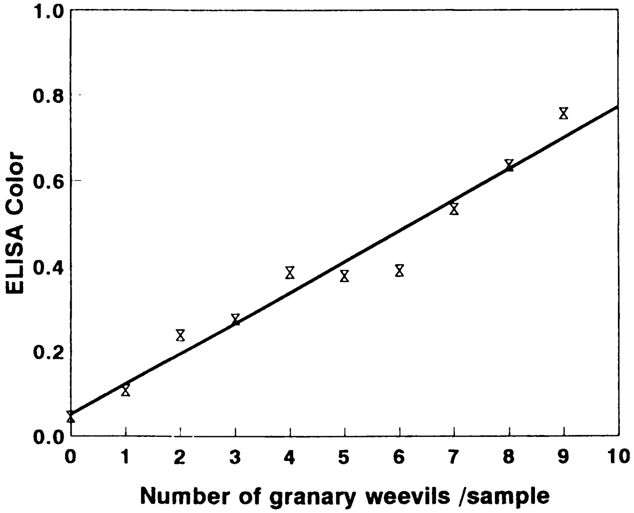

Figure 2: The response of the insect myosin ELISA to increasing levels of insect contamination. Samples of grain (50 g) were spiked with the indicated number of granary weevils.

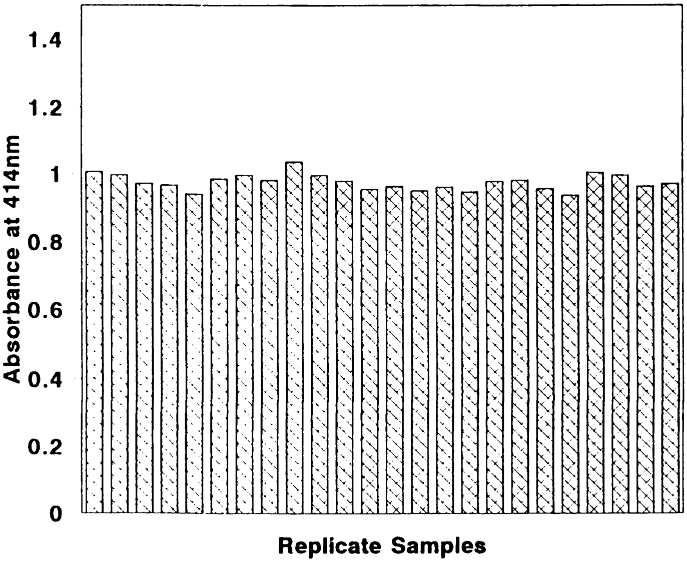

Figure 3: Replicability of the insect myosin ELISA. A test sample of contaminated grain was assayed multiple times on the same day.

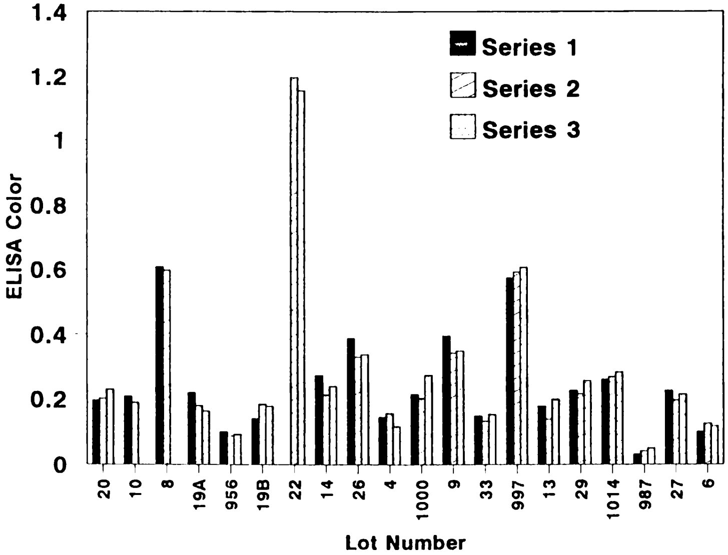

Figure 4: Reliability of insect myosin ELISA. Grain mill samples of varying levels of insect contamination were assayed two or three times over a two week period. Reproduced with permission from reference (19), CAB International, 1994.

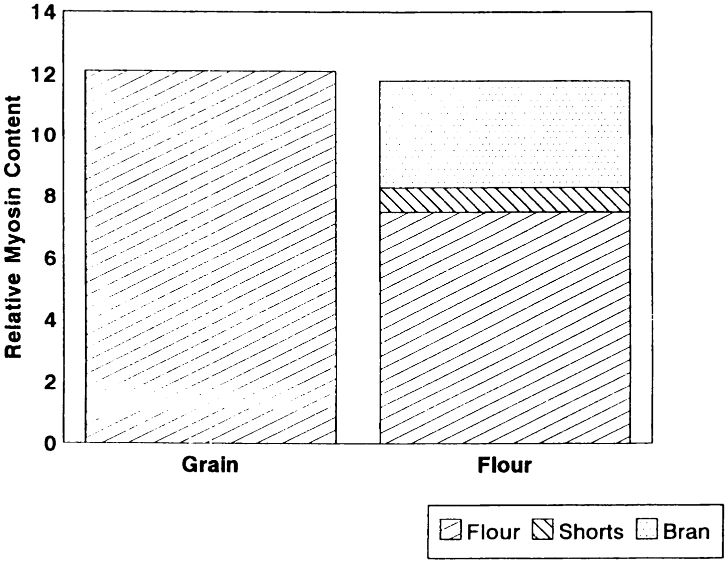

Figure 5: Use of the insect myosin ELISA to follow distribution of insect contamination throughout wheat processing. Assays of whole grain (left) showed the initial level of contamination and assays of the milled flour, shorts and bran fractions (right) indicated how insect material fractionated.

Much of the early work was restricted to the analysis of grain products, but we have recently shown that the insect myosin assay is applicable to a broader range of food products, such as peanuts, almonds, Brazil nuts, filberts, cashews and walnuts; dried fruits such as dates, figs, and raisins; and an extensive group of spices. Some of these materials require slight modifications in the extraction procedure, although in general the same sensitivity and linearity of the assay is obtained as with the grain analysis procedure (*19;* Behrens, P.; Heller, I.; Trieff, S.; Stephenson, J.; Kitto, G. B., The University of Texas at Austin, unpublished results). The insect myosin ELISA assay requires only a very modest outlay for equipment, the major expense being an ELISA reader costing in the range of $2,000–6,000 U.S. dollars. Technical personnel can be trained to high proficiency in one or two days. In cases where large numbers of samples need to be analyzed the procedure can readily be automated using off the shelf equipment employed in clinical laboratories.

While the assays described above provide a reliable quantitative means for checking food products for insect contamination in the laboratory, there are many occasions where it also would be desirable to have available very simple immunoassays that could be applied to single samples in a short period of time. Such tests could be used in loading and unloading docks, for spot-checking during processing, at warehouses or even in the field. For these reasons we have developed a rapid test strip procedure which can be completed within 15 min with the results being read using a low cost hand-held spectrophotometer. This test strip assay is in the final stages of laboratory development, with field trials planned for the near future.

Detecting Insects in Fruit. The transport of fruit, either within a country or through import and export, carries with it the threat of introducing noxious insect pests. This is true, for example, of the import of fruit from Hawaii into the mainland United States, with the potential for bringing in the Oriental fruitfly *Bactrocera dorsalis* or the Mediterranean fruitfly *Ceratitis capitata* (*20,21*). Similarly the export of stone fruits and apples to Japan from the U.S. is hampered by concerns about insect introductions (*22*). In addition to the use of certified fumigation procedures, inspection of fruit also is carried out at ports of entry. Typically such inspection involves slicing samples of the fruit and carrying out a visual inspection for insect larval and pupal stages. It would be desirable to have more quantitative biochemical techniques available which had the capabilities of being automated and could effectively test large numbers of samples.

Because the insect myosin ELISA procedure can detect an exceptionally broad range of insects we reasoned that it might be adapted for checking fruit for insect infestation. The myosin assay works well for detecting a range of Tephritid insect pests, including *Rhagoletis, Dacus, Ceratitis, Paracantha, Orellia, Aciurina* and *Neotephrita* species in a variety of fruits including grapefruit, oranges, lemons, limes, mangos, pears, tomatoes and watermelon. Life stages from first instar larvae through adults could be detected. Modifications to the extraction procedures of the fruit insect ELISA assays have to be made for each major type of fruit, to ensure neutralization of the fruit acids which can denature the insect antigen (*13;* Proske, P.; Lemburg, J.; Kitto, G. B., The University of Texas at Austin, unpublished results).

Although the insect myosin assay can detect the majority of fruit insect life stages it cannot detect insect eggs. For this purpose we have devised an immunoassay based on vitellin, a major protein in insect eggs. Rabbit polyclonal antisera were prepared against purified vitellin from *Anastrepha suspensa* eggs. In this case a sandwich ELISA using these antibodies proved highly specific for eggs from *Anastrepha* species, with little crossreactivity shown to *Dacus* and *Ceratitis* flies (*13*). This is most likely due to the fact that vitellin is a fairly rapidly evolving protein. While the specificity of the *A. suspensa* vitellin antibodies precludes the use of this particular assay as a general tool for detecting insect eggs, such species specific assays could be very helpful in identifying the particular type of insect involved in infestations (see for example *20*).

Monoclonal Antibody Based Immunoassays. Polyclonal antibodies are advantageous for immunoassay development since they can readily be prepared in large quantities at low cost. However, polyclonals typically exhibit a broad range of specificity. On the other hand monoclonal antibodies can be elicited which show exceptionally narrow specificity. We have taken advantage of monoclonal antibodies to develop a species specific test for the granary weevil *Sitophilus granarius*, a major stored grain pest (*15*). In this case the antigen was a specific, stable 60,000 dalton protein isolated from adult granary weevils. As shown in Figure 7, when used in combination with the myosin ELISA for estimating the total insect load of a food sample, the species-specific assay can accurately estimate the proportion of insect contamination contributed by granary weevils. In a similar manner a species-specific assay has been devised for the Kaphra beetle (*23*).

With increasing restrictions being applied to the use of fumigants and pesticides for controlling insect infestations of stored food materials alternate methods of control are being pursued with renewed vigor. Among the alternative techniques is the use of beneficial insects for reducing damage to grain and other foodstuffs which harbor destructive insect pests within the product (such as grain kernels or peanuts). The beneficial insects either directly kill or parasitize the destructive pests but are themselves easily removed from the product by airstream cleaning, since they are external feeders. We and our colleagues have developed monoclonal antibody based, species-specific immunoassays for several of the beneficial insects including *Bracon hebetor, Laelius pedatus, Xylocoris flavipes* and *Trichogramma pretiosum* (*24*; Lemburg, J.; Kedzieski, R.; Ross, C.; Kitto, G. B.,The University of Texas at Austin, unpublished results). These assays should prove useful for studying the dynamics of beneficial/destructive insect interactions and could be used in conjunction with the myosin assay (to measure total insect load) for appropriate timing of beneficial insect introductions.

Africanized bees first moved into the United States from Mexico in 1990 and since that time have moved into the southern states from Texas to California. These bees, because of their aggressive defensive behavior, pose a considerable threat to apiculture, particularly for managed pollination. The Africanized bees bear an extremely close physical resemblance to the common European bee, which makes the task of distinguishing between them extremely difficult. The primary identification methodologies employed today involve dissection of suspect bees, mounting of the

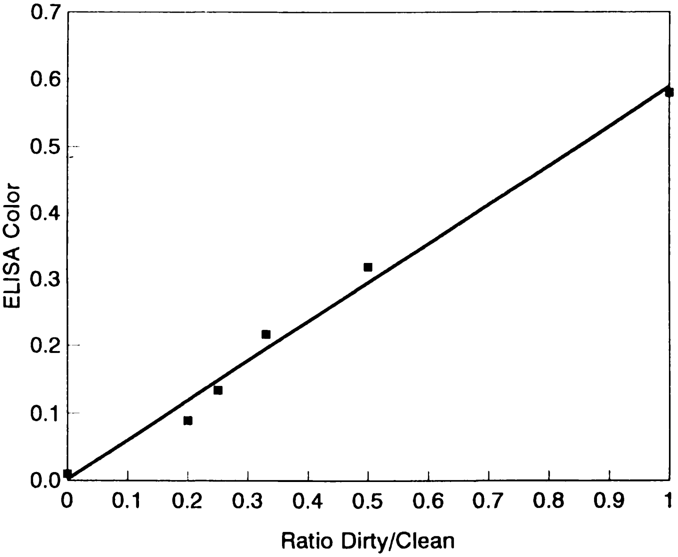

Figure 6: Illustration of how the insect ELISA procedure could be used for grain blending. Samples of clean and insect contaminated grain were mixed in varying proportions and assayed by the insect myosin ELISA.

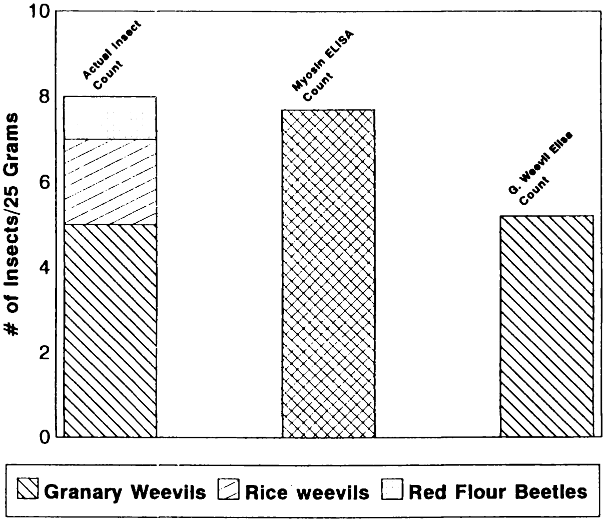

Figure 7: Use of the insect myosin and species-specific ELISA procedures to determine the proportion of insect contamination contributed by a given insect. Clean grain was spiked with the mixture of insects shown at the left. Total insect contamination was measured by the myosin ELISA (center) and the number of granary weevils was estimated by a monoclonal antibody based ELISA. The contaminated grain contained 5 granary weevils/50 g grain. Reproduced with permission from reference (19), CAB International, 1994.

parts of the bee on slides and projecting these slides so that measurements of the body parts can be made. These measurements are then analyzed by a computerized statistical procedure (*25,26*). Such testing services are available at only a few sites in the U.S.A.

We are engaged in the development of simple immunoassay procedures which should allow for ready discrimination of Africanized and European bees. Three related proteins which are unique to Africanized bees have been identified (*17*) and these have been used to prepare monoclonal antibodies. These antibodies have been employed for devising a sandwich ELISA procedure which, in the laboratory, provides the appropriate distinction between the bee species. Work is in progress to convert the ELISA procedure to a rapid test-strip format, using a simple bee squash, that would allow for identification of Africanized bees in the field.

Summary

As illustrated here and in other papers in the symposium immunoassays are finding increasing use for analytical purposes in agriculture for tasks ranging from pesticide residue detection to insect species identification. For many purposes these immunoassays provide major advantages over traditional wet chemistry procedures in terms of ease of use, sensitivity and replicability. Along with techniques such as high-performance liquid chromatography, mass spectrometry, capillary electrophoresis and gamma-ray spectrophotometry, immunological methods are finding a solid home in the armamentarium of the modern agricultural analyst.

Acknowledgments

This work was supported in part by grants from the U.S. Department of Agriculture, Millers' National Federation, the American Spice Trade Association, and the Texas Advanced Technology Program. We thank our many industry and U.S. Department of Agriculture colleagues for providing samples, participating in collaborative trials, offering valuable feedback and above all for their encouragement in developing these immunoassays. We also thank Susan Fullilove for her assistance in preparing this manuscript.

This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or a recommendation for its use by USDA.

References

1. Anonymous. FDA technical bulletin number 5, microanalytical procedures manual; Assoc. Off. Anal. Chem.: Arlington, VA, 1994.
2. Russell, G. E. *J. Food Protect.* **1988**, *51*, 547–533.
3. Trauba, R. L. *J. Assoc. Off. Anal Chem.* **1981**, *64*, 1408–1410.
4. Parker, P. E.; Brauwin, G. R.; Ryan, H. L. In *Storage of Cereal Grains and Their Products*, Am. Assoc. Cereal Chem.: St. Paul, MN, 1982.
5. Arteman, R. L. USDA Fed. Grain Insp. Serv. Rep. No. 81–22–1; USDA Fed. Grain Insp. Service: Washington, D.C., 1982.

6. U.S. Food and Drug Administration Compliance Policy Guides, Section 7104.06; Office of Enforcement, Division of Compliance Policy, Food and Drug Administration: Washington, D.C.
7. Kurtz, O'D.; McCormack, T. H. *J. Assoc. Off. Anal. Chem.* **1965**, *48*, 554–558.
8. Schatzki, T. F.; Fine, T. A. *Cereal. Chem.* **1988**, *65*, 233–239.
9. Webb, J. C. Ph.D. Dissertation, University of Tennessee: Knoxville, TN, 1973.
10. Webb, J. C.; Slaughter, D. C.; Litzkow, C. A. *Florida Entomol.* **1988**, *71*, 492–504.
11. Quinn, F. A.; Burkholder, W.; Kitto, G. B. *J. Econ. Entomol.* **1992**, *85*, 1463–1470.
12. Schatzki, T. F.; Wilson, E. K.; Kitto, G. B.; Heller, I. *J. Econ. Entomol.* **1993**, *86*, 1584–1589.
13. Proske, P. A. Masters' Thesis, University of Texas at Austin, Austin, TX, 1993.
14. Chen, W.-M. Masters' Thesis, University of Texas at Austin, Austin, TX, 1992.
15. Chen, W.-M.; Kitto, G. B. *Food Agric. Immunol.* **1993**, *5*, 165–175.
16. Davidson, F. I.; Udagawa, T.; Verdel, E.; Kitto, G. B. *Bee Sci.* **1992**, *2*, 193–199.
17. Verdel, E. F. O. Masters' Thesis, University of Texas at Austin, Austin, TX, 1994.
18. Cook, C.; Kao, C. Final Report QUARD-RD-12; USDA Fed. Grain Insp. Service, 1994
19. Kitto, G. B.; Quinn, F. A.; Burkholder, W. E. Stored Product Protection: Proc. 6th Internatl. Working Conf. Stored-Product Protection; Highey, E.; Wright, E. J.; Banks, H. J.; Champ, B. R., eds.; CAB International: Wallingford, UK, 1994; pp 415–420.
20. Liquido, N. J.; Chan, H. T.; McQuate, G. T. *J. Econ. Entomol.* **1995**, *88*, 85–96.
21. Lance, D. R.; Gates, D. B. *J. Econ. Entomol.* **1994**, *87*, 1377–1383.
22. Yokoyama, V. K.; Miller, G. T.; Hartsell, P. L. *J. Econ. Entomol.* **1993**, 87, 730–735.
23. Stuart, M. K.; Barak, A.V.; Burkholder, W. E. *J. Stored Prod. Res.* **1994**, 30, 9–16.
24. Stuart, M. K.; Burkholder, W. E. *Biol. Control* **1991**, *1*, 302–308.
25. Daly, H. V.; Balling, S. S. *J. Kansas Entomol. Soc.* **1978**, *51*, 857–869.
26. Sylvester, H. A.; Rinderer, T. E. *Am. Bee J.* **1987**, *127*, 511–515.

RECEIVED December 6, 1995

Chapter 22

Recent Studies on Immunoassays for Mycotoxins

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After a number of years of research, immunoassays have recently gained wide acceptance as analytical tools for mycotoxins. Antibodies against almost all the major mycotoxins are now available. Whereas quantitative immunoassays are still primarily used in the control laboratories, immunoscreening methods have been widely used for screening of mycotoxins in several commodities. Many immunoscreening kits which require less than 15 min to complete are commercially available. Several immunoassay protocols have been adopted as first action by the AOAC. The immunoaffinity method has become popular as a cleanup method in conjunction with other chemical methods and has included automated toxin analysis. This paper reviews the recent progress on immunoassays used for mycotoxins with discussions focusing on the advantages and disadvantages of various immunochemical methods, pitfalls encountered, approaches to overcoming these problems, improvements in the assay efficacy, and the outlook for the future.

Mycotoxins are low molecular weight, secondary metabolites produced by naturally occurring fungi (*1*–6). Since the discovery of aflatoxins in the early 1960s, developments in the last three decades have disclosed many new fungal poisons that are attracting attention because of their high toxicity and their association with foods and animal feeds (2). The presence of mycotoxins in foods and feeds is potentially hazardous to human and animal health. To decrease the risk of human exposure to mycotoxins, a rigorous program has been established for monitoring these toxins in foods. There is a need for more sensitive, rapid and accurate methods of toxin analysis. However, because only trace amounts of the toxin are present in the sample, analysis of mycotoxins in foods becomes a difficult task. Nevertheless, rapid progress in the area of mycotoxin analysis has been made during the last few years (*7–10*). Simplified sample cleanup protocols and new chromatographic methods, especially HPLC, and other chemical methods have been developed in recent years; these methods require extensive sample cleanup. To overcome

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the difficulties encountered with the chemical and biological methods, new immunochemical methods have been developed (*11–22*). Within the last few years, wide application of immunoassays for mycotoxins has been noted (*10,12,14–16,19–24*). There has been a rapid increase in publications in this area. For example, in the 1991 and 1994 AOAC mycotoxin-associated refereed reports, as many as 25–28% of the cited publications on mycotoxin analysis were immunoassay-related articles (*8,9,25,26*). Many immunoassay kits for mycotoxin analysis are also commercially available (*2,12,14,21,25*). The application of mycotoxin immunoassay is not limited to foods and feeds; it has been used as a sensitive approach to monitoring mycotoxins in body fluids and tissues or organs of humans and animals who have been exposed to mycotoxins (*10,14,16*). Thus, a new dimension of methodology for mycotoxin analysis as well as a new tool for diagnosis of mycotoxicoses in humans and animals has emerged since we first initiated research to develop an immunoassay for mycotoxins in the early 1970s (*11,13,14*). In this symposium, recent progress on the immunoassay of this group of mycotoxins will be highlighted. For a detailed discussion on immunoassays and earlier literature, several of the most recent reviews should be consulted (*10,11,13,16,19–21,24,27*). For the specificity of antibodies against various mycotoxins, see Chu (*14,16,23*).

Before further discussion on the progress of recent developments, four basic criteria on immunoassays for mycotoxin analysis should be considered: (1) Because most immunoassays for mycotoxins are based on the competition of binding between unlabeled toxin in the sample and labeled toxin in the assay system for the specific binding sites of antibody molecules, a well-labeled mycotoxin (as a marker) is needed in the assay system, in addition to a specific antibody (*10,13,14,16,23*). (2) For accurate quantification, a good method for the separation of free and bound forms of toxin is important. (3) Depending on the approaches that have been used for raising antibodies, the degree of cross-reactivity (specificity) of these antibodies with their respective structural analogs varies considerably; thus, one must be familiar with the specificity of the antibody to be used in the assay system. (4) Because there is always a possibility of the presence of some structurally related compounds in the sample that may react with the antibody, the sample matrix should be tested before the assay. In most of the immunoassays described below, sample cleanup is not necessary. Samples taken after extraction from the solid matrix could be used directly in the assay after appropriate dilution in the assay buffer. Nevertheless, the sensitivity increased after appropriate cleanup treatment. Thus, my discussion on recent progress will center around these subjects.

New Approaches to the Preparation of Mycotoxin–Protein Conjugates and Antibody Production

Mycotoxins are low molecular weight, secondary fungal metabolites that are not immunogenic. Thus, they must first be conjugated to a protein/polypeptide carrier before subsequent use in immunization (*10,16,17*). Considerable efforts in earlier studies were focused on the development of methods of preparation of mycotoxin derivatives and conjugation of mycotoxins to a protein or polypeptide carrier. Some of these methods, including a water soluble carbodiimide method and mixed anhydride methods, are still widely used. With more mycotoxins of diverse chemical structures being studied, other methods also have been used in recent years. For example, cyclopiazonic acid and ergot alkaloids were conjugated to proteins via the Mannich condensation method in the presence of formaldehyde (*28–30*). Fumonisin B_1 (FB_1) was cross-linked to a carrier protein after being activated with glutaraldehyde; the linkage was then stabilized with sodium borohydride (*31–36*). The activated ester method, including the formation of *N*-hydroxysuccinimide (NHS) esters (*37*), 1,1'-carbonylimidazole (*38*) and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester, also have been used (*34*). Conjugation of mycotoxins to a marker enzyme such as horseradish peroxidase (HRP) was generally done by the NHS, water soluble carbodiimide method (*16*), and the periodate method of Nakane and Kawaoi (*39*). While HRP was used most commonly in the ELISA, other enzymes such as alkaline phosphatase also have been used (*14*). To alleviate the problem that the antibodies may have nonspecific cross-reaction with the residues in the approximate linking-bridge region, carrier protein conjugates different from those used in the preparation of the immunogen are often used in the immunoassays.

Both polyclonal (Pab) and monoclonal (Mab) antibody techniques have been used for the production of antibodies against mycotoxins. While rabbits are still most commonly used as the animal species for production of Pab, other animals such as goats, pigs and sheep (*40–42*) also have been used. Production of antibodies against mycotoxins also has been achieved by immunizing hens; the antibodies (IgY) were then recovered from eggs (*43–46*). Clarke *et al.* (*43*) have developed an efficient protocol for the recovery of antibodies against ochratoxin A from eggs. Polyclonal antibodies against mycotoxins also have been obtained from the ascites fluid of BALB/c mice using the method of Kurpisz *et al.* (*47*). The murine antibodies were useful for immunoassay of fumonisins (F) (*32*). Antibodies against almost all the important mycotoxins are now available (Table I, ref. *48–72*). Details regarding their specificities can be seen from the original papers and from several reviews (*10,14,16,23*). In general, most of these antibodies are very specific for the respective mycotoxins.

From both toxicological and regulatory points of view, it is essential to have generic antibodies that are cross-reactive with a group of mycotoxins. Research in this area has not been very successful. One of the approaches that has been used in our laboratory involved generating antibodies against the acetylated trichothecenes so that once the toxins are acetylated, they will then react with the antibodies (*73–76*). These antibodies have been useful in determining the total type A and type B trichothecenes in naturally contaminated samples (*77–79*). However, this approach involves an additional chemical treatment of the sample before immunoassay. For aflatoxin, we used aflatoxin B_3 as the starting toxin in generating antibodies that have good cross-reactivities with other aflatoxins (*80,81*).

Other than mycotoxins, antibodies against specific fungi (*82–86*) as well as several enzymes involved in the biosynthesis of aflatoxin (*87,88*) and trichothecenes (*78,89*) have been generated. These antibodies have been used for identifying specific fungi in foods, for studies of the kinetics of enzymes involved in the biosynthesis of mycotoxins, and for cloning genes that encode the enzymes for mycotoxin synthesis.

Table I. Antibodies Against Mycotoxins[a–c]

Mycotoxins	*Type*
AAL toxin	Pab
Aflatoxins: B_1, B_2, G_1, G_2	Mab, Pab
Aflatoxin metabolites: B_{2a}, Q_1, M_1, aflatoxicol, DNA adducts	Mab, Pab
Citrinin	Pab
Cyclopiazonic acid	Mab, Pab
Ergot alkaloids	Mab, Pab
Fusarochromanone	Pab
Fumonisin	Mab, Pab
Kojic acid	Pab
Ochratoxin A	Mab, Pab
Patulin	Pab
Paxilline related	Mab, Pab
PR-toxin	Pab
Rubratoxin B	Pab
Secalonic acid	Pab
Sporidesmin	Mab, Pab
Sterigmatocystin	Pab
Trichothecenes: DAS, DON, FX, DOVE, AcDON, NIV, Roridin A, T-2 toxin and T-2 toxin metabolites (HT-2, T-2-tetraacetate 3'-OH-T-2, dep-T-2)	Mab, Pab
Versicolorin A	Pab
Zearalenone	Mab, Pab

[a] References for most mycotoxins: see Chu (*10,14,16,23*).

[b] Additional references: AAL toxin (*chapter by Szurdoki* et al., *this volume*), AFB (*44,48*), Citrinin (*49*), Cyclopiazonic acid (*28–30*), DON and acetyl-DON (*50–54, chapter by Schmitt* et al., *this volume*), Ergot alkaloids (*55–58, chapter by Shelby, this volume*), Fumonisin (*23,32–36,59–61, chapter by Maragos* et al., *this volume*), Fusarochromanone (*62*), OTA (*38,43,63*), Patulin (*64*), Paxilline related (*40–42*), Sporidesmin (*41,65,66*), Sterigmatocystin (ST; *67,68*), T-2 toxin (*69*), versicolorin A (*70*), Zearalenone (ZE; *69,71,72*).

[c] Abbreviations used: AF, aflatoxin; DAS, diacetoxyscirpenol; DON, deoxynivalenol; AcDON, Acetyl-DON; DOVE, deoxyverrucarol; FX, fusarenon-X; OTA, ochratoxin A; NIV, nivalenol; dep-T-2, de-epoxide T-2 toxin; Mab, monoclonal antibody; Pab, polyclonal antibody.

Anti-idiotype and Anti-anti-idiotype Antibodies for Selected Mycotoxins

The development of immunochemical methods for mycotoxin detection has led to a great demand for specific antibodies and related immunochemical reagents for the assay. An alternate approach to preparing immunochemical reagents is through generating anti-idiotype (anti-ID) antibodies (*90*). Anti-idiotype antibodies (Ab2) for large molecules have been well-developed and have been applied to clinical diagnosis and immunotherapy (*90*). Recent success in generating Ab2 against a number of small molecular weight haptens, including mycotoxins such as T-2 toxin (*91,92*), prompted our interest in generating anti-ID antibodies for other mycotoxins. We have successfully obtained various types of anti-ID and anti-anti-ID (Ab3) antibodies for aflatoxins (*45,93,94*) and fumonisin B_1 (FB_1) (*95*).

AB2 for mycotoxins were demonstrated in different animal species after immunization with the affinity-purified original idiotype antibodies (Ab1) (*93,95*). Ab2 were not only bound specifically to the original Ab1, but also were capable of being used as an immunogen in generating Ab3. Whereas Ab2 could not be used as a mycotoxin–protein conjugate in the indirect ELISA for the determination of aflatoxin and T-2 toxin, an Ab2-based indirect ELISA has been established for fumonisin analysis (*95*). Thus, these anti-idiotype antibodies are indeed the surrogates of mycotoxins. Recently, a hybridoma cell line that generates monoclonal Ab3 was obtained in our laboratory (Yu and Chu, in preparation). Table II compares the concentrations of free mycotoxins causing 50% inhibition (ID_{50} values) of binding of mycotoxin-marker to the Ab3 with the original Ab1. It is apparent that the anti-anti-idiotype antibodies have similar characteristics to the idiotype antibodies.

The availability of anti-idiotype and anti-anti-idiotype antibodies for mycotoxins has provided a new generation of immunochemical reagents, which could be used for both therapeutic and analytical purposes. Ab2, a surrogate of the toxin, could be used as the immunogen in generating antibodies for the toxin (thus, a vaccine), and also could be used in the immunoassay. In an *in vitro* study of the effect of antibodies on the binding of aflatoxin to DNA, Hsu (*96*) found that Ab2 were capable of inhibiting the binding of aflatoxin B_1 to DNA, but the inhibitory effect was not as high as the Ab1. Data from Table II indicates that Ab3 could be used as the antibody in the ELISA for toxin analysis.

Immunoassays for Screening and Detecting Mycotoxins in Foods and Feeds

With the availability of antibodies against mycotoxins, different immunoassays have been developed. While some of these methods could only be used in the screening tests, others provide quantitative information. A wide number of applications of immunoassays for mycotoxins have appeared in the last few years. Some of these methods also have been adopted as the first action by the AOAC (*7–9,21,25,26*).

Quantitative Immunoassays

Radioimmunoassay (RIA). Although radioimmunoassay was developed in the early phase of immunochemical studies, it is still used in some laboratories. RIA has been used for the analysis of aflatoxins in corn, wheat, peanuts, milk, serum, and eggs as well as for deoxynivalenol (DON) in corn and wheat, ochratoxin A (OTA) in serum and kidney, nivalenol in barley, PR toxin in cheese, and T-2 toxins in corn, wheat, serum, and urine (*11,13,14*). Mycotoxins labelled with ^{14}C, ^{3}H, and ^{125}I were used as the marker in most studies. Generally, RIA can detect 0.25–0.5 ng of purified mycotoxin in each analysis when tritiated mycotoxins are used as the markers. However, because of the sample matrix interference, the lower limit for mycotoxin detection in food or feed samples is about 2–5 ppb. Higher sensitivity, 0.004–0.1 ng/assay, could be achieved by using radioactive markers of high specific activity (*14*), for example, iodinated-mycotoxin marker. The sensitivity of RIA also can be improved by including a simple cleanup procedure after extraction. As newer solid-phase matrices and more immunochemical reagents became available, more efficient methods for the separation of free and bound toxin were developed (*14*). Thus, separation could be achieved by a simple filtration or centrifugation step. Although RIA provides accurate data for mycotoxin analysis, the method involves the use of a radioactive ligand. Thus, it has been primarily used in the laboratory for research purposes. Nevertheless, an RIA-based immunoassay kit for mycotoxin analysis is commercially available (*97*).

Competitive Enzyme-Linked Immunosorbent Assays (cELISA). Two types of ELISA have been used for the analysis of mycotoxins, and both types are heterogeneous competitive assays. In the competitive direct ELISA (cdELISA), antibodies against mycotoxins are coated on the ELISA plate. The mycotoxin-enzyme conjugate (marker) and free mycotoxin compete for the same binding site on the solid-phase antibody. In the competitive indirect ELISA (ciELISA), a mycotoxin–protein (or polypeptide) conjugate is first prepared and then coated to the microtiter plate before assay. The plate is then incubated with specific rabbit (or other type) antibody in the presence or absence of the homologous mycotoxin. The amount of antibody bound to the plate coated with mycotoxin–protein conjugate is then determined by reaction with goat antirabbit (or anti-other type) IgG-enzyme complex which is generally commercially available, and by subsequent reaction with the substrate. Thus, toxin in the samples and toxin in the solid-phase compete for the same binding site with the specific antibody in the solution. Although horseradish peroxidase (HRP) is most commonly used as the enzyme for conjugation, other enzymes such as alkaline phosphatase and beta-galactosidase also have been used (*10,14,19,21–24*).

In general, cELISA is approximately 10–100 times more sensitive than RIA when purified mycotoxins are used. As little as 2.5 pg of pure mycotoxin can be measured. Since a cleanup step is generally not necessary, many samples can be analyzed within a relatively short period. The sensitivity of cELISA for the determination of various mycotoxins in different commodities and body fluids is summarized in Tables III and IV. cdELISA can detect 0.05–50 ppb of mycotoxins in foods and feeds (*10,14*). Like RIA, the sensitivity of cELISA is improved when a cleanup treatment is included in the assay protocols (*14*). While many examples could be cited, a recent report demonstrated this problem well. Hongyo *et al.* (*98*) found a good correlation between the data obtained from a one-step ELISA of AF in corn with either HPLC or TLC, but the correlation between the ELISA data for AF in the mixed feed with HPLC and TLC was poor. In

Table II. ID_{50} Concentration (ng/mL) for AFB_1 and FB_1 Idiotype and Anti-anti-idiotype Antibodies*

Antibody types	*cdELISA*	*ciELISA*	*RIA*
AFB_1-Mab1	2.5	13.4	14.0
AFB_1-Pab1	0.1	0.6	7.8
AFB_1-Pab3	0.2	1.8	ND
AFB_1-Mab1	ND	ND	3.2
AFB_3-Mab1	ND	ND	5.3
AFB_3-Pab3	0.4	ND	0.9
AFB_1-IgY1	1.8	20.9	ND
AFB_3-IgY3	0.1	2.2	ND
FB_1-Mab1	ND	140	ND
FB_1-Pab3	ND	190	ND
FB_1-Mab3	ND	73	ND

*Abbreviations used: ID_{50}, concentration of toxin (such as AFB_1 or FB_1), causing 50% inhibition of the binding of antibody to the marker toxin. AFB_1, aflatoxin B_1; AFB_3, aflatoxin B_3; FB_1, fumonisin B_1; Mab_1, idiotype monoclonal antibody (original first antibody); Pab_1, idiotype polyclonal antibody; Mab_3, monoclonal anti-anti-idiotype antibody; Pab_3, polyclonal anti-anti-idiotype antibody; d, direct; i, indirect.

Table III. Sensitivity of Competitive Direct ELISA for Selected Mycotoxins[a,b]

Mycotoxins	*Foods/Feeds*	*Standard range (ng/assay)*	*Detection limits (μg/kg) or (μg/L)*
AFB/AFs	C, Wh, P, Pb	0.0025–1	1–10 (1)[c]
AFM	M, Ch	0.0012–1	0.10 (0.01)
DON	C, Wh	0.012–125	1000 (10)
15-Ac-DON	Wh	0.002–0.025	50–100
F	C, Mf	0.005–50	10–500
H-F	C	0.1–15	(5–10)
OTA	Wh, B	0.025–0.5	30 (1–2)
T-2	C, Wh	0.0025–0.2	2.5–50 (1)
Type A Tctc	C	0.0025–0.2	50–100
ZE	C	0.025–2.5	50

[a] Data and references: see Chu (*10,23*).

[b] Abbreviations used: B, barley; C, corn; Ch, cheese; F, fumonisin; H-F, hydrolyzed F; M, milk; Mf, mixed feed; P, peanuts; Pb, peanut butter; Wh, wheat; OTA, ochratoxin A; Tctc, trichothecenes; other abbreviations, see Table I.

[c] Value in parenthesis indicates that samples had been subjected to a cleanup treatment before immunoassay.

In Immunoassays for Residue Analysis; Beier, R., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

contrast, a good correlation was obtained when the mixed feed was subjected to column chromatography before ELISA. The efficacy of cELISA of fumonisin was also improved after a cleanup treatment of the samples (*23,36,61,95*). Due to the use of better antibody and toxin–enzyme conjugates, the time required to run the cELISA has improved considerably. Thus, the entire cELISA procedure can be completed within one hour (*14,19,21,97,99*). Better sensitivity also could be achieved by using different substrates. For example, a more sensitive substrate such as tetramethylbenzidine has been used for immunoassays using HRP as the marker enzyme (*36*). To save antibodies, Pesavento and Carter (*100*) have covalently conjugated antibodies against aflatoxin to the chemically activated hydrophilic membrane in the ELISA plate, which could be regenerated a number of times for repeated aflatoxin analysis.

The ciELISA also has been widely used for the analysis of a number of mycotoxins (*10,14,21,23*) with a sensitivity that is comparable to or slightly better than the direct cELISA in some cases (Table IV). This type of ELISA requires less antibody (100 times less) and does not require preparation of a toxin–enzyme conjugate. However, it requires more analytical time (2 h). To optimize the assay condition, the selection of secondary antibody–enzyme conjugate in the ciELISA is important. In a recent study for example, Okumura *et al.* (*101,102*) found that the sensitivity of their Mab-based ciELISA for aflatoxin M_1 was 50 times more sensitive if they used HRP-labeled anti-mouse antibody versus an alkaline phosphate-labeled system. To shorten the assay time for the ciELISA, two modifications were made by several investigators. One involved the conjugation of antibody to an enzyme, which is then used in the ELISA instead of using a second antibody–enzyme conjugate, and the other involved premixing the antibody with the second antibody–enzyme conjugate before the assay (*14,21,103,104*).

In addition to the antibody affinity, the efficacy of marker enzymes, the enzyme substrate and the sample matrices, the presence of an extraction solvent system also greatly affects the ELISA performance. Early studies showed that ELISAs could run in concentrations of methanol as high as 20–30% (*11,14*). Generally, samples contain 7–15% methanol in phosphate buffer. In the ELISA of hydrolyzed fumonisin B_1, Maragos *et al.* (*61*) found that more dilution was necessary for the corn samples extracted with acetonitrile than those extracted with methanol. This effect could be due to the solvent itself, or perhaps it was due to interference materials that were extracted by the acetonitrile.

A number of studies were carried out in recent years to investigate the efficacy of both direct and indirect immunoassays by comparing them with HPLC and TLC. Whereas good correlation has been found in most immunoassays (*10,14,21*), problems do exist for some assays. For example, data obtained from immunoassays of fumonisin were always higher than those obtained from chemical analysis (*23,105*). The problems encountered in the immunoassay of fumonisin was attributed to the cross-reaction of the antibodies with some structurally related compounds and could be overcome to some degree by using antibodies with higher affinity to the toxin (*98,106*). Collaborative studies for some ELISA protocols have been made. Several quantitative ELISA methods for the analysis or screening of mycotoxins have been adopted as the first action by AOAC (*8,9,25,26*).

Antibody Based-Immunoscreening Tests

By shortening the incubation time and adjusting the antibody and enzyme concentrations in the direct or modified indirect competitive microtiter plate ELISA assay system, it is possible to do a quick screening test at certain toxin levels (e.g., 20 ppb) (*2,12,14,99,107–111*). Based on the same principle as the competitive ELISA, several other types of immunoscreening tests with sensitivity similar to the ELISA also were developed. Rather than coating the antibody onto the microtiter plate, the antibody is immobilized on a paper disk or other membrane (*111–117*), which is used directly as a strip (*111*) or mounted either on a plastic card (card screen test), a plastic strip (as dipstick; *117,118*), in a plastic cup (Cup test and Cite), or in a syringe (Cite probe and Idexx probe; *112*). Antibodies also have been coated on polystyrene beads (*119,120*). The reaction is carried out on the wetted membrane disk. Thus, after reaction, the absence of color (or decrease in color), generally blue, at the sample spot indicates the presence of toxin in the sample. The reaction is generally very rapid and takes less than 10–15 min to complete. A monoclonal antibody-based dipstick immunoassay method was developed by Dewey *et al.* (*82*) for quick screening of *Penicillium islandicum* in rice grains.

Another screening test is the immunoaffinity method, which was originally designed for mycotoxins that fluoresce such as aflatoxins, ochratoxins, and zearalenone (*10,14,116,121–126*). In the assay, the sample extracts diluted in phosphate buffer are applied to the affinity columns in which specific antibody was covalently bound to the solid-matrix. After washing to remove the unbound materials, the specific mycotoxin is then eluted from the column with the appropriate solvent system and then subjected to other chemical analyses. For mycotoxins with native fluorescence such as aflatoxin, ochratoxin and zearalenone, the toxin level in the eluate could be directly determined fluorometrically or be determined after derivatization to enhance the fluorescence (*124–127*). For fumonisin screening, it is necessary to introduce a fluorophore to the materials eluted from the IAF column (*21,23*). The sensitivity to the IAC screening tests for aflatoxin B_1, fumonisin B_1, ochratoxin A and zearalenone was 2 ppb, 1 ppm, 5 ppb and 0.2 ppm, respectively.

The application of various screening tests to mycotoxins has been summarized by Chu (*10,14,23*) and Pestka (*21*), and most of the screening tests are commercially available as kits (*2,12,21,128*). All of the rapid screening test kits permit monitoring of mycotoxins semiquantitatively and have been found to be effective in screening mycotoxins in the field by FSIGS (*2,21,128,129*). Other evaluations of commercial kits also concluded that such kits could be used for screening tests (*12,26,71,130–133*). Collaborative studies for some of these immunoscreening tests have been made, and some of them have been adopted by the AOAC as first action for the screening of AFs in different commodities (*8,9,14,21,26,115,126,132*). With the increasing availability of commercial immunoassay kits, the AOAC International has established a Research Institute to evaluate the performance of different kits (*9,25,26*).

Complementing Chemical Analyses by Immunochemical Methods

Affinity Chromatography. With the availability of antibodies against various mycotoxins, immunoaffinity (IAF) columns were made by conjugating the antibodies to a solid-phase matrix. These columns are then used either in a screening test as discussed above or as a cleanup column for subsequent chemical analysis (*10,16,23*). An IAF column was first used in the RIA (*134*) and later for recovery of AFM_1 from urine and milk samples (*135*) for subsequent analyses. Although earlier application of this technique was primarily aimed at biological fluids (*123–125,136*), the IAF column has gained wide application as a clean-up tool for a number of mycotoxins and is not limited to fluid samples (*10,16,23*). Immunoaffinity columns for a number of mycotoxins also are commercially available. Table V summarizes some of the recent applications of IAF technology to mycotoxin analysis. A number of collaborative studies indicate that this technique is an efficient method for clean-up of aflatoxins (*10,127,142,143*). A combination of an IAF column and post-column derivatization (PsCD) has gained first action as the official method for screening and clean-up of AF in several commodities (*126*). Automation involving the use of an affinity column and HPLC was developed for routine analysis of AFM in milk (*137,138,159,160*), AFB in peanut butter (*144*), peanuts and corn (*161*), in other nuts (162) and OTA in cereal and animal products (*155*) and fumonisins (*133, 163, see chapter by Trucksess and Abouzied, this volume*).

Immunochromatography

With the availability of sensitive ELISA methods, this technique has proved effective as a postcolumn monitoring system for HPLC (*164*). This is especially useful for the analysis of compounds with no specific absorption, such as trichothecenes (TCTCs). For example, in the analysis of various type A TCTC mycotoxins, the sample extract with no clean-up treatment was first subjected to HPLC with a C_{18} reversed-phase column. Individual fractions eluted from the column were analyzed by ELISA using "generic" antibodies against group A trichothecenes. This approach can not only identify each individual group A TCTC, but also can determine their concentration quantitatively. As little as 2 ng of T-2 toxin and related TCTCs as well as their metabolites can be monitored by this method. A combination of HPLC and ELISA technology proved to be an efficient, sensitive, and specific method for the analysis of TCTC (*73,79*) and other mycotoxins (*67,68,165*). Likewise, immunoassay has been used in combination with TLC (*165*) in which the crude sample extract was applied to the TLC plate. After separation, each fraction in TLC was analyzed immunochemically.

Other Immunochemical Approaches

Several new immunochemical approaches were developed recently. In the so-called "hit-and-run" assay (*166,167*) for T-2 toxin, a T-2 toxin column was equilibrated with a fluorescein isothiocyanate (FITC)-labeled Fab fragment of IgG (anti-T-2 toxin). Samples containing T-2 toxin were injected onto the column. The FITC-Fab that eluted together

Table IV. Sensitivity of Competitive Indirect ELISA for Selected Mycotoxins[a,b]

Mycotoxins	*Foods/Feeds*	*Standard range (ng/assay)*	*Detection limits (μg/kg) or (μg/L)*
ciELISA			
AFs	C, P, Pb	0.0002–1	0.25–5
AFM	M	0.0001–0.005	0.005
DAS	C, Wh	0.005–5	300 (1)[c]
DON	C, Wh	0.010–100	1000 (10)
3-Ac-DON	R	0.005–1	1
F	C	1–100	200
NIV	B	0.05–5	(30)
OTA	B, K, Mf, Wh	0.005–10	0.06–50 (1)
T-2	M, S, U	0.002–0.2	5 (0.2–1)
HT-2	U	0.005–0.5	(0.5)
ST	S, U	0.01–1	5 (0.05)
ZE	C, W, Mf	0.05–2.5	1–60
Md-ciELISA			
AFB	B, P, Pb, C	0.01–1.5	10
T-2	B	0.05–5	(50)

[a] Data and references: see Chu (*10,23*).

[b] Abbreviations used: B, barley; C, corn; Ch, cheese; K, kidney; M, milk; Mf, mixed feed; P, peanuts; Pb, peanut butter; R, rye; S, serum; Wh, wheat; U, urine; i, indirect; Md, modified; other abbreviations, see Tables I–III.

[c] Value in parenthesis indicates that samples had been subjected to a cleanup treatment before immunoassay.

Table V. Immunoaffinity Chromatography of Mycotoxins

Mycotoxins	*Commodities*	*Analysis*	*References*
AFM	M[a]	Fl[b]	*123,137,138*
AFB	F, P	TLC	*139,140*
AFB	P, nuts, figs, etc	HPLC/PsCD	*141–144*
AFs	C, P, Ct, F, Pb	Fl/Br, HPLC/PCD	*124,126,129,145*
AFQ_1	U	HPLC	*146*
AF adducts	T, S, U	HPLC	*136,147–151*
AF-albumin	S	HPLC, ELISA	*150,152,153*
FB	C	HPLC	*133*
OTA	Cff, T, cereals	HPLC	*154–156*
ZE	M, U	GC/MS, ELISA	*157,158*

[a] Commodities tested: Cff, coffee; T, animal tissues; other abbreviations are described in Tables I–III.

[b] Methods for final analysis: Fl/Br and Fl represent fluorometric analysis of the solution eluted from the column with and without treatment with bromine solution, respectively.

with the samples containing T-2 toxin were then determined in a standard flow-through fluorometer. A similar approach was reported in which ribonuclease-labeled Fab was used as the indicator (*167*). Another approach that may lead to the development of a biosensor is a homogeneous immunoassay for T-2 toxin, which involves the use of liposomes and complement (*168,169*). However, neither of these two methods are very sensitive when compared to other immunochemical methods. An approach called HPTLC (high-performance TLC)-ELISAgram was introduced by Pestka (*170*). This method involves separation of mycotoxins using HPTLC, followed by blotting the chromatogram onto a nitrocellulose membrane coated with antibody, incubation with mycotoxin–enzyme conjugate, and a final incubation with substrate to develop the color. Although this method has good sensitivity, the need for a large amount of antibody limits its wide application. Instead of coating antibodies to the ELISA plate, Abouzied and Pestka (*171*) immobilized different monoclonal antibodies against aflatoxin B_1, fumonisin B_1 and zearaleneone, as multiple lines on the nitrocellulose strip; respective mycotoxin–peroxidase conjugates were used as the testing markers. In the assay, free mycotoxin and the mycotoxin–peroxidase are competing for the binding site of the antibody immobilized on the solid-phase nitrocellulose. After reacting with substrate, the color intensity was detected; a video- and computer-assisted system was used to estimate the toxin levels. These investigators were able to screen all three mycotoxins simultaneously with detection limits of 0.5, 500, and 3 ng/mL for aflatoxin B_1, FB_1 and zearalenone, respectively. Most recently, a time-resolved fluoroimmunoassay was developed for AF analysis (*172,173*). This method involves the use of Europium ion (Eu)-labeled antibodies and has a sensitivity similar to most ELISA methods with an I_{50} (50% inhibition of binding) concentration of 0.2 ng AFB_1/mL.

Cloning of Antibodies Against Mycotoxins

Several laboratories have initiated work on cloning of the antibodies against mycotoxins. A monoclonal antibody against aflatoxin was successfully cloned and expressed in *E. coli* by Tseng (*174*). However, details of methods used or properties of the antibody were not given. In our laboratory (Chu *et al.*, University of Wisconsin, in preparation), the Fab fragment of the heavy (H) and light (L) chains of IgG for a monoclonal antibody against aflatoxin B_1 were selectively amplified by PCR from the cDNA, cloned into a M13 phagemid vector and then expressed in *E. coli*. The DNA sequences encoding the variable regions of H and L chains of the Fab fragment of IgG for the monoclonal antibody were determined. Radioimmunoassay revealed that the reconstructed polypeptides were capable of binding aflatoxin (AFB_1). Free AFB_1 inhibited the binding of radioactive ^3H-AFB_1 at an ID_{50} of 2.43 ng/mL. The nucleotide sequence for the heavy chain and light chain variable regions of the anti-aflatoxin IgG were 90% and 92% similar to anti-cd cell surface phosphoprotein and anti-rheumatoid factor auto antibody, respectively. The nucleotide sequences for Mab against T-2 toxin also were determined. Sequence analysis revealed that nucleotide sequence for the heavy chain and light chain variable regions of this Mab were 93% and 92% similar to anti-human carcinoma and anti-RNA-auto antibody, respectively.

Concluding Remarks

During the last few years immunoassay techniques have gained wide acceptance as analytical tools for mycotoxins. Antibodies against almost all the important mycotoxins are currently available. Sensitivity, simple, and specific immunoassays have been established for the analysis of various mycotoxins in foods, feed, animal tissue and body fluids. Several immunoassay protocols have been adopted as first action by the AOAC. Immunoscreening methods have been widely accepted as a simple method for screening of aflatoxin in several commodities and many immunoscreening kits are commerically available. The immunoaffinity method also has become popular as a cleanup method in conjunction with other chemical methods. Immunochemical methods also have been used in various toxicological studies including immunohistological examination of patients' tissues (*175,176*), analysis of mycotoxin metabolites, and in the epidemiological studies to find a correlation between mycotoxin exposures and certain diseases. Recent developments have led to the production of antibodies against specific groups of fungi in foods which are used for the determination of specific molds that contaminate foods. Antibodies for the key enzymes involved in the biosynthesis of aflatoxin and trichothecenes have been raised and were used effectively in cloning genes for some of these enzymes. Such antibodies could be used in the future for controling toxin formation. Production of anti-idiotype and anti-anti-idiotype antibodies against several important mycotoxins has been been demonstrated. These antibodies were effective in an ELISA and could possibly be used as a vaccine in the future.

Since the sensitivity of many immunoassays is very high, the analyst needs to make a decision regarding whether a cleanup step is necessary to achieve a specific sensitivity. Improvements in the production of some immunoassay reagents and protocols are still needed. In most cases, a low antibody affinity for the analyte is considered by this reviewer to be one of the major factors attributed to such problems. Thus, future efforts should be directed at generating high affinity antibodies for some mycotoxins, e.g. fumonisins. Better labeling techniques, including fluorescent-labeled antibodies/mycotoxins, also should be tested to evaluate the possibility of achieving a more sensitive and rapid method for the toxin detection. With new approaches to the production of antibodies, including using anti-idiotype and anti-anti-idiotype antibodies and structural modulation (*177,178*) it is probable that better and high affinity antibodies can be obtained in the near future. Several laboratories, including our lab, have initiated work in cloning these antibodies. It will not be long before we understand how these antibodies react with these mycotoxins; thus, a new generation of antibodies could be made available through point mutation. With newer methods being used in the production of hapten–protein conjugates as described elsewhere in this book, cloning of antibodies as well as new developments in the biotechnology and biosensor areas, immunoassay for mycotoxins will be advanced to another new era. I hope that the present review will help to generate more interest in using immunochemical methods in the analytical, diagnostic and possibly therapeutic areas. I also hope that it will stimulate additional research in this rapidly progressing area to improve the methodology, to simplify the assay procedures, and to increase the sensitivity and specificity of the assay procedure in order to alleviate matrix interference problems.

Acknowledgements

This work was supported by the College of Agricultural and Life Sciences, the University of Wisconsin, Madison, WI. Part of the work described in this contribution was supported by a Public Health Service grant (CA 15064) from the National Cancer Institute, a contract (DAMD17-86-C-6173) from the U.S. Army Medical Research and Development Command of the Department of Defense, an USDA grant (58-6435-1-116) and an USDA North Central Regional Project (NC-129). The author thanks Carole Ayres and Barbara Cochrane for their help in preparing the manuscript.

Literature Cited

1. Busby, W. F.; Wogan, G. N. In *Mycotoxins and N-nitrosocompounds, Environmental Risks*; Shank, R. C., ed.; CRC Press: Boca Raton, FL, 1981, Vol. II; pp 3–45.
2. CAST. *Mycotoxins, Economic and Health Risks;* Council of Agricultural Science and Technology (CAST), Task force report No. 116, CAST: Ames, IA, 1989; 92 pp.
3. Cole, R. J.; Cox, R. H. *Handbook of Toxic Fungal Metabolites*; Academic Press: New York, NY, 1981; 937 pp.
4. Eaton, D. L.; Groopman, J. D. *The Toxicology of Aflatoxins—Human Health, Veterinary and Agricultural Significance*; Academic Press: New York, NY, 1994; 544 pp.
5. Miller, J. D.; Trenholm, H. L. *Mycotoxins in Grain: Compounds Other Than Aflatoxin*; Eagan Press: St. Paul, MN, 1994; 552 pp.
6. Sharma, R. P.; Salunkhe, D. K. *Mycotoxin and Phytoalexins in Human and Animal Health*; CRC Press: Boca Raton, FL, 1991.
7. Scott, P. M. In *Analysis of Oil Seeds and Fatty Acid*; Rossel, J. B.; Pritchard, J. L. R., eds.; Elsevier App. Sci.: New York, NY, 1991; pp 141–184.
8. Scott, P. M. *J. Assoc. Off. Anal. Chem.* **1992**, *75*, 95–102.
9. Scott, P. M. *J. AOAC Int.* **1993**, *76*, 112–119.
10. Chu, F. S. In *Analyzing Food for Nutrition Labeling and Hazardous Contaminants;* Jeon, I. J.; Ikins, W. G., eds.; Marcel Dekker, Inc.: New York, NY, 1995; pp 283–332.
11. Chu, F. S. In *Modern Methods in the Analysis and Structural Elucidation of Mycotoxins*; Cole, R. J., ed.; Academic Press: New York, NY, 1986; pp 207–237.
12. Chu, F. S. *Vet. Hum. Toxicol.* **1990**, *32* (Suppl.), 42–50.
13. Chu, F. S. In *Immunoassays for Trace Chemical Analysis: Monitoring Toxic Chemicals in Humans, Foods and the Environment*; Vanderlaan, M.; Stanker, L. H.; Watkins, B. E.; and Roberts, D. W., eds.; American Chemical Society: Washington, DC, 1991;ACS Symposium Series 451, pp 140–157.
14. Chu, F. S. In *Handbook of Applied Mycology, Mycotoxins*; Bhatnagar, D.; Lillehoj, E. B.; Arora, D. K., eds.; Marcel Dekker, Inc.: New York, NY, 1991, Vol. V; pp 87–136.
15. Chu, F. S. *J. Anim. Sci.* **1992**, 70, 3950–3963.

16. Chu, F. S. In *The Toxicology of Aflatoxins—Human Health, Veterinary and Agricultural Significance*; Eaton, D. L.; Groopman, J. D., eds.; Academic Press: New York, NY, 1994, pp 451–481.
17. Morgan, M. R. A. *J. Assoc. Pub. Anal.* **1985**, *23*, 59–63.
18. Morgan, M. R. A. *Tetrahedron* **1989**, *45*, 2237–2249.
19. Morgan, M. R. A.; Lee, H. A. In *Development and Application of Immunoassay for Food Analysis*; Rittenburg, J. H., ed.; Elsevier: New York, NY, 1990; pp 143–170.
20. Pestka, J. J. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 1075–1081.
21. Pestka, J. J. *Food Agric. Immunol.* **1994**, *6*, 219–234.
22. Wilkinson, A. P.; Ward, C. M.; Morgan, M. R. A. In *Modern Methods of Plant Analysis, New Series. Plant Toxin Analysis*; Linskens, H. F.; Jackson, J. F., eds.; Springer–Verlag: Berlin, 1992; pp 185–225.
23. Chu, F. S. In *Fumonisins in Food*; Jackson, L.; DeVries, J. W.; Bullerman, L. B., eds; Plenum Publishing Co.: New York, NY, 1996, in press.
24. Martlbauer, E.; Dietrich, R.; Terplan, G. *Arch. Lenbensmittel.* **1991**, *42*, 3–6.
25. Trucksess, M. W. *J. AOAC Int.* **1994**, *77*, 135–142.
26. Trucksess, M. W. *J. AOAC Int.* **1995**, *78*, 135–141.
27. Fukal, L.; Kas, J. *Trends Anal. Chem.* **1989**, *8*, 112–116.
28. Hahnau, S.; Weiler, E. W. *J. Agric. Food Chem.* **1991**, *39*, 1887–1891.
29. Hahnau, S.; Weiler, E. W. *J. Agric. Food Chem.* **1993**, *41*, 1076–1080.
30. Huang, X.; Chu, F. S. *J. Agric. Food Chem.* **1993**, *41*, 329–333.
31. Avrameas, S.; Ternynck, T. *Immunochemistry* **1969**, *56*, 1729–1733.
32. Azcona-Olivera, J. I.; Abouzied, M. M.; Plattner, R. D.; Norred, W. P.; Pestka, J. J. *Appl. Environ. Microbiol.* **1992**, *58*, 169–173.
33. Azcona-Olivera, J. I.; Abouzied, M. M.; Plattner, R. D.; Pestka, J. J. *J. Agric. Food Chem.* **1992**, *40*, 531–534.
34. Fukuda, S.; Nagahara, A.; Kikuchi, M.; Kumagai, S. *Biosci. Biotechnol. Biochem.* **1994**, *58*, 765–767.
35. Shelby, R. A.; Rottinghaus, G. E.; Minor, H. C. *J. Agric. Food Chem.* **1994**, *42*, 2064–2067.
36. Usleber, E.; Straka, M.; Terplan, G. *J. Agric. Food Chem.* **1994**, *42*, 1392–1396.
37. Kitagawa, T. In *Enzyme—Immunoassay*; Ishikawa, E.; Kawai, T.; Miyai, K., eds.; Igaku-shoin: Tokyo, Japan, 1981; p 136.
38. Clarke, J. R.; Marquardt, R. R.; Frohlich, A. A.; Pitura, R. J. *J. Food Protect.* **1994**, *57*, 991–995.
39. Nakane, P. K.; Kawaoi, A. *J. Histochem. Cytochem.* **1974**, 22, 1084–1091.
40. Garthwaite, I.; Briggs, L. R.; Miles, C. O; Munday, S. C.; Towns, N. R. The products of monoclonal antibodies to paxilline. Ann. Report of Mycotoxin Diseases Group, Ruakura Agric. Center, Ministry of Agriculture and Fisheries: New Zealand, 1991; pp 12–14.
41. Garthwaite, I.; Sprosen, J.; Briggs, L.; Collin, R.; Towers, N. R. *Food Agric. Immunol.* **1994**, *6*, 123–129.
42. Garthwaite, I.; Shearer, S.; Towers, N. R. *Food Agric. Immunol.* **1994**, *6*, 235–239.

43. Clarke, J. R.; Marquardt, R. R.; Frohlich, A. A.; Madrid, F. J.; Dawood, M. *J. Agric. Food Chem.* **1993**, *41*, 1784–1789.
44. Hsu, K. H.; Chu, F. S. *Food Agric. Immunol.* **1992**, *4*, 83–91.
45. Hsu, K. H.; Chu, F. S. *Food Agric. Immunol.* **1995**, *7*, 163–174.
46. Poulson, A. *Immunol. Invest.* **1990**, *19*, 253–258.
47. Kurpisz, M.; Gupta, S. K.; Fulgham, D. L.; Alexander, N. J. *J. Immunol. Methods* **1988**, *115*, 195–198.
48. Ward, C. M.; Wilkinson, A. P.; Bramham, S.; Lee, H. A.; Chan, W. S.; Butcher, G. W.; Hutchings, A.; Morgan, M. R. A. *Mycotoxin Res.* **1990**, *6*, 73–83.
49. Abramson, D.; Usleber, E.; Märtlbauer, E. *Appl. Environ. Microbiol.* **1995**, *61*, 2007–2009.
50. Mills, E. N. C.; Alcock, S. M.; Lee, H. A.; Morgan, M. R. A. *Food Agric. Immunol.* **1990**, *2*, 109–118.
51. Abouzied, M. M.; Azcona-Olivera, J. I.; Yoshizawa, T.; Pestka, J. *J. Appl. Environ. Microbiol.* **1993**, *59*, 1264–1268.
52. Nicol, M. J.; Lauren, D. R.; Miles, C. O.; Jones, W. T. *Food Agric. Immunol.* **1993**, *5*, 199–209.
53. Usleber, E.; Märtlbauer, E.; Dietrich, R.; Terplan, G. *J. Agric. Food Chem.* **1991**, *39*, 2091–2095.
54. Usleber, E.; Renz, V.; Märtlbauer, E.; Terplan, G. *J. Vet. Med. B* **1992**, *39*, 617–627.
55. Hill, N. S.; Thompson, F. N.; Dawe, D. L.; Stuedemann, J. A. *Am. J. Vet. Res.* **1994**, *55*, 419–424.
56. Shelby, R. A.; Kelley, V. C. *Food Agric. Immunol.*, **1991**, *3*, 169–177.
57. Shelby, R. A.; Kelley, V. C. *J. Agric. Food Chem.* **1990**, *38*, 1130–1134.
58. Shelby, R. A.; Kelley, V. C. *J. Agric. Food Chem.* **1992**, *40*, 1090–1092.
59. Maragos, C. M. *J. Agric. Food Chem.* **1995**, *43,* in press.
60. Maragos, C. M.; Richard, J. L. *J. AOAC Int.* **1994**, *77*, 1162–1167.
61. Maragos, C. M.; Plattner, R. D.; Miklasz, S. D. *Food Addit. Contam.*, **1995**, *12*, in press.
62. Yu, J.; Wei, R.-D.; Mirocha, C. J.; Chu, F. S. *Food Agric. Immunol.* **1990**, *2*, 55–64.
63. Breitholtzemanuelsson, A.; Dalhammar, G.; Hult, K. *J. AOAC Int.* **1992**, *75*, 5824–5829.
64. McElroy, L. J.; Weiss, C. M. *Can. J. Microbiol.* **1993**, *39*, 861–863.
65. Briggs, L. R.; Collin, R. G.; Miles, C. O.; Towers, N. R. Production of antibodies binding sporidesmin. Ann. Report of Mycotoxin Diseases Group, Ruakura Agric. Center, Ministry of Agriculture and Fisheries: New Zealand, 1991.
66. Briggs, L. R.; Towers, N. R.; Molan, P. C. *J. Agric. Food Chem.* **1994**, *42*, 2769–2777.
67. Olson, J. J.; Chu, F. S. *Food Agric. Immunol.* **1993**, *5*, 3–12.
68. Olson, J. J.; Chu, F. S. *J. Agric. Food Chem.* **1993**, *41*, 602–606.
69. Barna-Vetro, T.; Gyongyosi, A.; Solti, L. *Appl. Environ. Microbiol.* **1994**, *60*, 729–731.
70. Reynolds, G.; Pestka, J. J. *J. Food Prot.* **1991**, *54*, 105–108.
71. Bennett, G. A.; Nelsen, T. C.; Miller, B. M. *J. AOAC Int.* **1994**, *77*, 1500–1509.

72. Teshima, R.; Kasase, M.; Tanaka, T.; Hirai, K.; Sato, M.; Sawada, J. I.; Ikebuchi, H.; Ichinoe M.; Terao, T. *J. Agric. Food Chem.* **1990**, *38*, 1618–1622.
73. Chu, F. S.; Lee, R. C. *Food and Agric. Immunol.* **1989**, *1*, 127–136.
74. Wang, C. R.; Chu, F. S. *Appl. Environ. Microbiol.* **1991**, *57*, 1026–1030.
75. Wei, R. D.; Chu, F. S. *Anal. Biochem.* **1987**, *160*, 399–408.
76. Xu, Y. C.; Zhang, G. S.; Chu, F. S. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 945–949.
77. Chu, F. S.; Li, G. Y. *Appl. Environ. Microbiol.* **1994**, *60*, 847–852.
78. Park, J. J. *Immunochemical studies on trichothecenes*; Ph.D. dissertation: University of Wisconsin–Madison, Madison, WI, 1995.
79. Park, J. J.; Chu, F. S. *Mycopathologia* **1993**, *121*, 179–192.
80. Hefle, S. L.; Chu, F. S. *Food Agric. Immunol.* **1990**, *4*, 181–188.
81. Zhang, G. S.; Chu, F. S. *Experientia* **1989**, *45*, 182–183.
82. Dewey, F. M.; MacDonald, M. M.; Phillips, S. I.; Priestley, R. A. *J. Gen. Microbiol.* **1990**, *136*, 753–760.
83. Lu, P.; Marquardt, R. R.; Frohlich, A. A.; Mills, J. T. *Microbiology*, **1994**, *140*, 3267–3276.
84. Lu, P.; Marquardt, R. R.; Kierekjaszczuk, D. *Lett. Applied Microbiol.* **1995**, *20*, 41–45.
85. Tsai, G. J.; Cousin, M. A. *J. Dairy Sci.* **1990**, *73*, 3366–3378.
86. Tsai, G. J.; Cousin, M. A. *Appl. Environ. Microbiol.* **1993**, *59*, 2563–2571.
87. Lee, R. C.; Cary, J. W.; Bhatnagar, D.; Chu, F. S. *Food Agric. Immunol.* **1995**, *7*, 21–32.
88. Liu, B. H.; Keller, N. P.; Bhatnagar, D.; Cleveland, T. E.; Chu, F. S. *Food Agric. Immunol.* **1993**, *5*, 155–164.
89. Hohn, T. M.; McCormick, S. P.; Desjardins, A. E. *Curr. Genet.* **1993**, *24*, 291–295.
90. Nisonoff, A. *J. Immunol.* **1991**, *147*, 2429–2438.
91. Chanh, T. C.; Huot, R. I.; Schick, M. R.; Hewetson, J. F. *Toxicol. Appl. Pharmacol.* **1989**, *100*, 201–207.
92. Chanh, T. C.; Kennedy, R. C.; Hewetson, J. F. *Int. J. Clin. Lab. Res.* **1992**, *22*, 28–35.
93. Hsu, K.-H.; Chu, F. S. *J. Agric. Food Chem.* **1994**, *42*, 2353–2359.
94. Hsu, K. H.; Chu, F. S. *Food Agric. Immunol.* **1995**, *7*, 139–151.
95. Chu, F. S.; Huang, X.; Maragos, C. M. *J. Agric. Food Chem.* **1995**, *43*, 261–267.
96. Hsu, K.-H. *Idiotype, anti-idiotype and anti-anti-idiotype antibodies for aflatoxin: production, characterization and application.* Ph.D. dissertation: University of Wisconsin–Madison, Madison, WI, 1995.
97. Fukal, L.; Reisnerova, H. *Bull. Environ. Contam. Toxicol.* **1990**, *44*, 345–349.
98. Hongyo, K.-I.; Itoh, Y.; Hifumi, E.; Takeyasu, A. *J. AOAC Int.* **1992**, *75*, 307–312.
99. Chu, F. S.; Fan, T. S. L.; Zhang, G. S.; Xu, Y. C.; Faust, S.; McMahon, P. L. *J. Assoc. Off. Anal. Chem.* **1987**, *70*, 854–857.
100. Pesavento, M.; Carter, B. In *Use of Immobilized Biological Compounds*, Guilbault, G. G. Mascini, M., eds.; Kluwer Academic Publishers: Boston, 1993; pp 443–452.

101. Okumura, H.; Okimoto, J.; Kishimoto, S.; Hasegawa, A.; Kawamura, O.; Nakajima, M.; Miyabe, M.; Ueno, Y. *Food Agric. Immunol.* **1993**, *5*, 75–84.
102. Okumura, H.; Kawamura, O.; Kishimoto, S.; Hasegawa, A.; Shrestha, S. M.; Okuda, K.; Obata, H.; Okuda, H.; Haruki, K.; Uchida, T.; Ogsawara, Y.; Ueno, Y. *Carcinogenesis*, **1993**, *14*, 1233–1235.
103. Itoh, Y.; Nishimura, M.; Hifumi, E.; Uda, T.; Ohtani, K.; Kawamura, O.; Nagayama, S.; Ueno, Y. *Proc. Jpn. Assoc. Mycotoxicol.* **1986**, *24*, 25–29.
104. Sidwell, W. J. H.; Chan, W. S.; Morgan, M. R. A. *Food Agric. Immunol.* **1989**, *1*, 111–118.
105. Pestka, J. J.; Azcona-Olivera, J. I.; Plattner, R. D.; Minervini, F.; Doko, B.; Visconti, A. *J. Food Prot.* **1994**, *57*, 167–172.
106. Abouzied, M. M.; Askegard, S; Bird, C.; Miller, B. In *Fumonisins in Food*; Jackson, L.; DeVries, J. W.; Bullerman, L. B., eds; Plenum Publishing Co.: New York, NY, 1995, in press.
107. Lee, L. S.; Wall, J. H.; Cotty, P. J.; Bayman, P. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 581–584.
108. Park, D. L.; Miller, B. M.; Hart, P.; Yang, G.; McVey, J.; Page, S. W.; Pestka, J. J.; Brown, L. H. *J. Assoc. Off. Anal. Chem.* **1989**, *72*, 326–332.
109. Park, D. L.; Miller, B. M.; Nesheim, S.; Trucksess, M. W.; Vekich, A.; Bidigare, B.; McVey, J. L.; Brown, L. H. *J. Assoc. Off. Anal. Chem.* **1989**, *72*, 638–643.
110. Patey, A. L.; Sharman, M.; Wood, R.; Gilbert, J. *J. Assoc. Off. Anal. Chem.* **1989**, *72*, 965–969.
111. Schneider, E.; Dietrich, R.; Märtlbauer, E.; Usleber, E.; Terplan, G. *Food Agric. Immunol.* **1991**, *3*, 185–193.
112. Beaver, R. W.; James, M. A.; Lin, T. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 827–829.
113. Dorner, J. W.; Cole, R. J. *J. Assoc. Off. Anal. Chem.* **1989**, *72*, 962–964.
114. Stubblefield, R. D.; Greer, J. I.; Shotwell, O. L.; Aikens, A. M. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 530–532.
115. Trucksess, M. W.; Stack, M. E.; Nesheim, S.; Park, D. L.; Pohland, A. E. *J. Assoc. Off. Anal. Chem.* **1989**, *72*, 957–962.
116. Trucksess, M. W.; Young, K.; Donahue, K. F.; Morris, D. K.; Lewis, E. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 425–428.
117. Usleber, E.; Schneider, E.; Märtlbauer, E.; Terplan, G. *J. Agric. Food Chem.* **1993**, *41*, 2019–2023.
118. Sashidhar, R. B. *Environ. Health Perspect.* **1993**, *101* (Suppl. 3), 43–46.
119. Nieuwenhof, F. F. J.; Hoolwerf, J. D.; van den Bedem, J. W. *Milchwissenschaft* **1990**, *45*, 584–588.
120. Steimer, J.; Hahn, G.; Heeschen, W.; Bluthgen, A. *Milcheissenschaft* **1988**, *43*, 772–776.
121. Candlish, A. A.; Haynes, C. A.; Stimson, W. H. *Intl. J. Food Sci. Techn.* **1988**, *23*, 479–485.
122. Candlish, A. A. G.; Faraj, M. K.; Harran, G. Smith, *J. E. Biotechnol. Tech.* **1991**, *5*, 317–322.
123. Fremy, J. M.; Chu, F. S. In *Mycotoxins in Dairy Products*; van Egmond, H. P., ed.; Elsevier: Amsterdam, 1989; pp 97–125.

124. Groopman, J. D.; Donahue, K. F. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 861–867.
125. Hansen, T. J. *J. Food Protect.* **1990**, *53*, 75–77.
126. Trucksess, M. W.; Stack, M. E.; Nesheim, S.; Page, S. W.; Albert, R. H.; Hansen, J. T.; Donahue, K. F. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 81–88.
127. Garnder, C. R.; Whattam, M. M.; Taylor, P. J. L.; Stow, M. W. *J. Chromatogr.* **1993**, *648*, 485–490.
128. Lee, L. S. *J. Am. Oil Chem. Soc.* **1989**, *66*, 1398–1413.
129. Koeltzow, D. E.; Tanner, S. N. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 584–589.
130. Dorner, J. W.; Blankenship, P.D.; Cole, R. J. *J. AOAC Int.* **1993**, 76, 637–643.
131. Goto, T.; Manabe, M. *Rpt. Natl. Food Res. Inst.* **1988**, *52*, 53–59.
132. Trucksess, M. W.; Stack, M. E. *J. AOAC Int.* **1994**, *77*, 655–659.
133. Ware, G. M.; Umrigar, P. P.; Carman, A. S.; Kuan, S. S. *Anal. Lett.* **1994**, *27*, 693–715.
134. Sun, P.; Chu, F. S. *J. Food Safety* **1977**, *1*, 67–75.
135. Wu, S.; Yang, G.; Sun, T. *Clin. J. Oncol.* **1983**, *5*, 81–84.
136. Groopman, J. D.; Hasler, J. A.; Trudel, L. J.; Pikul, A.; Donahue P. R.; Wogan, G. N. *Cancer Res.* **1992**, *52*, 267–274.
137. Farjam, A.; van de Merbel, N. C.; Lingeman, H.; Frei, R. W.; Brinkman, U. A. T. *Int. J. Environ. Anal. Chem.* **1991**, *45*, 73–87.
138. Farjam, A.; van de Merbel, N. C.; Nieman, H.; Lingeman, H.; Brinkman, U. A. Th. *J. Chromatogr.* **1992**, *589*, 141–149.
139. Candlish, A. A. G.; Smith, J. E.; Stimson, W. H. *Biotech. Adv.* **1989**, *7*, 401–418.
140. Carvajal, M.; Mulholland, F.; Garner, R. C. *J. Chromatogr.* **1990**, *511*, 379–383.
141 Barmark, A. L.; Larsson, K. *J. AOAC Int.* **1994**, *77*, 46–52.
142. Patey, A. L.; Sharman, M.; Gilbert, J. *Food Addit. Contam.* **1990**, *7*, 515–520.
143. Patey, A. L.; Sharman, M.; Gilbert, J. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 76–81.
144. Sharman, M.; Gilbert, J. *J. Chromatogr.* **1991**, *543*, 220–225.
145. Holcomb, M.; Thompson, H. C., Jr. *J. Agric. Food Chem.* **1991**, *39*, 137–140.
146. Kussak, A.; Andersson, B.; Andersson, K. *J. Chromatgr. B - Biomedical Applications*, **1994**, *656*, 329–334.
147. Groopman, J. D.; Wild, C. P.; Hasler, J.; Chen, J.; Wogan, G. N.; Hasler, T. W. *Environ. Health Perspect.* **1993**, *99*, 107–113.
148. Harrison, J. C.; Garner, R. C. *Carcinogenesis* **1991**, *12*, 741–743.
149. Harrison, J. C.; Carvajal, M.; Garner, R. C. *Environ. Health Perspect.* **1993**, *99*, 99–105.
150. Sabbioni, G.; Ambs, G.; Wogan, G. N.; Groopman, J. D. *Carcinogenesis* **1990**, *11*, 2063–2066.
151. Wild, C. P.; Jiang, Y. S.; Sabbioni, G.; Chapot, B.; Montesano, R. *Cancer Res.* **1990**, *50*, 245–251.
152. Sheabar, F. Z.; Groopman, J. D.; Qian, G. S.; Wogan, G. N. *Carcinogenesis* **1993**, *14*, 1203–1208.
153. Wild, C. P.; Jiang, Y. Z.; Allen, S. J.; Hall, A. J.; Montesano, R. Carcinogenesis **1990**, *11*, 2271–2274.
154. Nakajima, M.; Terada, H.; Hisada, K.; Tsubouchi, H.; Yamamoto, K.; Uda, T.; Itoh, Y.; Kawamura, O.; Ueno, Y. *Food Agric. Immunol.* **1990**, *2*, 189–195.
155. Sharman, M.; MacDonald, S.; Gilbert, J. *J. Chromatogr.* **1992**, *603*, 285–289.

156. Zimmerli, B.; Dick, R. *J. Chromatogr.* **1995**, *666*, 85–99.
157. Azcona, J. I.; Abouzied, M. M.; Pestka, J. J. *J. Food Protect.* **1990**, *53*, 577–580.
158. Bagnati, R. M.; Oriundi, M. P.; Russo, V.; Danese, M.; Berti, F.; Fanelli, R. *J. Chromatogr.* **1991**, *564*, 493–502.
159. Carmen, A. S.; Kuan, S. S.; Ware, G. W.; Francis, O. J.; Miller, K. V. Abstract of the 103rd AOAC Annual International Meeting, St. Louis, MO, Sept. 25–28, 1989 Abstract No. 169; p 34.
160. Tuinstra, L. G. M. T.; Roos, A. H.; Van Trijp, J. M. P. *J. AOAC Int.* **1993**, *76*, 1248–1254.
161. Urano, T.; Trucksess, M. W.; Page, S. W. *J. Agric. Food Chem.* **1993**, *41*, 1982–1985.
162. Niedwetzki, G.; Lach, G.; Geschwill, K. *J. Chromatogr. A* **1994**, *661*, 175–180.
163. Jordan, L.; Hansen, T. L.; Zabe, N. A. *American Lab.* **1994**, *March*, 18–24.
164. Kramer, P. M.; Li, Q. X.; Hammock, B. D. *J. AOAC Int.* **1994**, *77*, 1275–1287.
165. Yu, J.; Chu, F. S. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 655–660.
166. Warden, B. A.; Allam, K.; Sentissi, A.; Cecchini, D. J.; Giese, R. W. *Anal. Biochem.* **1987**, *162*, 363–369.
167. Warden, B. A.; Sentissi, A.; Ehrat, M.; Cecchini, D. J.; Allam, K.; Giese, R. W. *J. Immunol. Methods* **1990**, *131*, 77–82.
168. Ligler, F. S.; Bredehorst, R.; Talebian, A.; Shriver, L. C.; Hammer, C. F.; Sheridan, J.; Vogel, P. C.; Gaber, B. P. *Anal. Biochem.* **1987**, *163*, 369–375.
169. Williamson, M. L.; Atha, D. H.; Reeder, D. L.; Sundaram, P. V. *Anal. Lett.* **1989**, *22*, 803–816.
170. Pestka, J. J. *J. Immunol. Methods* **1991**, *136*, 177–183.
171. Abouzied, M. M.; Pestka, J. J. *J. AOAC Int.* **1994**, *77*, 495–501.
172. Bacigalupo, M. A.; Ius, A.; Meroni, G.; Dovis, M.; Petruzzelli, E. *Analyst* **1994**, *119*, 2813–2815.
173. Degan, P.; Montagnoli, G.; Wild, C. P. *Clin. Chem.* **1989**, *435*, 2308–2310.
174. Tseng, T. C. *J. Toxicol. Toxin Rev.* **1994**, *13*, 229–241.
175. Hsieh, L. L.; Hsieh, T. T. *Cancer Res.* **1993**, *53*, 1278–1280.
176. Santella, R. M.; Zhang, Y. J.; Chen, C. J.; Hsieh, L. L.; Lee, C. S.; Haghighi, B.; Yang, G. Y.; Wang, L. W.; Feitelson, M. *Environ. Health Perspect.* **1993**, *99*, 199–202.
177. Beier, R. C.; Elissalde, M. H.; Stanker, L. H. *Bull. Environ. Contam. Toxicol.* **1995**, *54*, 479–487.
178. Elissalde, M. H.; Kamps-Holtzapple, C.; Beier, R. C.; Plattner, R. D.; Rowe, L. D.; Stanker, L. H. *Food Agric Immunol.* **1995**, *7*, 109–122.

RECEIVED August 30, 1995

Chapter 23

Detection of Acetylated Deoxynivalenol by Enzyme-Linked Immunosorbent Assay

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Specific polyclonal antisera against DON and acetylated derivatives were produced according to standard procedures. Several immunoassays could be established for specific toxin detection and quantification. Here, we describe a method for group-specific detection of DON and related compounds by combining an ELISA using a specific antiserum against 3-Acetyl DON with a sample preparation procedure, which converts non-reactive forms of DON into detectable forms by chemical modification. DON contents in naturally contaminated samples were determined by HPLC or GC/MS, and the results were found to correlate very well with those obtained by ELISA.

More than two decades have passed since Deoxynivalenol (DON) and it's precursor, 3-acetyldeoxynivalenol (3-AcDON) and 15-AcDON (3-AcDON) have been shown as contaminates of cereals colonized by certain *Fusarium* species (*1*). DON belongs to the trichothecene group of mycotoxins, produced mainly by *Fusarium* species. It is frequently found to contaminate cereals and foodstuffs world-wide. These toxins exhibit cytotoxic effects on cells and show dose-dependent response of feed refusal, diarrhea, and emesis, in animals fed contaminated feeds (*2–5*). The predominant metabolite in foodstuffs is DON, but the acetylated forms also are frequently isolated (6).

Currently, methods used for detection and identification of DON and derivatives are physico-chemical procedures ranging from thin-layer chromatography (TLC) to gas chromatography/mass spectrometry (GC/MS) (7,*8*). All these methods, including high pressure liquid chromatography (HPLC), require time- and labor-intensive sample clean-up as well as sophisticated instrumentation.

0097–6156/96/0621–0314$15.00/0

Despite an almost 10-year history of development, immunoassays for the detection of trichothecenes have not replaced other methods, which is in contrast to other mycotoxin tests; e.g., for the detection of aflatoxins. A major drawback of immunoassays for the detection of trichothecenes is their high degree of specificity, which limit their application as a screening test for toxin contamination (9–*11*). The difficulty to generate antibodies with broad specificity for the metabolites of DON can easily be explained by examining the chemical structure of the toxin (Figure 1, Table I). Antisera or monoclonal antibodies generated after immunization with immunogens produced by coupling DON via C-3 to a carrier should discriminate between structures with chemical variations in the opposite part of the molecule, thus discriminating between acetylated DON or non-acetylated DON at C-15 or C-7, as well as they will discriminate some other trichothecenes. Alternatively, immunization with DON, coupled via C-15 to a macromolecule, will elucidate antibodies recognizing DON derivatives resulting from changes at C-3. Generation of such monospecific antisera has been published by Usleber *et al.* (*12*).

To establish a group-specific ELISA for DON and acetylated forms, we combined the reaction of one of our antibodies specific to acetylated DON with a sample preparation which converts all DON in a sample to the acetylated form as described by Xu *et al.* (*10,13*).

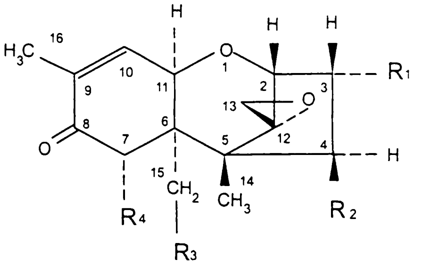

Figure 1. Structure of deoxynivalenol and related toxins.

Table I. Side-Chain Residues of the Derivatives of DON

Substance	R_1	R_2	R_3	R_4
DON	OH	H	OH	OH
3-AcDON	Ac	H	OH	OH
15-AcDON	OH	H	Ac	OH
Di-AcDON	Ac	H	Ac	OH
Tri-AcDON	Ac	H	Ac	Ac

Materials and Methods

The preparation of immunogens, immunization procedure and the characterization of antisera obtained have been published earlier (*12,14,15*).

Production of Immunogens. Briefly, 3-AcDON was reacted with glutaric anhydride at C-15 to 15-O-hemiglutaryl-3-AcDON. This was coupled to human serum albumin (HSA) by the mixed anhydride method.

Production of Antisera. For immunizations, 250 µg of the HSA-conjugate was emulsified in 2 mL Freund's complete adjuvant/distilled water and 3 female chinchilla bastard rabbits were immunized by using multiside intradermal injections. After 10 and 20 weeks, the same composition was applied subcutaneously for boostering.

Characterization of Antisera. The sera collected were precipitated by diluting dropwise with equal volume of $(NH_2)_2SO_4$ (70% saturation in distilled water) and extensively dialyzed against phosphate buffered saline. They then were characterized using competitive ELISAs. Dilutions of sera were adsorbed onto microtiter plates, and after blocking the remaining binding sites of the plate, they were incubated simultaneously with an appropriate dilution of 15-O-hemiglutaryl-3-AcDON coupled to horseradish peroxidase (HRP) by the activated ester method and with non-labelled trichothecenes or sample extracts.

RIDASCREEN DON ELISA. This enzyme immunoassay (R-Biopharm GmbH, Darmstadt, Germany) is conducted with the reagents mentioned above. The sample preparation procedure, chemical modification of prepared samples, and the comparative determination of naturally contaminated samples were done using this ELISA according to the manufacturer's instructions (*16*).

Sample Preparation. Representative samples were triturated and thoroughly mixed in a mixer. Each sample (2 g) was mixed with 10 mL of acetonitrile/distilled water (86:14), stirred for 2 h on a magnetic stirrer and then filtered with a folded paper filter. The filtered extract (1 mL) was reduced to dryness at 40–50 °C.

Acetylation of mycotoxins was performed with acetic anhydride and 4-dimethylaminopyridine as a catalyst in organic solvent as described earlier (*17,18*).

To start the acetylation reaction, 0.1 mL of reagent 1 and 0.1 mL of reagent 2 (which are provided ready to use with the ELISA kit) are pipetted into the dry residue. The reaction was allowed to proceed for 1 h at room temperature and then made up to 1 mL by adding distilled water (0.8 mL). This solution (50 µL) was diluted with buffer (450 µL) and employed in the test.

The acetylation reagents supplied with the RIDASCREEN DON kit were constituted to 95% and 96% (v/v) with Acetonitrile. Reagent 1 contains 5% (w/v) Dimethylaminopyridine (Aldrich, No 10,770–0), which is poisonous by inhalation and skin contact. Reagent 2 contains 4% (v/v) Acetic anhydride (Sigma, No A 3396), which is corrosive and may cause burns if it makes contact with the skin; it also is very flammable.

Acetylation. To prove the efficacy of the acetylation procedure, 20 aliquots of 1 µg DON each were acetylated with four time/temperature variations (n = 5 for each time/temperature combination). Acetylation was performed at 20 °C or 37 °C and for 1 or 2 hours. The acetylation reagents were diluted before use.

ELISA Procedure. The microtiter wells of the ELISA kit were coated with sheep antibodies directed against anti-acetyldeoxynivalenol rabbit IgG. Anti-acetyldeoxynivalenol antibodies (50 µL), acetyldeoxynivalenol enzyme conjugate (50 µL), and the acetylated deoxynivalenol standards or sample solution (50 µL) were added and incubated for 2 h at room temperature. Free acetylated deoxynivalenol and acetyldeoxynivalenol enzyme conjugate compete for the acetyldeoxynivalenol antibody binding sites (competitive enzyme immunoassay). At the same time, the acetyldeoxynivalenol antibodies also were bound by the immobilized rabbit antibodies. Any unbound enzyme conjugate was then removed in a washing step (3 times with 200 µL distilled water). Enzyme substrate and chromogen (urea peroxide and tetramethylbenzidine, 50 µL each) were added to the wells and incubated for 30 min at room temperature in the dark. Bound enzyme conjugate converts the colorless chromogen into a blue product. The addition of the stop reagent (100 µL) leads to a color change from blue to yellow. The measurement is made photometrically at 450 nm. (In the leaflet an optional reference wave length ≥ 600 nm is proposed). The absorption is inversely proportional to the deoxynivalenol concentration in the sample.

Standards. Standard solutions (6) are provided ready to use with the kit: 0 ppt (zero standard), 5 ppt, 25 ppt, 125 ppt, 625 ppt, 3125 ppt deoxynivalenol (acetylated) in 20 mM phosphate buffer with 150 mM NaCl, pH 7.4.

Cross-Reactivity. Compounds related to DON were serially diluted in the RIDASCREEN DON test. The relative cross-reactivity was calculated from the concentrations which inhibited the maximum absorption of the conjugate to 50%.

Results. The ELISA results were calculated using the cubic spline curve-fitting program RIDA Soft for DOS-microcomputers (R-Biopharm GmbH, Germany).

HPLC. HPLC analysis of fine floured wheat samples were performed at the Bayerische Landesanstalt für Bodenkultur und Pflanzenbau, Freising, Germany as described earlier (*19*). All samples have been collected in Germany and were naturally contaminated; they were divided and tested by ELISA at R-Biopharm in Darmstadt, Germany.

GC/MS. Barley samples harvested in 1994 in Canada were analyzed at Agriculture Canada, Winnipeg, Canada for DON analysis according to procedures published earlier (*20*). Samples were divided and sent to the Institute of Hygiene and Technology of Food of Animal Origin, Veterinary Faculty, University of Munich for comparison with the RIDASCREEN DON test.

Results and Discussion

From the immunization with 15-HG-HSA-3-AcDON we produced an antiserum against 3-Ac-DON with a high specificity against 3-AcDON that recognized DON 1000 times less than 3-AcDON, and 15-AcDON was recognized approximately 60 times less than 3-AcDON as determined by the 50% inhibition method. After introducing acetylation, the cross-reactivity of the test kit is as given in Table II.

There is no longer any difference between DON, 3-AcDON, and 15-AcDON with respect to their detectability after sample preparation which included the acetylation step. The standard curve of the assay (data not shown) ranges from 25 ppt (ng/kg) to 400 ppt, whereas the 25 ppt standard is significantly different from the zero standard; i.e., the inhibition value is approximately 10%. The sample preparation procedure leads to a total dilution factor of 50, which gives a calculated sensitivity of the assay of 1.25 ppb (µg/kg) and a measuring range up to 20 ppb. After acetylation, the sensitivity is equal for all natural derivatives of DON.

The efficacy of the acetylation procedure was monitored by acetylating DON under various conditions. The results are summarized in Table III.

Table II. Cross-Reactivity of the RIDASCREEN DON Assay.

Chemical	Percent
Deoxynivalenol (acetylated)	100.0
3-acetyldeoxynivalenol (acetylated)	100.0
15-acetyldeoxynivalenol (acetylated)	100.0
Acetyl-T2-Toxin	0.25
T2-tetraoltetraacetate	0.13
Nivalenol (acetylated)	0.1
T2-tetraol	0.07
3-Acetoxydiacetoxyscirpenol	0.03
Fusarenon X	0.01
Nivalenol	<0.01
T2-Toxin	<0.01
Diacetoxyscirpenol	< 0.01

Table III. Influence of Time and Temperature on Acetylation of DON.

Time/Temperature	Average (ppt)	CV (%)	n	Average (% Inhibition)
1 h/20 °C	121.4	6.54	5	35.54
1 h/37 °C	116.2	7.3	5	36.54
2 h/20 °C	116.6	11.8	5	36.74
2 h/37 °C	118.5	6.11	5	26.16

For each time/temperature combination, DON was acetylated 5 times in parallel. After dilution 1:10, an ELISA was performed under standard conditions. The differences between the 4 groups are statistically not significant (1% probability level). Therefore, the procedure proposed (1 h at room temperature) converts DON quantitatively into the acetylated form.

The comparison of the RIDASCREEN DON ELISA with established methods included 39 barley samples assayed with ELISA and GC/MS and 50 wheat flour samples tested with ELISA and HPLC. The results are summarized in Table IV and Table V.

Table IV. Comparison of ELISA with Other Methods for the Determination of DON.

HPLC versus ELISA			GC/MS versus ELISA		
Sample No.	ELISA ppb	HPLC ppb	Sample No.	ELISA ppb	GC/MS ppb
1	460	460	51	2570	3800
2	60	40	52	1450	1200
3	140	40	53	590	600
4	140	40	54	3330	3000
5	45	40	55	2840	3900
6	90	40	56	2430	2100
7	50	40	57	1110	1300
8	20	0	58	2260	1700
9	120	90	59	520	600
10	140	60	60	3190	1600
11	470	550	61	680	400
12	80	0	62	860	200
13	120	140	63	2540	2300
14	500	600	64	3870	4200
15	300	180	65	1850	2500
16	140	50	66	2410	2400
17	75	40	67	3230	4000
18	170	90	68	3030	3300
19	90	40	69	1230	1000
20	410	390	70	5890	6600
21	380	230	71	780	1300
22	240	40	72	3430	4200
23	160	90	73	2190	2400
24	200	80	74	2460	2100
25	350	290	75	1530	1700
26	250	150	76	1530	1800
27	310	310	77	5480	5900
28	180	90	78	13530	15100
29	110	40	79	4640	3700
30	90	40	80	2630	2700
31	120	60	81	1360	1500
32	100	50	82	3550	3900
33	440	480	83	180	300
34	80	40	84	2290	2600
35	120	40	85	240	100
36	15	40	86	170	300
37	250	260	87	670	800

Continued on next page

Table IV *Continued*

38	340	320	88	3060	4200
39	280	230	89	6190	5600
40	310	160			
41	470	320			
42	180	150			
43	90	70			
44	200	160			
45	90	90			
46	170	150			
47	60	40			
48	110	90			
49	160	120			
50	420	310			

Table V. Statistics of the Comparative Determination of DON.

	HPLC vs ELISA		GC/MS vs ELISA	
	ELISA	HPLC	ELISA	GC/MS
No. of samples	50	50	39	39
mean (ppb)	197.9	149.4	2610	2740
min (ppb)	15	0	170	100
max (ppb)	500	600	13530	15100
R^2	0.85		0.95	

Regarding the results in total, we have to notice that the wheat flours were less contaminated than the barley samples. However, all results are in reasonable ranges. Only one result was very high for a barley sample, which should be retested; it was high in both assays. In two samples, DON was not detectable by HPLC; the corresponding ELISA results were 80 and 20 ppb, respectively. This discrepancy is at a very low range of DON contamination and is acceptable considering, that only 1 sample in this series was tested below 20 ppb with the ELISA. If the higher value (80 ppb) obtained with ELISA is due to variation of the assay, the result still is in the lower range of contamination found (35 samples were measured above 80 ppb).

The specific determination of 15-AcDON and 3-AcDON by means of specific ELISA in barley samples is ongoing. So far, 16 samples have been tested positive for 15-AcDON with a maximum value of 200 ppb (data not shown). In this sample, 15-AcDON contributes only approximately 5% of the total toxin. At this time, we do not believe, that the correlations will change significantly after addition of these results.

In conclusion, the RIDASCREEN DON ELISA is a reliable tool for the determination of DON in cereals. The correlation to results obtained with physico-chemical methods is acceptable. The assay is used as a screening assay, and 6 standards are provided with the kit. The kit leads to reliable results with an average interassay CV being lower than 10%. However, the test is constructed as a competitive ELISA which requires a confirmation of positives.

Literature Cited

1. Morooka, N.; Uratsuji, N.; Yoshizawa, T.; Yamamoto, H. *J. Food Hyg. Soc. Jpn.* **1972**, *13*, 368–375.
2. Vesonder, R. F.; Ciegler, A.; Jensen, A. H. *J. Appl. Microbiol.* **1973**, *26*, 1008–1110.
3. Yoshizawa, T.; Morooka, N. *J. Food Hyg. Soc. Jpn.* **1974**, *15*, 261–269.
4. Coppock, R. W.; Swanson, S. W.; Gelberg, H. B.; Koritz, G. D.; Hoffmann, W, E.; Buck, W. B.; Vesonder, R. F. *Am. J. Vet. Res.* **1985**, *46*, 169–174.
5. Abbas, H. K.; Mirocha, C. J.; Tuite, J. *J. Appl. Environ. Microbiol.* **1986**, *51*, 841–843.
6. ApSimon, J. W.; Blackwell, B. A.; Blais, L.; Fielder, D. A.; Greenhalgh, R.; Kasitu, G.; Miller, J. D. Savard, M. *Pure Appl. Chem.* **1990**, *62*, 1339–1346.
7. Miller, J. D.; Young, J. D.; Trenholm, H. L. *Can. J. Bot.* **1983**, *61*, 3080–3087.
8. Kamimura, H.; Nishijima, M.; Yasuda, K.; Saito, K.; Ibe, A.; Nagayama, T.; Ushiyama, H.; Naoi, Y. *J. Assoc. Off. Anal. Chem.* **1981**, *64*, 1067–1073.
9. Chu, F. S. *Vet. Hum. Toxicol.* **1990**, *32*, 42–50.
10. Xu, Y.-C.; Zhang, G. S.; Chu, F. S. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 945–949.
11. Mills, E. N. C.; Alcock, S. M.; Lee, H. A.; Morgan, M. R. A. *Food Agric. Immunol.* **1990**, *2*, 109–118.
12. Usleber, E.; Märtlbauer, E.; Dietrich, R.; Terplan, G. *J. Agric. Food Chem.* **1991**, *39*, 2091–2095.
13. Xu, Y.-C.; Zhang, G. S.; Chu, F. S. *J. Assoc. Off. Anal. Chem.* **1986**, *69*, 967–969.
14. Usleber, E.; Renz, V.; Märtlbauer. E.; Terplan, G. *J. Vet. Med.* **1992**, *B 39*, 617–627.
15. Usleber, E.; Schneider, E.; Märtlbauer, E.; Terplan, G. *J. Agric. Food Chem.* **1993**, *41*, 2019–2023.
16. RIDASCREEN DON; Instruction leaflet, R-Biopharm GmbH: Darmstadt, 1994.
17. Wei, R.-D.; Bischoff, W.; Chu, F. S. *J. Food Prot.* **1986**, *49*, 267–271.
18. Bamburg, J. R.; Riggs, N. V.; Strong, F. M. *Tetrahedron* **1968**, *24*, 3329–3336.
19. Engelhardt, G.; Schuster, M.; Lepschy, J.; Wallnöfer, P. R. *Z. Lebensm. Unters. Forsch.* **1986**, *182*, 123–126.
20. Nowicki, T. W.; Gaba, D. G.; Dexter, J. E.; Matsuo, R. R.; Clear, R. M. *J. Cereal Sci.* **1988**, *8*, 189–202.

RECEIVED September 7, 1995

Chapter 24

Comparison of Direct and Indirect Enzyme Immunoassays for the Detection of the Mycotoxin Citrinin

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Polyclonal antisera against the mycotoxin citrinin, produced in rabbits after immunization with a citrinin-keyhole limpet hemocyanin conjugate, were used to establish competitive direct and indirect enzyme immunoassays (EIA). A citrinin-horseradish peroxidase conjugate was used as the labelled antigen in a direct EIA, a citrinin-glucose oxidase conjugate was employed as the solid phase antigen in indirect EIA. The antibodies used in this study were highly specific for citrinin, having no measurable cross-reactivity with austdiol, alternariol, deoxynivalenol, or ochratoxin A. After optimization of test parameters, the detection limits for citrinin in buffer solutions by direct and indirect EIA were 5–10 ng/mL and 1–2 ng/mL, respectively.

The mycotoxin citrinin (Figure 1) is produced by several *Aspergillus* and *Penicillium* species (*1, 2*). It was originally isolated as an antibiotic in 1931, but its utility, as such, was negated due to its nephrotoxicity (*3*). Citrinin often occurs together with another common mycotoxin, ochratoxin A, as a natural contaminant in various cereals (*4–9*). Citrinin, like ochratoxin A, acts primarily as a nephrotoxin (*10*) and teratogen.

Although a number of physico-chemical methods has been developed for the detection of citrinin in foods, feeds, and biological fluids, including thin-layer chromatography (*6, 11–13*) and liquid chromatography (*14–16*), so far no satisfactory routine analytical method for this toxin is available (*17, 18*). Immunochemical approaches to the detection of mycotoxins have been of increasing importance throughout the last decade. In particular, enzyme immunoassays have been established for many mycotoxins as a convenient alternative for detecting these substances in foods and feeds (*19*). Recently we have described the production of polyclonal antibodies against citrinin and their use in indirect EIA for citrinin in wheat flour (*20*). Here we describe the development of a direct EIA for citrinin,

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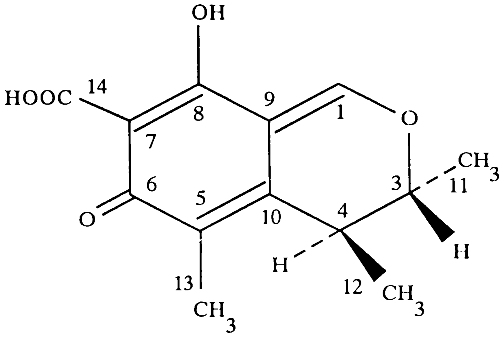
OH
HOOC
14
9
8
1
O
7
CH3
11
6
3
5
O
10
4
H
H
13
12
CH3
CH3

Figure 1. Structure of citrinin.

using a citrinin-horseradish peroxidase conjugate. Important parameters which influence the test practicability both of the direct and indirect formats are compared.

Materials and Methods

Materials. Citrinin, ochratoxin A, austdiol, alternariol, deoxynivalenol, formaldehyde 37% solution, 3,3',5,5'-tetramethylbenzidine (TMB), casein (sodium salt) and polyoxyethylenesorbitan monolaurate (Tween-20) were purchased from Sigma-Aldrich Vertriebs GmbH, Deisenhofen, Germany. Glucose oxidase (GOX) from *Aspergillus niger* van Tieghem (E.C. 1.1.3.4), molecular weight 186,000, and keyhole limpet hemocyanin (KLH), molecular weight 3−7.5 million, from *Megathura crenulata* L. were obtained from Boehringer Mannheim GmbH Biochemicals, Mannheim, Germany. EIA grade horseradish peroxidase (HRP), molecular weight 40,000 (EC 1.11.1.7) also was obtained from Boehringer. Affinity chromatography purified sheep anti-rabbit immunoglobulin G (IgG) was used as described earlier (*21*). Goat anti-rabbit IgG-HRP conjugate was purchased from Sigma-Aldrich.

Anti-Citrinin Antisera. Details of production of antisera against citrinin have been described earlier (*20*). In brief, three rabbits were intradermally immunized with a citrinin-keyhole limpet hemocyanin conjugate prepared by formaldehyde condensation. Subcutaneous booster injections were given at week 15 after primary immunizations. Blood samples were taken at 2−3 week intervals, and the serum was obtained by centrifugation. Sera of each rabbit, collected from week 22 to 27, were individually pooled (each 70−80 mL) and stored frozen at -18 °C without further treatment.

Production of a Citrinin-Horseradish Peroxidase Conjugate. A citrinin-HRP conjugate was prepared for use in direct competitive EIA. HRP (2.0 mg) was dissolved in 0.8 mL sodium acetate buffer (0.1 M; pH 4.2). Citrinin was dissolved in methanol to give a 5 mg/mL solution, and 0.2 mL added to the HRP-sodium acetate solution. A 100 μL aliquot of 37% formaldehyde solution was then added, and the mixture incubated for 24 h at 37 °C. Afterwards, the citrinin-HRP was dialyzed at 4 °C for three days against three changes of phosphate buffered saline (PBS; 0.01 M phosphate buffer, pH 7.2, containing 0.1 M NaCl) and stored at -18 °C.

Production of a Citrinin-Glucose Oxidase Conjugate. Citrinin was conjugated to the glycoprotein GOX for use as the solid phase antigen in indirect competitive EIA (*20*). In brief, GOX (3.7 mg) was dissolved in 0.8 mL sodium acetate buffer (0.1 M; pH 4.2) and mixed with citrinin solution (1 mg/mL methanol; 0.2 mL). A 324 μL aliquot of 37% formaldehyde solution was then added, and the mixture incubated for 72 h at ambient temperature (22 °C). Afterwards, the citrinin-GOX was dialyzed at 4 °C for three days against three changes of PBS and stored at -18 °C.

Titration of Antisera with Citrinin-HRP. Optimum concentrations of antiserum and enzyme conjugate were determined by twofold checkerboard titration with and without addition of citrinin. A microtiter plate was coated (100 μL/well) with goat anti-rabbit IgG (10 μg/mL carbonate-bicarbonate buffer [0.05 M; pH 9.6]) overnight at ambient temperature in a humid chamber. The solution was removed, and free protein-binding sites of the wells were blocked with casein sodium salt, 2% in PBS, for 30 min at ambient temperature. The plate was washed three times with a 0.85% NaCl solution containing Tween-20 (250 μL/L) and drained. Then 35 μL toxin standard buffer solution (PBS containing 10% methanol), toxin-free or containing 100 ng citrinin/mL was added to the wells of each half of the plate, followed by 35 μL of a serial dilution (in PBS) of antiserum pool (rabbit #1, #2, #3), and 35 μL of a serial dilution of citrinin-HRP (in PBS containing 1% casein sodium salt), and incubated for 2 h at ambient temperature.

Then each plate was washed as above, and 100 μL of enzyme substrate/chromogen solution (*22*) containing H_2O_2 (3 mM) and TMB (1 mM) in potassium citrate buffer (0.2 M; pH 3.9) were added per well. After 15 min the enzyme reaction was stopped with 1 M H_2SO_4 (100 μL per well) and the absorbance measured at 450 nm. A combination giving the desired results, i.e., absolute absorbance values of the toxin-free wells of ≥1.0 and absorbance reduction in the corresponding toxin-containing well of ≥80 %, was chosen for establishment of the direct EIA.

Competitive Direct EIA. A microtiter plate was coated (100 μL/well) with anti-rabbit IgG (10 μg/mL carbonate-bicarbonate buffer) overnight at ambient temperature in a wet chamber. The solution was removed, and free protein-binding sites of the wells were blocked with casein sodium salt, 2% in PBS, for 30 min at ambient temperature. The plate was washed three times with a 0.85% NaCl solution containing Tween-20 (250 μL/L) and drained. To each well, 35 μL citrinin standard solution (in PBS containing 10% methanol), 35 μL antiserum against citrinin (pool rabbit #1, diluted 1:2000 with PBS), and 35 μL citrinin-HRP (diluted 1:400 with PBS containing 1% casein sodium salt) were added and incubated for 2 h at ambient temperature. Then each plate was washed as above, and further treated with enzyme substrate solution as described above. All standards solutions were analyzed in quadruplicate.

After absorbance measurement, the test was evaluated using an on-line PC and an EIA calculation software developed by Märtlbauer (*23*), which uses a cubic spline function for calculation of the standard curve. The program also determines the detection limit (students *t*, n=4; 95% confidence limit) and the 50% inhibition dose. The measuring range of the standard curve usually is from 20% to 80% relative binding (B/B_0 x 100).

Competitive Indirect EIA. The indirect EIA was performed essentially as described earlier (*20*). In brief, the plates were coated with 100 μL/well of the citrinin-GOX conjugate (1:1000 in sodium carbonate buffer) overnight at ambient temperature. The citrinin-GOX solution was removed, free protein-binding sites of the wells were blocked with 2% casein sodium salt/PBS, then the plate was washed and drained. Fifty μL each of citrinin standard solution (in 10 %methanol/PBS) and the anti-citrinin antiserum (pool rabbit #3; diluted 1:1000 with PBS) were added to each well and the plate was incubated for 1 h at room temperature. The plate was washed, and goat anti-rabbit IgG-HRP conjugate (1:5000 in 1% casein sodium salt in PBS;

100 μL per well) was added. After 1 h at ambient temperature, the plate was washed and further treated with enzyme substrate/chromogen solution as described above.

Results and Discussion

By using formaldehyde, a common conjugation reagent reactive with amines, amides, guanidino and phenolic groups (*24*), citrinin was successfully bound to KLH, GOX, and HRP. Conjugation ratio (moles of citrinin per mole of carrier protein) in the citrinin-GOX were found to be approximately 3:1 by UV spectroscopy (*20*). Conjugation ratios in the citrinin-KLH and citrinin-HRP could not be determined because of extensive precipitation of the former and ambiguous UV spectra obtained from the latter. However, in past experiments slightly turbid or even fully precipitated hapten-KLH conjugates have been found to be very powerful immunogens, which gave useful antibodies against a number of problem haptens (*25−27*).

In all three rabbits dosed with the citrinin-KLH conjugate high specific serum titers could be detected as early as five weeks after immunization. All sera gave standard curves in indirect EIA which enabled specific detection of citrinin in the low ng/mL range. No cross-reactivity was found with the structurally similar mycotoxins austdiol and alternariol, nor with the mycotoxins ochratoxin A and deoxynivalenol which are likely to co-occur together with citrinin in high concentrations (*20*).

For standardization of the indirect EIA, serum pool of rabbit #3 was chosen because it gave the most sensitive test system, with a detection limit for citrinin buffer solutions in the range of 1−2 ng/ml. The dilutions of antiserum (1:1000) and solid phase citrinin-GOX (1:1000) were sufficiently high to enable their use in routine analysis, considering the serum pool volume of 75 mL and the simple conjugation protocol for citrinin-GOX.

In contrast, for direct EIA the serum pool #3 and the citrinin-HRP had to be used at dilutions of approximately 1:100 to give absorbance values of ~ 1.0 units. Therefore, serum of rabbit #1, which had a higher serum titer (Figure 2) and showed stronger binding to the citrinin-HRP, was used for the direct EIA in a 1:2000 dilution. The amount of citrinin-HRP necessary for this EIA (dilution 1:400) was still comparatively high, corresponding to a peroxidase working concentration of approximately 2−3 μg/mL. The standard curve detection limit of this assay for citrinin (5 − 10 ng/ml) was slightly higher than that of the indirect test format (Figure 3). Further work will aim at improving the conjugation of citrinin to HRP, because the direct EIA format is in practice more convenient to perform than the indirect test, which requires one additional incubation step.

However, both tests provided simple reliable procedures with low intra-plate coefficients (1.5−7%; n=4). Plates coated with citrinin-GOX (indirect EIA) or anti-rabbit IgG could be stored for at least 3−4 weeks at 4 °C, ready for use. The sensitivity and specificity of the EIA described above would probably be sufficient to assay citrinin in agricultural commodities. Initial tests, using the indirect EIA for analysis of citrinin in artificially contaminated wheat flour, had shown that at toxin levels ranging from 200−2000 ng/g good recoveries of 89 − 104% could be achieved with coefficients of variation (n=4) of 6.9−13% (*20*). The immunoassays, with their advantages of speed and simplicity, could conveniently be used for screening samples followed by confirmation of positive results by physicochemical methods.

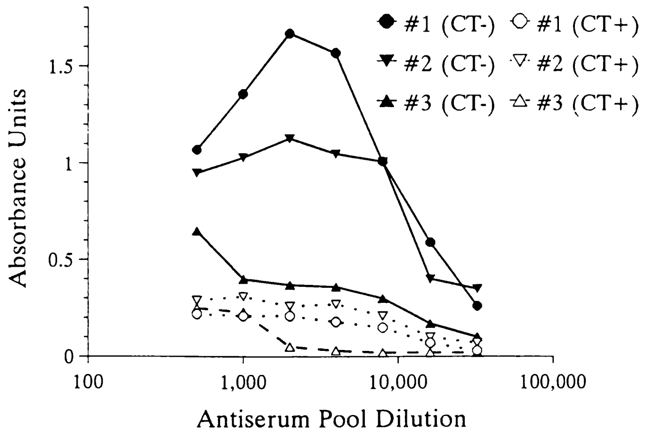

Figure 2. Comparison of antisera pools of rabbits #1, #2, and #3, using two-fold checkerboard titration (Results are shown for one citrinin-HRP dilution [1:400] only) in direct EIA. Serial dilutions of antiserum were incubated with the citrinin-HRP and either toxin-free (CT-) or toxin-containing (CT+; 100 ng/mL) standard buffer solutions.

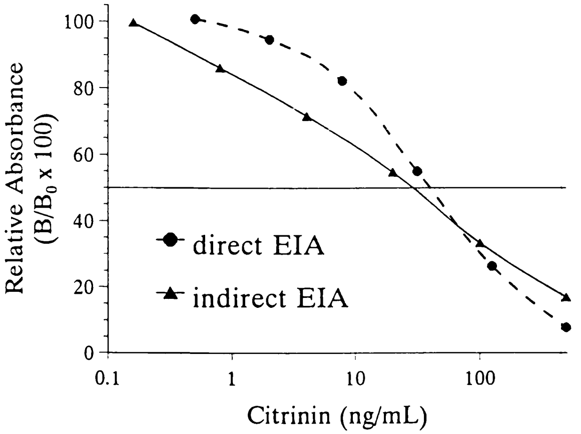

Figure 3. Comparison of standard curves for competitive EIA detection of citrinin, using direct EIA or indirect EIA format, respectively. The x axis shows the logarithm of mycotoxin concentration. The y axis shows the corresponding absorbance value B relative to the absorbance of the negative control B_0, expressed as (B/B_0) x 100. B_0 values were 1.0 (direct EIA) and 1.1 (indirect EIA) absorbance units. Each point represents the mean of four determinations.

Acknowledgments

We thank External Affairs and International Trade Canada for travel funding, and M. Straka, M. Lorber, and D. Smith for technical assistance.

Literature Cited

1. Betina, V. 1989. In *Mycotoxins: chemical, biological and environmental aspects*; Betina V., ed.; Elsevier: Amsterdam, 1989; pp 174−191.
2. Wilson, D. M. In *Biotoxins, Biodegradation and Biodeterioation Research*; O'Rear, C. E.; Llewelyn, G. C., eds.; Plenum Press: New York, NY, 1994, pp 65−73.
3. Hetherington, A. C.; Raistrick, H. *Thom. Phil. Trans. Roy. Soc. London Ser. B* **1931**, *220*, 269−295.
4. Scott, P. M.; van Walbeek, W.; Kennedy, B.; Anyeti, D. *J. Agric. Food Chem.* **1972**, *20*, 1103−1109.
5. Krogh, P.; Hald, B.; Pedersen, E. J. *Acta. Pathol. Microbiol. Scand. Sect. B* **1973**, *81*, 689−695.
6. Chalam, R. V.; Stahr, H. M. *J. Assoc. Off. Anal. Chem.* **1979**, *62*, 570−572.
7. Hökby, E.; Hult, K.; Gatenbeck, S.; Rutqvist, L. *Acta. Agric. Scand.* **1979**, *29*, 174−178.
8. Osborne, B. G. *Food Cosmet. Toxicol.* **1980**, *18*, 615−617.
9. Nelson, T. S., Kirby, L. K.; Beasley, J. N.; Johnson, Z. B; Ciegler, A. *Poult. Sci.* **1985**, *64*, 464−468.
10. Phillips, R. D.; Berndt, W. O; Hayes, A. W. *Toxicology* **1979**, *12*, 285−298.
11. Stubblefield, R. D. *J. Assoc. Off. Anal. Chem.* **1979**, *62*, 201−202.
12. Gimeno, A.; Martins, M. L. *J. Assoc. Off. Anal. Chem.* **1983**, *66*, 85−91.
13. Gimeno, A. *J. Assoc. Off. Anal. Chem.* **1984**, *67*, 194−196.
14. Lepom, P. *J. Chromatogr.* **1986**, *355*, 335−339.
15. Marti, L. R., Wilson D. M.; Evans, B. D. *J. Assoc. Off. Anal. Chem.* **1978**, *61*, 1353−1358.
16. Phillips, R. D.; Hayes, A. W.; Berndt, W. O. *J. Chromatogr.* **1980**, *190*, 419−427.
17. Scott, P. M. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 120−128.
18. Trucksess, M. W. *J. AOAC Int.* **1994**, *77*, 135−141.
19. Chu, F. S. In *Residue Analysis in Food Safety: Applications of Immunoassay Methods*; Beier, R. C.; Stanker, L. H., eds.; American Chemical Society: Washington, DC, 1996 (this ACS symposium volume).
20. Abramson, D.; Usleber, E.; Märtlbauer, E. *Appl. Environ. Microbiol.* **1995**, *61*, 2007−2009.
21. Märtlbauer, E.; Gareis, M.; Terplan, G. *Appl. Environ. Microbiol.* **1988**, *54*, 225−230.
22. Gallati, H.; Pracht, J. *J. Clin. Chem. Clin. Biochem.* **1985**, *23*, 453−460.
23. Märtlbauer, E. *Enzymimmuntests für antimikrobiell wirksame Stoffe;* Ferdinand Enke Verlag: Stuttgart, Germany, 1993; pp 206−222.
24. Wong, S. S. *Chemistry of protein conjugation and cross—linking*; CRC Press: Boca Raton, FL, 1993; pp 101−103.

25. Usleber, E.; Straka, M.; Terplan, G. *J. Agric. Food Chem.* **1994**, *42*, 1392–1396.
26. Renz, V.; Terplan, G. *Arch. Lebensmittelhyg.* **1988**, *39*, 30-33.
27. Schneider, E.; Usleber, E.; Märtlbauer, E. In *Residue Analysis in Food Safety: Applications of Immunoassay Methods*; Beier, R. C.; Stanker, L. H., eds.; American Chemical Society: Washington, DC, 1996 (This ACS symposium volume).

RECEIVED August 16, 1995

Chapter 25

Development of an Enzyme Immunoassay for *Alternaria alternata* f.sp. *lycopersici* Toxins

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AAL toxins and fumonisins are sphinganine analog mycotoxins secreted by the fungal pathogens *Alternaria alternata* f.sp. *lycopersici* and *Fusarium moniliforme*, respectively. Consumption of fumonisin-contaminated maize has been reported to cause several animal diseases and has been correlated with high incidence of human esophageal cancer. Recent studies also demonstrate that both fumonisins and AAL compounds are phytotoxic to tomato, cytotoxic to cultured mammalian cells, and inhibit ceramide synthase in animals. Preliminary evidence indicates that AAL toxins are present in field-grown tomatoes, destined for processing, which show signs of the blackmold disease. An enzyme-linked immunosorbent assay (ELISA) would provide a rapid and inexpensive analytical tool for screening large numbers of food samples for AAL toxins. We report here the development of a class-selective ELISA for detection of AAL toxins at ppb to low ppm levels. Mice were inoculated with various protein conjugates of AAL compound TA and the elicited polyclonal antibodies were used for assay development. No cross-reactivities were found with sphinganine, the structurally related sphingoid base, or with fumonisin B_1. The sensitivity and selectivity of the assay produced in this preliminary study indicate a high potential for development of ELISAs for the determination of AAL toxins in food and feed samples.

Gilchrist and Grogan (*1*) demonstrated that the fungal pathogen *Alternaria alternata* f.sp. *lycopersici* produces AAL toxins (Figure 1). These toxins are the primary chemical determinants of Alternaria stem canker disease of tomato cultivars homozygous-recessive for the *Asc* gene (*asc/asc*) (*2*). Another class of mycotoxins, the fumonisins (Figure 2), are secreted by several species of *Fusarium* including *Fusarium moniliforme* Sheldon, an economically important pathogen of maize and other grains (*3*, *4*). In tomato, the *Asc* gene regulates sensitivity to both AAL toxins and the fumonisins (*2*).

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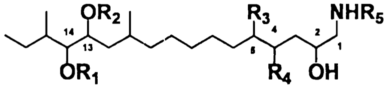
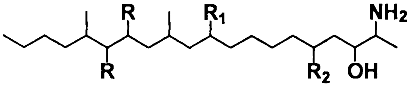


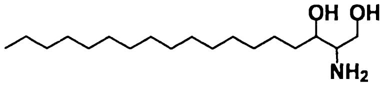
Toxin	R_1	R_2	R_3	R_4	R_5
TA_1	H	R	OH	OH	H
TA_2	R	H	OH	OH	H
TB_1	H	R	H	OH	H
TB_2	R	H	H	OH	H
TC_1	H	R	H	H	H
TC_2	R	H	H	H	H
TD_1	H	R	H	OH	$COCH_3$
TD_2	R	H	H	OH	$COCH_3$
TE_1	H	R	H	H	$COCH_3$
TE_2	R	H	H	H	$COCH_3$

R-: $HO_2C\text{-}CH_2\text{-}CH(CO_2H)\text{-}CH_2\text{-}CO\text{-}$

Figure 1. Structures of AAL toxins.



Toxin	R	R_1	R_2
FB_1	$O_2C\text{-}CH_2\text{-}CH(CO_2H)\text{-}CH_2\text{-}CO_2H$	OH	OH
FB_2	$O_2C\text{-}CH_2\text{-}CH(CO_2H)\text{-}CH_2\text{-}CO_2H$	H	OH
FB_3	$O_2C\text{-}CH_2\text{-}CH(CO_2H)\text{-}CH_2\text{-}CO_2H$	OH	H



Sphinganine

Figure 2. Structures of fumonisins and sphinganine.

AAL Toxins

Isolation of the five main structural congeners of AAL toxin (TA, TB, TC, TD, and TE) and elucidation of their gross chemical structures have been reported by Bottini *et al.* (*5*) and Caldas *et al.* (*6*, *7*). Each toxin fraction is an isomeric-mixture of an aminopolyol backbone esterified by a terminal carboxyl group of tricarballylic acid at the C-13 or C-14 hydroxyl groups (Figure 1). The absolute configuration of the main chain of compound TA was determined independently by two groups (*8*, *9*). Recently, the configuration of the tricarballylic acid residue was also reported (*10*). The NMR studies and MM2 calculations indicated the presence of strong hydrogen bonds between NH → 2-O and 4-OH → 5-O positions which stabilizes the unique conformation of the segment around the amino-group of the TA molecule even in aqueous solution (*9*).

Structural and Biochemical Similarity of AAL Toxins and Fumonisins

Fumonisins (e.g., FB_1, Figure 2) (*11*–*13*) are structurally related to AAL compounds (Figure 1). Compounds FB_1, FB_2, and FB_3 are diesters of a family of aminopolyols with both hydroxyl groups at C-14 and C-15 esterified with the terminal carboxyl group of tricarballylic acid (Figure 2). Both the fumonisins and AAL toxins bear structural similarity to sphingoid bases such as sphinganine (Figure 2). The most recent stereochemical analyses (*8*, *10*, *14*) demonstrate that FB_1 and TA have identical configurations at all common stereocenters throughout the aminopolyol backbones and tricarballylic ester side chains.

In animals, the fumonisins were shown to cause a variety of serious or fatal diseases. Although FB_1 does not appear to be genotoxic, it is carcinogenic in rat liver (*15*), and exhibits marked activity as a cancer promoter (*16*, *17*). Leukoencephalomacia (ELEM) in equines, porcine pulmonary edema (PPE), hepatotoxicity and nephrotoxicity in rats, and hepatocellular hyperplasia in turkey poults are animal disease conditions produced by the fumonisins (*4*, *15*, *17*–*22*). Epidemiological data also link the consumption of *F. moniliforme*-contaminated maize with a high incidence of human esophageal cancer in the Transkei region of southern Africa (*23*). Both fumonisin FB_1 and AAL-toxin TA were found to be cytotoxic in mammalian cell cultures (*24*, *25*) and phytotoxic to susceptible tomato varieties (*asc*/*asc*) (*2*, *26*).

Both the AAL toxins and the fumonisins are potent inhibitors of sphinganine (sphingosine) *N*-acyltransferase (ceramide synthase), and thereby disrupt sphingolipid metabolism, which may account for a number of their biological effects (*15*, *27*–*31*). It is well established that sphingolipids are of great importance in cellular signaling systems, and interference of fumonisins and AAL toxins with sphingolipid metabolism may result in cellular deregulation (*15*). Exposure to these toxins leads to elevated concentrations of free sphingoid bases (e.g., sphinganine, Figure 2) in both animals (*15*) and plants (*29*), which correlates with cell and tissue damage. Fumonisins contaminate maize and maize-based human and animal foodstuffs usually in low ppb to low ppm levels worldwide (*4*, *7*, *32*–*34*). Consumption of foodstuffs with relatively high concentrations of fumonisins involves potential health hazards (*32*).

To our knowledge, no detailed animal toxicity data are available for *Alternaria* toxins. Nevertheless, the structural similarity and common mode of biochemical action of AAL compounds and fumonisins are sufficient reason for concern about the presence of AAL toxins in the food chain (*2*, *6*, *25*). Risk assessment for both groups of compounds, including studies on situations where they occur together or with other natural toxins (*35*) in foods is warranted. Several investigations on the combined toxicological effects and interactions have already been performed (*19*, *20*, *36*).

Analytical Methods for AAL Compounds and Fumonisins

Field tomato samples and commercial tomato-based food products are often contaminated with AAL toxins in the low ppb to low ppm concentration range (Gilchrist, D. G., University of California, Davis, unpublished results). Currently, only instrumental analyses such as high-performance liquid chromatography (HPLC) (*37*) and electrospray ionization-mass spectrometry (ESI-MS) (*7*) are available for quantitative determination of AAL compounds in food. An enzyme-linked immunosorbent assay (ELISA) would provide scientists and regulatory agencies with a rapid and inexpensive analytical method for large-scale monitoring of *Alternaria* toxins. A further advantage of the immunochemical analysis would be the potential to develop an immunoaffinity cleanup method using the anti-AAL antibodies which could remarkably enhance instrumental analyses as it was demonstrated with the commercial immunoaffinity columns for fumonisins (*38*–*40*).

Thin layer chromatography (*4*, *41*) and instrumental methods like HPLC, ESI-MS, gas chromatography-MS (*4*, *7*, *32*, *40*, *42*–*44*), capillary electrophoresis (CE) (*45*) and CE-ESI-MS (*46*) are used most often for the analysis of fumonisins. These analytical methods are highly sensitive and selective; however, they have a number of shortcomings such as need for expensive apparatus, trained personnel, and laborious sample preparation. Hence, these methods are not useful for low-cost screening of large numbers of samples and for on-site analyses. In an attempt to address these limitations, immunoassays have recently been devised for the detection of ppb to ppm concentrations of fumonisins (*33*, *41*, *42*, *47*–*52*). These assays are class-selective for the fumonisins, they do not cross-react with the AAL toxins.

Methods

Modified procedures (*53*–*55*) were used for conjugation of TA toxin to carrier proteins to obtain immunogens and coating antigens (see below). Polyclonal mouse antisera were raised following standard protocols (*53*, *56*). Indirect ELISA in the coating antigen format was similar to that of Bekheit *et al.* (*57*). Briefly, the mixture of the competitor and the mouse anti-TA antibody was incubated in the coated wells. The wells were then treated with enzyme-labeled goat anti-mouse antibody, and finally with the chromogen enzyme-substrate. Photometric detection of the colored product, generated by the enzyme, allowed to obtain standard curves with the analytes. The full account of our studies including experimental details and results with further immunogens will be given elsewhere.

Results and Discussion

Synthesis of Immunogens. Most of the fumonisin-immunoassays are based on immunogens prepared by the conventional glutaraldehyde (GA) coupling (cf. Figure 3) (*33*, *47*, *48*, *51*). This one-step method is combined with the reduction of the formed Schiff base by sodium borohydride to stabilize the fumonisin-protein conjugate in several procedures (*47*, *48*, *51*). Another approach by Elissalde *et al.* (*49*) used a more elaborate synthesis: carrier proteins were thiolated with 2-iminothiolane (2-IMT), and FB_1 was then conjugated to these protein derivatives by means of sulfosuccinimidyl 4-(4-(*N*-maleimido)phenyl)butyrate (sulfo-SMPB) (*54*), a heterobifunctional cross-linker (cf. Figure 4).

In the beginning of our studies, we failed to elicit useful antisera with protein-AAL toxin conjugates obtained by a one-step GA method (Figure 3) combined with sodium borohydride-reduction, a procedure similar to what was used in the first published fumonisin-ELISAs (*47*, *48*). In this and the following conjugation reactions, AAL toxin TA was used as the ligand. The one-step GA method, in which the ligand, protein, and GA are mixed, is poorly controlled and usually results in a complex mixture of polymerized products. However, an improved, two-step GA coupling (*53*), permitting formation of better defined, stable conjugates without reductive post-treatment, has been successfully applied for the synthesis of highly immunogenic protein-toxin conjugates (Figure 3). In this procedure, the carrier protein is first treated with an excess of GA, which is eliminated before the reaction with the toxin. The application of sulfo-SMPB (*55*), the heterobifunctional cross-linker used previously in the development of a fumonisin-assay (*49*, *54*), also led to useful immunogens (Figure 4).

Antibody Production and ELISA Development. Polyclonal mouse antisera were raised against both sets of protein conjugates using routine protocols (*53*, *56*). Heterologous ELISAs in the immobilized antigen format were developed with the resulting high-titer antisera in the early phases of the immunization schedules. On the basis of these preliminary competitive immunoassay data, further immunogens were designed. (The results obtained with these new synthetic immunogens will be presented elsewhere.) By using several mice for each conjugate, we were able to evaluate our coupling methods with reduced cost and time. We found this approach to be more feasible than obtaining rabbit polyclonal antisera when used with a number of immunogens.

Assays using the best antiserum-coating antigen combinations detected the AAL compounds at ppb to low ppm concentrations and displayed class-selectivity for the *Alternaria* toxins. The performance of one of our ELISAs is illustrated in Table I and Figure 5. The high sensitivity (Table I and Figure 5) for TE, a containing an *N*-acetyl-group (Figure 1), is likely due to recognition of the linker attached to the amino-group of TA in the structure of the immunogen conjugate (TA-GA-KLH, see Figures 1 and 3). No cross-reactivity with structurally similar natural products (FB_1 and sphinganine, see Figure 2) was found.

In this study, we devised an ELISA for the selective detection of the entire set of AAL toxins. The sensitivity and selectivity of the assay hold promise for the analysis AAL toxins in food samples. The best immunogens will be used to develop

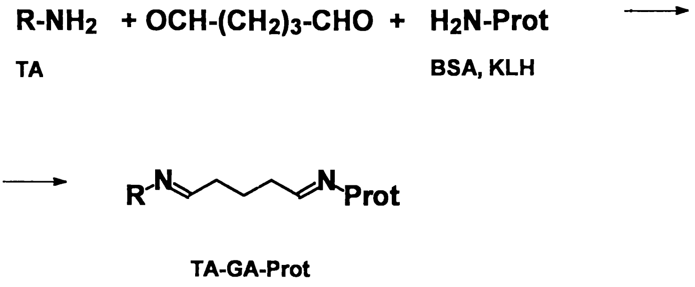

R-NH2 + OCH-(CH2)3-CHO + H2N-Prot
TA
BSA, KLH
R
N
N
Prot
TA-GA-Prot

Figure 3. Glutaraldehyde coupling. Carrier proteins: BSA, KLH.

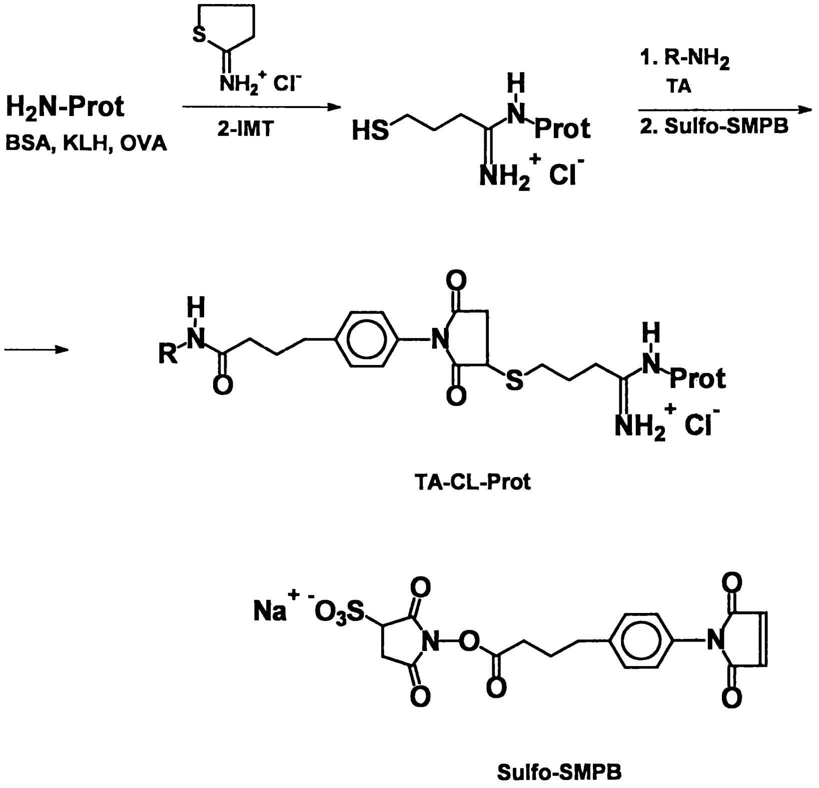

H2N-Prot
BSA, KLH, OVA
S
NH2+ Cl-
2-IMT
HS
H
N
Prot
NH2+ Cl-
1. R-NH2
TA
2. Sulfo-SMPB
H
R
N
O
N
O
S
H
N
Prot
NH2+ Cl-
TA-CL-Prot
Na+ -O3S
O
N
O
O
O
N
O
O
Sulfo-SMPB

Figure 4. Synthesis of conjugates by protein thiolation with 2-IMT and then by using heterobifunctional cross-linker sulfo-SMPB. Carrier proteins: BSA, KLH, OVA.

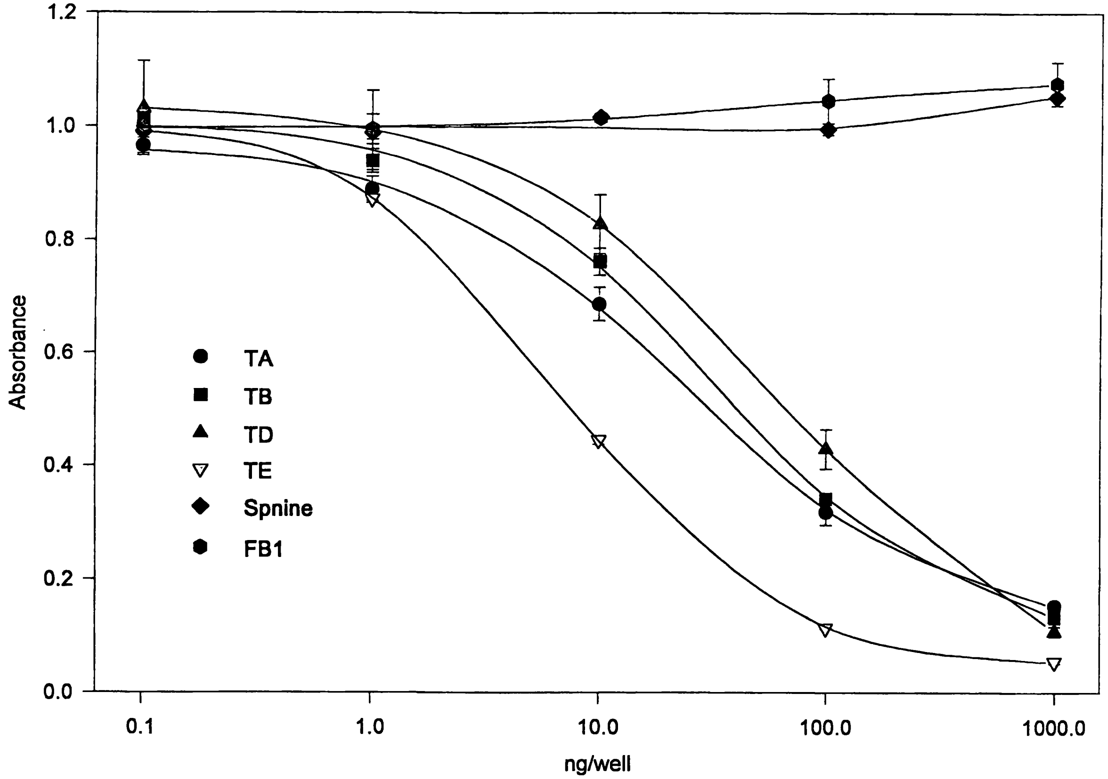


Figure 5. Standard curves obtained by the ELISA system involves mouse antiserum raised against TA-GA-KLH and coating antigen TA-CL-OVA (cf. Table 1). Concentration: ng/well = ng in 50 μL. Spnine: sphinganine.

additional polyclonal antisera and monoclonal antibodies. Studies are currently underway with novel conjugation chemistries, syntheses of immunogens from other AAL toxins and fumonisins, ELISA development for fumonisins, and with real samples.

Table I. Competitive ELISA results with AAL Toxins.

		TA	TB	TD	TE
IC_{50}[a,b]	(ng/well)	24.5	33.0	65.7	6.9
IC_{50}	(ppb)	490	660	1314	138
CR[c]	(%)	100	74	37	355

[a] ELISA system involves mouse antiserum raised against TA-GA-KLH and coating antigen TA-CL-OVA. TA-GA-KLH was obtained by the two-step GA coupling (Figure 3). TA-CL-OVA was synthesized by protein thiolation and then by using sulfo-SMPB, a heterobifunctional cross-linker (Figure 4). No competition was observed with FB_1 and sphinganine (Figure 2).

[b] IC_{50}: analyte concentration required for 50% inhibition.

[c] CR: cross-reactivity (%) = 100 x IC_{50}[TA]/IC_{50}[analyte].

Acknowledgments

Financial support by California Tomato Board, Campbell Research, EPA Center for Ecological Health Research (CR819658), Idetek Inc., NIEHS Grant (ES02710), and NIH Center for Environmental Health Sciences at UC Davis (1P30 Es-05707) Pilot Project is greatly appreciated. The authors thank E.D. Caldas and A.D. Jones for instrumental analyses and C. Kamps-Holtzapple for helpful discussions.

Literature Cited

1. Gilchrist, D. G.; Grogan, R. G. *Phytopathol.* **1976**, *66*, 165–171.
2. Gilchrist, D. G.; Ward, B.; Moussato, V.; Mirocha, C. *J. Mycopathologia* **1992**, *117*, 57–64.
3. Nelson, P. E.; Plattner, R. D.; Shackelford, D. D.; Desjardins, A. E. *Appl. Environ. Microbiol.* **1992**, *58*, 984–989.
4. Nelson, P. E.; Desjardins, A. E.; Plattner, R. D. *Annu. Rev. Phytopathol.* **1993**, *31*, 233–252.
5. Bottini, A. T.; Bowen, J. R.; Gilchrist, D. G. *Tetrahedron Lett.* **1981**, *22*, 2723–2726.
6. Caldas, E. D.; Jones, A. D.; Ward, B.; Winter, C. K.; Gilchrist, D. G. *J. Agric. Food Chem.* **1994**, *42*, 327–333.

7. Caldas, E. D.; Jones, A. D.; Winter, C. K.; Ward, B.; Gilchrist, D. G. *Anal. Chem.* **1995**, *67*, 196–207.
8. Boyle, C. D.; Harmange, J.-C.; Kishi, Y. *J. Amer. Chem. Soc.* **1994**, *116*, 4995–4996.
9. Oikawa, H.; Matsuda, I.; Kagawa, T.; Ichihara, A; Kohmoto, K. *Tetrahedron* **1994**, *50*, 13347–13368.
10. Boyle, C. D.; Kishi, Y. *Tetrahedron Lett.* **1995**, *36*, 5695-5698.
11. Bezuidenhout, S. C.; Gelderblom, W. C. A.; Gorst-Allman, C. P.; Horak, R. M.; Marasas, W. F. O.; Spiteller, G.; Vleggar, R. *J. Chem. Soc. Chem. Commun.* **1988**, 743–745.
12. Gelderblom, W. C. A.; Marasas, W. F. O.; Vleggaar, R.; Thiel, P. G.; Cawood, M. E. *Mycopathologia* **1992**, *117*, 11–16.
13. Plattner, R. D.; Weisleder, D.; Shackelford, D. D.; Peterson, R.; Powell, R. G. *Mycopathologia* **1992**, *117*, 23–28.
14. Blackwell, B. A.; Edwards, O. E.; ApSimon, J. W.; Fruchier, A. *Tetrahedron Lett.* **1995**, *36*, 1973–1976.
15. Riley, R. T.; Voss, K. A.; Yoo, H.-S.; Gelderblom, W. C. A; Merrill, A. H., Jr. *J. Food Prot.* **1994**, *57*, 638–645.
16. Gelderblom, W. C. A.; Jaskiewicz, K.; Marasas, W. F. O.; Thiel, P. G.; Horak, R. M.; Vleggaar, R.; Kriek, N. P. *J. Appl. Environ. Microbiol.* **1988**, *54*, 1806–1811.
17. Norred, W. P.; Voss, K. A. *J. Food Prot.* **1994**, *57*, 522–527.
18. Voss, K. A.; Chamberlain, W. J.; Bacon, C. W.; Norred, W. P. *Natural Toxins* **1993**, *1*, 222–228.
19. Diaz, G. J.; Boermans, H. *J. Vet. Human Toxicol.* **1994**, *36*, 548–555.
20. Weibking, T. S.; Ledoux, D. R.; Bermudez, A. J.; Rottinghaus, G. E. *Poultry Sci.* **1994**, *73*, 1517–1525.
21. Beier, R. C.; Elissalde, M. H.; Stanker, L. H. *Bull. Environ. Contam. Toxicol.* **1995**, *54*, 479–487.
22. Voss, K. A.; Chamberlain, W. J.; Bacon, C. W.; Herbert, R. A.; Walters, D. B.; Norred, W. P. *Fundam. Appl. Toxicol.* **1995**, *24*, 102–110.
23. Sydenham, E. W.; Thiel, P. G.; Marasas, W. F. O.; Shephard, G. S.; van Schalkwyk, D. J.; Koch, K. R. *J. Agric. Food Chem.* **1990**, *38*, 1900–1903.
24. Shier, W. T.; Abbas, H. K.; Mirocha, C. *J. Mycopathologia* **1991**, *116*, 97–104.
25. Mirocha, C. J.; Gilchrist, D. G.; Shier, W. T.; Abbas, H. K.; Wen, Y.; Vesonder, R. F. *Mycopathologia* **1992**, *117*, 47–56.
26. Lamprecht, S. C.; Marasas, W. F. O.; Alberts, J. F.; Cawood, M. E.; Gelderblom, W. C. A.; Shephard, G. S.; Thiel, P. G.; Calitz, F. *J. Phytopathol.* **1994**, *84*, 383–391.
27. Norred, W. P.; Wang, E.; Yoo, H.; Riley, R. T.; Merrill, A. H., Jr. *Mycopathologia* **1992**, *117*, 73–78.
28. Merrill, A. H., Jr.; Wang, E.; Gilchrist, D. G.; Riley, R. T. *Adv. Lipid Res.* **1993**, *26*, 215–234.
29. Abbas, H. K.; Tanaka, T.; Duke, S. O.; Porter, J. K.; Wray, E. M.; Hodges, L.; Sessions, A. E.; Wang, E.; Merrill, A. H., Jr.; Riley, R. T. *Plant Physiol.* **1994**, *106*, 1085–1093.

30. Abbas, H. K.; Duke, S. O.; Paul, R. N.; Riley, R. T.; Tanaka, T. *Pestic. Sci.* **1995**, *43*, 181–187.
31. Wu, W.-I; McDonough, V. M.; Nickels, J. T., Jr.; Ko, J.; Fischl, A. S.; Vales, T. R.; Merrill, A. H., Jr.; Carman, G. M. *J. Biol. Chem.* **1995**, *270*, 13171–13178.
32. Thiel, P. G.; Marasas, W. F. O.; Sydenham, E. W.; Shephard, G. S.; Gelderblom, W. C. A. *Mycopathologia* **1992**, *117*, 3–9.
33. Usleber, E.; Straka, M.; Terplan, G. *J. Agric. Food Chem.* **1994**, *42*, 1392–1396.
34. Doko, M. B.; Rapior, S.; Visconti, A.; Schjoth, J. E. *J. Agric. Food Chem.* **1995**, *43*, 429–434.
35. Chamberlain, W. J.; Bacon, C. W.; Norred, W. P.; Voss, K. A. *Food Chem. Toxicol.* **1993**, *31*, 995–998.
36. Kubena, L. F.; Edrington, T. S.; Kamps-Holtzapple, C.; Harvey, R. B.; Elissalde, M. H.; Rottinghaus, G. E. *Poultry Sci.* **1995**, *74*, 306–313.
37. Siler, D. J.; Gilchrist, D. G. *J. Chromatogr.* **1982**, *238*, 167–173.
38. Ware, G. M.; Umrigar, P. P.; Carman, A. S., Jr.; Kuan, S. S. *Anal. Lett.* **1994**, *27*, 693–715.
39. Scott, P. M.; Delgado, T.; Prelusky, D. B.; Trenholm, H. L.; Miller, J. D. *J. Environ. Sci. Health* **1994**, *B29*, 989–998.
40. Trucksess, M. W.; Stack, M. E.; Allen, S.; Barrion, N. *J. AOAC Int.* **1995**, *78*, 705–710.
41. Shelby, R. A.; Rottinghaus, G. E.; Minor, H. C. *J. Agric. Food Chem.* **1994**, *42*, 2064–2067.
42. Pestka, J. J.; Azcona-Olivera, J. I.; Plattner, R. D.; Minervini, F.; Doko, M. B.; Visconti, A. *J. Food Prot.* **1994**, *57*, 169–172.
43. Akiyama, H.; Miyahara, M.; Toyoda, M.; Saito, Y. *J. Food Hyg. Soc. Japan* **1995**, *36*, 77–81.
44. Wilkes, J. G.; Sutherland, J. B.; Churchwell, M. I.; Williams, A. J. *J. Chromatogr. A* **1995**, *695*, 319–323.
45. Maragos, C. M. *J. Agric. Food Chem.* **1995**, *43*, 390–394.
46. Hines, H. B; Brueggemann, E. E.; Holcomb, M.; Holder, C. L. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 519–524.
47. Azcona-Olivera, J. I.; Abouzied, M. M.; Plattner, R. D.; Norred, W. P.; Pestka, J. J. *Appl. Environ. Microbiol.* **1992**, *58*, 169–173.
48. Azcona-Olivera, J. I.; Abouzied, M. M.; Plattner, R. D.; Pestka, J. J. *J. Agric. Food Chem.* **1992**, *40*, 531–534.
49. Elissalde, M. H.; Kamps-Holtzapple, C.; Beier, R. C.; Plattner, R. D.; Rowe, L. D.; Stanker, L. H. *Food Agric. Immunol.* **1995**, *7*, 109–122.
50. Abouzied, M. M.; Pestka, J. J. *J. AOAC Int.* **1994**, *77*, 495–501.
51. Fukuda, S.; Nagahara, A.; Kikuchi, M.; Kumagai, S. *Biosci. Biotech. Biochem.* **1994**, *58*, 765–767.
52. Chu, F. S.; Huang, X.; Maragos, C. M. *J. Agric. Food Chem.* **1995**, *43*, 261–267.
53. Tijssen, P. *Practice and Theory of Enzyme Immunoassays (Laboratory Techniques in Biochemistry and Molecular Biology. Vol. 15)*; Burdon, R. H., van Knippenberg, P. H., eds.; Elsevier: Amsterdam, The Netherlands, 1985.

54. Kamps-Holtzapple, C.; Carlin, R. J.; Sheffield, C.; Kubena, L.; Stanker, L.; DeLoach, J. R. *J. Immunol. Methods* **1993**, *164*, 245–253.
55. Kamps-Holtzapple, C.; Stanker, L. H.; DeLoach, J. R. *J. Agric. Food Chem.* **1994**, *42*, 822–827.
56. Peeters, J. M.; Hazendonk, T. G.; Beuvery, E. C.; Tesser, G. I. *J. Immunol. Methods* **1989**, *120*, 133–143.
57. Bekheit, H. K. M.; Lucas, A. D.; Szurdoki, F.; Gee, S. J.; Hammock, B. D. *J. Agric. Food Chem.* **1993**, *41*, 2220–2227.

RECEIVED October 3, 1995

Chapter 26

Comparison Study of a Fumonisin Enzyme Immunoassay and High-Performance Liquid Chromatography

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80799 Munich, Germany

Corn samples collected from German retail stores were tested for fumonisin using a commercially available enzyme-linked immunosorbent assay (RIDASCREEN Fumonisin) and HPLC. The recoveries for fumonisin B_1 from artificially contaminated corn at levels of 50 and 500 ng/g were 59.7% and 73.2% after the enzyme immunoassay (EIA) analysis of the raw extracts; the corresponding values after SAX extraction and HPLC analysis were 62.3% and 65.6%. The comparison of both methods showed a slight overestimation of the EIA versus HPLC, but the RIDASCREEN Fumonisin can be characterized as a powerful and very acceptable screening method.

Fumonisins (Figure 1) are a group of mycotoxins mainly produced by *Fusarium moniliforme* and have shown to be of high importance as naturally occurring contaminants in corn worldwide *(1,2)*.

The known toxic effects like equine leukoencephalomalacia outbreaks, porcine pulmonary edema and heptocarcinogenicity in rats *(3,4,5)*, as well as the naturally occurring levels of fumonisins in corn, present a potential threat to human and animal health. This potential threat suggests the need for screening and analytical methods to routinely monitor for the presence of fumonisins in foods and feeds. Currently, the most frequently used analytical methods for fumonisins are high-performance liquid chromatographic (HPLC) systems *(6,7)*. Costs and time requirements, especially for the sample clean-up steps, make these methods unsuitable to process large numbers of samples.

In such a situation, immunochemical approaches have been shown to be good alternatives for the screening of cereals for fumonisins. So far, four enzyme immunoassays have been described which are monoclonal *(12–14)*, and also polyclonal *(8)*, and anti-idiotype/anti-anti-idiotype *(15)* antibodies have been developed. A membrane-based test for fumonisin B_1 (FB_1) also has been described *(16)*. The development of such an immunological test by Usleber *et al.* (*8*) was used in a

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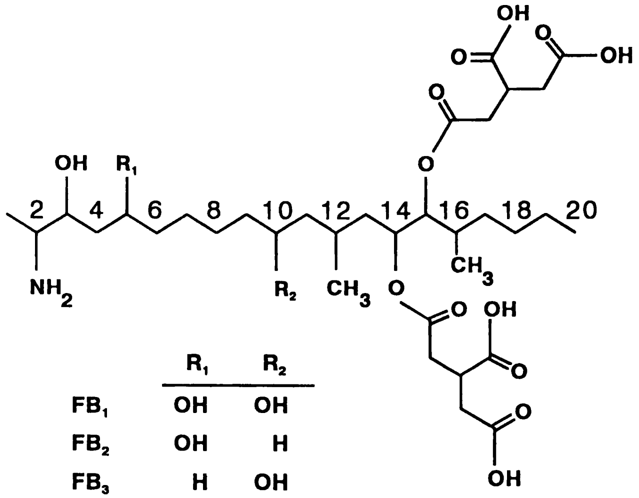
OH
O
OH
O
O
O
OH R$_1$
2 4 6 8 10 12 14 16 18 20
NH$_2$
R$_2$
CH$_3$
O
CH$_3$
O OH
O
O
OH

	R$_1$	R$_2$
FB$_1$	OH	OH
FB$_2$	OH	H
FB$_3$	H	OH

Figure 1. Structures of fumonisins B_1, B_2 and B_3 *(11)*.

commercially available test kit, RIDASCREEN Fumonisin. This report describes the specifications of RIDASCREEN Fumonisin. The results of this test were compared to those obtained by a HPLC method which is essentially based on a method originally described by Shephard *et al.* (*6*) and improved by Sydenham *et al.* (*9*).

Materials and Methods

FB_1 was purchased from Sigma Chemicals, Deisenhofen, Germany. RIDASCREEN Fumonisin kits were obtained from R-Biopharm GmbH, Darmstadt, Germany. All chemicals and solvents used were of at least analytical grade.

Source of Samples. A total of 19 samples, collected in German retail stores, were analyzed for fumonisins. Some of these samples were imported from another European country.

HPLC. A method originally described by Shephard *et al.* (*6*) and improved by Sydenham *et al.* (*9*) was used for HPLC detection of FB_1 with some modifications. A Merck LichroCart 125–4 column, filled with LiChrospher 100 RP-8 material, was used as the stationary phase. The mobile phase was methanol (669 mL)/0.1 M NaH_2PO_4 (340 mL), adjusted to a pH of 3.4 with orthophosphoric acid and pumped at a flow rate of 1 mL/min. For toxin derivatization, 50 µL of standard FB_1 (in methanol) was mixed with 200 µL of *o*-phthaldialdehyde (OPA) solution (*9*), and then 20 µL was injected into the HPLC system using a Waters Model 712 WISP injector. Derivatized FB_1 (retention time 12.8 min) was detected using a Shimadzu RF 535 fluorescence detector (excitation at 335 nm; emission at 440 nm). Toxin concentrations (injection range from 2 to 40 ng) were calculated from peak areas using Nelson EG software (LKB, Bromma, Sweden).

RIDASCREEN Fumonisin ELISA. The Fumonisin-ELISA was performed according to the instruction of the producer. The basis of the test is the antigen-antibody reaction. The microtiter wells are coated with antibodies directed against FB_1. Fumonisin enzyme conjugate, fumonisin standard solutions or sample solutions are added. Free FB_1 and fumonisin enzyme conjugate compete for the fumonisin binding sites (competitive enzyme immunoassay). Any unbound enzyme conjugate is then removed in a washing step. Enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine) were added to the wells and incubated. Bound enzyme conjugate converts the colorless chromogen into a blue product. The addition of the stop reagent (1 M H_2SO_4) leads to a color change from blue to yellow, which was measured photometrically at 450 nm. The toxin content was quantified using an on-line PC and enzyme immunoassay (EIA) software (*10*), which uses a cubic spline function for calculation of the standard curve. The measuring range of the curve is usually from 20–80% relative binding (B/B_0 x 100). To determine the test specificity, fumonisins B_1, B_2 and B_3 were tested for competition with FB_1-HRP (horseradish peroxidase) under the conditions of the RIDASCREEN instructions. The absorbance values were plotted point-to-point and the relative cross-reactivity of each toxin was calculated on the basis of the concentration necessary to inhibit 50% FB_1-HRP binding. Typical results for FB_1, FB_2 and FB_3 are 100%, 40% and 100%.

Corn Sample Extraction. Ground corn samples (25 g) were extracted with 50 mL of methanol/water (75/25) for 20 min on a magnetic stirrer. For the spiking experiments, the corresponding FB_1 concentrations (50 or 500 ng/g) were added to the weighed corn samples and immediately thereafter extracted. The extract was centrifuged and the supernatant filtered through a paper filter. An aliquot of this raw extract was adjusted to a methanol content of 10% with PBS, further diluted at least 1:3 in 10% methanol/PBS, and directly assayed by RIDASCREEN Fumonisin EIA. An aliquot of the raw extract (10 mL) was further purified using strong anion exchange (SAX) cartridges [Adsorbex (Merck); 400 mg per cartridge] according to the detailed instructions by Sydenham *et al.* (*9*). The final volume of the purified and concentrated extract was 0.2 mL of methanol, corresponding to 5 g of sample. For the determination of FB_1 by HPLC, purified extract (50 µL) was added to 200 µL of OPA solution and further treated as described for toxin standards. Recovery (EIA, HPLC) of FB_1 from artificially contaminated corn samples was studied at concentrations of 50 and 500 ng/g, respectively. Food samples (popcorn; corn grit; corn semolina, in Germany kown as polenta) were purchased from local retail stores from October 1992 to March 1993 and analyzed by EIA and HPLC. Five of these samples were imported products from another European country.

Results and Discussion

The intraassay and interassay coefficients of variations of the RIDASCREEN Fumonisin EIA were usually below 7% and 12%, respectively. The mean detection limit and 50% dose were found at 0.2 and 2 ng/mL, respectively. A typical standard curve is shown in Figure 2. When specificity was checked under the conditions of the EIA, the relative cross-reactivity with FB_1, FB_2 and FB_3 were found to be 100%, 40% and 100%, respectively.

Due to the high sensitivity of the assay, a simplified sample preparation procedure was sufficient for EIA analysis, resulting in a detection limit for FB_1 in corn of about 10 ng/g (10 ppb). To check the data obtained by EIA using this rapid extraction procedure, the raw extracts were additionally analyzed by HPLC after SAX purification.

The recoveries for FB_1 at the 50 and 500 ng/g level, as determined by the two analyses, are listed in Table I. In general, the data shows overall agreement between both sample preparation procedures and methods. In our experiments, magnetic stirring of the sample-solvent mixture for 20 min was not very efficient (recovery 59–73%) but gave consistent results within the concentration range of interest. It also was very convenient for the simultaneous extraction of more than 10 samples; an important aspect for a large-scale screening method.

The EIA values of naturally contaminated samples (Table II) were somewhat higher than those obtained for SAX extracts and HPLC. At least for the lower FB_1 values (< 100 ng/g), it cannot be excluded that some samples had a higher matrix effect which led to an overestimation of the toxin content as previously mentioned. For higher toxin concentrations, however, the sample extracts were diluted 1:1000 and

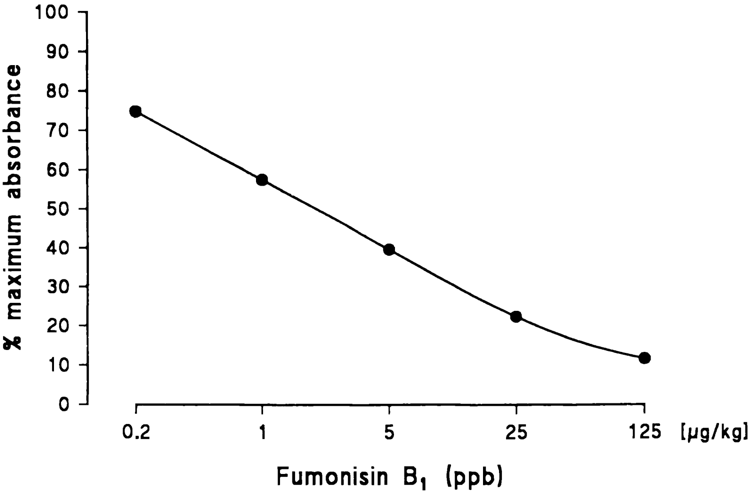

Figure 2. Typical standard curve for RIDASCREEN Fumonisin. The x-axis (log-scale) indicates the toxin concentration. The y-axis indicates the relative binding of FB_1-HRP (B/B_0).

Table I. Comparison of the Recovery of Fumonisin B_1 from Artificially Contaminated Corn Samples Using SAX-Purified Extracts (HPLC) and Raw Methanolic Extracts (EIA).

FB_1 Added (ng/g)		FB_1 Found (ng/g) HPLC (SAX)	EIA (Raw Extracts)
0		< 10	< 10
50		37.4	33.6
50		27.2	25.3
50		25.0	28.3
50		35.9	31.8
50		30.2	30.2
	mean ± SD	31.1 ± 5.4	29.8 ± 3.2
	recovery (%)	62.3	59.7
500		335	320
500		270	360
500		379	418
500		358	349
500		296	381
	mean ± SD	328 ± 44.5	366 ± 36.6
	recovery (%)	65.6	73.2

higher for EIA analysis, thus eliminating unspecific effects. As fumonisins B_2 and B_3 were not determined by HPLC, no data was available as to whether or not these toxins were present in some samples. Since the RIDASCREEN Fumonisin EIA strongly cross-reacts with these toxins, and maybe with other (unknown) fumonisins, this cross-reactivity could result in obtaining higher EIA values.

The analysis of food samples (n = 19) from Munich retail stores showed that corn meal and popcorn both were frequently (84%) contaminated with FB_1, although most samples had toxin levels of less than 100 ng/g. Maximum values in corn semolina exceeded 1 µg/g FB_1. Only one sample, which had 20 ng of FB_1/g by EIA, was negative by HPLC. The overall agreement for the positive samples between HPLC and EIA was acceptable. It is interesting to note that all samples exceeding FB_1 values of 200 ng/g were imported corn semolina.

In conclusion, the RIDASCREEN Fumonisin is both sensitive and easy to perform. The assay could be used as a screening method for fumonisins in corn and corn-based products, as a large number of samples could be analyzed without time-consuming sample extractions.

The frequent contamination of corn for human consumption as found in this study shows the need to routinely screen corn-based foods for these toxins. There is a great need for more information concerning the levels of fumonisins in various corn products along with more toxicology studies to identify the levels of fumonisins allowable in foods.

Table II. Comparison of the FB_1 Values in Naturally Contaminated Corn Samples Determined in Purified Extracts by HPLC and in Raw Extracts by EIA.

Sample	HPLC[a] Value[b] (ng/g)	Raw Extract EIA Value[c] (ng/g)
popcorn D[d]	< 10.0	< 10.0
popcorn D	< 10.0	20.8
popcorn D	12.3	35.4
popcorn D	46.8	51.6
popcorn D	114.0	175.0
popcorn D	104.0	146.0
corn grit D	< 10.0	< 10.0
corn grit D	13.9	58.9
semolina D	19.9	26.9
semolina D	17.3	63.3
semolina D	20.7	32.8
semolina D	27.5	60.6
semolina D	< 10.0	< 10.0
semolina O[e]	364.0	354.0
semolina O	520.0	679.0
semolina O	45.6	284.0
semolina O	1230.0	1280.0
semolina O	650.0	1000.0
semolina O	737.0	889.0

[a] SAX-purifed extract. [b] Single determinations. [c] Serial dilutions (fourfold determinations) of the extracts analyzed. The dilution that gave absorbance values closest to the 50% dose (40–60%) was used for calculation. [d,e] Presumable country of origin: [d] D, Germany, [e] O, other European country.

Literature Cited

1. Norred, W. P. *J. Toxicol. Environ. Health.* **1993**, *38*, 309–328.
2. Scott, P. M. *Int. J. Food Microbiol.* **1993**, *18*, 257–270.
3. Ross, F. P.; Rice, L. G.; Osweiler, G. D.; Nelson, P. E.; Richard, J. L.; Wilson, T. M. *Mycopathologia* **1992**, *117*, 109–114.
4. Thiel, P. G.; Shephard, G. S.; Sydenham, E. W.; Marasas, W. F. O.; Nelson, P. E.; Wilson, T. M. *J. Agric. Food Chem.* **1991b**, *39*, 109–111.
5. Cawood, M. E.; Gelderblom, W. C. A.; Vleggaar, R.; Behrend, Y.; Thiel, P. G.; Marasas, W. F. O. *J. Agric. Food Chem.* **1991**, *39*, 1958–1962.
6. Shephard, G. S.; Sydenham, E. W.; Thiel, P. G.; Gelderblom, W. C. A. *J. Liq. Chromatogr.* **1990**, *13*, 2077–2087.
7. Scott, P. M.; Lawrence, G. A. *J. AOAC Int.* **1992**, *75*, 829–934.
8. Usleber, E.; Straker, M.; Terplan, G. *J. Agric. Food Chem.* **1994**, *42*, 1392–1396.
9. Sydenham, E. W.; Thiel, P. G.; Marasas, W. F. O.; Shephard, G. S.; Van Schalkwyk, D. J.; Koch, K. R. *J. Agric. Food Chem.* **1990**, *38*, 1900–1903.
10. Märtlbauer, E. *Enzymimmuntests für antimikrobiell wirksame Stoffe;* Ferdinand Enke Verlag: Stuttgart, 1993; pp 206–222.
11. Plattner, R. D.; Weisleder, D.; Shackelford, D. D.; Peterson, R.; Powell, R. G. *Mycopathologia* **1992**, *117*, 23–28.
12. Azcona–Olivera, J. I.; Abouzied, M. M.; Plattner, R. D.; Pestka, J. J. *J. Agric. Food Chem.* **1992**, *40*, 531–534.
13. Fukuda, S.; Nagahara, A.; Kikuchi, M.; Kumagai, S. *Biosci. Biotechnol. Biochem.* **1994**, *58*, 765–767.
14. Elissalde, M. H.; Kamps-Holtzapple, C.; Beier, R. C.; Plattner, R. D.; Rowe, L. D.; Stanker, L. H. *Food Agric. Immunol.* **1995**, *1*, 109–122.
15. Chu, F. S.; Huang, X.; Magaros, C. M. *J. Agric. Food Chem.* **1995**, *43*, 261–267.
16. Abouzied, M. M.; Pestka, J. J. *J. AOAC Int.* **1994**, *77*, 495–501.

RECEIVED September 7, 1995

Chapter 27

Monoclonal Antibody-Based Competitive Enzyme-Linked Immunosorbent Assays for the Hydrolysis Product of Fumonisin B_1 (HFB_1)

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Fumonisin B_1 (FB_1) a mycotoxin produced by certain Fusarium molds, consists of two tricarballylic acid groups esterified to a 20 carbon aminopentol backbone (HFB_1). The occurrence of HFB_1 in corn based foods is suspected, primarily because of the ubiquitous nature of FB_1 in corn. A competitive direct enzyme linked immunosorbent assay (CD-ELISA) was developed for the rapid analysis of HFB_1. The CD-ELISA was based upon a monoclonal antibody, prepared against HFB_1, that cross reacts with the hydrolysis products of the fumonisins B_2, B_3, and B_4. The antibody did not react with the intact fumonisins, sphingosine, sphinganine, or tricarballylic acid. When applied to extracts of ground corn, the performance of the CD-ELISA was affected by the level of solvent and by matrix components. By accommodating these factors through sample cleanup and dilution, the CD-ELISA provides a sensitive screening method for HFB_1 in foods.

The fumonisin mycotoxins have been isolated from feeds suspected to have caused both leukoencephalomalacia in horses and a form of pulmonary edema in swine. The mode of action of fumonisins has not been established, however alteration of sphingolipid biosynthesis is one manifestation of exposure (*1*). The intact fumonisins inhibit the enzyme ceramide synthase and profoundly alter the amounts and relative proportions of sphingosine and sphinganine (*1,2*). Fumonisins may also accelerate sodium/calcium exchange (*3*). Under alkaline conditions (*4*) or through metabolism one or both of the tricarballylic acid (TCA) groups present in the intact fumonisins can be lost. Removal of both TCA groups from fumonisin B_1 (FB_1) yields a hydrolysis product (HFB_1) that inhibits the incorporation of serine into sphingosine in primary hepatocytes (*4*). The extent to which HFB_1 may contribute to fumonisin-induced toxicity is unknown. Long term, whole-animal, toxicity studies using purified HFB_1 have not been published. A short-term (21 day) test of the carcinogenic potential of HFB_1 to primary rat hepatocytes indicated HFB_1 was inactive at this target. Interestingly, in the same study, HFB_1 was found to be more cytotoxic than FB_1 (*5*). HFB_1 was also cytotoxic to poultry

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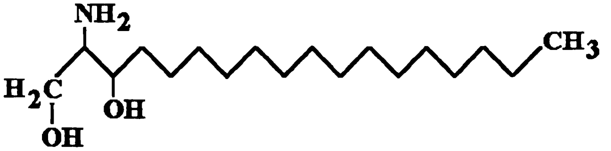
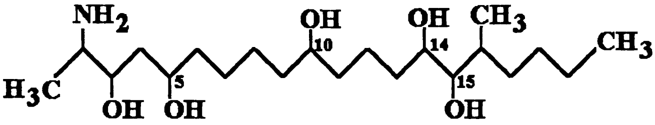
In Immunoassays for Residue Analysis; Beier, R., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

lymphocytes (Dombrink-Kurtzman, M., USDA-ARS-NCAUR, personal communication, 1994). Furthermore, corn containing FB_1 that was treated with calcium hydroxide contained predominantly HFB_1 and remained toxic when fed to male F344/N rats (*6*). When vervet monkeys were exposed to FB_1 much of the fumonisin passed through the animals unmetabolized, however small amounts of HFB_1, as well as the partially hydrolyzed product (lacking one TCA group), were detected in the feces (*7*). These factors suggest HFB_1 is itself toxic, and that exposure can occur both from HFB_1 already present in food and from HFB_1 formed *in vivo* by metabolism of FB_1.



Sphinganine



Hydrolyzed Fumonisin B_1

Since the discovery of fumonisins, the levels present in foods and feeds have been of substantial interest. The intact fumonisins occur predominantly in corn at concentrations that can vary considerably from year to year (*8–11*). Analytical approaches for the intact fumonisins include TLC (*12*), HPLC (*9, 13, 14*), GC-MS (*15*), liquid secondary ion mass spectrometry (*16*) and immunochemical methods (*17–23*). The levels present in corn-based foods were reviewed recently by Nelson et al. (*24*). To our knowledge there is only one report describing detection of the hydrolysis product (*25*) and one report of the possible occurrence of HFB_1, in corn-based foods processed with $Ca(OH)_2$ (*26*). The latter method used tandem strong anion exchange and C_{18} columns for isolation of HFB_1 from methanolic extracts, followed by derivatization with a fluorophore and separation by HPLC.

The development of immunochemical methods for the intact fumonisins has been very successful (*17–23*). Several ELISAs are available commercially, as are affinity columns for isolation of intact fumonisins from corn (*22*). ELISAs using both polyclonal (*21, 23, 27*) and monoclonal (*17, 18, 20, 23*) antibodies have been developed and are reviewed elsewhere in this volume.

None of the antibodies produced against the intact fumonisins have been reported to react with the hydrolysis products. To monitor human exposure to HFB_1 immunochemical methods for the hydrolysis products of FB_1 are needed that provide the sensitivity attained using organic extraction, but which are less labor intensive than HPLC methods. A confirmatory method for the presence of intact FB_1, following hydrolysis would also be useful. For these reasons, an affinity based method for the determination of HFB_1 was developed (*28*). Polyclonal and monoclonal antibodies specific for HFB_1 were developed and formed the basis for a competitive direct ELISA for determination of HFB_1 in corn.

Experimental

Immunizations. Purified FB_1 was hydrolyzed using sodium hydroxide as described by Norred et al. (*4*). After checking for complete hydrolysis by HPLC, the HFB_1 was conjugated to cholera toxin (CT) for immunization of Balb/C mice. The immunogen (HFB_1-CT) was prepared using a glutaraldehyde linkage through the primary amine of HFB_1 according to a method similar to that described previously for FB_1-CT (*27*). Two milligrams of cholera toxin were dissolved in water, and dialyzed against PBS to remove impurities. HFB_1 was added in 50-fold molar excess and an equal volume of 2% glutaraldehyde was added at 10 °C and held at this temperature for 1 h. Aggregates formed during this time. The reaction was warmed to room temperature and sodium borohydride was added to a level of 10 mg/mL. The reaction was cooled to 10 °C and held for 1 h. The reaction mixture was dialyzed against PBS (10 mM phosphate buffer containing 8.5% sodium chloride, pH 7.4) before use as an immunogen.

Five 10 wk-old female mice were given primary intraperitoneal (i.p.) injections of 50 µg HFB_1-CT. Mice were immunized with 100 µL volumes of a 1:1 mixture of HFB_1-CT emulsified into Freund's complete adjuvant. All subsequent booster injections, administered every 3 to 4 weeks, used Freund's incomplete adjuvant. Whole blood was collected from the retro-orbital venous plexus and allowed to clot at 4 °C for 20 min. The sample was then centrifuged at 10,000 rpm (Microfuge, Eppendorf) to sediment the cellular fraction. The serum was removed and frozen at -20 °C until screening by competitive indirect ELISA. Sera were collected at the fifth and eighth week after injection.

Competitive Indirect ELISA. Mouse sera were screened for antibodies directed against HFB_1 using a competitive indirect ELISA (CI-ELISA). In this assay HFB_1 in solution and an HFB_1-ovalbumin conjugate (HFB_1-OA) competed for binding to available antibodies. The HFB_1-OA conjugate was prepared by a method similar to that described above for cholera toxin with the exception that 10 mg of ovalbumin and 2 mg HFB_1 were used (molar ratio of 20:1 HFB_1:OA). After dialysis against PBS containing 0.05% sodium azide the HFB_1-OA was lyophilized and stored at -20 °C.

HFB_1-OA was noncovalently coated to polystyrene microtiter plates by adding 100 µL of HFB_1-OA conjugate, concentration 1.0 µg/mL in 0.05 M sodium carbonate buffer (pH 9.6), to the plates and allowing to stand overnight at 4 °C. Concentrations of HFB_1-OA between 0.25 and 2.0 µg/mL provided acceptable displacement curves. After washing 4 times with 0.32 mL Tween-PBS (0.02% Tween-20 in 0.01 M phosphate buffered saline, pH 7.4), 0.32 mL of BSA-PBS (1% bovine serum albumin in 0.01 M PBS) was added and allowed to incubate at 37 °C for 30 min. The plate was washed as described above and 50 µL of HFB_1 was added followed by 50 µL of serum. The competition reaction was allowed to proceed for 1 h at 37 °C, after which the plate was washed and 100 µL of goat anti-mouse peroxidase conjugate (diluted 1:3,000 in BSA-PBS) was added. The plate was incubated for 30 min at 37 °C then washed with Tween-PBS before addition of 100 µL of the substrate, *o*-phenylenediamine (OPD). After 10 minutes at 37 °C the reaction was stopped by the addition of 0.1 mL of 1 N hydrochloric acid. Color development was determined from the absorbance at 490 nm. Sera from all five of the mice given injections with HFB_1-CT showed titer to HFB_1-OA and were displaced with free HFB_1.

Hybridoma Production. Mice with sera containing antibodies reactive to HFB_1 were aseptically splenectomized and the splenocytes isolated for use in cell fusions (*29*). Splenocytes were fused to a Balb/c non-immunoglobulin secreting mouse myeloma cell line, SP2/0-Ag14 and were plated in HAT medium, as per Kennett et al. (*29*). After 2–3 weeks, HAT resistant cultures were isolated and screened for anti-HFB_1 activity by competitive indirect ELISA. Positive cultures were subsequently cloned, expanded and frozen at -70 °C. Cloning was performed by limiting dilution (1 cell/well) and microscopically scored to assure monoclonality.

The mouse with the serum giving the best displacement (mouse O) was given a booster injection of HFB_1-CT and used for lymphocyte fusion. None of the 81 fusion products from this animal could be displaced with free HFB_1. Fusion of lymphocytes derived from the mouse with the second best displacement (mouse L) resulted in 79 products, two of which could be displaced by HFB_1. Unfortunately neither of the positive fusion products could be recovered in viable form after they were frozen. Fusion products were obtained from a third mouse (mouse RR). Of the 192 products obtained, five were displaced with HFB_1. Three of these were subcloned; two of the three were negative. The remaining clone yielded 16 subclones, 15 of which were able to be displaced with free HFB_1. One of the subclones, the cell line P2F11-3-H7, produced an antibody (IgG_1, kappa light chain) that was the basis for further immunoassay development.

Ten-week-old female Balb/c mice were given i.p. injections of 0.5 mL of 2,6,10,14-tetramethylpentadecane (Pristane) seven days before they were given an i.p. injection of $3x10^6$ cells of the hybridoma cell line suspended in 1.0 mL PBS, pH 7.2 (*30*). Following a 2–3 week waiting period ascitic fluid was collected, every other day for a period of 12 days, via intraperitoneal paracentesis. The fluid was delipidated (*31, 32*) and the antibody precipitated with a final concentration of 50% saturated ammonium sulfate. The monoclonal antibody P2F11-3-H7 was used as the basis for a competitive direct (CD) ELISA for HFB_1.

Competitive Direct ELISA. In this assay, free HFB_1 and HFB_1 labeled with horseradish peroxidase (HFB_1-HRP) competed for limited amounts of the P2F11-3-H7 antibody attached to the plate. The HFB_1-HRP was prepared by the sodium periodate method of Nakane and Kawaoi (*33*). Briefly, free amine groups of HRP were blocked by the addition of 2,4-dinitro-fluorobenzene (FDNB). Hydroxyl groups on HRP were then oxidized by adding sodium periodate, and allowing to stand for 30 min. The reaction was quenched by the addition of ethylene glycol and the mixture was incubated 1 h. The resulting HRP solution was dialyzed against 0.01 M sodium carbonate prior to the addition of HFB_1 to achieve a molar ratio of 25:1 (HFB_1:HRP). After incubation of the reaction mixture for 3 h at room temperature, sodium borohydride was added to achieve a level of 1.25 mg/mL and allowed to react overnight at 4 °C. Following dialysis against PBS the HFB_1-HRP conjugate was diluted 1:3 in a stabilizer solution (SuperFreeze, Pierce, Rockford IL) and stored at -20 °C. The conjugate was diluted 1:2,000 in BSA-PBS before use.

The solid phase of the CD-ELISA was prepared by transferring 0.1 mL of diluted antibody (75 µg antibody/mL coating buffer) to the wells of a polystyrene microtiter plate and allowing to bind overnight at 4 °C. After washing 4 times with 0.32 mL Tween-PBS, the wells were blocked with 0.32 mL of BSA-PBS and incubated at 37 °C for 30 min. The wells were washed and 25 µL of BSA-PBS was added, followed by 50 µL of sample (or standard) and 50 µL of HFB_1-HRP. The plates were incubated 1 h at 37 °C and the bound enzyme conjugate was determined as described above.

Corn samples (25 g) were extracted with 100 mL of a mixture of 80% methanol/20% phosphate buffer solution and concentrated using C_{18} Sep-Pak cartridges (Maragos et al., in press). On the day of analysis dried extracts, equivalent to 2.5 g of corn, were solubilized with 0.2 mL of 80% methanol and diluted to 2.0 mL with BSA-PBS. Samples were then analyzed by CD-ELISA.

Results and Discussion

The P2F11-3-H7 antibody was remarkably specific for HFB_1, and was reactive with the hydrolysis products of FB_2, FB_3, and FB_4. These products (HFB_2, HFB_3, HFB_4) are similar in structure to the aminopentol HFB_1 but lack either the hydroxyl group at C10 (HFB_2), the hydroxyl group at C5 (HFB_3), or both (HFB_4). The antibody did not bind significantly with any of the four intact fumonisins or with sphingosine, sphinganine, or tricarballylic acid. The reactivity of the antibody provided insight into the actual site of attachment to the molecule. In the competitive direct ELISA, the lowest IC_{50} (36 ng/mL) was observed with HFB_1, followed by HFB_3 (174 ng/mL), HFB_2 (331 ng/mL) and HFB_4 (1700 ng/mL). This indicated binding was best when both the C5 and C10 hydroxyl groups were present. The lack of reactivity with the intact fumonisins (especially FB_1) indicated the presence of both the C5 and C10 hydroxyls was not sufficient for binding. The binding of the antibody in the C5–C10 region of the intact fumonisins is apparently inhibited by the bulky tricarballylic groups present at C14 and C15.

The CD-ELISA was sensitive for HFB_1, with a lower limit of detection of 2 ng/mL in BSA-PBS. At the IC_{50} the amount of HFB_1 present in the microtiter well was 1.8 ng (4.4 pmol). The ELISA was applied to the analysis of HFB_1 in corn over the range of 5–1000 ng/g. Recovery of HFB_1 after extraction and concentration using a C_{18} Sep-Pak cartridge averaged 79% (Maragos et al., in press). The limit of quantification for the method was 10 ng HFB_1/g, with an IC_{50} of 41 ng/g. Reproducibility below 20 ng/g was poor, but improved substantially above this level (coefficient of variation of 8.1% between 50 and 500 ng/g).

The diluent used to prepare the HFB_1 standard curve influenced the sensitivity of the method and was important for accurate quantitation. Quantification was most accurate when samples were compared to a standard curve prepared in a control (low fumonisin) corn extract that was passed through a C_{18} Sep-Pak cartridge. Several components of the extract were capable of influencing the standard curve: in particular the solvent concentration and the amount of corn matrix present.

Preparation of standards in BSA-PBS containing 8% methanol produced an assay with an IC_{50} of 126 ng/mL, roughly three-fold higher than when methanol was absent. Further examination indicated methanol or acetonitrile can adversely affect the sensitivity of the assay (Table I).

Table I. Effect of Solvent Upon the CD-ELISA

Solvent (%)[a]	*Relative IC_{50}*[b] Methanol	Acetonitrile
2.5	NT[c]	1.51
5.0	1.23	2.18
7.5	1.96	3.00
10.0	2.38	3.83
12.5	NT	4.81
15.0	2.77	11.64
20.0	4.23	NT
25.0	8.97	NT

[a] Percentage of solvent used to prepare the standard curve.
[b] The IC_{50} observed with solvent divided by the IC_{50} observed with BSA-PBS (71 ng/mL).
[c] NT: not tested.

The antibody was more susceptible to acetonitrile than to methanol. As a result, corn extracted with acetonitrile/water mixtures must be diluted more than if methanol/water extracts are used, and sensitivity will be correspondingly reduced when acetonitrile is used. Because the solvent content directly influenced the sensitivity of the assay, the solvent concentration in extracts applied to the CD-ELISA must be rigidly controlled, particularly if quantitation of an unknown amount of HFB_1 is desired.

The CD-ELISA was also influenced by constituents of the corn matrix itself. Components present in the corn extract reduced the binding of HFB_1-HRP both in the presence and absence of HFB_1. To separate the effects of solvent and corn constituents an extract of corn was diluted to various degrees with a solution of 8% methanol and 1% BSA in PBS. The methanol content of all samples were equivalent (8%), yet, the maximum color development was reduced and the competition reaction was inhibited as the concentration of corn matrix was increased (Figure 1). The IC_{50}'s ranged from 96 ng/mL (extract diluted 1:5) to 196 ng/mL (undiluted extract). While dilution of the extract enhanced the IC_{50} of the assay, in practice the sensitivity with corn was reduced because the gain in sensitivity provided by the improved IC_{50} was offset by the extra sample dilution. For example, diluting the sample 1:3 resulted in a roughly twofold improvement in IC_{50}, whereas a threefold improvement would be required to justify making the dilution. Dilution with BSA-PBS, rather than 8% methanol-BSA-PBS, may improve the IC_{50} enough to justify the use of a threefold dilution. The matrix effect may vary with the source of corn, and therefore the highest dilution of extract that can be used, while still maintaining adequate sensitivity, is encouraged. Currently under investigation are an immunoaffinity column instead of a C_{18} cartridge cleanup, and application of the CD-ELISA to matrices other than corn, such as serum, where sample pretreatment may not be required.

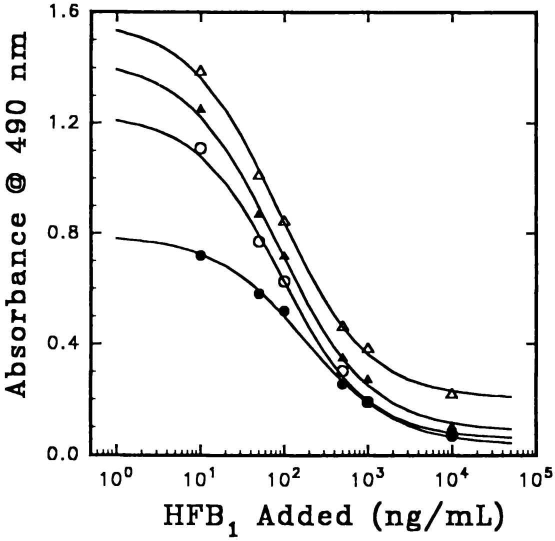


Figure 1. Effect of corn matrix upon the performance of the HFB_1 CD-ELISA. Corn matrix, obtained after cleanup of a corn sample by C_{18} solid-phase extraction, was diluted to various degrees with 8% methanol, 1% BSA in phosphate buffer as described in the text, then spiked with HFB_1. Curves are for undiluted extract, equivalent to 1.25 g corn/mL (●); extract diluted 1:3 (O); extract diluted 1:5 (▲); and extract diluted 1:10 (Δ).

Conclusions

Sensitive immunoassays for the detection of hydrolyzed fumonisins, in particular HFB_1, have been developed. The CD-ELISA provides a rapid method for screening corn for HFB_1 at levels above 5 ng/g, and for quantification above 20 ng/g. Variables such as solvent content and matrix constituents play a role in quantitation and must be addressed by sample cleanup or dilution.

Acknowledgments

We thank Judy Greer at the USDA-ARS-NCAUR and Amy Hamilton at the University of Illinois-Urbana for excellent technical assistance. Mention of companies or products does not imply their endorsement by the US Department of Agriculture over others not cited.

Literature Cited

1. Wang, E.; Norred, W. P.; Bacon, C. W.; Riley, R. T.; Merrill, A. H. Jr. *J. Biol. Chem.* **1991**, *266*, 14486–14490.
2. Merrill, A. H. Jr.; Van Echten, G.; Wang, E.; Sandhoff, K. *J. Biol. Chem.* **1993**, *268*, 27299–27306.

3. Sauviat, M. P.; Laurent, D.; Kohler, F.; Pellegrin, F. *Toxicon,* **1991**, *29*, 1025–1031.
4. Norred, W. P.; Wang, E.; Yoo, H.; Riley, R. T.; Merrill, A. H. Jr. *Mycopath.* **1992**, *117*, 73–78.
5. Gelderblom, W. C. A.; Cawood, M. E.; Snyman, S. D.; Vleggaar, R.; Marasas, W. F. O. *Food Chem. Toxicol.* **1993**, *31*, 407–414.
6. Hendrich, S.; Miller, K. A.; Wilson, T. M.; Murphy, P. A. *J. Agric. Food Chem.* **1993**, *41*, 1649–1654.
7. Shephard, G. S.; Thiel, P. G.; Sydenham, E. W.; Vleggaar, R.; Alberts, J. F. *Food Chem. Toxicol.* **1994**, *32*, 23–29.
8. Sydenham, E. W.; Gelderblom, W. C. A.; Thiel, P. G.; Marasas, W. F. O. *J. Agric. Food Chem.* **1990**, *38*, 285–290.
9. Ross, P. F.; Rice, L. G.; Plattner, R. D.; Osweiler, G. D.; Wilson, T. M.; Owens, D. L.; Nelson, H. A.; Richard, J. L. *Mycopathologia* **1991**, *114*, 129–135.
10. Thiel, P. G.; Shephard, G. S.; Sydenham, E. W.; Marasas, W. F. O.; Nelson, P. E.; Wilson, T. M. *J. Agric. Food Chem.* **1991**, *39*, 109–111.
11. Richard, J. L., Bennett, G. A., Dombrink-Kurtzman, M. A.; Maragos, C. M. Proceedings of the 48th Annual Corn and Sorghum Industry Research Conference. Session II, **1993**, 125–138.
12. Rottinghaus, G. E.; Coatney, C. E.; Minor, H. C. *J. Vet. Diagn. Invest.* **1992**, *4*, 326–329.
13. Shephard, G. S.; Sydenham, E. W.; Thiel, P. G.; Gelderblom, W. C. A. *J. Liq. Chrom.* **1990**, *13*, 2077–2087.
14. Bennett, G. A.; Richard, J. L. *J. AOAC Intl.* **1994** *77*, 501–506.
15. Plattner, R. D.; Norred, W. P.; Bacon, C. W.; Voss, K. A.; Peterson, R.; Shackelford, D. D.; Weisleder, D. *Mycologia* **1990**, *82*, 698–702.
16. Voss, K. A.; Norred, W. P.; Plattner, R. D.; Bacon, C. W. *Food Chem. Toxicol.* **1989**, *27*, 89–96.
17. Azcona-Olivera, J. I.; Abouzied, M. M.; Plattner, R. D.; Pestka, J. J. *J. Agric. Food Chem.* **1992**, *40*, 531–534.
18. Fukuda, S.; Nagahara, A.; Kikuchi, M.; Kumagai, S. *Biosci. Biotech. Biochem.* **1994**, *58*, 765–767.
19. Pestka, J. J.; Azcona-Olivera, J. I.; Plattner, R. D.; Minervini, F.; Doko, M. B.; Visconti, A. *J. Food Prot.* **1994**, *57*, 169–172.
20. Shelby, R. A.; Rottinghaus, G. E.; Minor, H. C. *J. Agric. Food Chem.* **1994**, *42*, 2064–2067.
21. Usleber, E.; Staka, M.; Terplan, G. *J. Agric. Food Chem.* **1994**, *42*, 1392–1396.
22. Ware, G. M.; Umrigar, P. P.; Carman, A. S. Jr.; Kuan, S. S. *Anal. Lett.* **1994**, *27*, 693–715.
23. Chu, F. S.; Huang, X.; Maragos, C. M. *J. Agric. Food Chem.* **1995**, *43*, 261–267.
24. Nelson, P. E.; Desjardins, A. E.; Plattner, R. D. *Ann. Rev. Phytopathol.* **1993**, *31*, 233–252.
25. Hopmans, E. C.; Murphy, P. A. *J. Agric. Food Chem.* **1993**, *41*, 1655–1658.
26. Scott, P. M.; Lawrence, G. A. Poster presented at the 108th Meeting of the AOAC International: Portland, OR, September 12–15, 1994; Poster no. 24-001.
27. Azcona-Olivera, J. I.; Abouzied, M. M.; Plattner, R. D.; Norred, W. P.; Pestka, J. J. *Appl. Environ. Microbiol.* **1992**, *58*, 169–173.

28. Maragos, C. M.; Plattner, R. D.; Miklasz, S. D. *Food Add. Contam.*, in press.
29. Kennett, R. H.; McKearn, T. J.; Bechtol, K. B. *Monoclonal Antibodies: A New Dimension in Biological Analyses.* Plenum Press: New York, NY, 1982; pp. 365–367.
30. Hoogenraad, N.; Hewman, T.; Hoogenraad, J. *J. Immun. Meth.* **1983**, *61*, 317–320.
31. Allen, D. C.; Hill, E. A. *Plasma Proteins*; Blackwell Scientific Publications Ltd.: Oxford, UK, 1977; p. 222.
32. Walton, K. W.; Scott, P. J. *J. Clin. Path.* **1964**, *17*, 627–643.
33. Nakane, P. K.; Kawaoi, A. *J. Histochem. Cytochem.* **1974**, *22*, 1084–1091.

RECEIVED June 21, 1995

Chapter 28

Evaluation and Application of Immunochemical Methods for Fumonisin B_1 in Corn

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Two immunochemical methods were evaluated and compared with a strong anion exchange (SAX) method for the determination of fumonisin B_1 (FB_1) in corn. The two methods were the enzyme-linked immunosorbent assay (ELISA) and the monoclonal antibody-affinity column (IAC) method. The ELISA test is a direct competitive microtiter-well assay in which a strip reader, which measures the color of an enzymatic reaction, is combined with a log/logit data transformation program and linear regression calibration to determine FB_1 concentration. FB_1 in test samples from IAC or SAX isolation is derivatized with *o*-phthaldialdehyde, and the derivative is separated from other impurities by reversed-phase high-performance liquid chromatography (HPLC) and then quantitated by fluorescence detection. Recoveries of FB_1 from corn spiked over the range of 1–4 μg/g were 73–106, 79–83, and 64–92% for the ELISA, IAC, and SAX methods, respectively. Results of analysis of the same extract from naturally contaminated corn by the three methods were similar. HPLC-electrospray mass spectrometry was used to positively identify the FB_1 isolated with the microtiter wells from an extract of a naturally contaminated corn.

Mycotoxins are natural toxins produced by fungi. The occurrence of mycotoxins in foods and feeds is a worldwide problem. The five most important toxins from an agricultural perspective are the aflatoxins, deoxynivalenol, zearalenone, ochratoxin A, and the fumonisins (*1*). Analytical methods are well established for the first four toxins. The fumonisins represent the most recently discovered family of Fusarium toxins (2). They are water-soluble metabolites produced on corn by *Fusarium moniliforme*, *F. proliferatum*, and several other fungi (*3,4*). The most abundant of the fumonisins are fumonisin B_1 (FB_1) and fumonisin B_2

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(FB_2). The FB_1/FB_2 ratio in corn is usually about 3:1 (*5*). FB_1 was found to cause equine leucoencephalomalacia (*2*), porcine pulmonary edema (*6*), and rodent hepatotoxicity (*7*) and has been implicated in human esophageal cancer on the basis of epidemiological data (*8,9*). The International Agency for Research on Cancer, Working Group on the Evaluation of Carcinogenic Risks in Humans, evaluated the available toxicological data and classified the toxins derived from *F. moniliforme*, which include FB_1 and FB_2, as possible carcinogens to humans (*10*). Therefore, great interest has been generated to develop methods of analysis for these toxins.

Fumonisins can be separated and identified by high-performance liquid chromatography (HPLC) (*11*), thin-layer chromatography (*12*), and gas chromatography-mass spectrometry (GC-MS) (*13*). Immunochemical methods also have been developed (*14-16*). With the availability of commercial immunoassay kits, monitoring for fumonisin contamination can be moved from the laboratory to the production facilities. Immunoassays can be used to obtain data quickly, so that immediate action can be taken when necessary.

In order to meet the demand for minimum laboratory sample handling and increased numbers of analyses, we evaluated two immunochemical methods: an enzyme-linked immunosorbent assay (ELISA) method (Veratox Quantitative Fumonisin Test, Neogen Corp., Lansing, MI 48912, 1995) and an immunoaffinity column method (IAC) (*17*). The parameters, such as the capacity, specificity, accuracy, and precision of the two methods, were compared with those obtained for a strong anion exchange column (SAX) method (*11*). An advantage of the ELISA method is that it does not require derivatization of the toxin with *o*-phthaldialdehyde (OPA) and reversed-phase HPLC separation for quantitation with a fluorescence detector. We also investigated the cross-reactivities of the antibodies and the use of an MS technique to confirm the identity of FB_1 isolated from the ELISA antibody-coated wells.

Experimental

Standards. FB_1 and FB_2 were isolated from cultured corn in our laboratory. On the basis of MS ion ratio data, the purities of the compounds were 96 and 90%, respectively.

Samples. Corn analyzed by a reference method (*11*) and shown to contain FB_1 at a level of 150 ng/g was used for the recovery study.

Extraction. Test portions of corn spiked at 1, 2, and 4 μg/g and of naturally contaminated corn (50 g) were extracted with 250 mL methanol-water (70/30). The extracts were then diluted as described in the three methods. Five replicates of each test solution (diluted extract) were analyzed by the ELISA method. Three replicates of each test solution were analyzed by the other two methods.

ELISA Method. The polyclonal antibodies against FB_1 were produced in rabbits after immunization with an FB_1-keyhole limpet hemocyanin conjugate (*18*). The assay used a microtiter-well format. The corn extracts (100 μL) were diluted with 3.9 mL methanol-water (10/90). The diluted extracts and standard solutions were added to the wells, which contained FB_1-horseradish peroxidase (HRP) conjugate in 100 μL buffer. After mixing, the contents of the wells were transferred to the antibody-coated wells. (The wells were coated with sheep anti-fumonisin polyclonal antibodies in 0.1 M carbonate buffer (pH 9.6) overnight at 40 °C. The wells were washed with deionized water. Nonspecific binding was minimized by blocking the unbound sites of the microtiter wells with 300 μL of 1% polyvinyl alcohol (w/v) in phosphate-buffered saline for 30 min at 37 °C. After washing with deionized water, strips were dried and packed in foil pouches with desiccant, sealed, and stored at 4 °C until used.) The wells were incubated at room temperature for 20 min with mixing for 30 s at 5 min intervals. The wells were then washed five times with water, and 100 μL tetramethylbenzidine-peroxide substrate solution was added. After 10 min the reaction was stopped by adding 100 μL diluted sulfuric acid solution, and the toxins were quantitated from the absorbance, which was measured at 450 nm with an ELISA reader coupled to a computer using log/logit software.

IAC Method. The column (Vicam, 29 Mystic Ave., Somerville, MA 02145) employed monoclonal antibody-coated agarose beads. The corn extract (10 mL) was diluted with 40 mL diluting solution (12.5 g sodium chloride, 2.5 g sodium bicarbonate, and 2 drops Tween 20 in 500 mL water). After filtering, 5 mL of the diluted extract (equivalent to 0.2 g test sample) was added to the column. The column was washed with 5 mL diluting solution and 5 mL water, and the toxin was eluted by washing two times with 0.8 mL methanol-water (80/20). The eluate was evaporated, and the residue was redissolved in 200 μL methanol, derivatized, and analyzed by HPLC.

SAX Method. The cartridge was packed with 0.5 g quaternary amine (strong anion exchange) in a polypropylene tube with a 10 mL reservoir (Varian). The cartridge was conditioned with 5 mL methanol and 5 mL methanol-water (70/30). The corn extract (10 mL, equivalent to 2 g test sample) was applied to the cartridge. The cartridge was washed with 5 mL methanol-water (70/30) and 3 mL methanol. The toxin was eluted with 10 mL methanol-acetic acid (99/1), and the solvent was evaporated at 60 °C under a stream of nitrogen. The residue was dissolved in 200 μL methanol, derivatized, and analyzed by HPLC.

Derivatization and HPLC Analysis. The derivatization reagent consisted of 40 mg OPA, 1 mL methanol, 5 mL 0.1 M sodium tetraborate, and 50 μL mercaptoethanol. A Waters 710 Plus autoinjector was used to deliver 100 μL derivatization reagent to 25 μL test solution, and to mix the solution and inject it onto a 25 cm x 3.9 mm, 5 μm Waters μBondapak C_{18} column. The mobile phase was acetonitrile-water-acetic acid (50/50/1); the flow rate was 1 mL/min.

A Waters 470 fluorescence detector was used at an excitation wavelength of 355 nm and an emission wavelength of 440 nm.

Confirmation of Identity of FB_1. Test solution (100 μL) and 100 μL buffer were added to each of the five antibody-coated wells. After incubating at room temperature for 20 min with mixing for 1 min at 3 min intervals, the wells were washed five times with water. The toxin was eluted by adding 100 μL methanol to each well and mixing for 1 min. The combined methanol was transferred to a 1.5 mL Eppendorf tube and evaporated on a steam bath under a stream of nitrogen. The wells were rinsed twice with methanol. The methanol was added to the same tube and evaporated. The residue was redissolved and analyzed by HPLC–electrospray MS by using a Finnigan TSQ 7000 mass spectrometer (Musser, S. M., Food and Drug Administration, personal communication, 1995).

Results and Discussion

Various ratios of methanol/water or acetonitrile/water have been used to extract fumonisins from corn and corn-based products. Although the ELISA, IAC, and SAX methods use methanol/water in ratios of 7/3, 8/2, and 7.5/2.5 for extractions, respectively, the test portions in this evaluation were extracted with methanol/water (7/3). The same extract was used for the three methods to eliminate variability in extraction efficiency and sampling.

We examined the immunochemical methods for their cross-reactivity with structurally related analytes, cross-reactivity with dissimilar analytes, and capacity. We compared the accuracy and precision of the results from analyses of spiked corn by these methods and by the reference method. The correlation coefficients of the immunochemical methods and the reference method were determined from comparisons of results obtained from analyses of naturally contaminated corn.

For the ELISA method, the relative cross-reactivities of FB_1, FB_2, and FB_3 were found to be 100, 24, and 30%, respectively. In Figure 1 the x-axis (log scale) indicates the toxin concentration. The y-axis indicates the relative binding of toxin-HRP (B/B_o) (absorbance of standard toxin concentration/absorbance of toxin negative buffer solution) x 100. The relative cross-reactivity of each fumonisin was calculated on the basis of concentration necessary to inhibit 50% of the FB_1-HRP binding.

The relative cross-reactivities of the fumonisins on the immunoaffinity columns were not provided by the manufacturer. The recoveries of FB_1 and FB_2 added to the columns are shown in Table I. The average recovery of FB_1 added to methanol/water was 89% in the range of 0.3–1.0 μg, whereas the average recovery of FB_2 for the same range was 79%. The average recovery of FB_1 added to corn extract (same levels as in methanol/water) was 90%, whereas the average recovery of FB_2 was 77%. The recoveries of both toxins added to methanol/water and to corn extract were similar from the immunoaffinity column, indicating that the matrix did not affect the antibodies. This finding also

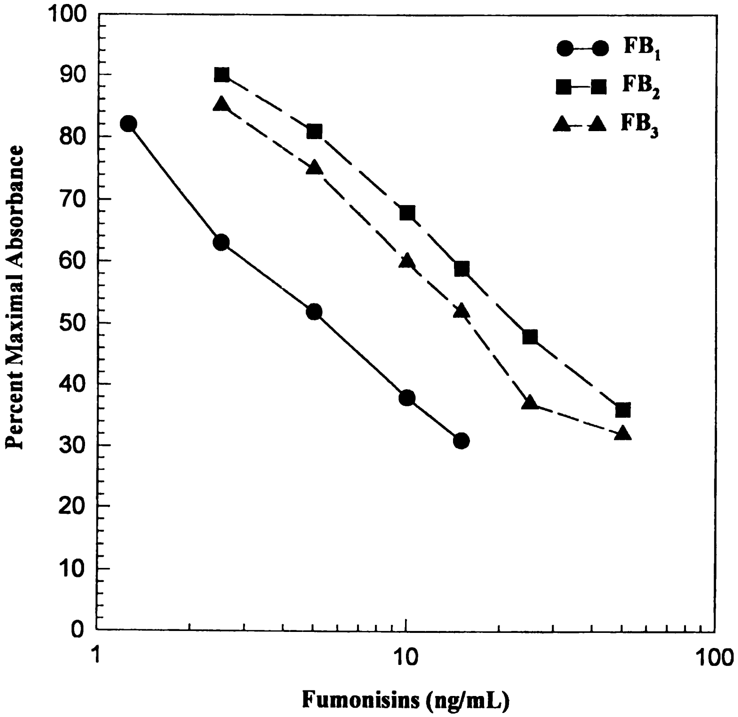

Figure 1. Cross-reactivities of FB_1 (●-●), FB_2 (■-■), and FB_3 (▲-▲) in the ELISA.

Table I. Recoveries of FB_1 and FB_2 Added to Methanol/Water and to Corn Extract from Immunoaffinity Column

Matrix	*Amount Added (μg)*		*Recovery (%)*	
	FB_1	*FB_2*	*FB_1*	*FB_2*
MeOH/H_2O	1.0	0	83	--
	0.7	0.3	90	83
	0.5	0.5	90	76
	0.3	0.7	93	83
	0	1.0	--	75
			Av. **89**	**79**
Corn extract	1.0	0	97	--
	0.7	0.3	87	74
	0.5	0.5	88	84
	0.3	0.7	87	74
	0	1.0	--	76
			Av. **90**	**77**

suggested that the antibodies might have similar cross-reactivity with FB_1 and FB_2.

It is important to demonstrate that the analyte of concern can be identified in the presence of dissimilar compounds. More than one mycotoxin can be found in the same grain. Many other Fusarium toxins, such as deoxynivalenol and zearalenone, are often found in corn; cross-reactivity with these compounds is undesirable. The ELISA method provided similar results from test solutions containing FB_1, FB_1 and deoxynivalenol, FB_1 and zearalenone, and all three fumonisins. The IAC also showed no binding with deoxynivalenol and zearalenone.

The applicable range of the ELISA method was 0.1-2.5 ng/mL, which is equivalent to 0.5-10 μg/g corn. The IAC has a maximum binding capacity of 1 μg FB_1 according to the manufacturer. When an extract (equivalent to 1 g) of corn contaminated with FB_1 at 1.90 μg/g was added to the immunoaffinity column, the corn was found to contain FB_1 at 0.92 μg/g. However, when an extract (equivalent to 1 g corn) containing <50 ng FB_1/g was spiked with FB_1 at 0.3-1.0 μg/g, the average recovery was 90%. From this information, the capacity of the column was estimated to be about 1.1 μg FB_1. Similar capacity of the column was determined by Ware et al. (*19*). The maximum loading capacity of the column was equivalent to 2 g corn. The limit of determination was 25 ng/g. The lower and upper limits of determination depend on the amount of extract applied to the column and the amount of the toxin derivative injected

Table II. Recoveries of Fumonisin B_1 from Corn by Ion Exchange (SAX), ELISA, and Immunoaffinity Column (IAC) Methods

FB_1 Added (µg/g)	*Recovery (%)*[a]		
	SAX	*ELISA*	*IAC*
1.0	64	73	83
2.0	92	85	79
4.0	89	106	81
RSD_r (%)[b]	4.7-8.9	4.9-7.2	0.9-3.5

[a] Corrected for background levels of 0.15, 0.10, and 0.13 µg/g obtained by SAX, ELISA, and IAC, respectively.
[b] RSD_r = repeatability relative standard deviation.

onto the HPLC column. The manufacturer recommends application of the equivalent of 0.2 g extract to the column. For corn containing >5 µg FB_1/g, the analysis should be repeated using the equivalent of <0.2 g extract.

The applicable range for the SAX method was about 25–15,000 ng/g. This was based on loading the equivalent of 5 g extract on the SAX cartridge.

The fluorescence intensity of the OPA derivative is time dependent. The maximum intensity is obtained within 1 min after the addition of the OPA reagent to the extract. The fluorescence intensity response decreased 15% after 10 min and 30% after 1 h (*17*). When the autoinjector was used, it took about 10 min to prepare the derivative. The day-to-day variability of the fluorescence intensity of the OPA derivative was about 10%. Within the same day, the variation of the fluorescence intensity of the OPA derivative of the same standard solution was about 3%, which is the sum of the variation of the derivative and the variation of the injector. When the same OPA reagent was used after 8 days, the fluorescence intensity of the OPA derivatives declined more than 20%, compared with that of OPA derivatives prepared with freshly made reagent.

The recoveries of FB_1 at the 1, 2, and 4 µg/g levels, obtained by the three methods, are shown in Table II. The accuracy and precision of the methods were similar. The methods compare very favorably.

Results of analyses of corn-based products purchased in Washington, DC, area grocery stores are shown in Table III. All 10 samples were contaminated with FB_1. Results obtained by ELISA were about 38% higher than those obtained by the SAX method. This difference probably was caused by the cross-reactivity of the antibodies with the FB_1 structurally related compounds or the loss of FB_1 in the solid-phase purification prior to HPLC analysis. The slope of

Table III. Analysis of Corn Meal for Fumonisin B_1 (μg/g) by Ion Exchange (SAX), ELISA, and Immunoaffinity Column (IAC) Methods

Sample No.	*SAX* *FB_1*	*ELISA* *Total Fumonisins*	*IAC* *FB_1*
1	0.61	0.50	0.44
2	2.86	3.94	2.76
3	0.15	0.24	0.19
4	0.61	0.96	0.30
5	0.17	0.26	0.26
6	0.27	0.46	0.60
7	1.48	2.28	0.95
8	2.99	4.02	1.95
9	0.40	0.80	0.50
10	4.28	6.02	3.08

the ELISA concentration vs. the SAX concentration was 1.3765 ($Y = 1.3765X + 0.0456$); the correlation coefficient was 0.9964. Results obtained by the IAC method were about 71% of those obtained by the SAX method. The slope of the IAC concentration vs. the SAX concentration was 0.7135 ($Y = 0.7135X + 0.1169$); the correlation coefficient was 0.9674. Since the SAX and IAC methods are for FB_1 alone, and the ELISA is for "total" fumonisins, we considered the overall agreement of the results obtained by the three methods to be acceptable.

We confirmed the identity of FB_1 isolated from antibody-coated wells, using an MS technique. Six test solutions were added to the wells: (1) buffer containing FB_1-HRP and 10% methanol, (2) buffer containing FB_1-HRP and diluted corn extract, (3) diluted corn extract, (4) 20 μg FB_1 standard, (5) buffer (no FB_1-HRP) and diluted naturally contaminated corn extract (3.8 μg/g, 0.1 mL extract was added to 1.9 mL 10% methanol), and (6) buffer (no FB_1-HRP). MS analysis showed the FB_1 molecular ion in test solutions 1, 2, and 5. This response indicated that the buffer containing the FB_1 conjugate could not be used as a negative control, and that FB_1 would not bind to the antibody in the absence of the buffer solution. This is the first time that a mycotoxin isolated from antibody-coated wells was positively identified by an MS technique.

In summary, the immunochemical methods for determining FB_1 in corn gave results that are similar to those obtained by the reference method, and they are compatible with the HPLC method, as indicated in Table IV. We also demonstrated for the first time that the ELISA device can be used to isolate an

Table IV. Comparison of Ion Exchange (SAX), ELISA, and Immunoaffinity Column (IAC) Methods for the Analysis of Corn for Fumonisin B_1

Factor	*SAX*	*ELISA*	*IAC*
Range (ng/g)	25-15,000	500-6,000	25-5000
Accuracy (%), 1-4 μg/g[a]	64-92	73-106	79-83
Precision (%), 1-4 μg/g	4.7-8.9	4.9-7.2	0.9-3.5
Applicability	corn	corn	corn
Cross-reactivity	NA[b]	B_1:B_2=100:24	B_1:B_2=100:90
Time (min)/analysis	50	35	50
Equipment	LC	ELISA reader	LC
Location	laboratory	field	laboratory
Temperature	ambient	>23 °C	ambient
MS confirmation	yes	yes	yes

[a] Recovery.
[b] NA = not applicable.

analyte for MS analysis. Thus, the immunochemical methods not only can provide a rapid on-site field test, but they also can effectively complement the standard HPLC procedure currently used in routine monitoring for fumonisins in foods.

Literature Cited

1. Miller, J. D. In *The Toxicology Forum*; Caset Assoc. Ltd.: Fairfax, VA, 1994; pp 3-9.
2. Gelderblom, W. C. A.; Jaskiewicz, K.; Marasas, W. F. O.; Thiel, P. G.; Horak, R. M.; Vleggaar, R.; Kriek, N. P. J. *Appl. Environ. Microbiol.* **1988**, *54*, 1806-1811.
3. Nelson, P. E.; Plattner, R. D.; Shackelford, D. D.; Desjardins, A. E. *Appl. Environ. Mircobiol.* **1992**, *58*, 984-989.
4. Thiel, P. G.; Marasas, W. F. O.; Sydenham, E. W.; Shephard, G. S.; Gelderblom, W. C. A.; Nieuwenhuis, J. *Appl. Environ. Microbiol.* **1991**, *57*, 1089-1093.
5. Pohland, A. E. In *The Toxicology Forum*; Caset Assoc. Ltd.: Fairfax, VA, 1994; pp 186-196.
6. Harrison, L. R.; Colvin, B. M.; Greene, J. T.; Newman, L. E.; Cole, J. R. *J. Vet. Diagn. Invest.* **1990**, *2*, 217-221.

7. Gelderblom, W. C. A.; Kriek, N. P. J.; Marasas, W. F. O.; Thiel, P. G. *Carcinogenesis* **1991**, *12*, 1247–1251.
8. Sydenham, E. W.; Thiel, P. G.; Marasas, W. F. O.; Shephard, G. S.; van Schalkwyk, D. J.; Koch, K. R. *J. Agric. Food Chem.* **1990**, *38*, 1900–1903.
9. Thiel, P. G.; Marasas, W. F. O.; Sydenham, E. W.; Shephard, G. S.; Gelderblom, W. C. A. *Mycopathologia* **1992**, *117*, 3–9.
10. *International Agency for Research on Cancer*, Monograph 56, 1993; pp 445–466.
11. Sydenham, E. W.; Shephard, G. S.; Thiel, P. G. *J. AOAC Int.* **1992**, *75*, 313–318.
12. Rottinghaus, G. E.; Coatney, C. E.; Minor, H. C. *J. Vet. Diagn. Invest.* **1992**, *4*, 326–329.
13. Plattner, R. D.; Branham, B. E. *J. AOAC Int.* **1994**, *77*, 525–532.
14. Azcona-Olivera, J. I.; Abouzied, M. M.; Plattner, R. D.; Pestka, J. J. *J. Agric. Food Chem.* **1992**, *40*, 531–534.
15. Shelby, R. A.; Rottinghaus, G. E.; Minor, H. C. *J. Agric. Food Chem.* **1994**, *42*, 2064–2067.
16. Usleber, E.; Straka, M.; Terplan, G. *J. Agric. Food Chem.* **1994**, *42*, 1392–1396.
17. Trucksess, M. W.; Stack, M. E.; Allen, S.; Barrion, N. *J. AOAC Int.* **1995**, *78*, 705–710.
18. Avrameas, S.; Ternynck, T. *Immunochemistry* **1969**, *56*, 1729–1733.
19. Ware, G. M.; Umrigar, P. P.; Carman, A. S.; Kuan, S. S. *Anal. Lett.*, **1994**, *27*, 693–715.

RECEIVED December 4, 1995

Chapter 29

Molecular Modeling Studies of the Fumonisin Mycotoxins

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The fumonisins are mycotoxins that are produced by the corn pathogen, *Fusarium moniliforme*, and have been shown to cause a wide range of toxic effects in numerous animals. They have been shown to have cancer-promoting activity in animals, and have been linked to human esophageal cancer. To date, the only biochemical findings show that the fumonisins are inhibitors of the sphingosine and sphinganine *N*-acyltransferase system. No other biochemical effect of the fumonisins has been established. This paper discusses the molecular models of the lowest energy conformations obtained for fumonisin B_1, B_2, B_3, and B_4 (FB_{1-4}) and the FB_1 backbone. The structure with the lowest energy conformation of FB_1 in relation to iron ligand formation is investigated. The lowest energy conformation of FB_1 based on the stereostructure of FB_1 (SS-FB_1) also is presented. These modeled structures provide insight into why antibodies produced to FB_1 have lower affinities for FB_1 than expected. They also produce insight into other potential activities that may be associated with these molecules.

Fusarium moniliforme Sheldon is an important ear rot pathogen of maize (*Zea mays* L.) and other grains world wide (*1*). Four biologically active fumonisins have been isolated from *F. moniliforme* cultures and contaminated grains, and characterized. Fumonisins B_1 and B_2 (FB_1 and FB_2), Figure 1 (*2*), and fumonisins B_3 and B_4 (FB_3 and FB_4), Figure 1 (*3,4*), were isolated from culture material and characterized.

F. moniliforme culture material and the pure fumonisins have a wide degree of biological activity in different animal species. When corn infected with *F. moniliforme* was fed to horses, clinical signs and lesions were produced typical of the fatal neurotoxic syndrome, equine leukoencephalomalacia (ELEM) (*5,6*). ELEM is characterized pathologically by liquefaction necrosis of the white matter of one or both cerebral hemispheres (*7*). Marasas *et al.* (*8*) presented the first experimental

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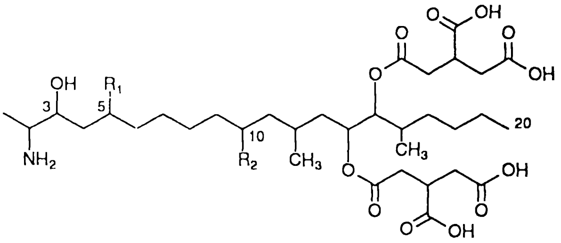

OH
R1
3
5
NH2
10
R2
CH3
O
O
OH
O
O
OH
20
CH3
O
OH
O
O
O
OH

Fumonisin B_1: $R_1 = OH$, $R_2 = OH$

Fumonisin B_2: $R_1 = OH$, $R_2 = H$

Fumonisin B_3: $R_1 = H$, $R_2 = OH$

Fumonisin B_4: $R_1 = H$, $R_2 = H$

Figure 1. Chemical structures of the fumonisins B_1-B_4.

evidence that FB_1 causes ELEM. FB_2 in cultured *Fusarium proliferatum* also was shown to cause ELEM (*9*). Since the early 1900's ELEM has been reported in the U.S. and it also is known in Africa, Brazil, China, Egypt, Japan, and in Europe (*8,10–13*). Corn samples associated with ELEM caused hepatotoxicity and renal toxicity in male Sprague-Dawley rats (*14*). *F. moniliforme* cultures caused porcine pulmonary edema (PPE) (*15,16*). Outbreaks of PPE syndrome occurred at the same places and times as ELEM outbreaks, and PPE syndrome can be caused by IV injection of FB_1 (*17*). Either PPE or liver failure emerged as distinct pathogenic expressions of FB_1 toxicoses over a series of pig studies (*18*). However, young female pigs fed a diet containing FB_1 developed nodular hyperplasia of the liver, and hyperplastic plaques within the distal esophageal mucosa (*19*). FB_1 undergoes enterohepatic cycling in the pig, and has affinity for specific tissues, particularly the liver (*20*).

F. moniliforme cultures caused acute nephrosis and hepatosis in sheep, and cirrhosis, intraventricular cardiac thrombosis, and nephrosis in rats (*15*). FB_1 is foetotoxic to rats by both suppressing growth and fetal bone development (*21*). Male white leghorn chickens fed diets containing MRC 826 (a strain of *F. moniliforme* isolated from corn) culture material resulted in decreased bursa weights, and these chickens were immunosuppressed in both the primary and secondary immune response (*22*). But, laying hens were not adversely affected from FB_1 exposure at 2 mg/kg (*23*). The effects of FB_1 (obtained from Indian maize) was studied on peritoneal exudate cells (macrophages) collected from 4-week-old female chickens. FB_1 caused a significant reduction in the viability and phagocytic potential of the female chicken peritoneal macrophages (*24*). It was hypothesized that FB_1 exposure may be a potential problem in India. FB_1 caused reduced body weights in turkey poults, but the relative weights of the liver and pancreas increased (*25,26*).

MRC 826 cultures were shown to have cancer-promoting activity (*27*). Dietary concentrations of MRC 826 culture material fed at 2% to rats caused hepatocellular carcinoma in 80% and ductular carcinoma of the liver in 63% of the surviving rats. Many of the rats also had basal cell hyperplasia of the esophageal epithelia (*28*). MRC 826 cultures caused a significant enhancement of nitrosamine-induced esophageal carcinoma in rats (*29*). The mammalian cell line, MDCK dog kidney epithelial cells, was sensitive to FB_1 and FB_2 (IC_{50} = 2.5 and 2 μg/mL, respectively). There was sufficient sensitivity of the assay to detect 200 ng FB_1 or FB_2. The MDCK cells exhibited morphological changes within 24 h of treatment (*30*). MRC 826 culture material was highly toxic to vervet monkeys (*Ceropithecus pygerythus*) and caused acute, subacute, and chronic toxic hepatitis (*31*). FB_1 treated vervet monkeys showed no hydrolysis products in the bile; however, in the gut the tricarballylic acid moiety at C-14 was hydrolyzed (*32*).

The main staple food of the Republic of Transkei, Union of South Africa is corn. Transkei has a high incidence of esophageal and liver cancer in humans (*33*). The Fusarium mycotoxins were hypothesized as playing a role in the development of tumors of the digestive tract (*34–37*). A positive correlation was found between food contaminated with *F. moniliforme* (*38,39*), the incidence of *F. moniliforme* in homegrown maize (*28*), and with the esophageal cancer risk in Transkei.

FB_1 and FB_2 are the first discovered naturally occurring inhibitors of sphingosine and sphinganine *N*-acyltransferase (ceramide synthase) in rat hepatocytes (*40*).

It was shown that FB_1 and FB_2 inhibits proliferation and are cytotoxic to renal epithelial cells ($LLC\text{-}PK_1$). Soon after FB_1 was removed from the exposed cells, they then experienced normal growth kinetics (*41*). FB_1 inhibited ceramide synthase in microsomes of mouse cerebellar neurons. Again, the inhibition of sphingolipid synthesis was reversible (*42*). Because of the inhibition due to the fumonisins, the formation of complex sphingolipids was blocked leading to the accumulation of sphinganine. Increased levels of sphinganine were observed in ponies (*43*), and fumonisins also increased the sphinganine/sphingosine ratio in pigs (*44*), in chickens and turkeys (*45*), and in catfish (*46*). It was suggested that the disruption of the *de novo* pathway of sphingolipid biosynthesis may be a critical link between fumonisin exposure and the onset of various diseases (*40*). The review by Merrill *et al.* (*47*) presents a number of mechanistic considerations for the effect of fumonisin inhibition on sphingolipid biosynthesis.

The many documented occurrences of health effects in animals and humans from naturally occurring fumonisin contaminated grains, makes a clear statement that the fumonisins may pose a health problem to society in general. Therefore, it would be appropriate to screen large numbers of samples to obtain exposure data for animals and humans by an ongoing analysis method. A number of methods for detection of the fumonisins have been reported as described in a review by Beier and Nigg (*48*). Most of these methods are based on TLC, HPLC, or GC/MS and are work intensive and time consuming. Because antibody-based assays have reduced requirements for sample preparation, sample size, equipment, and quantities of organic solvents which are required for most physicochemical procedures (*49–52*), the immunoassay would be an appropriate method for the required analyses of fumonisins. The immunoassay is rapidly gaining acceptance as a screening tool and a quantitative method (*53–56*).

Polyclonal (*57,58*) antibodies (Pabs) and monoclonal (*55,59–61*) antibodies (Mabs) have been developed for use in screening methods for fumonisins in the enzyme-linked immunosorbent assay (ELISA) format. The Mab developed by Shelby and Kelley (*59*) was used for detection of FB_1 in maize (*62*). A competitive indirect (ci) ELISA was used, and the results consistently reported higher levels of FB_1 than obtained by TLC. The Mab obtained to the FB_1 hapten cross reacted equally well with FB_{1-4}. Therefore, the immunoassay would be expected to give a determination of all fumonisins present, not just the levels of FB_1. As a result, the ciELISA shows a higher value for FB_1 than did other instrumental methods. The Mab developed by Azcona-Olivera *et al.* (*57*) was used in a comparative assessment study of fumonisin in grain-based foods (*63*), and in a simultaneous screening study of FB_1, aflatoxin B_1, and zearalenone by line immunoblot (*64*). The comparison of a competitive direct (cd) ELISA, GC-MS, and HPLC determinations for FB_1 in grain-based foods gave higher estimates for FB_1 by cdELISA than by GC-MS or HPLC (*63*). Only FB_1 was determined by GC-MS and HPLC, but the cdELISA provided a result based on all the fumonisins present in the sample. This result correlated well with the results obtained by Shelby *et al.* (*62*). Anti-idiotype and anti-anti-idiotype antibodies (See chapter by Chu, this volume) against FB_1 also have been produced. (*65*). The anti-idiotype has an epitope with an internal image of FB_1.

We set out to produce Mabs to FB_1 (*61*) that hopefully would have a higher sensitivity than those previously reported. Some of those results were explained by the use of molecular models of FB_1 (*61*). An earlier molecular modeling study of FB_{1-4} suggested that the fumonisins may be good chelators (*66*). This paper presents the stereoscopic molecular model of FB_1 chelating Fe^{2+}, and a refined stereoscopic molecular model for FB_1 using it's stereostructure (SS-FB_1), along with its electrostatic potential energy surface.

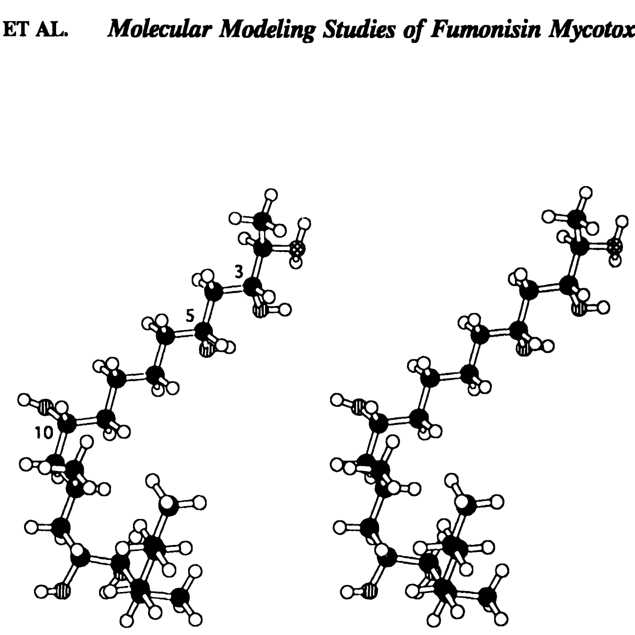
Materials and Methods

A good description of molecular mechanics and its underlying philosophy is presented by Boyd and Lipkowitz (*67*), and Lipkowitz and Peterson (*68*). Molecular modeling studies were performed using a CAChe WorkSystem™ with release 3.5 software on a Macintosh Quadra 700 computer equipped with a RP88 coprocessor board and a CAChe stereoscopic display (CAChe Scientific, Beaverton, OR). The method of calculation used was similar to that reported by Beier *et al.* (*66*). Briefly, minimum potential energy conformations of all compounds were calculated by molecular mechanics using Allinger's standard MM2 force field parameters (*69*) augmented to contain force field parameters for cases not addressed by MM2 (CAChe Scientific). Molecular mechanics calculations utilized the conjugate gradient optimization technique. Following the initial geometrical optimization, a one-pass sequential search to determine the lowest potential energy conformations was performed by rotating all dihedral angles. Following the initial sequential searches, multiple multipass sequential searches of four dihedral angles at a time were conducted to determine the lowest potential energy for the interacting systems of atoms. A final multipass sequential search of each set of dihedral angles was conducted for refinement of the optimum geometries. At completion of each sequential search a geometrical optimization was performed on each of the 5 to 15 selected structures having the lowest potential energies. The resultant lowest potential energy structure was then used for the next refinement calculation. The set of structures resulting from each sequential calculation were viewed using the CAChe Visualizer+ graphics application.

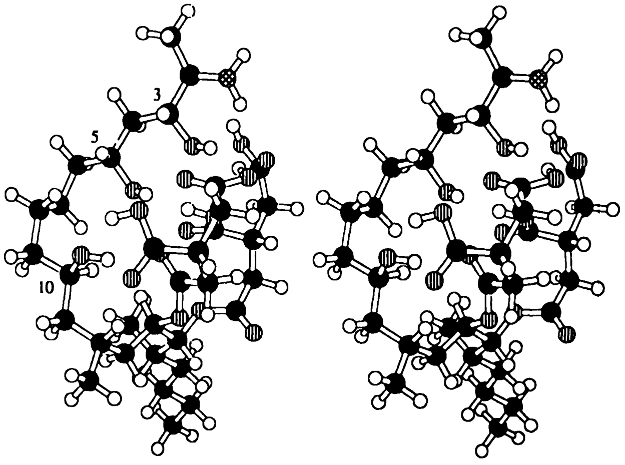
Results and Discussion

Azcona-Olivera *et al.* (*55*) reported a mean IC_{50} for FB_1 of 63 ng/well (ng/100 µL). They conjugated FB_1 via a 5 atom cross-linker, glutaraldehyde, to the ε-amino groups of lysine on Cholera toxin. We were looking to improve the antibody sensitivity by conjugating FB_1 via the 16 atom cross-linker sulfo-SMPB to thiolated ovalbumin (*61*). An IC_{50} of 30 ng/well (ng/100 µL) was obtained for the antibody selected during the study. Thus, only a twofold improvement in sensitivity (at the mean IC_{50}) was observed as compared to that found by Azcona-Olivera *et al.* (*55*) using glutaraldehyde as the cross-linker. We were striving to obtain Mabs to FB_1 with an IC_{50} on the order of 0.33 ng/100 µL, as was obtained for salinomycin Mabs (*70*), or 0.19 ng/100 µL, as was obtained for halofuginone Mabs (*71*).

Molecular modeling of the fumonisins was attempted to reveal features of the molecule that might help explain the different specificities to FB_{1-3} that were observed for the Mabs that we produced (*61*). The molecular mechanics calculated structures for the FB_1 backbone, FB_1, FB_2 and FB_3 (Figures 2 and 3) are represented

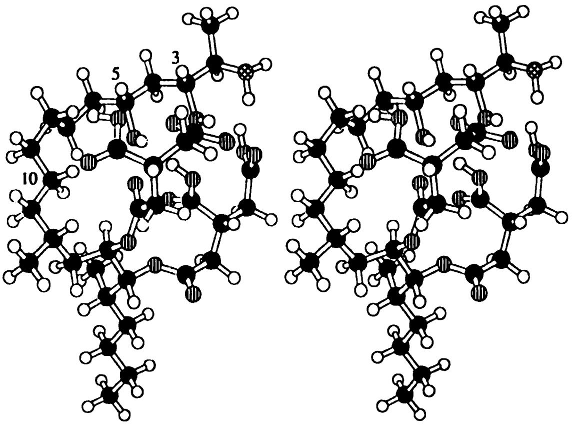


B_1 Backbone

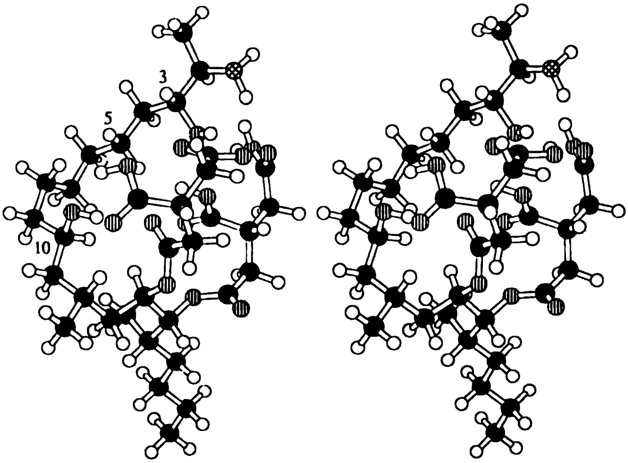


Fumonisin B_1

Figure 2. Ball and cylinder stereoscopic models of the minimum energy conformations of FB_1 and the FB_1 backbone. Carbon atoms are depicted as black spheres, hydrogen atoms are open spheres, nitrogen atoms are cross-hatched spheres, and oxygen atoms are represented as spheres with vertical lines (Reprinted with permission from ref. 61, AFRC Institute of Food Research, 1995, and ref. 66, Springer-Verlag, 1995).



Fumonisin B$_2$



Fumonisin B$_3$

Figure 3. Ball and cylinder stereoscopic models of the minimum energy conformations of FB_2 and FB_3. Carbon atoms are depicted as black spheres, hydrogen atoms are open spheres, nitrogen atoms are cross-hatched spheres, and oxygen atoms are represented as spheres with vertical lines (Reprinted with permission from ref. 61, AFRC Institute of Food Research, 1995, and ref. 66, Springer-Verlag, 1995).

as ball and cylinder stereomodels. Carbon atoms are depicted as black spheres, hydrogen atoms are open spheres, nitrogen atoms are cross-hatched spheres, and oxygen atoms are represented as spheres with vertical lines. The use of a stereoscope gives each set of molecules a three dimensional appearance. FB_{1-3} each have a unique folded structure that one might liken to that of a folded peptide, and this result is dramatically different from the calculated structure of the FB_1 backbone, devoid of the tricarballylic ester side chains (Figure 1).

The three-dimensional models of these molecules show the amine backbone folded with the two esterified trimethylpropane-1,2,3-tricarboxylic acid side chains to form a cage-like structure. Because of this folding, the four acid groups and the three hydroxyl groups of FB_1 are in close proximity to each other, and extend into the cage-like structure of FB_1. It is easy to observe that the FB_1 backbone, which has both trimethylpropane-1,2,3-tricarboxylic acid moieties at C-14 and C-15 removed (Figure 2), does not possess the three-dimensional cage-like structure of FB_{1-4} (Figures 2, 3 and 4). The epitope of the cage-like structure of FB_1 would be expected to be dramatically different from an epitope on the FB_1 backbone. Therefore, the binding site of the antibodies developed to the FB_1 antigen would be expected to be much different than that required for the FB_1 backbone.

The substantial decrease in the binding of the antibodies with the FB_1 backbone (Table I) shows the importance of the acid side-chains to the antigen-antibody interaction. FB_2 varies structurally from FB_1 only by the absence of a hydroxyl group at C-10 (Figures 1, 2 and 3). The presence or absence of this hydroxyl group produced a small, but varied effect on the affinity of the different Mabs for FB_1 and FB_2, 30–67 and 30–94 ng/well, respectively (Table I). FB_3 varies structurally from FB_1 by the absence of a hydroxyl group at C-5. The absence of this hydroxyl group decreased the affinity of the Mabs by an average of threefold, but varied greatly between Mabs, 81–274 ng/well (Table I). The difference in the interaction of FB_1 and FB_2 compared with FB_3 indicated the importance of the hydroxyl group on C-5 in antigen-antibody binding. However, since these hydroxyls are primarily internalized due to the folding of the fumonisin structures, these molecules appear relatively similar by the antibodies as seen by the small differences in binding affinities (threefold, *61*).

A striking result from the fumonisin molecular modeling studies was that linking FB_1 to carrier proteins in the ways that have been attempted would most likely not result in antibodies with high affinities for the free fumonisins. To date, all immunoassay studies have linked fumonisin B_1 through the backbone amine group to a protein. Figures 2 and 3 show clearly that the folding of the fumonisins into a cage-like structure results in placing the amine in close proximity to both acid side chains. It is this acid functionality which is presumed part of the epitope for the fumonisin antibodies. Mab binding studies suggested that this region of the molecule contains the epitope to which the antibodies bind (*61*). The molecular models suggest that linking through the amine would present the portion of the fumonisin molecule that is less hydrophilic to the immune system; whereas one would expect that the epitope for the free fumonisins would be the hydrophilic region. Linking through the amino group would also be expected to change the hydrophilic region of FB_1. A conjugation site at C-20 distal to the nitrogen should present the portion of the molecule that would be considered part of the epitope for FB_1 (Figure 2) unchanged to the immune

Table I. IC_{50}s for FB_1, FB_2, FB_3 and the FB_1 backbone with different Mabs produced to a FB_1-conjugate.[a]

Antibodies	Fumonisin (ng/well)			
	FB_1	FB_2	FB_3	FB_1 backbone
FAb01	61	94	109	4259
FAb02	67	63	207	3455
FAb03	51	33	200	3273
FAb04	56	37	167	10496
FAb06	43	26	185	2783
FAb07	46	30	83	766
FAb08	45	51	274	6040
FAb09	48	59	97	1887
FAb10	49	35	95	1386
FAb11	53	32	105	2690
FAb12	46	32	161	3733
FAb13	34	26	81	2333
FAb14	35	30	109	518
FAb15	30	69	83	783

[a]Adapted from ref. *61*.

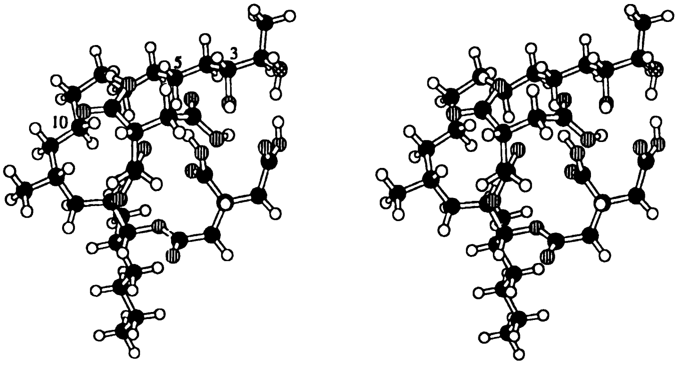


Figure 4. Ball and cylinder stereoscopic model of the minimum energy conformation of FB_4. Carbon atoms are depicted as black spheres, hydrogen atoms are open spheres, nitrogen atoms are cross-hatched spheres, and oxygen atoms are represented as spheres with vertical lines (Reprinted with permission from ref. *66*, Springer-Verlag, 1995).

system; that portion of the molecule with the greatest number of hydrophilic groups. Such a conjugate may result in antibodies with greater affinities for free FB_1.

The use of the molecular modeling appeared even more interesting, since during the calculation of the structure for FB_4 (Figure 4), it became clear to us that the fumonisins could possibly be good chelators, and also possibly disrupt membrane function (*66*).

The molecular mechanics calculated structure for the Fe•FB_1 complex is shown in Figure 5 as a ball and cylinder stereomodel. Carbon atoms are depicted as black spheres, nitrogen atoms are cross-hatched spheres, oxygen atoms are spheres with vertical lines, and iron is represented as a sphere with checker coding. This Fe•FB_1 complex also has a unique folded structure similar to that seen for free FB_1 (Figure 2).

There are a number of possibilities for FB_1 to form a complex with Fe^{2+}. Figure 5 depicts Fe^{2+} being bound to the two acid groups on the tricarballylic ester side chain at C-14 of FB_1 (arrow). This site was chosen for the initial calculation of the Fe•FB_1 complex based on the apparent close proximity of the tricarballylic ester side chain on C-15 to the fumonisin backbone. However, Fe^{2+} could possibly be bound in different ways to the acid groups between the two tricarballylic ester side chains. Fe^{2+} could be bound to the two acid groups on the tricarballylic ester side chain on C-15 of FB_1. Fe^{2+} could possibly be bound between acid groups on both of the tricarballylic ester side chains at C-14 and C-15. Also, two Fe^{2+} atoms may be bound to both of the acid groups on the side chains at C-14 and C-15. If structures for all possible sites were calculated then the conformational energy of the different structures could be compared. This would provide an evaluation of the most probable structure of Fe•FB_1. It can be pointed out that the three dimensional structure calculated for Fe•FB_1 still retains a folded structure similar to that of FB_1, and that FB_1 incorporates Fe^{2+} readily.

The possibility that the fumonisins may have a role in binding iron or other ions has important consequences. Chelation of metals like iron is known to increase the lethality of bacteria. Pyochelin is an iron-binding compound isolated from culture media of *Pseudomonas aeruginosa* (*72,73*). It also is known to bind zinc (*74,75*). *P. aeruginosa* causes infections in patients that are immunocompromised (*76,77*), have cystic fibrosis (*76,78*) or burns (*76*). Pyochelin is known to increase the lethality of virulent bacteria (*79*). Therefore, with a potential to bind metal ions the fumonisins also may have detrimental health effects due to ion binding. Research and epidemiological studies should be conducted to evaluate the possibility of the fumonisins causing problems in immunocompromised people.

If chelation is occurring due to the fumonisins it may be some other metal than iron that is important. We chose iron for modeling purposes because it is commonly bound by biological molecules. As is shown above, the compound, pyochelin, is very important in the lethality of the infections caused by the bacterium, *P. aeruginosa*, as a result of binding iron (*79*). But other metals like, Ca, Na, K, Mn, Co, Ni, Cu, or Zn may be important.

Since publishing our original models of FB_{1-4} and the FB_1 backbone (*61,66*) the total stereostructure of FB_1 (SS-FB_1) has been deduced (Figure 6). The absolute configuration at each stereogenic center in the FB_1 backbone was determined by

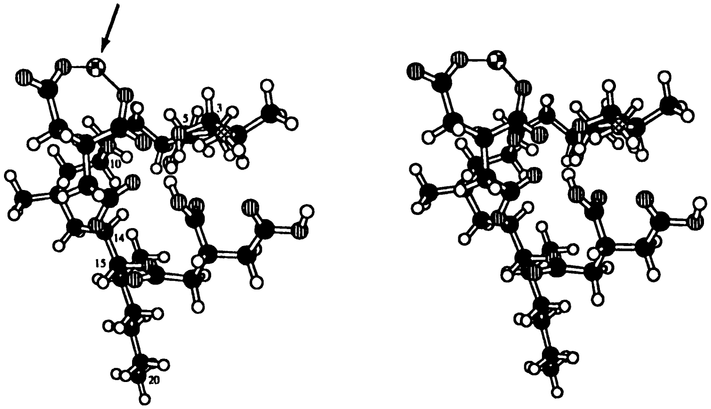

5
3
10
14
15
20

Figure 5. Ball and cylinder stereoscopic model of the minimum energy conformation of FB_1 chelated with Fe^{2+}. Carbon atoms are depicted as black spheres, hydrogen atoms are open spheres, nitrogen atoms are cross-hatched spheres, oxygen atoms are spheres with vertical lines, and iron atoms are represented as checker board spheres.

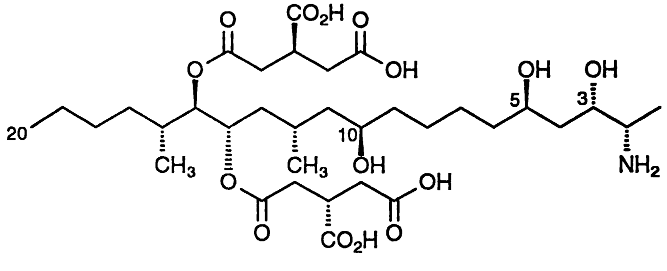

O
CO2H
O
O
OH
OH
OH
5
3
20
10
CH3
CH3
OH
O
OH
NH2
O
CO2H
O

Figure 6. Stereostructure of FB_1 (SS-FB_1).

Hoye *et al.* (*80*). After the preparation of the appropriate derivatives they used a combination of ^{1}H NMR studies and chiral gas chromatography to determine all the stereocenters in the FB_1 backbone. These conclusions are consistent with the determination of C-2 and C-3 by Poch *et al.* (*81*) using ^{1}H NMR to compare the appropriate model compounds to an oxazoline derivative of the FB_1 backbone. They also are consistent with the determination of the C-1 to C-5 fragment by ApSimon *et al.* (*82,83*) using ^{1}H and ^{13}C NMR analyses of a carbamate and *N-p*-bromobenzyl carbamate derivatives of FB_1. Furthermore, the conclusions of Hoye *et al.* (*80*) also are consistent with the determination of Boyle *et al.* (*84*) using ^{13}C NMR comparisons of the appropriate model compounds as well as the configuration for C-1 to C-5 of the AAL toxin by Oikawa *et al.* (*85*) utilizing a degradative study followed by ^{1}H NMR. The same relative configuration of the C-10 to C-16 fragment of the FB_1 backbone also was determined by Blackwell *et al.* (*86*) by forming a 10,14-cyclic ether derivative and then using ^{1}H and ^{13}C NMR analysis. The stereostructure of FB_1 was completed with the determination of the absolute configuration of the two propane-1,2,3-tricarboxylic acid side chains. Shier *et al.* (*87*) showed that the acid group at the C-3' position in the side chains was in the *S* configuration for both FB_1 and AAL toxin T_A by chiral gas chromatography of the appropriate derivatives. This completed the stereostructure as shown in Figure 6. However, recently Boyle and Kishi have shown unambiguously via comparison of the ^{1}H NMR for related compounds that the stereochemistry at the tricarballylic acid centers of FB_1 and of AAL toxin T_A to be *R* (*88*). This is the opposite stereochemistry for those centers than was earlier proposed by Shier *et al.* (*87*). An interesting observation pointed out by Poch *et al.* (*81*), was that the relative stereochemistry at C-2 and C-3 of fumonisin B_1 is opposite to that of sphingosine.

Figure 7 shows the ball and cylinder stereoscopic model of the minimum energy conformation of the stereostructure of FB_1 (SS-FB_1). This model is somewhat different than the earlier FB_1 model (Figure 2), as a result of having the stereostructure of FB_1 (Figure 6) available. However, the stereoscopic model of SS-FB_1 retains the characteristic folding that forms a cage-like structure as was earlier seen with the stereoscopic models of FB_{1-4}. The calculated global minimum of SS-FB_1 was –79.718 kcal/mol. At about –35 to –40 kcal/mol the overall cage feature began to form, and after it started forming the overall structure progressed very quickly to the final conformation. These calculations suggest that the cage-like feature of SS-FB_1 is quite stable. This is born out by NMR studies conducted on FB_1. ApSimon *et al.* (*83*), following NMR studies of FB_1 stated "that the fumonisin backbone forms a more rigid structure in solution than might be expected from this lipid-like molecule." The molecular modeling and NMR methods corroborate each other on the stability of the FB_1 conformation. The hydrophobic end of the backbone along with the hydrophilic cage region may allow these molecules to disrupt membrane function. Further calculations of the FB_1 stereostructure will be made using the recent stereochemical assignments of the tricarballylic acid moieties presented by Boyle and Kishi (*88*).

Figure 8 shows a stereoview of the electrostatic potential energy surface of SS-FB_1. The use of a stereoscope gives these surfaces a three dimensional appearance. These surfaces are composed of two parts: A negative potential energy surface, which is indicated as 'a' in Figure 8, and a positive potential energy surface, which is indi-

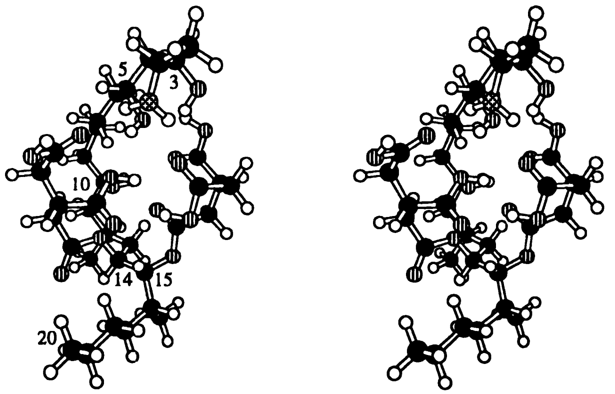


Figure 7. Ball and cylinder stereoscopic model of the minimum energy conformation of the stereostructure of FB_1 ($SS-FB_1$). Carbon atoms are depicted as black spheres, hydrogen atoms are open spheres, nitrogen atoms are cross-hatched spheres, and oxygen atoms are spheres with vertical lines.

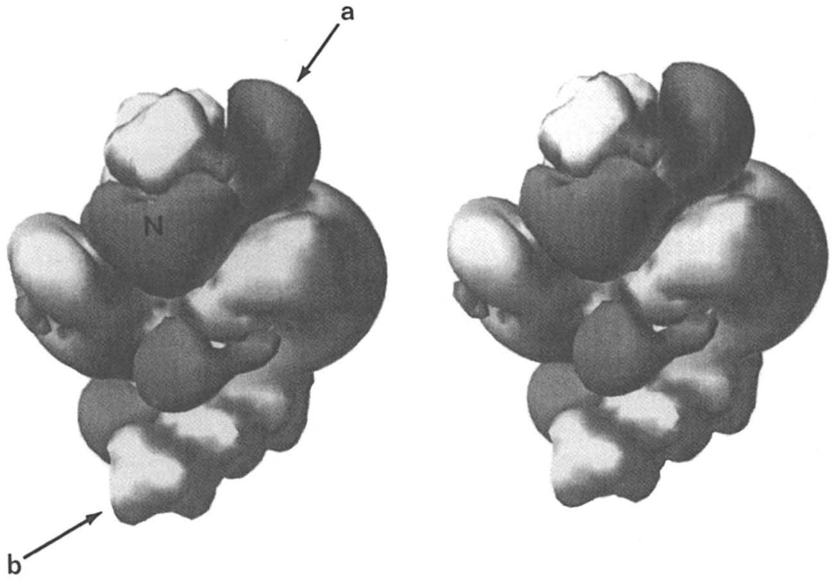


Figure 8. Left and right stereoviews of the electrostatic potential energy surface of the molecular model of $SS-FB_1$. The 'a' surfaces are darker and correspond to a negative potential energy, which is attractive to a proton. The 'b' surfaces are lighter and correspond to a positive potential energy, which is repulsive to a proton. The letter N is in close proximity to the location of the $SS-FB_1$ NH_2 group.

cated as 'b'. The negative potential energy surface is attractive to a proton, and the positive potential energy surface is repulsive to a proton. The hidden surface, or back side of the molecule is primarily made up only of positive potential energy. The overall surface shows that the major exterior of SS-FB_1 appears to be very hydrophobic in nature. Only a small amount of hydrophilic surface is observed on SS-FB_1. These calculations suggest that FB_1 is clearly a globular molecule; not just a linear lipid as is easy to conclude from the structures in Figures 1 and 6. The electrostatic potential energy surface of SS-FB_1 also bears out the observations that were made from the ball and cylinder model of FB_1 in Figure 2 (*61*). The position of attachment of FB_1 to proteins (though the NH_2 group) is in close proximity to the acid side chains, and therefore, the hydrophilic region. The letter N on the electrostatic potential energy surface in Figure 8 is in close proximity to the location of the NH_2 group. This potential energy surface clearly shows that linking through the amino group would change the hydrophilic region of FB_1.

Conclusions

The molecular modeling of the fumonisins has produced stereoscopic models of FB_{1-4} and SS-FB_1 that have allowed the interpretation of why antibodies produced to FB_1 have lower affinities for free FB_1 than anticipated. The surface of FB_1 was shown to be mainly hydrophobic, and it is a globular molecule. It is hypothesized that the hydrophilic and hydrophobic qualities of the fumonisins may allow them to disrupt membrane function. FB_1 also may affect the sodium-potassium or calcium permeability of the cell, and thus alter the homeostasis of the cell. The possibility of the fumonisins playing a role in iron or other metal ion binding may have important consequences. We suggest that epidemiological studies be conducted concerning immuno-compromised people and exposure to the fumonisins.

Acknowledgments

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Literature Cited

1. Booth, C. *The Genus Fusarium*; Commonwealth Mycological Institute: Kew, Surrey, England, 1971.
2. Bezuidenhout, S. C.; Gelderblom, W. C. A.; Gorst-Allman, C. P.; Horak, R. M.; Marasas, W. F. O.; Spiteller, G.; Vleggaar, R. *J. Chem. Soc. Chem. Commun.* **1988**, 743–745.
3. Gelderblom, W. C. A.; Marasas, W. F. O.; Vleggaar, R.; Thiel, P. G.; Cawood, M. E. *Mycopathologia* **1992**, *117*, 11–16.
4. Plattner, R. D.; Weisleder D.; Shackelford, D. D.; Peterson, R.; Powell, R. G. *Mycopathologia* **1992**, *117*, 23–28.
5. Wilson, B. J.; Maronpot, R. R. *Vet. Rec.* **1971**, *88*, 484–485.

6. Wilson, B. J.; Maronpot, R. R.; Hildebrandt, P. K. *J. Am. Vet. Med. Assoc.* **1973**, *163*, 1293–1295.
7. Wilson, T. M.; Nelson, P. E.; Ryan, T. B.; Rouse, C. D.; Pittman, C. W.; Neal, T. P.; Porterfield, M. L.; Saunders, G. K. *Equine Prac.* **1985**, *80*(11), 63–69.
8. Marasas, W. F. O.; Kellerman, T. S.; Gelderblom, W. C. A.; Coetzer, J. A. W.; Thiel, P. G.; van der Lugt, J. J. *Onderstepoort J. Vet. Res.* **1988**, *55*, 197–203.
9. Ross, P. F.; Nelson, P. E.; Owens, D. L.; Rice, L. G.; Nelson, H. A.; Wilson, T. M. *J. Vet. Diagn. Invest.* **1994**, *6*, 263–265.
10. Buck, W. B.; Haliburton, J. C.; Thilsted, J. P.; Lock, T. F.; Vesonder, R. F. *Proc. Ann. Meet. - Am. Assoc. Vet. Lab. Diagn.* **1979**, *22*, 239–258.
11. Haliburton, J. C.; Buck, W. B. *Curr. Top. Vet. Med. Anim. Sci.* **1986**, *33*, 75–79.
12. McCue, P. M. *Compendium* **1989**, *11*, 646–651.
13. Sydenham, E. W.; Marasas, W. F. O.; Shephard, G. S.; Thiel, P. G.; Hirooka, E. Y. *J. Agric. Food Chem.* **1992**, *40*(6), 994–997.
14. Voss, K. A.; Norred, W. P.; Plattner, R. D.; Bacon, C. W. *Food Chem. Toxicol.* **1989**, *27*(2), 89–96.
15. Kriek, N. P. J.; Kellerman, T. S.; Marasas, W. F. O. *Onderstepoort J. Vet. Res.* **1981**, *48*, 129–131.
16. Ross, P. F.; Nelson, P. E.; Richard, J. L.; Osweiler, G. D.; Rice, L. G.; Plattner, R. D.; Wilson, T. M. *Appl. Environ. Microbiol.* **1990**, *56*(10), 3225–3226.
17. Harrison, L. R.; Colvin, B. M.; Greene, J. T.; Newman, L. E.; Cole, J. R. *J. Vet. Diagn. Invest.* **1990**, *2*, 210–212.
18. Colvin, B. M.; Cooley, A. J.; Beaver, R. W. *J. Vet. Diagn. Invest.* **1993**, *5*, 232–241.
19. Casteel, S. W.; Turk, J. R.; Cowart, R. P.; Rottinghaus, G. E. *J. Vet. Diagn. Invest.* **1993**, *5*, 413–417.
20. Prelusky, D. B.; Trenholm, H. L.; Savard, M. E. *Nat. Toxins* **1994**, *2*(2), 73–80.
21. Lebepe-Mazur, S.; Bal, H.; Hopmans, E.; Murphy, P.; Hendrich, S. *Vet. Human Toxicol.* **1995**, *37*(2), 126–130.
22. Marijanovic, D. R.; Holt, P.; Norred, W. P.; Bacon, C. W.; Voss, K. A.; Stancel, P. C.; Ragland, W. L. *Poult. Sci.* **1991**, *70*, 1895–1901.
23. Vudathala, D. K.; Prelusky, D. B.; Ayroud, M.; Trenholm, H. L.; Miller, J. D. *Nat. Toxins* **1994**, *2*(2), 81–88.
24. Gelderblom, W. C. A.; Jaskiewicz, K.; Marasas, W. F. O.; Thiel, P. G.; Horak, R. M.; Vleggaar, R.; Kriek, N. P. J. *Appl. Environ. Microbiol.* **1988**, *54*(7), 1806–1811.
25. Kubena, L. F.; Edrington, T. S.; Kamps-Holtzapple, C.; Harvey, R. B.; Elissalde, M. H.; Rottinghaus, G. E. *Poult. Sci.* **1995**, *74*, 306–313.
26. Kubena, L. F.; Edrington, T. S.; Kamps-Holtzapple, C.; Harvey, R. B.; Elissalde, M. H.; Rottinghaus, G. E. *Poult. Sci.* **1995**, *74*, 1295–1303.
27. Chatterjee, D.; Mukherjee, S. K. *Lett. Appl. Microbiol.* **1994**, *18*, 251–253.
28. Marasas, W. F. O.; Kriek, N. P. J.; Fincham, J. E.; van Rensburg, S. J. *Int. J. Cancer* **1984**, *34*, 383–387.

29. van Rensburg, S. J.; Marasas, W. F. O.; Gelderblom, W. C. A.; Thiel, P. G.; Rabie, C. J. *Mycotoxins and oesophageal cancer*; Proc. Vth Int. IUPAC Symp. Mycotoxins and Phycotoxins: Vienna, Austria, 1982; pp 265–268.
30. Shier, W. T.; Abbas, H. K.; Mirocha, C. J. *Mycopathologia* **1991**, *116*, 97–104.
31. Jaskiewicz, K.; Marasas, W. F. O.; Taljaard, J. J. F. *J. Comp. Pathol.* **1987**, *97*, 281–291.
32. Shephard, G. S.; Thiel, P. G.; Sydenham, E. W.; Savard, M. E. *Nat. Toxins* **1995**, *3*(3), 145–150.
33. Rose, E. F.; Fellingham, S. A. *Afr. J. Sci.* **1981**, *77*, 555–561.
34. Schoental, R. *Cancer* **1977**, *40*(October), 1833–1840.
35. Schoental, R.; Joffe, A. Z. *J. Pathol.* **1974**, *112*, 37–42.
36. Schoental, R.; Joffe, A. Z.; Yagen, B. *Br. J. Cancer* **1976**, *34*, 310.
37. Schoental, R.; Joffe, A. Z.; Yagen, B. *Br. J. Cancer* **1978**, *38*, 171.
38. Marasas, W. F. O. In *Cancer of the Oesophagus*; Pfeifer, C. J., ed.; CRC Press: Boca Raton, FL, 1982, Vol. 1; pp 29–40.
39. Marasas, W. F. O.; Wehner, F. C.; van Rensburg, S. J.; van Schalkwyk, D. J. *Phytopathology* **1981**, *71*, 792–796.
40. Wang, E.; Norred, W. P.; Bacon, C. W.; Riley, R. T.; Merrill, A. H., Jr. *J. Biol. Chem.* **1991**, *266*(22), 14486–14490.
41. Yoo, H.-S.; Norred, W. P.; Wang, E.; Merrill, A. H., Jr.; Riley, R. T. *Toxicol. Appl. Pharmacol.* **1992**, *114*, 9–15.
42. Merrill, A. H., Jr.; van Echten, G.; Wang, E.; Sandhoff; K. *J. Biol. Chem.* **1993**, *268*(36), 27299–27306.
43. Wang, E.; Ross, P. F.; Wilson, T. M.; Riley, R. T.; Merrill, A. H., Jr. *J. Nutr.* **1992**, *122*, 1706–1716.
44. Riley, R. T.; An, N.-H.; Showker, J. L.; Yoo, H.-S.; Norred, W. P.; Chamberlain, W. J.; Wang, E.; Merrill, A. H., Jr.; Motelin, G.; Beasley, V. R.; Haschek, W. M. *Toxicol. Appl. Pharmacol.* **1993**, *118*, 105–112.
45. Wiebking, T. S.; Ledoux, D. R.; Bermudez, A. J.; Turk, J. R.; Rottinghaus, G. E.; Wang, E.; Merrill, A. H., Jr. *Poult. Sci.* **1993**, *72*, 456–466.
46. Goel, S.; Lenz, S. D.; Lumlertdacha, S.; Lovell, R. T.; Shelby, R. A.; Li, M.; Riley, R. T.; Kemppainen, B. W. *Aquat. Toxicol. (Amst.)* **1994**, *30*, 285–294.
47. Merrill, A. H., Jr.; Wang, E.; Gilchrist, D. G.; Riley, R. T. *Adv. Lipid Res.* **1993**, *26*, 215–234.
48. Beier, R. C.; Nigg, H. N. In *Foodborne Disease Handbook, Diseases Caused by Hazardous Substances*; Hui, Y. H.; Gorham, J. R.; Murrell, K. D.; Cliver, D. O., eds.; Marcel Dekker, Inc.: New York, NY, 1994, Vol. 3; pp 67–68.
49. Haagsma, N.; van der Water, C. In *Analysis of Antibiotic Drug Residues in Food Products of Animal Origin*; Agarwal, V. K., ed.; Plenum Press: New York, NY, 1992; pp 81–97.
50. Lee, H. A.; Wilkinson, A. P.; Ward, C. M.; Mills, E. N. C.; Morgan, M. R. A. In *Food Safety and Quality Assurance: Applications of Immunoassay Systems*; Morgan, M. R. A.; Smith, C. J.; Williams, P. A., eds.; Elsevier Applied Science: New York, NY, 1992; pp 85–92.

51. Gazzaz, S. S.; Rasco, B. A.; Dong, F. M. *Crit. Rev. Food Sci. Nutr.* **1992**, *32*(3), 197–229.
52. Nugent, P. A. In *Emerging Strategies for Pesticide Analysis*; Cairns, T.; Sherma, J., eds.; CRC Press: Boca Raton, FL, 1992; pp 247–258.
53. Hammock, B. D.; Mumma, R. O. In *Pesticide Analytical Methodology*; Harvey, J., Jr.; Zweig, G., eds.; American Chemical Society: Washington, DC, 1980, ACS Symposium Series No. 136; pp 321–352.
54. Greirson, B. N.; Allen, D. G.; Gare, N. F.; Watson, I. M. *J. Agric. Food Chem.* **1991**, *39*(12), 2327–2331.
55. Azcona-Olivera, J. I.; Abouzied, M. M.; Plattner, R. D.; Pestka, J. J. *J. Agric. Food Chem.* **1992**, *40*(3), 531–534.
56. Shelby, R. A.; Kelly, V. C. *J. Agric. Food Chem.* **1992**, *40*(6), 1090–1092.
57. Azcona-Olivera, J. I.; Abouzied, M. M.; Plattner, R. D.; Norred, W. P.; Pestka, J. J. *Appl. Environ. Microbiol.* **1992**, *58*(1), 169–173.
58. Usleber, E.; Straka, M.; Terplan, G. *J. Agric. Food Chem.* **1994**, *42*(6), 1392–1396.
59. Shelby, R. A.; Kelley, V. C. *Phytopathology* **1992**, *82*(4), 500 (Abstr.)
60. Fukuda, S.; Nagahara, A.; Kikuchi, M.; Kumagai, S. *Biosci. Biotech. Biochem.* **1994**, *58*(4), 765–767.
61. Elissalde, M. H.; Kamps-Holtzapple, C.; Beier, R. C.; Plattner, R. D.; Rowe, L. D.; Stanker, L. H. *Food Agric. Immunol.* **1995**, *7*(2), 109–122.
62. Shelby, R. A.; Rottinghaus, G. E.; Minor, H. C. *J. Agric. Food Chem.* **1994**, *42*(9), 2064–2067.
63. Pestka, J. J.; Azcona-Olivera, J. I.; Plattner, R. D.; Minervini, F.; Doko, M. B.; Visconti, A. *J. Food Prot.* **1994**, *57*(2), 169–172.
64. Abouzied, M. M.; Pestka, J. J. *J. AOAC Int.* **1994**, *77*(2), 495–501.
65. Chu, F. S.; Huang, X.; Maragos, C. M. *J. Agric. Food Chem.* **1995**, *43*(1), 261–267.
66. Beier, R. C.; Elissalde, M. H.; Stanker, L. H. *Bull. Environ. Contam. Toxicol.* **1995**, *54*, 479–487.
67. Boyd, D. B.; Lipkowitz, K. B. *J. Chem. Educ.* **1982**, *59*, 269–274.
68. Lipkowitz, K. B.; Peterson, M. A. *Chem. Rev.* **1993**, *93*, 2463–2486.
69. Allinger, N. L. *J. Am. Chem. Soc.* **1977**, *99*, 8127–8134.
70. Elissalde, M. H.; Beier, R. C.; Rowe, L. D.; Stanker, L. H. *J. Agric. Food Chem.* **1993**, *41*(11), 2167–2171.
71. Rowe, L. D.; Beier, R. C.; Elissalde, M. H.; Stanker, L. H. *J. Agric. Food Chem.* **1994**, *42*(5), 1132–1137.
72. Cox, C. D.; Graham, R. *J. Bacteriol.* **1979**, *137*(1), 357–364.
73. Cox, C. D.; Rinehart, K. L., Jr.; Moore, M. L.; Cook, J. C., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*(7), 4256–4260.
74. Cuppels, D. A.; Stipanovic, R. D.; Stoessl, A.; Stothers, J. B. *Can. J. Chem.* **1987**, *65*(9), 2126–2130.
75. Beier, R. C.; Stipanovic, R. D. *Biomed. Environ. Mass Spectrom.* **1989**, *18*(3), 185–191.
76. Wood, R. E. *Hosp. Pract.* **1976**, *11*, 91–100.

77. Bodey, G. P.; Bolivar, R.; Fainstein, V.; Jadeja, L. *Rev. Infect. Dis.* **1983**, *5*, 279–313.
78. Sokol, P. A. *J. Clin. Microbiol.* **1986**, *23*(3), 560–562.
79. Cox, C. D. *Infect. Immun.* **1982**, *36*(1), 17–23.
80. Hoye, T. R.; Jiménez, J. I.; Shier, W. T. *J. Am. Chem. Soc.* **1994**, *116*(20), 9409–9410.
81. Poch, G. K.; Powell, R. G.; Plattner, R. D.; Weisleder, D. *Tetrahedron Lett.* **1994**, *35*(42), 7707–7710.
82. ApSimon, J. W.; Blackwell, B. A.; Edwards, O. E.; Fruchier, A.; Miller, J. D.; Savard, M.; Young, J. C. *Pure Appl. Chem.* **1994**, *66*(10/11), 2315–2318.
83. ApSimon, J. W.; Blackwell, B. A.; Edwards, O. E.; Fruchier, A. *Tetrahedron Lett.* **1994**, *35*(42), 7703–7706.
84. Boyle, C. D.; Harmange, J.-C.; Kishi, Y. *J. Am. Chem. Soc.* **1994**, *116*(11), 4995–4996.
85. Oikawa, H.; Matsuda, I.; Ichihara, A.; Kohmoto, K. *Tetrahedron Lett.* **1994**, *35*(8), 1223–1226.
86. Blackwell, B. A.; Edwards, O. E.; ApSimon, J. W.; Fruchier, A. *Tetrahedron Lett.* **1995**, *36*(12), 1973–1976.
87. Shier, W. T.; Abbas, H. K.; Badria, F. A. *Tetrahedron Lett.* **1995**, *36*(10), 1571–1574.
88. Boyle, C. D.; Kishi, Y. *Tetrahedron Lett.* **1995**, *36*(32), 5695–5698.

RECEIVED October 6, 1995

Chapter 30

Immunoassay for Mercury in Seafood and Animal Tissues

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The measurement of mercury in swordfish, tuna, scallop, bass, shark, and alligator tissue by immunoassay correlated well with cold-vapor atomic absorption. Methylmercury, which bioaccumulates in fish and other animals, was extracted from the tissue with a procedure optimized for the immunoassay. Since methylmercury is extremely toxic, it is important to identify fish or animal tissues with mercury levels considered unsafe for human consumption. Current analytical methods for mercury are expensive and time-consuming, and they must be performed in a laboratory. The mercury immunoassay can be adapted for quick, on-site screening of large numbers of tissue samples.

Mercury contamination of rivers, lakes, and oceans originates from both natural and human-related sources (*1,2*). Elemental mercury is distributed throughout the atmosphere and deposited as inorganic mercury in precipitation, thereby introducing mercury into remote bodies of water (*3*). Microbial methylation of inorganic mercury produces highly toxic methylmercury, which bioaccumulates in fish and other animals (*1,4–6*). Continued consumption of food containing methylmercury results in damage to the human central nervous system (*1*). Mercury in many marine and freshwater fish exceeds the 1 μg/g (ppm) action level established by the U.S. Food and Drug Administration.

Given the extreme toxicity of methylmercury, it is important to identify fish or other animals with mercury levels above those considered fit for human consumption. Cold-vapor atomic absorption spectrophotometry (CVAAS) and cold-vapor atomic fluorescence are two methods in current use, but these methods are expensive and must be performed in a laboratory. A rapid and inexpensive mercury-specific immunoassay developed by BioNebraska has been used to detect mercury in water and soil in the field (*7–9*). This paper presents the results of experiments in which this immunoassay was used to measure mercury in animal tissue. Mercury was extracted

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from tissue samples using a procedure designed for use with CVAAS, but with modifications that make it amenable to the immunoassay.

Material and Methods

Tissue Samples. Alligator and bass samples were obtained from the Florida Game and Fresh Water Fish Commission. Shark, swordfish, scallop, and tuna samples were obtained from the Environmental Research Institute, Storrs, CT. Cormorant liver samples were obtained from the Florida Keys Wild Bird Center, Tavernier, FL. Mercury was extracted from the tissues and analyzed by both cold-vapor atomic absorption and the BiMelyze mercury immunoassay.

Extraction Procedure. Tissue samples were kept frozen at -20 °C until used. Prior to extraction, they were thawed and cut into small pieces (approximately 0.2 cm^3) using a clean razor blade, and 5 g were placed in acid-washed 125-mL glass bottles with plastic lids. Controls consisted of a reagent blank and Dorm-1 (National Research Council of Canada), a lyophilized certified reference material of dogfish muscle containing 0.798 μg/g total mercury.

Tissue digestion procedures involving strong acids and oxidizing agents were performed in a fume hood by personnel wearing appropriate protective clothing. The procedure used to prepare samples for the immunoassay was modified from that described by Delany *et al.* (*4*). Concentrated nitric acid (5 mL) was added to 5 g of tissue. The bottles were capped, incubated for 2 h at room temperature with occasional agitation, then placed in a water bath at 95 °C for 2 h. After cooling to room temperature, 30% H_2O_2 (7 mL) was added. The samples were heated again for 2 h at 95 °C. The extracts were allowed to cool, and the total volume of the extract was measured. The Dorm-1 powder was treated similarly except that 20 mL of acid per gram was used to allow for complete mixing, and 14 mL of 30% H_2O_2 was added.

This modified extraction procedure was compared with EPA Method 245.6, in which 4 mL of concentrated sulfuric acid and 1 mL of concentrated nitric acid were added to 0.25 g of tissue. After a 30 min incubation at 80 °C, the sample was cooled at 4 °C to prevent excessive bubbling when 15 mL of 5% potassium permanganate and 8 mL of 5% potassium persulfate were added. The sample was then heated for 90 min at 30 °C and diluted with 55 mL of deionized water. Excess permanganate was reduced by adding 6 mL of a solution containing 12% sodium chloride and 12% hydroxylamine sulfate.

Cold-Vapor Atomic Absorption. Tissue extracts were assayed for mercury using an LDC Analytical Model 1255 Mercury Monitor equipped with a Linear Scientific Model 1201 chart recorder. Extracts were diluted 1:100 or 1:25 with 1.6 M HNO_3. Standards of 20, 10, 5, 2, and 1 ppb mercury were prepared by diluting the certified standard, SRM 3133 (National Institute of Standards and Technology, 10 mg Hg/mL in 10% HNO_3), in 1.6 M HNO_3. Ten milliliters of 5% tin(II) chloride dihydrate in 0.5 N HCl was added to the reservoir of the bubbler assembly, and 0.7 mL of standard or diluted extract was injected. High purity grade nitrogen was used to carry the elemental mercury vapor to the detection cell. After the recorder pen returned to the baseline position, the trap of the bubbler assembly was emptied, 10 mL of fresh tin chloride solution was added, and the next sample was injected. The concentration of mercury in the samples was calculated on a wet-weight basis.

Immunoassay Procedure. Digests also were analyzed with the BiMelyze Mercury Assay Plate Kit following a 1:1,000 dilution with 0.1 M HEPES, pH 7. A standard curve of 8, 2, 0.5, 0.25, and 0.125 ppb mercury in 0.1 M HEPES, pH 7, was made from SRM 3133. For the analysis of cormorant liver samples, for which 0.5 g samples were used, the standard curve was prepared by adding mercury to a 1:1,000 dilution of a low-mercury liver digest in 0.1 M HEPES, pH 7. Mercury Assay Plates were prepared by coating each well of a 96-well polystyrene plate with a glutathione-bovine serum albumin conjugate (GSH-BSA) (*8*) in phosphate buffered saline. Samples and standards (100 µL) were added to each of two wells of the Mercury Assay Plate and incubated for 15 min at room temperature. The plate was rinsed three times with deionized water, then 100 µL of a solution containing a mercury-specific monoclonal antibody was added to each well and incubated for 10 min at room temperature. The plate was washed three times with a buffered rinse solution and three times with water, then 100 µL of a solution containing goat anti-mouse IgG horseradish peroxidase conjugate was added to each well. Following a 10 min incubation at room temperature, excess reagent was washed away as before, and 100 µL of ABTS substrate solution (2,2'-azinobis[3-ethylbenzothiazoline]-6 sulfonic acid diammonium salt) was added to each well. Color development was stopped after 10 min by the addition of 100 µL of 1% sodium dodecyl sulfate to each well. Absorbencies measured at 405 nm using a BioTek EL311 microplate autoreader were proportional to the log of the mercury concentration.

Detection Limit Determination. The lower detection limit (LDL) of the immunoassay for mercury in tissue was determined using a scallop tissue extract which did not contain detectable mercury. The extract was diluted 1:1000 in 0.1 M HEPES, pH 7, and then mercury from SRM 3133 was added to final concentrations of 0.5, 0.25, 0.125, 0.062, 0.031, 0.016, and 0.008 ppb. The mercury dilutions and diluted extract without added mercury were then analyzed with the immunoassay.

Results and Discussion

The basic procedure used to measure Hg^{2+} in solution is diagrammed in Figure 1. After tissue digestion, samples were diluted in pH 7 buffer and added to plates which were coated with glutathione-bovine serum albumin (GSH-BSA). After mercury was bound to the GSH-BSA, the mercury-specific antibody was added, and it binds to the immobilized mercury. Horseradish peroxidase (HRP)-conjugated antibody was added next, followed by the addition of ABTS substrate. Cleavage of substrate resulted in color formation.

The HNO_3/H_2O_2 digestion procedure converts methylmercury to Hg^{2+}, the form recognized by the mercury-specific antibody. This procedure was modified slightly so that analyses by CVAAS and immunoassay could be completed on the same day. In the original procedure, samples were digested with nitric acid for 2 h, heated at 95 °C for 4 h, and heated at 95 °C for an additional 2 h following addition of hydrogen peroxide. In the modified procedure, the 4-h heating step was shortened to 2 h, and this procedure adequately extracted mercury from the tissue as determined by CVAAS (data not shown). These extracts were then analyzed by the immunoassay. The results from these initial experiments showed that mercury concentrations determined by the immunoassay were lower than those determined by CVAAS. Experiments were performed to determine optimal HNO_3 and H_2O_2 concentrations for sample digestion and mercury detection with the immunoassay. The effect of H_2O_2 concentration was examined with five different tissue samples digested with 70% HNO_3 and 5, 9, or 13% H_2O_2 (Table I). Mercury concentrations determined by immunoassay (IA) were closest to 100% of CVAAS values for samples digested with 13% H_2O_2, so this concentration was used in subsequent extractions.

Table I. Effect of Hydrogen Peroxide on Detection of Mercury in Tissue

Sample	H_2O_2 (%)	(IA / CVAAS) x 100 (%)
1	5	47
	9	67
	13	88
2	5	40
	9	45
	13	90
3	5	50
	9	62
	13	75
4	5	60
	9	80
	13	100
5	5	50
	9	60
	13	114

Many tissue digestion procedures are available, but the high acid concentration and strong oxidizing agents used are not amenable to the immunoassay. An important issue, therefore, is the development of an efficient extraction procedure which is also compatible with the immunoassay. The procedure utilizing 70% HNO_3 and 13% H_2O_2 was compared to EPA Method 245.6 which employs chemicals that interfere with the immunoassay. Tissue samples were digested with both procedures and analyzed by CVAAS. The HNO_3/H_2O_2 procedure recovered 64, 75, 68, and 69% of the mercury from shark, swordfish, tuna, and alligator tissue compared to the EPA

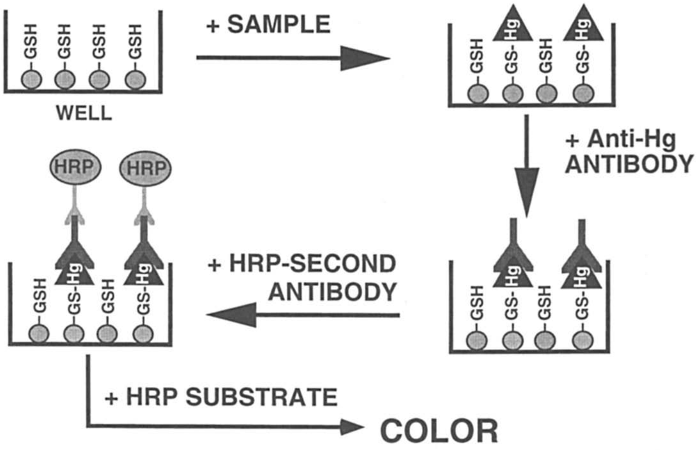
-GSH
WELL
+ SAMPLE
GS-Hg
+ Anti-Hg ANTIBODY
+ HRP-SECOND ANTIBODY
HRP
+ HRP SUBSTRATE
COLOR

Figure 1. Mercury immunoassay procedure.

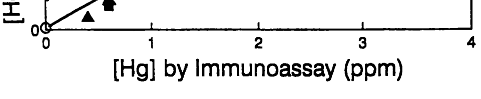

Figure 2. Detection of mercury in tissue by immunoassay and CVAAS. alligator (■), bass (△), scallop (○), shark (●), swordfish (□), tuna (▲)

from tissue, it was incompatible with the immunoassay because the small sample size used in this procedure and the dilution required for neutralization resulted in low sensitivity. Subsequent immunoassays in this study were performed on tissue samples digested with HNO_3 and H_2O_2.

The FDA has established an action level of 1 ppm for mercury in fish (*10*), and some states have issued consumption advisories for fish containing greater than 0.5 ppm mercury. The immunoassay must be able to measure mercury at or below these concentrations in order to be useful as a screening test. The immunoassay's lower detection limit (LDL) was determined by analyzing a diluted scallop extract spiked with different concentrations of mercury. The LDL was considered to be the concentration of mercury which gave an absorbance equal to the absorbance of the negative control plus 3 times the standard deviation. An LDL of 0.03 ppb was obtained, which corresponds to 0.1 ppm of scallop tissue after the dilution of the extract is taken into consideration. With a detection limit of 0.1 ppm, this test possesses adequate sensitivity for the identification of samples which contain mercury at levels which are considered unsafe.

The immunoassay's ability to measure mercury from various tissue samples was assessed. Swordfish, tuna, scallop, bass, shark, and alligator tissue was digested with the modified procedure and analyzed by both CVAAS and immunoassay (Figure 2). Linear regression analysis gave a slope of 1.02 and an r value of 0.97. The quantitation limit for mercury in these tissue samples was approximately 0.1 ppm for CVAAS and approximately 0.4 ppm for immunoassay. The immunoassay quantitation limit of 0.125 ppb in 0.1 M HEPES, pH 7, corresponds to a quantitation limit of 0.4 ppm in tissue after the mercury is extracted and diluted 1:1000. In addition, preliminary results indicate that the immunoassay can measure mercury in cormorant liver tissue (data not shown). Immunoassay reproducibility was assessed by performing five separate immunoassays of shark, swordfish and tuna tissue. CV's of 11.4, 5.7, and 9.2% were obtained for these samples, respectively. With this limited number of samples, the immunoassay reproducibly measured mercury in a variety of tissues.

Mercury in food is a serious threat to human health, but the complexity of current analytical methods makes widespread screening difficult. The mercury-specific immunoassay can be adapted for quick, portable analysis of mercury in tissue. A good correlation was obtained between the immunoassay and CVAAS for tissue samples digested with 70% HNO_3 and 13% H_2O_2. Since this extraction procedure is lengthy and somewhat inefficient, the development of a quick, efficient, and safe extraction procedure which also is amenable to the immunoassay is a goal of future research. Once such a procedure is developed, the immunoassay can be used for quick, on-site determination of mercury in tissue.

Literature Cited

1. *Environmental Health Criteria 101. Methylmercury;* World Health Organization: Geneva, Switzerland, 1990.
2. *Environmental Health Criteria 86. Mercury-Environmental Aspects;* World Health Organization: Geneva, Switzerland 1989.
3. Fitzgerald, W. F. *The World & I* **1993**, *8*(10), 192–199.

4. Delaney, M. F.; Bell, J. U.; Sundlof S. F. *J. Wildlife Dis.* **1988**, *24*(1), 62–66.
5. Grieb, T. M.; Driscoll, C. T.; Gloss, S. P.; Schofield, C. L.; Bowie, G. L.; Porcella, D. B. *Environ. Toxicol. Chem.* **1990**, *9*, 919–930.
6. Driscoll, C. T.; Yan, C.; Schofield, C. L.; Munson, R.; Holsapple, J. *Environ. Sci. Technol.* **1994**, *28*(3), 136A–143A.
7. Wylie, D. E.; Carlson, L. D.; Carlson, R.; Wagner, F. W.; Schuster, S. M. *Anal. Biochem.* **1991**, *194*, 381–387.
8. Wylie, D. E.; Lu, D.; Carlson, L. D.; Carlson, R.; Funda Babacan, K.; Schuster, S. M.; Wagner, F. W. *Proc. Nat. Acad. Sci. U.S.A.* **1992**, *89*, 4104–4108.
9. *U.S. Department of Energy Methods for Evaluating Environmental and Waste Management Samples, Rev. 2;* Goheen, S. C.; McCulloch, M.; Thomas, B. L.; Riley, R. G.; Sklarew, D. S.; Mong, G. M.; Fadeff, S. K., eds.; Pacific Northwest Laboratory: Richland, Washington, 1994, MB100.
10. U.S. Food and Drug Administration. *Federal Register* **1979**, *44*, 4012.

RECEIVED July 26, 1995

Chapter 31

Immunochemical Approaches to the Analysis of Paralytic Shellfish Poisoning Toxins

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Using different paralytic shellfish poisoning (PSP)-protein conjugates several monoclonal antibodies against saxitoxin (STX) as well as polyclonal antibodies against STX and neosaxitoxin have been produced. The sensitivity and particularly the specificity of the antibodies will be discussed in detail, their use in microtiter plate EIA's and in immunoaffinity columns for the analysis of contaminated mussels is described.

Paralytic shellfish poisoning (PSP) toxins are produced by certain marine algae known as dinoflagellates. Contamination of shellfish has been associated with harmful algae blooms throughout the world. The first PSP component to be chemically characterized was saxitoxin (STX). Three groups of PSP toxins, *N*-sulfocarbamoyl, carbamate and decarbamoyl toxins are known (Figure 1). All 18 toxins are naturally occurring; thus, meaning that a mixture of different toxins is usually present in a contaminated sample (*1*).

PSP toxins act through a potent, reversible blockage of the sodium conductance in nerve and muscle membranes. The lethal dose for humans is 1–4 mg expressed as saxitoxin equivalents. Each of the PSP toxins has its characteristic toxicity expressed as mouse units (MU) per μmole. The toxicity of *N*-sulfocarbamoyl derivatives is relatively low (18–430 MU), that of the carbamate toxins is significantly higher (673–2045 MU) and the decarbamoyl toxins exhibit intermediate toxicity (530–1220 MU).

The classical method for the analysis of PSP toxins is the mouse-bioassay, based on the lethal effect of intraperitoneally administered PSP toxins. In most countries this method is still used for monitoring purposes. But in addition to objections to the use of experimental animals, sensitivity of the mouse bioassay is too close to the regulatory limit, which is in the range of 40–80 μg per 100 g shellfish (2). The assay also lacks specificity.

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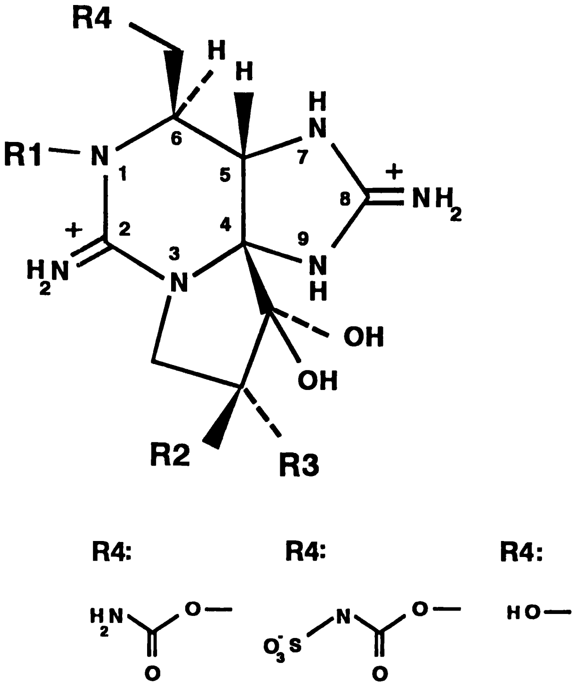
R4
H
H
H
N
7
+
8
NH2
R1
N
1
6
5
+
2
4
H2N
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9
N
H
OH
OH
R2
R3
R4:
R4:
R4:
H2N
O
O
O3-S
N
O
O
HO

R 1	R 2	R 3	Carbamate Toxins	N-Sulfo-carbamoyl Toxins	Decarbamoyl Toxins
H	H	H	STX	B 1	dc-STX
OH	H	H	neoSTX	B 2	dc-neoSTX
OH	H	OSO_3^-	GTX 1	C 3	dc-GTX 1
H	H	OSO_3^-	GTX 2	C 1	dc-GTX 2
H	OSO_3^-	H	GTX 3	C 2	dc-GTX 3
OH	OSO_3^-	H	GTX 4	C 4	dc-GTX 4

Figure 1. Structures of paralytic shellfish poisoning (PSP) toxins.

The following is a brief overview of the characteristics of antibodies against PSP toxins produced in our laboratory with some examples for the applicability of immunochemical methods used in analyzing for these algal toxins.

Antibodies Against PSP Toxins

Although the first antibodies against STX were described in 1966 by Johnson and Mulberry (*3*), immunochemical methods still play only a minor part in the field of PSP toxin analysis. This is primarily due to the multiplicity of STX analogs that makes it unlikely or even impossible, that a single antibody reactive with all PSP toxins could be produced. For screening purposes, however, it would be sufficient, if the immunochemical methods used in a monitoring program were able to detect only the highly toxic compounds, namely the decarbamoyl and carbamate toxins.

Polyclonal Antibodies. Using a previously described formaldehyde condensation procedure (*4,5*) for the preparation of the immunogen, STX was coupled to keyhole limpet hemocyanin (KLH). After immunization of rabbits, highly sensitive antibodies were obtained and a competitive indirect enzyme immunoassay (EIA) was established, in which STX-bovine serum albumin (BSA) served as coating antigen (*6*). A 10-fold increase of the test sensitivity was observed after development of a direct EIA using STX coupled to horseradish peroxidase via a modified periodate reaction as the enzyme conjugate (*7*). The detection limit of this test system was 7 pg STX/mL. Taking into account that the tolerance level for PSP toxins is set at 40 to 80 μg per 100 g shellfish, the sensitivity of both EIA's is more than sufficient for all analytical purposes.

But, as mentioned above, the usefulness of immunochemical test systems in the field of PSP analysis is a question of the specificity of the antibodies, particularly, recognition of the highly toxic carbamate and decarbamoyl toxins. Until recently, the determination of cross-reactivity was nearly impossible as a result of the lack of commercially available pure standards. During the last few years, however, a Canadian group has isolated several PSP toxins, in particular STX, neosaxitoxin (neoSTX), a mixture of the gonyautoxins-2/3 (GTX-2/3) and GTX-1/4, decarbamoyl-STX (dc-STX) and a mixture of the *N*-sulfocarbamoyl toxins C-1/2 (*8*). Using these well characterized standards, the polyclonal antibodies against STX showed cross-reactivities of 27.8% and 12.1% with dc-STX and GTX-2/3, respectively; whereas, their reactivity towards neoSTX and particularly GTX-1/4 and C1/2 was very low (Table I). But, as a result of the extremely high sensitivity of the test system, the detection limit for C1/2 is still less than 1 ng/mL.

Studying the effect of using a heterologous PSP toxin-enzyme conjugate on the cross-reactivity, we observed that mainly the detection limit for neoSTX could be improved (*9*). In a direct EIA with dc-STX-HRP, the detection limits (25% inhibition dose) for STX, neoSTX, dc-STX, and GTX-2/3 were 6.2, 68.0, 24.3, and 96.1 pg/mL, respectively. Compared with the homologous assays, the recognition of the toxins was more uniform, thus reducing the risk of an over- or underestimate of the total toxin content in an unknown mixture. Low cross-reactivities with neoSTX in assays using polyclonal antibodies against STX also were described by other authors (*5,10*).

In order to facilitate the detection of the *N*-1-hydroxy derivatives (neoSTX and the corresponding structural analogues), rabbits were immunized with neoSTX conjugated to the glycoprotein glucose oxidase (GlcOx) using the periodate method. Competitive indirect EIA, using STX-BSA as coating antigen, revealed that the antibodies are specific for neoSTX, but that they also have substantial cross-reactivity with GTX-1/4 (Table I). The detection limits are 18 pg neoSTX/mL and 26 pg GTX-1/4/mL, respectively. Considering the complementary cross-reactivities of the polyclonal antibodies against STX and neoSTX it is obvious, that a combination of these two test systems could be very useful for monitoring purposes. The only other polyclonal antiserum against neoSTX, desribed by Chu and Huang (*11*), mainly cross-reacts with STX (11.4%), but no data were given concerning the reactivity of these antibodies with gonyautoxins.

Table I. Relative Cross-Reactivities (RCR) of the Polyclonal Antibodies against Saxitoxin and Neosaxitoxin.

	Antibodies against			
	Saxitoxin		*Neosaxitoxin*	
Toxin	*50% dose (pg/mL)*	*% RCR*[a]	*50% dose (pg/mL)*	*%RCR*[a]
Saxitoxin	15.0	100	2,420	3.0
dc-Saxitoxin	47.5	27.8	42,054	0.2
Gonyautoxin-2/3	163.5	12.1	2,886	3.3
C-1/2	5,053	0.5	202,350	0.1
Neosaxitoxin	510.0	3.1	76.1	100
Gonyautoxin-1/4	6,242	0.3	111.2	88.5

[a] Cross-reactivity was calculated on a molar basis.

Monoclonal Antibodies. All monoclonal antibodies described so far show very low affinities for STX and therefore are not suitable for the detection of PSP toxins in food (*12*). In our first attempts to produce monoclonal antibodies against STX we used the STX-KLH and other STX-protein conjugates all produced by the Mannich reaction (*4,5*). Unfortunately, all of these conjugates proved to be poor immunogens in mice. Much better results could be achieved by using another coupling procedure, namely the modified periodate reaction. Coupling STX to the glycoprotein glucose oxidase yielded a highly immunogenic antigen-carrier preparation. After only a single immunization, 10 out of 12 mice showed high serum antibody titers against STX.

Using the STX-glucose oxidase immunogen, 9 hybridoma cell lines, all secreting antibodies of the IgG1 subclass, were established. Table II shows the characteristics of the monoclonal antibodies (Mab) designated 5F7, 7H11, 1E8, each representing a group of antibodies different in terms of sensitivity and particularly in their specificity relative to STX. The monoclonal antibodies were less sensitive than the corresponding polyclonal antibodies, but all showed considerable cross-reactivity to GTX-2/3. Only weak cross-reactivity to the corresponding *N*-sulfocarbamoyl toxins C-1/2 was observed indicating that the R4 side chain had great influence on the affinity of the monoclonal antibodies. Additionally, Mab 1E8 showed very low reactivity with dc-STX, whereas Mabs 7H11 and 5F7 exhibited cross-reactivities of 11.4 and 18.2%, respectively. Also, each change in the side chains reduces the reactivity of Mab 5F7 by a factor of 10, whereas, a change at side chain R2/3 had little influence (86.3% cross-reactivity with GTX-2/3) on the affinity of Mab 7H11. Additionally, this Mab showed remarkable cross-reactivity with all tested carbamate and decarbamoyl toxins; e.g., neoSTX, GTX-1/4, GTX-2/3 and dc-STX.

Considering the cross-reactivity of both the polyclonal antibodies against neoSTX and the monoclonal antibodies against STX, there is strong evidence that using the periodate method for the preparation of PSP toxin-carrier conjugates, resulted in antibodies with improved cross-reactivities with the respective, structural related gonyautoxin.

Application of Immunochemical Methods for the Analysis of PSP Toxins

Using highly sensitive and well characterized antibodies, immunochemical methods could be very useful at different stages of an integrated analytical system for the analysis of PSP toxins. Depending on the required result, immunochemical test systems can be designed as rapid qualitative (*7,13*) or quantitative assays. Additionally, immunochemical methods may be combined with physico-chemical methods, thus improving both sensitivity and specificity of traditional chromatographic techniques.

Quantitative Test. The microtiter plate assay has the potential to replace the mouse-bioassay as the screening assay. Currently, a comparison study between the two assays is being performed using the EIA based on the polyclonal antibodies against STX as an immunochemical method. So far 64 samples (mussels, King scallops and Queen scallops) were analyzed, and 44 were positive in the mouse-bioassay (Donald, M., MAFF, Aberdeen, UK, personal communication, 1995). Generally, analysis by EIA resulted in lower values (28–370 μg STX equivalents per 100 g shellfish) than the bioassay (37–657 μg STX equivalents per 100 g shellfish), but all samples which reacted positive in the mouse-bioassay were also positive in the enzyme immunoassay. Nearly all bioassay negative samples also yielded negative results or very low values ($<$ 10 μg/100 g) in the EIA. The differences between the two methods of analysis can be simply explained: lower values in the immunochemical assay are caused by the low cross-reactivity of the EIA with toxic analogues, and higher values occur, if high concentrations of relatively non-toxic *N*-sulfocarbamoyl toxins are present in the sample material.

Table II. Relative Cross-Reactivities (RCR) of the Monoclonal Antibodies Against Saxitoxin.

		5F7		7H11		1E8	
Toxin	mol wt	50% dose (ng/mL)	% RCR[a]	50% dose (ng/mL)	% RCR[a]	50% dose (ng/mL)	% RCR[a]
Saxitoxin	299	1.5	100.0	1.5	100.0	0.2	100.0
dc-Saxitoxin	256	11.8	11.4	7.2	18.2	18.1	0.9
Gonyautoxin-2/3	394	14.0	14.1	2.2	86.3	0.7	33.1
C-1/2	473	> 500.0	< 0.4	76.5	3.0	161.3	0.2
Neosaxitoxin	315	15.9	10.0	17.9	8.5	7.1	2.6
Gonyautoxin-1/4	410	163.8	1.3	13.2	15.1	4.6	5.1

[a] Cross-reactivity was calculated on a molar basis.

In view of these first and preliminary results, it seems possible to replace the mouse-bioassay by an enzyme immunoassay or at least by a combination of immunoassays with complementary specificity like the STX and neoSTX-EIA described above.

Immunoaffinity Chromatography (IAC). As for every screening assay, positive samples should be reanalyzed by physico-chemical methods at least for legal or statutory purposes. In the analysis of PSP toxins the common method is HPLC with fluorescence detection after pre- or postchromatographic oxidation (*14,15*). The problems encountered with this method of analysis are mainly of a chromatographic nature. To separate the 18 structurally related compounds, sophisticated gradient elution programs and mobile phases were developed, but clean-up methods for the purification of shellfish extracts are barely described. To circumvent drawbacks of chromatographic analyses through sample interferences we developed immunoaffinity chromatographic columns using the broadly cross-reactive Mab 7H11 (*16*).

The antibody was coupled to CNBr activated Sepharose 4B, mini-columns containing 200 μL of the immunosorbent were used in all of our studies. Each immunoaffinity-column had a capacity of approx. 3 μg PSP toxin. In order to determine recovery rates for the available toxin standards, 2 μg of each toxin in a concentration of 100 ng per mL were passed through the column and subsequently the bound toxin was eluted with acetic acid. For STX, all GTX toxins and dc-STX the recovery rates were in the range of 70–90%. NeoSTX and C-1/2 also were retained by the immunoaffinity columns, but to a minor extent. In order to check the applicability of the IAC columns for analysis of shellfish extracts, we passed uncontaminated, artificially contaminated and naturally contaminated mussel extracts through the column and analyzed the corresponding eluates by HPLC according to (14). The chromatograms of uncontaminated samples (Figure 2) showed the excellent clean-up provided by the IAC columns. No sample peaks could be observed. The recovery of PSP toxins from mussel extracts were in a similar range as observed applying the pure standards to the IAC columns. The analysis of naturally contaminated mussels gives evidence, that in addition to the known reactivity, the Mab 7H11 also binds dc-GTX-2/3 and the *N*-sulfocarbamoyl toxin B1 (Figure 2).

Conclusions

The presented results strongly support the use of immunochemical methods for improved analysis of the PSP toxins. In our laboratory work is still in progress to obtain more data concerning the correlation between bioassay and enzyme immunoassay and to improve the clean-up of mussel samples using IAC columns.

Acknowledgments

We thank B. Minich and M. Straka for excellent technical assistance.

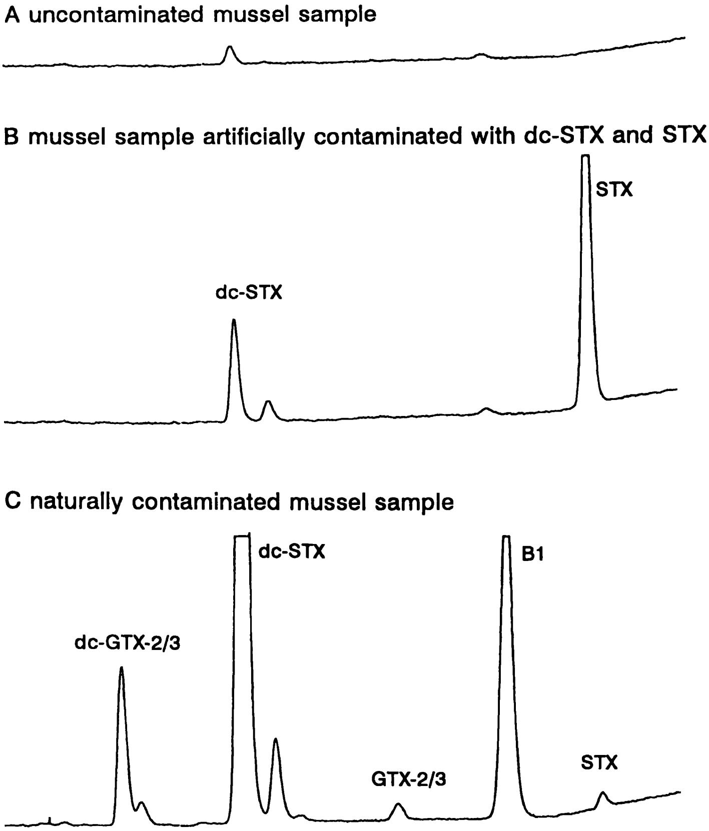

Figure 2. HPLC chromatograms of extracts of (A) uncontaminated, (B) artificially contaminated (100 ng/g STX and 25 ng/g dc-STX) and (C) naturally contaminated mussels. All extracts were purified by IAC columns. HPLC conditions were exactly as described by Lawrence *et al.* (*14*). The retention time of STX was 13.8 min.

Literature cited

1. Hall, S.; Strichartz, G.; Moczydlowski, E.; Ravindran, A.; Reichhardt, P. B. In *Marine Toxins: Origin, Structure and Molecular Pharmacology*; Hall, S.; Strichartz, G., eds.; American Chemical Society: Washington, DC, 1990, ACS Symposium Series No. 418; pp 29–65.
2. Van Egmond, H. P.; Aune, T.; Lassus, P.; Speijers, G. J. A.; Waldcock, M. *J. Nat. Toxins* **1993**, *2*, 41–83.
3. Johnson, H. M.; Mulberry, G. *Nature* **1966**, *211*, 747.
4. Johnson, H. M.; Frey, P. A.; Angelotti, R.; Campell, J. E.; Lewis, K. H. *Proc. Soc. Exp. Biol. Med.* **1964**, *117*, 425–430.
5. Chu, F. S.; Fan, T. S. L. *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 13–16.
6. Renz, V.; Terplan, G. *Arch. Lebensmittelhyg.* **1988**, *39*, 30–33.
7. Usleber, E.; Schneider, E.; Terplan, G. *Lett. Appl. Microbiol.* **1991**, *13*, 275–277.
8. Laycock, M. V.; Thibault, P.; Ayer, W.; Walter, J. A. *Nat. Toxins* **1994**, *2*, 175–183.
9. Usleber, E.; Dietrich, R.; Märtlbauer, E.; Terplan, G. *Lett. Appl. Microbiol.* **1994**, *18*, 337–339.
10. Cembella, A.; Parent, Y.; Jones, D.; Lamourex, G. In *Toxic Marine Phytoplankton*; Graneli, E.; Sundstrom, B.; Edler, L.; Anderson, D. M., eds.; Elsevier Science Publishing: New York, NY, 1990, pp 339–344.
11. Chu, F. S.; Huang, X. *J. AOAC Int.* **1992**, *75*, 341–345.
12. Huot, R. I.; Armstrong, D. L.; Chanh, T. C. *J. Toxicol. Environ. Health* **1989**, *27*, 381–393.
13. Usleber, E.; Schneider, E.; Terplan, G.; Laycock, M. V. *Food Addit. Contam.* **1995**, *12*, 405–413.
14. Lawrence, J. F.; Menard, C. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 1006–1012.
15. Sullivan, J. J. In *Marine Toxins: Origin, Structure and Molecular Pharmacology*; Hall, S.; Strichartz, G., eds.; American Chemical Society: Washington, DC, 1990, ACS Symposium Series No. 418; pp 66–77.
16. Dietrich, R.; Usleber, E.; Terplan, G. *107th Annual AOAC Meeting*, Washington, DC, 1993; p 143.

RECEIVED October 3, 1995

Chapter 32

Comparison of Immunoassay, Cellular, and Classical Mouse Bioassay Methods for Detection of Neurotoxic Shellfish Toxins

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Shellfish occasionally present a health risk to consumers due to bioaccumulation of natural marine toxins. One such class of toxin is the brevetoxins (PbTx) which are responsible for neurotoxic shellfish poisoning. Monitoring of commercial produce presently involves the evaluation of toxicity by intraperitoneal injection of a crude lipid extract of shellfish into mice. We have compared a newly developed ELISA assay for brevetoxins, with a detection limit of 0.25 mg brevetoxin-2 (PbTx-2) per kg shellfish flesh (2.5 ng/mL in the assay), a neuroblastoma-based sodium channel enhancement assay (detection limit 15 ng/mL), and the standard mouse bioassay for evaluation of shellfish samples collected during recent New Zealand algal blooms. Discrepancies among the assays were observed due to the presence of a 'new' lipid soluble, sodium channel active biotoxin.

Neurotoxic shellfish poisoning (NSP) occurs when shellfish that have accumulated the lipid-soluble brevetoxins are eaten. Symptoms include incoordination, respiratory distress, paralysis and convulsions. To protect human health, many governments have set maximum permissible limits for the toxins, with the regulations set by the US FDA being the most widely accepted. Attempts are currently being made worldwide to replace the nonspecific mouse bioassays specified in these regulations with more humane, specific assays. Here we compare two possible alternatives.

In the summer of 1992–3, New Zealand (NZ) experienced its first NSP event (*1*), with shellfish samples showing positive in the mouse bioassay. A portion of the samples collected during this and subsequent outbreaks, including a number of samples which gave irregular results in the mouse bioassay, have now been analysed for brevetoxin by ELISA and by a neuroblastoma assay which detects toxins activating the cellular sodium channel.

0097–6156/96/0621–0404$15.00/0

Materials and Methods

Shellfish Material. Shellfish samples were collected at a number of sites around the New Zealand coastline as part of the Marine Biotoxin Monitoring Programme. The majority of samples represented mussel (*Perna canaliculus*), scallop (*Placopecen magellanicus*), and oyster (*Tiostrea chilensis*, *Crassostrea gigas*), although some samples of tuatua (*Paphies subtriangulata subtriangulata*), pipi (*Paphies australis*) and cockles (*Bassina yatei*) were also analysed.

Extraction. Shellfish material was prepared according to (2). Briefly, shellfish were shucked and the flesh (100 g) homogenised with 300 mL acetone for 20 s. After filtration through Whatman 541 paper the solid residue was reextracted with a further 200 mL acetone, and the filtered extracts were pooled. Acetone was removed by rotary evaporation at 35 °C and the aqueous residue partitioned twice against dichloromethane. The lower (dichloromethane) fraction was passed through anhydrous Na_2SO_4 and dried *in vacuo* to a constant weight. This fraction was termed the lipid extract.

Mouse Bioassay. The mouse bioassay, which involves the evaluation of toxicity by intraperitoneal injection of the crude lipid extract of shellfish into mice, was performed according to the method of (*3*), except that the lipid extract was suspended in a solution of 1% Tween 60 in 0.85% NaCl or in vegetable oil and diluted to a final concentration equivalent to 10 g sample per mL for intraperitoneal injection. Results were calculated as mouse units as described by (*3*). All animal manipulations were approved by animal ethics committees established under the Animal Protection (code of ethical conduct) Regulations Act, 1987.

Neuroblastoma Assay. The neuroblastoma assay was performed as described by (*4*) with minor modifications. Stock cultures of the mouse neuroblastoma cell lines Neuro-2A and NB41A3 were maintained in a humidified incubator (5% CO_2) at 37 °C in RPMI medium (RPMI 1640, Gibco BRL 31800-022, New York, NY) supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM pyruvate, 100 U/mL penicillin and 100 U/mL streptomycin. Monolayer cultures ($5x10^4$ cells/well) were prepared and incubated for 9–25 h with the sample extract (10 μL) or brevetoxin standard (PbTx-2) in both the presence (test wells) and absence (controls) of ouabain (final assay concentration of 600 μM for Neuro-2A, 850 μM for NB41A3) and veratridine (final assay concentration of 60 μM for Neuro-2A and 85 μM for NB41A3); total test volume was 230 μL/well. Cell viability was determined by aspiration of the medium and incubation of the cell layer with 3-[4,5-dimethylthiazol-2-yl]-5,5-diphenyltetrazolium bromide (MTT) dye solution. A 5 mg/mL stock solution of MTT in 0.01 M phosphate buffer pH 7.4 containing 0.15 M NaCl (PBS) was diluted 1:6 with RPMI and 60 μL added to each well. The cells were incubated for 20 min with the MTT solution, which was then removed by aspiration. The precipitated dye was resuspended in 100 μL/well DMSO and absorbance at 595 nm determined using a microplate spectrophotometer. Lipid extracts of shellfish material were either applied to the assay as a suspension in 1% Tween 60 solution (as per mouse bioassay), or resuspended in methanol to a concentration representing 1 g original shellfish meat per mL and diluted 1:9 in serum-free medium. A minimum of three replicates of standards and samples were used for each determination of toxin concentration.

ELISA. Antibodies were raised against brevetoxin-BSA conjugate (PbTx-3-BSA) as described in (*5*).

Brevetoxin standard (PbTx-2) and brevetoxin-horseradish peroxidase conjugates (PbTx-3-HRP) were purchased from Chiral Corp, Miami, FL. Amplification reagent (ELAST) was obtained from Dupont-NEN, Germany.

The direct competitive ELISA was performed as follows:

ELISA plates (NUNC Immunoplate I, Denmark) were coated with antiserum in 0.05 M sodium bicarbonate buffer pH 9.4 (50 µL, 50 µg/mL) overnight at 20 °C. After a wash in PBS, additional binding sites were blocked by incubation with 1% BSA solution (300 µL, 1h, 20–25 °C). Plates were washed once in PBS and either used immediately or stored at 4 °C for up to 7-days. For assay, 50 µL sample or standard was added to the wells together with 50 µL brevetoxin-horseradish peroxidase solution (100 µg/mL). After incubation at 20–25 °C for 3 h wells were washed four times with PBS + 0.05% Tween 20 (PBST), four times with PBS, and the amplification/substrate reagents sequentially added to the wells as follows. Biotinyl tyramide solution (100 µL/well) was incubated for 15 min, wells were aspirated, washed four times with PBST and four times with PBS. Streptavidin-horseradish peroxidase (100 µL/well) was then added and incubated for 30 min, before the wells were again aspirated and washed four times with PBST and four times with PBS. 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate solution, prepared by adding 100 µL TMB stock (10 mg/mL in DMSO) to 10 mL sodium acetate buffer 0.1 M, pH 5.5 containing 0.005% H_2O_2 , was then added and incubated for 15 min, before the reaction was stopped by the addition of 50 µL 2 M H_2SO_4 and absorbance at 450 nm determined using a microplate spectrophotometer. Standards and samples were prepared for ELISA by dilution of a methanol suspension with PBS to a maximum methanol concentration of 10% (v:v). Samples were analysed in triplicate over a range of dilutions.

Results - Part I - Assay Performance

Mouse Bioassay. With the exception of a small number of assays run at Ruakura to confirm the toxicity of bulk extracts prepared in our laboratory for oral dosing trials and other work, all mouse bioassays were run by the Communicable Disease Centre, Institute of Environmental Science & Research, Porirua, as part of the NZ biotoxin monitoring programme. Subsamples of extracts prepared for mouse bioassay were obtained and used for the comparative assays described below.

Neuroblastoma Assay. The neuroblastoma assay gave reproducible curves for the brevetoxin PbTx-2 standard against which the toxin concentration of sample extracts could be assessed. The standard assay could be completed with incubation times as brief as 9 h, with low intraassay variability. Extended incubation times up to 24 h gave parallel standard curves (Figure 1). The standard working range was slightly wider for the NB41A3 cell line than for the Neuro-2A. Interassay and intraassay variances were similar for the two lines (Table I). NB41A3 was the preferred cell line because it gave the most reproducible monolayer culture. NB41A3 cells exhibited neuroblast-like morphology in comparison to the mixed neuron-like and amoeboid-like cell morphology of the Neuro-2A line.

When analyzing shellfish material, resuspension of the lipid extract in methanol was found to be the best method of sample preparation. The use of a Tween suspension of sample increased the viable cell count in control wells, in a non reproducible manner, above that determined for the no-toxin standard wells. The methanol procedure did not give these artifacts in the control and was thus adopted for the remainder of this study. Employing this methanol resuspension, reproducible interassay results were obtained for both assay (with ouabain/veratridine) and control (no ouabain/veratridine) samples. Coefficients of variance for these samples were in the order of 10–20%. Samples were quantified in these assays as 'brevetoxin (PbTx-2) equivalents' with a detection limit of 15 ng/mL in the sample extract, equivalent to 1.5 mg/kg in the shellfish tissue.

ELISA. The ELISA assay (Figure 2) was optimised by titrating concentrations of all reagents and amplification system components to give the final assay setup described above. The standard working range and detection limits were determined, along with interassay and intraassay variances (Table I). Methanol resuspension of samples were employed in all ELISA analyses. Methanol was found to give a matrix effect, although this was consistent for all samples and standards. The use of a methanol concentration of 10% throughout for both samples and standards was therefore sufficient to give reproducible results. Brevetoxin-free shellfish material showed no cross-reaction in the ELISA. Spike recovery experiments have yet to be performed.

Table I. Comparison of Assay Sensitivity and Precision.

Toxin concentration ng/mL	**Neuro 2A** Coefficient of variance (%)		**NB41A3** Coefficient of variance (%)		**ELISA** Coefficient of variance (%)	
	Intraassay	Interassay	Intraassay	Interassay	Intraassay	Interassay
1000	11.65	12.15	21.57	19.63	10.38	11.96
100	15.86	16.38	14.56	15.68	5.21	5.07
10	8.87	10.43	8.22	8.23	3.29	5.89
1	-	-	-	-	5.47	11.37
Working range	15–200 ng/mL		15–200 ng/mL		2.5–75 ng/mL	

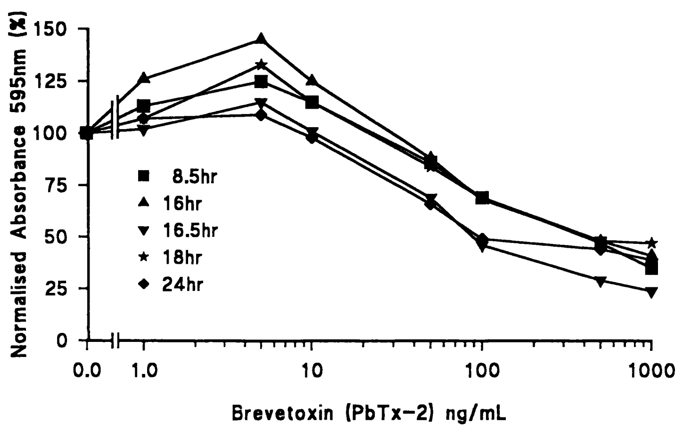

Figure 1. Effect of Incubation Time on Neuroblastoma Assay For Brevetoxin.

Brevetovin standard was incubated with the NB41A3 cell line for 8.5 to 24 h. Parallel curves are observed for PbTx-2 over this period indicating consistent response. Absorbance was normalized as percentage of no-toxin sample (including ouabain/veratridine). Absorbance for this Zero sample ranged from 0.822 Absorbance units (8.5 h) to 0.556 (24 h).

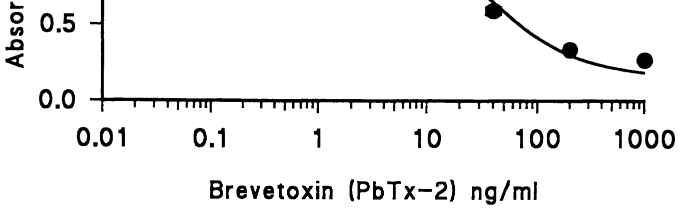

Figure 2. Standard curve for Brevetoxin ELISA.

The brevetoxin (PbTx-2) standard curve prepared in 10% methanol/PBS has linear working range (I_{85} to I_{15}) of 2.5 to 75 ng/mL, I_{50} = 15 ng/mL. Maximum absorbance is 1.5 Absorbance units.

Interassay and intraassay coefficients of variation (calculated as standard deviation/mean x 100 (%)), was assessed over triplicate samples for each assay. The linear working range and minimum detection limits are also shown.

Results - Part II - Analysis of Shellfish Material

Are The New Zealand NSP Toxins Brevetoxins ? Extracts prepared from shellfish collected during the summer of 1993–4 and found to contain NSP toxins by the mouse bioassay were strongly positive in both the ELISA and the neuroblastoma assay systems, indicating the presence of compounds with both brevetoxin-like structure and biological activity. Brevetoxin derivatives were subsequently isolated from NZ shellfish and purified to homogeneity by Prof. Yasumoto (Yasumoto, T., Tohoku University, Japan, personal communication, 1994). Table II shows the result of analysis of two of these toxins in the two assay systems. The ELISA data shows that both toxins cross-react with the antibody, and thus have a considerable degree of structural homology with the brevetoxin standard PbTx-2. The neuroblastoma assay indicates that the new toxins have a very much higher biological activity, the 'slow acting' toxin being some 90 times more potent than brevetoxin (PbTx-2).

Table II. Analysis of Purified NZ NSP Toxins by ELISA and Neuroblastoma Assay.

	100 µg 'Fast acting' Toxin	100 µg 'Slow acting' Toxin
ELISA	65 µg PbTx-2 Equiv.	20 µg PbTx-2 Equiv.
Neuroblastoma	457 µg PbTx-2 Equiv.	9000 µg PbTx-2 Equiv.

Values indicate the equivalent amount of brevetoxin required to give the same level of response in the assay.

Irregular Results...Non-Brevetoxin NSP Toxins?

In 1994–5 a number of oyster samples collected from the South Island of New Zealand gave results in the mouse bioassay indicative of NSP toxin levels greater than 660 mouse units per 100 g shellfish flesh, a level 33 times the action limit. These results were questioned, however, as the observed symptoms were different from those normally associated with NSP toxins. Further, unlike the brevetoxin-containing extracts, these samples did not show the expected proportional reduction in toxicity on dilution. Indeed, sample extracts diluted beyond 1:5 did not lead to death.

A number of these samples were analysed by ELISA and although the ELISA detected the presence of brevetoxins in some extracts, the concentrations were extremely low and could not account for the positive mouse bioassay results. In the neuroblastoma assay no effect was observed over incubation periods ranging from 9 to 18 h, an incubation period normally sufficient to detect brevetoxins. In assays incubated for 25 h, however, marked sodium channel activity was observed.

To resolve the apparently conflicting results with these samples, the regulators repeated the mouse bioassay analyses in parallel using the NZ acetone extraction (*2*) and the APHA ether extraction (*3*) procedures. The high toxicities of the acetone extracts were not observed in the ether extracts of the same shellfish sample. Indeed, most of the ether extracts analyzed returned toxicities below the action level. Furthermore, the results of the mouse bioassay using ether extraction now agreed with the more specific ELISA and short incubation neuroblastoma assays.

A bulk extract of shellfish material containing 200 MU/100 g tissue was prepared and used to compare the three assay methods. The toxin content of 100 g oyster flesh was determined to be 208 MU by the mouse bioassay (acetone); less than 10 ng of PbTx-2 equivalents by ELISA; and 455 µg of PbTx-2 equivalents by long incubation neuroblastoma assay. It should be noted that in preliminary studies this material produced no signs of toxicity when administered to rats (up to 63 g/100 g liveweight) and mice (up to 126 g/100 g liveweight) by gavage (Towers, N.; Garthwaite, I.; Smith, B. L.; Munday, R.; AgResearch Ruakura, New Zealand, unpublished data)

The acetone soluble 'biotoxin' has subsequently been isolated by Yasumoto, and its structure is under investigation. Preliminary results indicate that the toxin is not a poly-ether and is structurally unrelated to the brevetoxins (Yasumoto, T., Tohoku University, Japan, unpublished data.). Samples of this purified material have been analysed by ELISA and neuroblastoma assay. The antibody used in the ELISA was shown to have no cross-reactivity with the material. The neuroblastoma assay showed no effect over standard incubation periods of 9 to 18 h, but marked sodium channel activity at 25 h (Figure 3).

Conclusions

In this paper we have described the development of a brevetoxin-specific ELISA, and compared this and the recently described neuroblastoma-based assay with the classical mouse bio-assay for the analysis of the compounds responsible for neurotoxic shellfish poisoning. These analytical methods have helped us understand the toxins present in recent algal bloom incidents around the NZ coastline and have exemplified some of the problems in the use of nonspecific bioassays in assessing the potential toxicity of shellfish samples.

Ideally the procedure used in screening shellfish for harmful toxins should show a direct correlation between the toxicity (or toxin concentration) recorded in test systems and severity of the toxic effects on ingestion of the contaminated food or extracts of the food. However, published data relating test results with oral toxicity are not available for any of the three assay systems for NSP (brevetoxins) and all three assays circumvent a major component of the 'real' situation – the digestive process leading to uptake of toxin.

Currently the mouse bioassay is the procedure most widely accepted by regulatory authorities for the detection of brevetoxins in shellfish. However, the

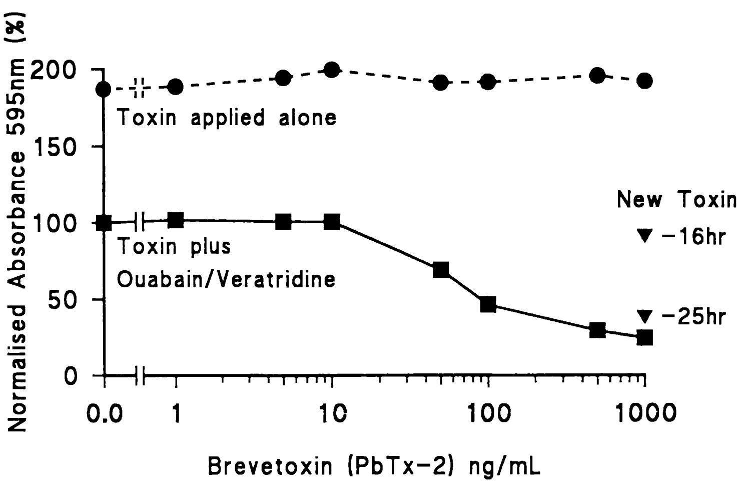

Figure 3. Neuroblastoma Assay of The New 'Biotoxin'

Neuroblastoma assays employing line NB41A3 were developed for 16 and 25 h in the presence of brevetoxin standard or 30 μg/mL new biotoxin. Lower absorbance, indicative of increased sodium influx is observed with the new biotoxin after 25 h incubation.

mouse bioassay gave a number of false positives when acetone was used for the extraction solvent and could not readily and unequivocably distinguish between brevetoxin and the new biotoxin. Furthermore the test is relatively expensive of technician time and is unacceptable on animal ethics grounds in some countries.

The neuroblastoma assay is a significant move away from the use of live animal toxicity assessment. It measures a specific toxic effect on the sodium channel at the cellular level as the inclusion of the no ouabain/veratridine controls enables the detection of cell death due to nonspecific cytotoxicity, differentiating this from any specific effect on the sodium channel. Although the assay can distinguish between brevetoxin and the new biotoxin by virtue of the differences in the incubation periods required for the expression of toxicity, this assay must still be regarded as nonspecific as it detects an 'activity' rather than a specific (molecular) structure. This may be considered an advantage so long as the activity detected is correlated with oral toxicity. In the case of the new biotoxin found in oysters, activity in the neuroblastoma assay does not appear to be associated with oral toxicity.

The ELISA method was superior to the neuroblastoma assay in both reproducibility and assay sensitivity. In addition the ELISA was quicker and easier to perform than the neuroblastoma assay and did not require the ongoing maintenance of the cell cultures. This assay is also the most specific, giving positive results only with the brevetoxins. The unique specificity of the antibody reagents used, which bind only to molecules containing the brevetoxin backbone, gives confirmation of the presence of these toxins in a sample. The ELISA system reported here is the first ELISA available for the routine analysis of shellfish material. Previous competitive

ELISA methods for brevetoxin (6) have been unsuitable due to low absorbance in the detection system. The incorporation of ELAST amplification systems in the current assay has resolved this problem.

Given that the current NSP mouse bioassay is nonspecific and has yielded false positives it would seem prudent that while it is required by regulatory authorities it should be augmented by the use of a more specific method such as the ELISA. This should enhance the protection of the consumer against known brevetoxins and the shellfish industry against unnecessary closure of commercial shellfish beds. In addition it would also alert the shellfish producers and health regulators to the presence of compounds that give positive responses in the mouse bioassay, which may or may not be toxic when taken orally, and which merit further investigation.

Acknowledgments

The authors acknowledge the assistance of staff of the Communicable Disease Centre, Institute of Environmental Science & Research, Porirua, who provided sub-samples of shellfish extracts prepared for the screening toxicity by mouse bioassay.

Literature Cited

1. Smith, P.; Chang, F. H.; Mackenzie, L. In *Marine Toxins and New Zealand Shellfish: Proceedings of a Workshop on Research Issues, 10-11 June 1993.* Jasperse, J. A., ed.; The Royal Society of New Zealand:, Wellington, NZ, **1993**, Miscellaneous Series 24; pp 11–17.
2. Hannah, D. J.; Till, D. G.; Deverall, T.; Jones, P. D.; Fry, J. M. *J. Assoc. Off. Anal. Chem.* **1995**, *78*, 480–483.
3. Irwin, N. In *Recommended procedures for the examination of seawater and shellfish*; American Public Health Association, Inc: **1970**, 4th Edition; pp 61–65 and Table 4.4.
4. Manger, R. L.; Leja, L. S.; Lee, S. Y.; Hungerford, J. M.; Wekell, M. M. *Anal. biochem.* **1993**, *214*, 190–194.
5. Poli, M. A.; Hewetson, J. F. In *Proc. 3rd Intl. Conf. on Ciguatera Fish Poisoning*; Tosteson, T. R., ed.; Polyscience Publications; Quebec, Canada, 1992.
6. Trainer, V. L.; Baden, D. G. In *Toxic marine phytoplankton: proceedings of the Fourth International Conference on Toxic Marine Phytoplankton held June 26-30 in Lund Sweden*; Granéli, E.; Sundström, B.; Edler, L.; Anderson, D. M., eds.; Elsevier: New York, NY,1990; pp 430–435.

RECEIVED July 26, 1995

Chapter 33

Production and Characterization of Antibodies Against Histamine

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Rabbits were immunized with histamine conjugated to keyhole limpet hemocyanin (KLH). Antibodies against histamine were detected in a competitive direct enzyme immunoassay (EIA), using a histamine-horseradish peroxidase conjugate as the labelled antigen. The detection limit for histamine was 230 ng/mL buffer solution. The assay was very specific for underivatized histamine. Based on the concentrations required for 50% binding inhibition (histamine: 520 ng/mL), relative cross-reactivity of these antibodies with histidine, 1-methylhistamine, 3-methylhistamine, and *N*ω-acetylhistamine was ≈0.015%, 0.14%, 0.4%, and 0.75%, respectively. The EIA was used to analyse histamine in artificially contaminated salmon meat. The detection limit was in the range of 10 μg/g, recoveries of histamine at levels of 10–1000 μg/g were between 83% and 97%, respectively.

Importance of exogenous (dietary) histamine (Figure 1) as a potential risk for human health has been discussed controversely during the last years (*1,2*). It still is not clear whether histamine, other biogenic amines, or a completely different agent is involved in certain food intoxications such as, e.g., scombrotoxicosis after consumption of mackerel, tuna and related fish (*3*). However, maximum tolerance levels of 200 mg histamine per kilogram fish tissue have been set within the European Union. A tolerance level for histamine in alcoholic beaverages of 2 mg/L has been suggested. Other foods in which high histamine levels are quite common include certain cheeses and sauerkraut (*4*).

Several physico-chemical methods for histamine, in particular liquid chromatography and fluorescence detection after derivatization with o-phtaldialdehyde or dansyl chloride have been described and so far they are the most commonly used routine assays for biogenic amines in foods (*5,6*). These methods are usually costly, require extensive sample clean-up, and have a low sample throughput. In order to enhance food control measurements, and possibly to help

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clarifying the role of dietary histamine for human health, rapid and easy analytical methods for this compound would be beneficial. Several immunochemical approaches for the detection of histamine in human serum and other biological fluids have been described (*7–11*).

So far, only one report (*12*) describes the application of an enzyme immunoassay for the detection of histamine in foods, and two other tests are commercially available (*13,14*). However, since the antibodies used in these tests are not reactive with the parent compound but with a histamine adduct, derivatization of histamine is required before analysis. In this paper we describe a simple method to produce specific polyclonal antibodies against the parent compound, free histamine, and their use in direct competitive EIA of histamine in salmon homogenate.

Materials and Methods

Materials. Histamine, histidine (D- and L-form), *N*α-acetylhistidine, 1-methylhistamine, 3-methylhistamine, *N*ω-acetylhistamine, tyramine, imidazole, tryptamine, 5-hydroxytryptamine (serotonin), 1-methylimidazole-acetic acid, imidazole 4-acetic acid, tryptophan (D- and L-form), glutaraldehyde 25% (v/v) solution, sodium periodate, sodium borohydride, 3,3',5,5'-tetramethylbenzidine (TMB), casein (sodium salt) and polyoxyethylenesorbitan monolaurate (Tween-20) were purchased from Sigma-Aldrich Vertriebs GmbH, Deisenhofen, Germany. Keyhole limpet hemocyanin (KLH), molecular weight 3–7.5 million, from *Megathura crenulata L.* was obtained from Boehringer Mannheim GmbH Biochemicals, Mannheim, Germany. EIA-grade horseradish peroxidase (HRP), molecular weight 40,000 (EC 1.11.1.7) was also from Boehringer. Affinity chromatography purified sheep anti-rabbit immunoglobulin G (IgG) was used as described earlier (*15*). Salmon meat homogenate with low histamine level (<3 μg/g; determined by HPLC) was kindly provided by L. Walther, Landesuntersuchungsamt für das Gesundheitswesen Südbayern, Oberschleißheim, Germany.

Immunogen Synthesis. Histamine was coupled to KLH with glutaraldehyde (*16*). Histamine (18 mg) was dissolved in 3 mL phosphate buffered saline (PBS, pH 7.3, 0.01 M phosphate buffer containing 0.1 M NaCl). This solution was added to 30 mg lyophilized KLH. A 10% (v/v) aqueous solution of glutaraldehyde (100 μL) was added and the mixture stirred at room temperature for 3 h. The conjugate was dialyzed against 3 x 5 L PBS and stored frozen at -18 °C.

Preparation of a Histamine-Horseradish Peroxidase Conjugate. For use as the enzyme-labelled antigen in direct competitive EIA, histamine was coupled to horseradish peroxidase (HRP) via the periodate method (*17*). HRP (27 mg), dissolved in 2 mL distilled water, was reacted with 0.4 mL of an aqueous sodium periodate solution (21.4 mg/mL) for 20 min. The activated HRP was dialyzed against 1 mM acetate buffer (pH 4.4), then 2 mg histamine (dissolved in 1 mL distilled water) was added. The pH of this mixture was adjusted to 9.5 with 0.1 M NaOH and incubated for 2 h at room temperature. Then, 0.1 mL sodium borohydride solution (4 mg/mL distilled water) was added and incubated at 4 °C for one hour. The conjugate was dialyzed against PBS, and stored lyophilized at

-18 °C. The peroxidase concentration of the conjugate, determined photometrically from the absorbance value at 403 nm, was 4.5 mg/mL.

Antiserum Production. For immunization, a portion of the histamine-KLH conjugate, including precipitate, was diluted 1:10 with distilled water, and 1.5 mL were emulsified with 4.5 mL complete Freund's adjuvant. Three rabbits (female chinchilla bastard; Savo Ivanova GmbH, Kisslegg, Germany) were each injected intradermally with 2 mL portions (corresponding to approximately 0.5 mg of KLH) of this immunogen. Intramuscular restimulation with the same composition of the immunogen was given at week 12 after primary immunization.

Antibody titers were controlled using a double antibody EIA technique: a microtiter plate was coated (100 μL/well) with sheep anti-rabbit IgG (10 μg/mL carbonate-bicarbonate buffer [0.05 M; pH 9.6]) overnight at ambient temperature in a humidified chamber. The solution was removed, and free protein-binding sites of the wells were blocked with casein sodium salt, 2% (w/v) in PBS, for 30 min at ambient temperature. The plate was washed three times with a 0.85% (w/v) NaCl solution containing 0.025 % (v/v) Tween-20 and drained. Then 50 μL of a serial dilution (in PBS) of antiserum, and 50 μL of histamine-HRP (diluted 1:1000 with PBS containing 1% (w/v) caseinate) was added and incubated for 2 h at ambient temperature. Then each plate was washed as above, and 100 μL of enzyme substrate/chromogen solution (*18*) containing H_2O_2 (3 mM) and TMB (1 mM) in potassium citrate buffer (0.2 M; pH 3.9) were added per well. After 15 min the enzyme reaction was stopped with 1 M H_2SO_4 (100 μL per well) and the absorbance measured at 450 nm.

Titer was defined as the antiserum dilution giving absorbance readings of 0.3 under these conditions. Sera of one rabbit, collected from week 14 to 29, were pooled (170 mL). A portion was precipitated with ammonium sulfate (*19*), dialyzed against PBS, and stored at -18 °C.

Direct Competitive Enzyme Immunoassay. The wells of a microtiter plate were coated with 100 μL/well anti-histamine antiserum (diluted 1:1000 with carbonate-bicarbonate buffer) overnight. Free protein binding sites were blocked with a solution of 2% (w/v) sodium caseinate in PBS (150 μL per well) for 30 min. The plate was washed three times and drained. Then histamine standard or sample extract solution (in PBS; 50 μL/well) was added, followed by histamine-HRP (in 1% (w/v) sodium caseinate/PBS; 50 μL/well) solution in a dilution of 1:20,000 (225 ng HRP/mL). After incubation for 2 h at room temperature, the plate was washed again and further treated with enzyme substrate/chromogen solution as described above. All standard and sample extract solutions were performed in quadruplicate.

Specificity of the EIA was determined using a range of substances structurally similar to histamine as listed in Table I. The concentration of each compound required for 50% inhibition of histamine-HRP binding to the anti-histamine antibodies was used to calculate relative cross-reactivities.

Analysis of Histamine in Salmon Meat Homogenate. Salmon meat homogenate was artificially contaminated using histamine standard solutions (in PBS). Portions of salmon flesh (2 g) were mixed with 10 mL PBS and extracted by magnetic stirring for 30 min. The mixture was centrifuged (3000 x g) for 20 min. The supernatant was further diluted with PBS (at least 1:4) and analysed by EIA.

Results and Discussion

Antibodies against histamine could be detected in the sera of all three rabbits immunized with the histamine-KLH conjugate, although antibody titer levels were low in two animals. However, the anti-histamine antiserum pool produced in this experiment (170 mL) provided a stock of antiserum which is sufficient to coat > 10,000 microtiter plates.

With a molecular weight of 111, histamine is a very small compound, even compared with other haptens (molecular weight usually > 250) against which antibodies have been produced. KLH was chosen as the protein carrier because of its outstanding immunogenicity in bastard rabbits as found in earlier studies (*20–22*). This is probably due to precipitate formation during most conjugation reactions, resulting in a long immunogen release period after injection. Characterization of hapten-protein conjugation ratios of such precipitate-containing immunogens is not possible. However, immunizing an animal always means introducing a "black box" into the experiment, and poor immune response in rabbits immunized with well-defined conjugates is a common observation for those working in this field. Although we have only empirical evidence, we recommend to try hapten-KLH conjugates if other carriers failed to induce immune response, or if weak anti-hapten antibody production is expected.

As free histidine is found in many foods, a most important factor in the development of an EIA for histamine is that high antibody specificity is required. Even a moderate cross-reactivity with free histidine would make the test system useless for analysis of food samples. In particular several fish species, including tuna, mackerel, and herring may contain free histidine in high concentrations (1-14 mg/g), whereas maximum histamine levels are in the range of 1-5 mg/g (*5,23*). EIA cross-reactivity with histidine must therefore be sufficiently low, to avoid false positive results even in the presence of high histidine levels.

The antibodies described here were found to be very specific for histamine (Table I), having only weak cross-reactivities in EIA with three metabolites (*i.e.*, < 1%). Histamine metabolites are found in human serum (*11*), but little is known whether they occur in foods (*24*). As the standard materials used have a purity of only 98%, it cannot be excluded that the cross-reactivity is due to histamine impurities.

The apparent minimal cross-reactivity with histidine (0.015%) in a concentration range of 3–10 mg/mL buffer solution is not necessarily specific histidine binding, but could also be caused by impurities of the histidine standard (purity: 99%), or by nonspecific interference on EIA performance in a buffer solution series containing a histidine gradient of 0–1.0% (*i.e.*, 0–10,000 μg/mL).

With a detection limit of the standard curve (Figure 2) for histamine (in buffer solutions) of 230 ng/mL, the EIA described here has a moderate sensitivity which

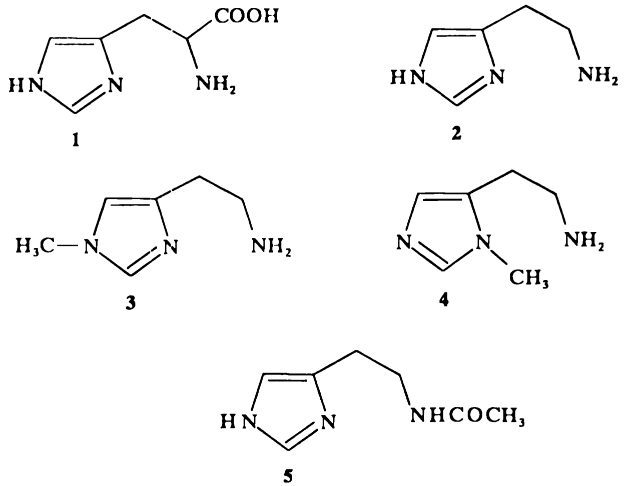

Figure 1. Structures of histidine (1), histamine (2), 1-methylhistamine (*Nτ*-methylhistamine) (3), 3-methylhistamine (*Nπ*-methylhistamine) (4), and *Nω*-acetylhistamine (5).

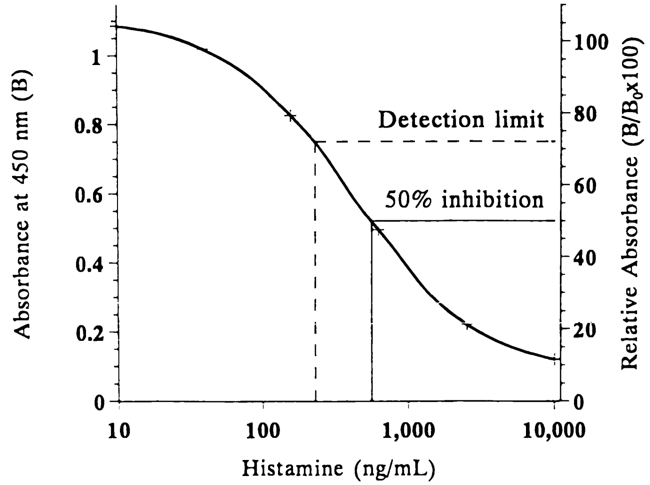

Figure 2. Typical standard curve of the competitive direct EIA for histamine. Each point represents the mean of four determinations. The intraassay CV's are usually between 1.5% and 8%. Mean 50% inhibition dose and detection limit of the test system, calculated from 8 standard curves performed on eight different days, were at 520 ng/mL and 230 ng/mL, respectively. Mean interassay CV was 7.3%.

corresponds favourably with the high levels of histamine which are of interest in food analysis.

Table I. Specificity of the Competitive Direct Enzyme Immunoassay for Histamine

Compound	50% Inhibition Dose (μg/mL)	Cross-Reactivity[a] (%)
histamine	0.52	100
D-histidine	≈ 3500[b]	≈ 0.015
L-histidine	≈ 3500[b]	≈ 0.015
1-methylhistamine	360	0.14
3-methylhistamine	130	0.4
*N*ω-acetylhistamine	69	0.75

[a] Values not corrected for molecular weight. [b] Possibly non-histidine related (purity of standard is 99%) or nonspecific inhibition. Other substances tested with cross-reactions of less than 0.05% were: *N*α-acetylhistidine, cimetidine, 5-hydroxytryptamine (serotonin), imidazole, imidazole 4-acetic acid, 1-methylimidazole acetic acid, D- and L-tryptophan, tryptamine, and tyramine.

A preliminary study, to check the applicability of the EIA for the detection of histamine in salmon meat homogenate, showed that levels as low as 10 μg histamine per gram tissue could be detected (Table II). High recoveries between 83 and 97% were obtained in a concentration range from 10−1000 μg/g, using a simple and rapid sample preparation procedure. An extract dilution which corresponds to 50 mg of sample per mL was necessary to avoid nonspecific sample matrix interference.

Table II. Recovery of Histamine in Salmon Meat by EIA (n=5)

Histamine Added (μg/g)	Histamine Found Mean (μg/g)	SD (μg/g)	CV (%)	Recovery (%)
0	--[a]			
10	8.3	1.5	18	83
100	97	16	16.5	97
1000	870	160	19	87

[a] Histamine negative extracts gave mean %B/B_0 values of 89%±8.8, outside the detection limit of the standard curve (%B/B_0 ≤ 75%; Figure 2).

Considering the sample dilution factor of 1:20, histidine concentrations of at least 70 mg/g would be required to give false-positive reactions, a value which is quite above the normal histidine level (6−7 mg/g) in salmon tissue (*25*). For histamine determination near the tolerance level of 200 μg/g, optimum sample dilutions for EIA are ten times higher (ca 1:200) and correspond to approximately 5 mg of sample per mL. False positive results due to reaction with free histidine can therefore be excluded.

In conclusion, the test system for histamine described here could be useful as a simple and inexpensive screening method for histamine in fish and fish products. Further test evaluation, using naturally contaminated sample material and comparing EIA results with those of other methods (e.g, HPLC), are under study.

Acknowledgment

We thank Dr. L. Walther, LUA Südbayern, Oberschleißheim, Germany, for providing the histamine-negative salmon sample material, and M. Djeffal for excellent technical assistance.

Literature Cited

1. Clifford, M. N.; Walker, R.; Ijomah, P.; Wright, J.; Murray, C. K.; Hardy, R. *Food Addit. Contam.* **1991**, *8*, 641−652.
2. Morrow, J. D.; Margolis, G. R.; Rowland, J.; Roberts, L. J. *New Engl. J. Med.* **1991**, *324*, 716−720.
3. Clifford, M. N.; Walker, R.; Ijomah, P.; Wright, J.; Murray, C. K.; Hardy, R.; Märtlbauer, E. P.; Usleber, E.; Terplan, G. *Food Addit. Contam.* 1993, 9, 657−666.
4. Halász, A.; Báráth, A.; Simon-Sarkadi, L.; Holzapfel, W. *Trends Food Sci. Technol.* 1994, *5*, 42−49.
5. Stockemer, J. *Z. Lebensm.-Unters. Forsch.* **1982**, *174*, 108−113.
6. Kirschbaum, J.; Luckas, B.; Beinert, W.-D. *J. Chromatogr.* **1994**, *661*, 193−199.
7. Buckler, R. T.; Ficalora, J. A.; Gavin, J. J.; Pluckett, G. A. European Patent Application 86108512.4. Publication Number 0 208 953 A1, 1986.
8. Chevrier, D.; Guesdon, J.-L.; Mazié, J.-C.; Avrameas, S. *J. Immunol. Methods* **1986**, *94*, 119−125.
9. Peyret, L. M.; Moreau, P.; Dulluc, J.; Geffard, M. *J. Immunol. Methods* **1986**, *90*, 39−45.
10. Guesdon, J.-L.; Chevrier, D.; Mazié, J.-C.; David, B.; Avrameas, S. *J. Immunol. Methods* 1986, *87*, 69−78.
11. Hammar, E.; Berglund, A.; Hedin, A.; Rustas, K.; Ytterström, U.; Åkerblom, E. *J. Immunol. Methods* **1990**, *128*, 51−58.
12. Rauch, P.; Rychetsky, P.; Hochel, I.; Bilek, R.; Guesdon, J.-L. *Food Agric. Immunol.* **1992**, *4*, 67−72.
13. Transia Diffchamb, Lyon, France. Produktinformation 1994.
14. Immuno Biological Laboratories (IBL), Hamburg, Germany. Produktinformation 1994.
15. Märtlbauer, E.; Gareis, M.; Terplan, G. *Appl. Environ. Microbiol.* **1988**, *54*, 225−230.
16. Avrameas, S.; Ternynck, T. *Immunochemistry* **1969,** *56*, 1729−1733.
17. Wilson, M. B.; Nakane, P. In *Immunofluorescence and related staining*

techniques; Knapp, W., ed., Biomedical Press; Elsevier: Amsterdam, 1978; pp 215−224.

18. Gallati, H.; Pracht, J. *J. Clin. Chem. Clin. Biochem.* **1985**, *23*, 453−460.
19. Hebert, G. A.; Pelham, P. L.; Pittman, B. *Appl. Microbiol.* **1973**, *25*, 26−36.
20. Renz, V.; Terplan, G. *Arch. Lebensmittelhyg.* **1988**, *39*, 30−33.
21. Usleber. E.; Straka, M.; Terplan, G. *J. Agric. Food Chem.* **1994**, *42*, 1392−1396.
22. Abramson, D.; Usleber, E.; Märtlbauer, E. *Appl. Environ. Microbiol.* **1995**, *61*, 2007−2009.
23. Untermann, F. *Ernährungs-Umschau* **1986**, *33*, 278−281.
24. Rice, S. L.; Eitenmiller, R. R.; Koehler, P. E. *J. Milk Food Technol.* **1976**, *5*, 353−358.
25. Souci, S. W.; Fachmann, W.; Kraut, H. *Food composition and nutrition tables 1989/90.* Wissenschaftliche Verlagsgesellschaft: Stuttgart, Germany, 1989; pp 382−383.

RECEIVED July 26, 1995

Chapter 34

Coupling Enzyme Immunoassay with Supercritical Fluid Extraction

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The coupling of enzyme immunoassay with supercritical fluid extraction (SFE) is an attractive technique for analysts faced with decreasing the use of hazardous solvents, due to their adverse impact on the environment. This chapter will describe the development of supercritical fluid extraction techniques which can be combined with enzyme immunoassays for the detection of pesticide residues and similar toxicants in food and environmental samples. The use of static versus dynamic SFE will be contrasted with respect to speed of analysis, equipment requirements, and quantitative vs. qualitative analysis. Detection of the presence of pesticides in meat matrices was accomplished using different commercial test kits. Removal of various interferences from the sample extract prior to EIA is necessary to achieve quantitative results, due to the presence of lipid coextractives in the extract. The above techniques have been successfully employed to determine pesticide residue content in meat products and other matrices below their specified tolerance limit set by regulatory agencies.

The use of enzyme immunoassay (EIA) for the determination of trace levels of toxicants in food and environmental matrices is now well documented in the literature (*1,2*). EIA offers the possibility of rapid determination, both qualitatively and quantitatively, of a variety of pesticides, drugs, and mycotoxins that can contaminate the food supply of the United States (*3–6*). Many EIA protocols also utilize aqueous solutions or a minimal amount of organic solvent while performing the test assay; making them environmentally-compatible in both a laboratory or field/plant setting.

Supercritical fluid extraction (SFE), a sample preparation technique, has developed concurrently in time with EIA over the past ten years (*7*). Performing SFE with supercritical carbon dioxide (SC–CO_2), can eliminate many of the problems associated with the use of organic solvents in extractions, namely flammability,

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toxicity, cost of purchase and disposal, and adverse impact on the environment. It also has been demonstrated that analytical SFE can yield shorter extraction times and more precise recovery of analytes relative to Soxhlet extractions for analyte concentrations at the parts per billion (ppb) level; i.e., pesticides (*8,9*), mycotoxins (*10*), and environmental contaminants (*11,12*).

Coupling SFE with analysis techniques, such as EIA or capillary electrophoresis (CZE), which use minimal amounts of solvents, provides the analyst with tandem methodologies that have several mutual advantages. Since both techniques (SFE and EIA) are environmentally-compatible, and if the two methods could be coupled, then on-site testing becomes possible, eliminating the need to transport samples to a laboratory with the associated loss of time. Analysis time can further be minimized if both techniques can be performed relatively fast. Within limits, EIA offers the possibility of screening large numbers of samples qualitatively for the presence of target analytes. This eliminates the need for an analyses that uses expensive equipment and/or reagents, and the associated labor costs to perform sophisticated instrumental analysis. For these reasons, we have conducted research on behalf of the USDA's Food Safety and Inspection Service (FSIS) for the past two years to couple SFE with EIA for the analysis of pesticide residues in meat products (*13,14*).

Several research teams have complemented our research efforts and utilized SFE for specific purposes. Earlier research by Wong *et al.* (*15*) indicated that SFE could be combined with EIA for the determination of parathion and its oxidation product, 4-nitrophenol, in environmental matrices such as soil. Further research by Lopez-Avila and Van Emon (*16,17,* See chapter by Lopez-Avila *et al.*, this volume) has illustrated the general applicability of the SFE/EIA technique to the determination of pesticides and polychlorinated biphenyls in soils, and recently these investigators have reported an extension of their methods for the analysis of drugs in tissue samples (*18*). The recent availability of several portable analytical SFE modules (*19–21*) also should further the application base for SFE/EIA in field or plant environments.

In this chapter, we shall emphasize factors that are crucial for the successful coupling of SFE with EIA since our expertise lies first and foremost in analytical SFE. The reported methods development research has been centered principally on applying SFE/EIA for the analysis of pesticides in foods, such as meats, containing a large amount of potential coextractives (fats) that also dissolve in SC–CO_2. Hence, we will describe here the development procedure for SFE/EIA assays, emphasizing ways of minimizing coextractives, or dealing with their effect in the subsequent EIA test. However, some basic principles of SFE need to be understood by the reader and these are discussed below.

Some Basic Principles of SFE

Analytical SFE exhibits many advantages over conventional sample preparation methods for the isolation of toxicant residues from a variety of sample matrices. It has been demonstrated that quantitative extractions of many environmental toxicants can be achieved via SFE for environmental pollutants, pesticides, and naturally occurring toxicants, such as mycotoxins. SFE permits extractions to be accomplished in a shorter time interval, and with better precision, relative to Soxhlet extraction for trace analytes down to the part-per-billion level. Analytical SFE is not normally concerned with the extraction of large quantities of material; consequently, it can be applied for the analysis of a wide variety of analytes (encompassing a large polarity range) at trace levels in different sample matrices.

Figure 1 depicts the SFE process, with an accompanying graph depicting the solubility of a frequently studied solute, naphthalene, in SC–CO_2 as a function of pressure and temperature. The SFE process is inherently simple: the components or matrix to be extracted are placed in the extraction vessel, a compressor or pump supplies the supercritical fluid to the extraction vessel, the dissolved solutes pass through a pressure reduction device (valve or restrictor) and are collected in some type of separator device (solvent, sorbent, empty vessel), by a reduction in pressure and/or temperature, usually to ambient conditions. The choice of extraction and/or separation conditions is to a first approximation, based on the solubility of the target analyte(s) in the supercritical fluid. An example of this principle for naphthalene in SC–CO_2 is shown in the right hand portion of Figure 1. Here naphthalene will dissolve to the extent of 5.2 mole% in SC–CO_2 at 300 bar and approximately 55 °C, conditions which typically would be used for the extraction step (E_1). A partial reduction in naphthalene's solubility in SC–CO_2 can then be affected in the separator (S_1, S_2) by reducing the pressure to 90 atm at approximately 45 °C, which yields a naphthalene concentration of 0.2 mole%, or alternatively to 1.2 mole% by reducing the temperature along the isobar to approximately 20 °C. Obviously, even greater reductions in solubilities can be achieved by performing the separation at ambient conditions. This should result in a good recovery of naphthalene, providing the trapping (separator) device is optimized for collecting the analyte.

Perhaps the key parameter in understanding the efficacy of SFE is the effect of extraction pressure on solute solubility in the supercritical fluid phase. Figure 2 illustrates a typical solubility curve for the solute, naphthalene, in SC–CO_2 under isothermal conditions. Here the onset of naphthalene's solubility in SC–CO_2; i.e., its "threshold pressure" (*22*), occurs slightly before 75 atm. As can be seen in Figure 2, the solubility of naphthalene rises precipitously over a narrow interval of pressure, resulting in a relatively high solubility for this particular solute at pressures under 200 atm. This behavior is fairly typical for many solutes undergoing SFE, but can be moderated substantially by the type of matrix from which the solute is extracted (*23*).

Similar solubility trends for lipid solutes (triglycerides) in SC–CO_2 have been recorded (*24*). Consequently, if there is not a sufficient difference in the threshold pressures of a target analyte (i.e., naphthalene) and a typical coextractive, such as triglycerides (i.e., fats/oils); then total separation is impossible, resulting perhaps in undesirable interferences being present in the final analytical assay method (EIA). Differences in solute threshold pressures and solubilities in supercritical fluids are attenuated by significant differences in the chemical structure (i.e., polarity) of solutes, their relative volatilities, or large differences in their respective molecular masses. Unfortunately in many SFEs, the above conditions with respect to the solutes being extracted do not exist, resulting in some contamination of the desired analyte with interfering coextractives. Hence, the resultant extract may or may not require some degree of cleanup, depending on the specificity of the analysis method for the analyte(s). This step also may be ignored if the coextractives do not interfere in the

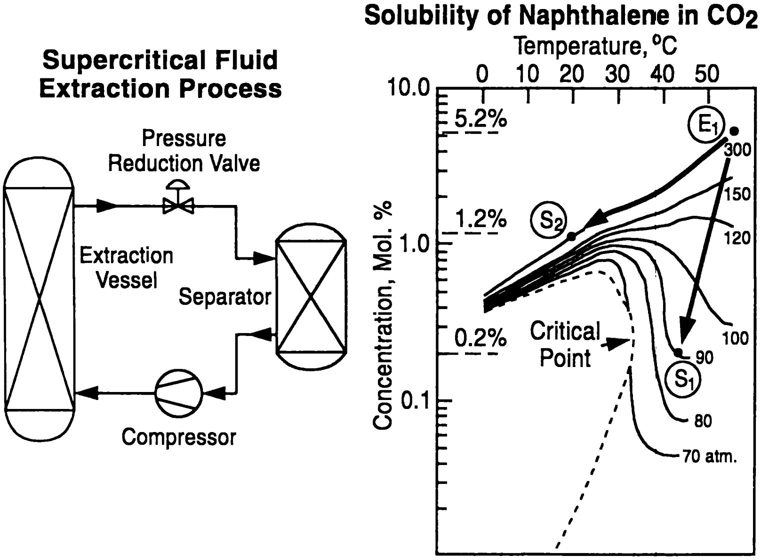

Figure 1. The basic SFE process and solubility of naphthalene in SC–CO_2 as a function of temperature and pressure (E_1 = extraction conditions; S_1, S_2 = separator conditions).

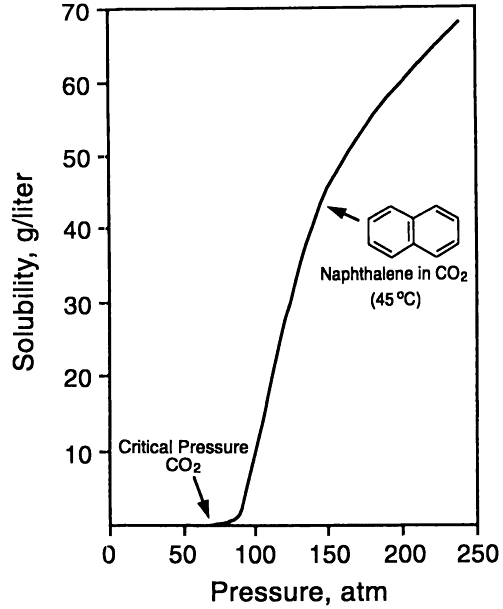

Figure 2. Solubility of naphthalene in SC–CO_2 as a function of pressure at 45 °C.

final assay method or contribute to contamination of the measuring device (instruments).

Figure 3 shows the solubility curve for the pesticide, alachlor, in SC–CO_2 as a function of pressure at 55 °C. The threshold pressure in this case is well below 100 atm, and alachlor's solubility in CO_2 is substantial; approximately 20 g/100 g of CO_2 at 250 atm. This is more than enough analyte solubility for detection by most EIA techniques, suggesting that high extraction pressures would not be required for most SFE/EIA couplings based on solubility considerations alone. Such an observation opens up the possibility of applying SFE/EIA for the qualitative assay of many analytes at trace levels, since high analyte solubilities in the extractant phase are not required for trace analysis. In addition, the extraction of trace analytes does not require a large quantity of CO_2 at extremely high pressures to affect removal of the analyte from some sample matrices, opening the way for the use of a small, portable extraction device that does not require access to a cylinder source for fluid supply or a large, cumbersome electrically- or pneumatically-driven pump to deliver a compressed fluid to the extractor proper.

Approaches for Coupling SFE with EIA

As noted in the introduction, one of the major advantages in using immunoassay is the ability to apply the technique in the field for rapid quantification of contaminants, such as pesticides or mycotoxins. This seminal goal has guided our approach to coupling SFE with EIA, and we shall describe in this section the generic experimental approach which has been utilized in developing several novel devices applicable to field assay work. Further detail of the associated experimental apparatus and technique are described in the literature (*13,14*) and the emphasis here will be on the integration of the two techniques, including several new methods not previously described.

The design of the experimental apparatus and protocols embodied several features associated with both techniques. Water was chosen as the collection solvent because most of current EIA kits are designed to function in aqueous media. In addition, water also is a compatible solvent for use in a food processing plant environment. Initial studies also utilized low extraction pressures to limit the extraction of lipids which might interfere in the EIA. Dry ice was examined as a CO_2 source since it is readily available and could be used in a processing plant. Although the purity of most dry ice would be inadequate for some SFE processes when coupled off- or on-line with classical detection methods (i.e., ECD/GC analysis); however, this proved to be a negligible problem with EIA detection methods due to their high specificity. We also have found that dry ice can aid in homogenizing tissue samples as shown by Benville and Tindle (*25*).

The initial "static" system used in our SFE/EIA studies is shown in Figure 4. This system used an extraction cell that was fabricated out of high pressure tubing (Autoclave Engineers, Erie, PA) (*13*), sealed on one end to provide a reservoir, not only for the sample, but also for the extraction fluid (CO_2). Dry ice was then added to cool the cell, followed by additional dry ice for use as the extraction fluid (CO_2). At this point the sample was introduced into the cell before capping the vessel with an assembly consisting of a pressure gauge, on/off valve, and restrictor. Pressure for the

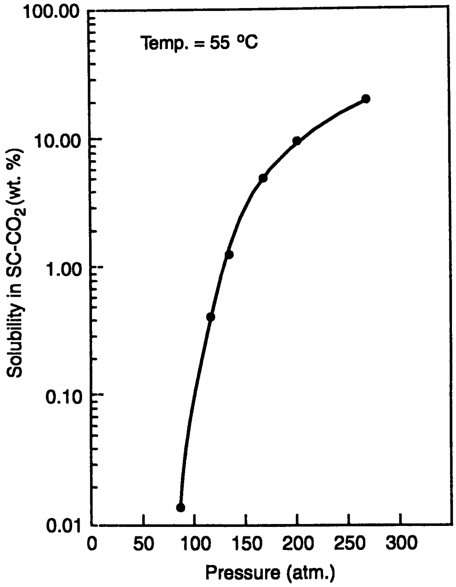

Figure 3. Solubility of alachlor in SC–CO_2 as a function of pressure at 55 °C.

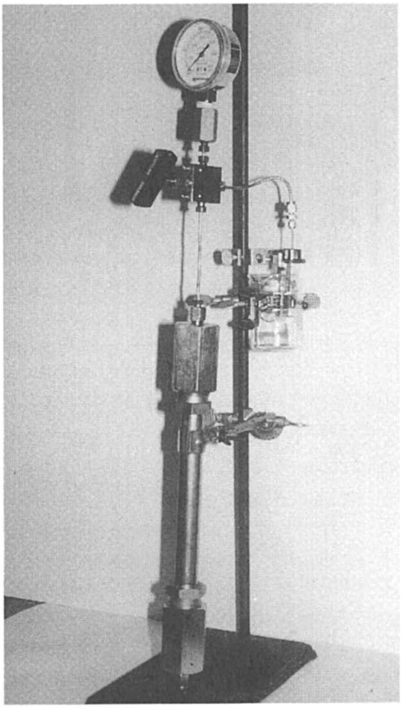

Figure 4. Static, pumpless SFE system.

extraction was developed by means of applying heat to the extraction cell, thereby expanding the CO_2 in a fixed volume. Static holds of 15 min at the developed pressures (86–118 atm) have been sufficient to extract enough analyte for qualitative determination of pesticide residues using commercial EIA kits. The vessel is simply vented at the end of this single stage SFE through an on/off valve into a collection vial filled with water via a 0.127 mm id restrictor made of PEEK tubing. Freezing of the water due to the Joule–Thomson expansion effect of CO_2 was moderated by placing the extract collection vial containing 5 mL of water into another beaker containing water. Both the beaker and the extraction cell can be heated with the aid of heating mantles (Glas-Col Company, Terre Haute, IN).

Using the above described system greatly simplifies the number of components needed in the field for conducting the qualitative SFE. Results for the determination of alachlor down to a 5 ppb level in fortified lard and poultry tissue are given in Table I, using a Resi-I Quant alachlor kit (Immunosystems, Scarborough, ME) for detection of the analyte. Similar experiments also were conducted using CO_2 extraction with a small quantity of methanol added as a cosolvent to the extraction cell.

Table I. Static SFE/EIA Screening Results.

Sample	Alachlor Detected[a]
Dry Ice (x 3)	–
Lard (x 3) (0.5–2.1 g)	–
Alachlor, (20 ng)	+
Lard (g), spiked with alachlor (ppb)	
1.9 g, 5 ppb Alachlor	–
2.4 g, 10 ppb Alachlor	+
2.5 g, 25 ppb Alachlor	+
1.1 g, 50 ppb Alachlor	+
2.3 g, 50 ppb Alachlor	+
0.5 g, 100 ppb Alachlor	+
Poultry Tissue	
10.1 g, No spike	–
12.0 g, 4.2 ppb Alachlor	+

[a] The + = pesticide detected (concentration of alachlor in collection water is above detection limit); the – = pesticide not detected (concentration of alachlor in water is below detection limit).

As shown in Table II, alachlor spikes could be detected in both lard and liver matrices, although sensitivity of the EIA kit was inhibited somewhat by the presence of methanol.

TABLE II. SFE/EIA Screening Results on Lard and Bovine Liver Samples (with Methanol Addition).

Sample	Alachlor Detected
No Sample	–
0.68 g Lard	–
0.52 g Lard, 100 ppb Alachlor	+
2.52 g Liver	–
2.16 g Liver, 23 ppb Alachlor	+

A more sophisticated CO_2 filling scheme has been developed using a siphon tube CO_2 cylinder to fill the extraction cell. In this case, initial cooling of the cell is provided externally by an ice bath to avoid CO_2 loss before warming. This unit uses a commercial GC oven for heating a CO_2-containing reservoir upstream from the sample cell, thereby allowing larger amounts of CO_2 to be employed for the SFE. The unit has been used for the detection of spiked carbofuran in frankfurters and liver using a Enzytec (Kansas City, KS) enzyme inhibition assay (*13*).

Of course commercial SFE units can be used to continuously deliver larger quantities of SC–CO_2 for more exhaustive extraction of target analytes at high extraction pressures (*26*). We have used a Dionex Model 703 SFE unit (Dionex, Sunnyvale, CA) in conjunction with an EIA kit for the detection of the organochlorine pesticide, dieldrin, spiked in poultry fat. The Model 703 unit provides for simultaneous, parallel extraction of up to eight samples at extraction pressures up to 680 atm. Figure 5 is a schematic of the unit which consists of a pumping unit that distributes extraction fluid through a manifold for distribution to the individual cells, followed by pressure reduction through a heated restrictor element into collection vials. To integrate the sample cleanup step with SFE, the 3.5 mL cells were sealed on the exit end and filled with approximately 1.8 g of deactivated alumina (neutral, Brockman I) (*27*) for retention of the co-solubilized fat. Then 0.2 g of fat containing the pesticide was placed on top of the alumina mini-cleanup column. Extraction conditions were as follows: pressure = 250 atm, temperature = 50 °C, time = 60 min. A total CO_2 volume of 10–15 L (on an expanded basis) was adequate for eluting the organochlorine pesticide.

Collection of the extract was accomplished in an empty vial at 0 °C, followed by solubilization of the analyte with 2 mL of 0.01% Tween 20 in water. The Resi-I-Imune kit for cyclodiene pesticides (ImmunoSystems, Scarborough, ME) was used to sense the presence or absence of the dieldrin by colorimetric assay. Approximately 160 mL of the above solution was placed in each microtiter well for an absorbance reading. Figure 6 shows the results, a definitive color response for the negative control sample and a qualitative reduction in color for the extracts containing dieldrin.

Multi-vessel SFEs also can be performed using a pumpless extractor of our own design. Figure 7 is a schematic of an extractor design which can process two samples simultaneously. Liquefied CO_2 from a siphon tube source was connected to the extraction cells of the same design described previously by means of a three-way

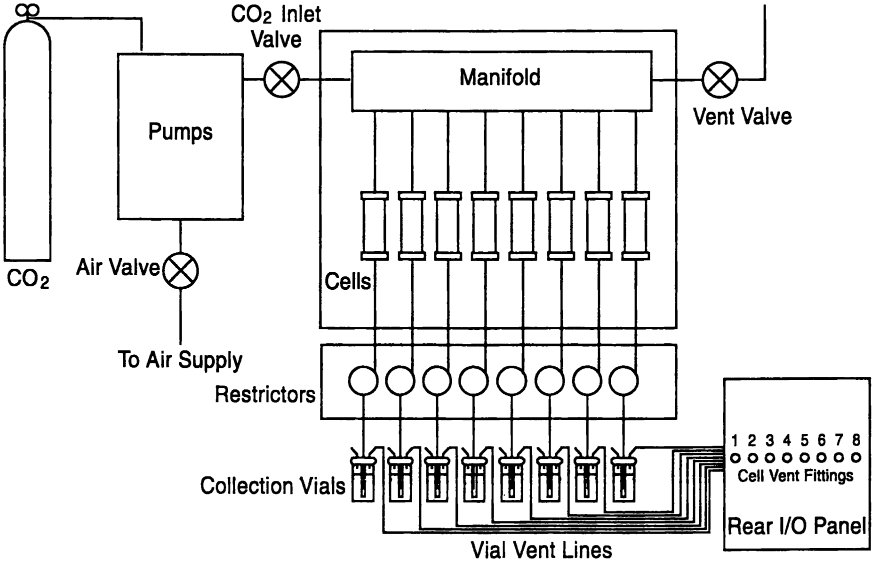
CO2 Inlet Valve
Manifold
Vent Valve
Pumps
CO2
Air Valve
Cells
To Air Supply
Restrictors
1 2 3 4 5 6 7 8
Cell Vent Fittings
Rear I/O Panel
Collection Vials
Vial Vent Lines

Figure 5. Schematic diagram of a continuous SFE system (Dionex 703).

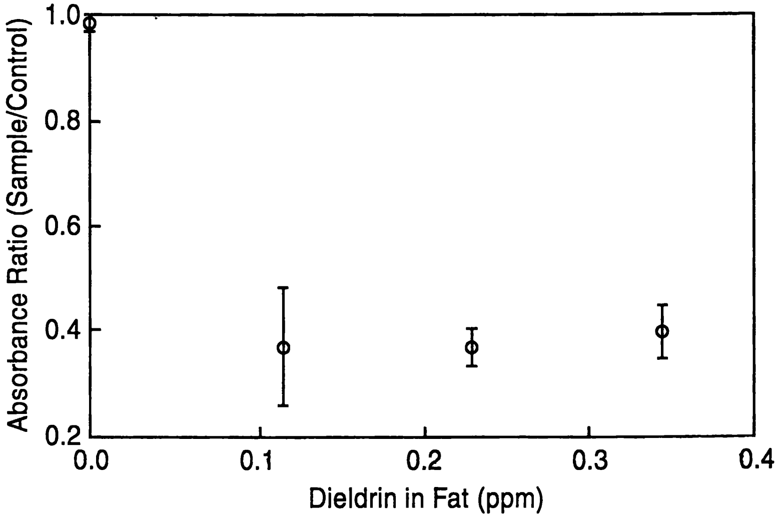

Figure 6. Detection of dieldrin in poultry fat by EIA using SFE with *in-situ* cleanup.

valve, so that either cell could be filled or jettisoned of its fluid content independently. Instead of using ice as an external coolant on the cells, the cells are immersed in a bucket of dry ice so that the CO_2 in the cell remains in its liquid state during the filling stage. After filling, the use of quick connect fittings permits the entire extraction cell assembly to be transported into a heated water bath where the extraction pressure is developed. Connection of the extraction cell device to a tandem collection assembly consisting of dual, heated micrometering valves attached to collection tubes permits extraction and collection to be affected in 40–70 min (*14*).

Figure 8 shows the results of extracting alachlor from a bovine liver sample as a function of extraction time. Figure 8 indicates that a plateau is reached after approximately 1 h or less of extraction time. Loss of analytes from the collection vessel was monitored and shown to be negligible for water or aqueous solutions containing up to 20% of methanol by volume. Use of a heated ultrasonic bath also improved analyte recovery. However, the limitations imposed by a pumpless extractor of this design, with regard to the achievable extraction pressure and/or quantity of CO_2 delivered, limits the amount of target analyte that can be extracted. A comparison of spiked alachlor extracts from bovine liver using 5 g of liver tissue mixed with 3–5 g of Hydromatrix (*28*), using methanol as a cosolvent (modifier), clearly shows the advantage of a continuous (dynamic) SFE system (Figure 9). A pumpless system using dry ice as a CO_2 source produces about a 40% yield of alachlor from the liver matrix, while the higher extraction pressure achieved using a pumpless system and liquefied CO_2 fill permits over 80% recovery of the alachlor, which is primarily due to the higher achievable extraction pressures at 50 °C (up to 600 atm). Experiments run on the Dionex extraction system with the continuous cosolvent addition module (Model 723) adding 5 mole% of methanol to the SC–CO_2 yielded quantitative recovery of alachlor from bovine liver at spiking levels up to 200 ppb at an extraction temperature and pressure of 50 °C and 450 atm, respectively. For both Figures 8 and 9, alachlor levels were determined by use of a magnetic bead-based immunoassay technique (Ohmicron, Newtown, PA).

Initial determinations of alachlor and other pesticides with the Ohmicron kits indicated a serious problem from coextracted lipid matter which interferred with quantitative EIA utilizing dynamic SFE on the Dionex unit. In contrast to the static extractions, control (blank) meat samples gave false positive readings that were above the detection limit for pesticides such as alachlor and carbofuran. Unfortunately, the previously described alumina mini-column cleanup method that can be performed *in-situ* for chlorinated pesticide assays is not applicable for the more polar pesticides. Consequently, post extraction cleanup of the extracts was explored. Three cleanup methods were tried (*14*): solid phase extraction cartridges, liquid–liquid partition, and membrane disk filtration. The main objective in the cleanup step was to remove the turbidity associated with the presence of coextracted lipids in the diluent buffer mixture in the EIA test procedure. Solid phase extraction using a C_{18} column cleanup performed on SFE extracts containing alachlor and carbofuran, yielded 103 and 97% recovery, respectively. Cleanup using an acetonitrile/hexane partitioning scheme gave 109 and 93% recovery, respectively, of the above two analytes. However, simple micro-extraction with a membrane filter, a 0.5 micron Millex-LCR membrane filter (Millipore, New Bedford, MA) proved more than sufficient yielding

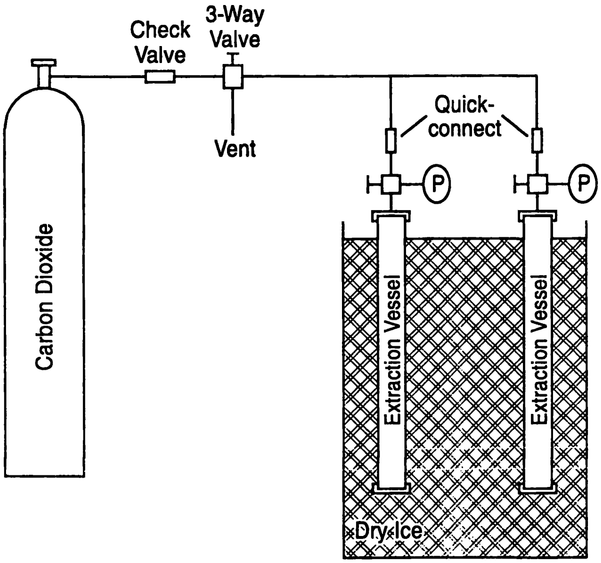
Check Valve
3-Way Valve
Vent
Quick-connect
P
P
Carbon Dioxide
Extraction Vessel
Extraction Vessel
Dry Ice

Figure 7. Schematic diagram showing a CO_2 charging system for a dual vessel, pumpless extractor.

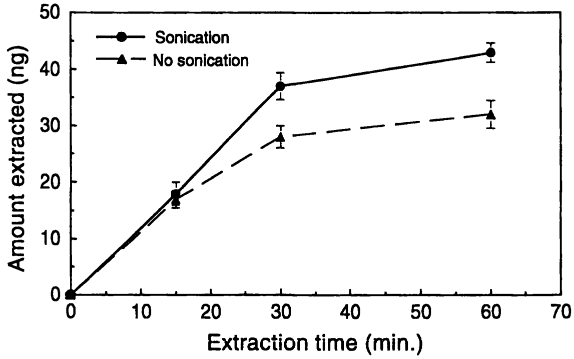

Figure 8. Extraction efficiency of alachlor from bovine liver samples using a pumpless SFE system.

100% recovery for both carbamate pesticides. This is nicely illustrated in Figure 10, where the EIA B/B_0 values between the spiked meats and blank samples were clearly distinguishable at the residue tolerance limits for alachlor (20 ppb). The general applicability of this approach is further illustrated in Figure 11 for three different pesticides assayed with the Ohmicron kits in three different sample matrices: liver, ground beef, and lard.

Quantitative results obtained with five of the Ohmicron EIA kits are presented in Table III for the pesticide analytes. These results are the mean of six individual analytical determinations for the previously mentioned sample matrices. The SFE/EIA determined recoveries and their precision indicates that all matrices are acceptable at the concentration levels of the analytes in meat or fat samples (*29,30*). Further, the minimum detection limit (MDL) achieved by the SFE/EIA method is well below the lower detection limits and residue action (tolerance) limits specified by FSIS.

Table III. SFE/EIA Results for Dynamic Extraction of Pesticide-Fortified Meat Products.

	SFE/EIA		FSIS Residue Program	
Compound	Recovery +/– SD (%)	MDL (ppb)	LDL (ppb)	Residue Limit (ppb)
Alachlor	118 +/– 13	1		20
Carbofuran	93 +/– 10	3	5	50
Atrazine	98 +/– 2	1	5	20
Benomyl	101 +/– 7	5	50	100
2,4-D	140 +/– 35	14	200	200

This indicates that the SFE/EIA methods can be used for quantitative monitoring of pesticide residues as well as the rapid screening of meat products for pesticide residue contamination.

Conclusions

The above studies indicate that SFE can be successfully integrated with a number of commercially available EIA-based kits for the detection of pesticide residues in meat and probably other associated food products. This paves the way for application of the method in food production plants where the presence of large quantities of hazardous chemicals associated with normal chemical laboratory operations cannot be tolerated. One of the major advantages of coupling SFE and EIA technologies is the speed with which toxicant contamination problems can be ascertained, thereby avoiding the expense and time associated with the routing of samples to a conventional analytical laboratory. However, even in a conventional laboratory setting SFE/EIA has the potential of reducing the number of elaborate chemical testing methods normally used in pesticide screening programs.

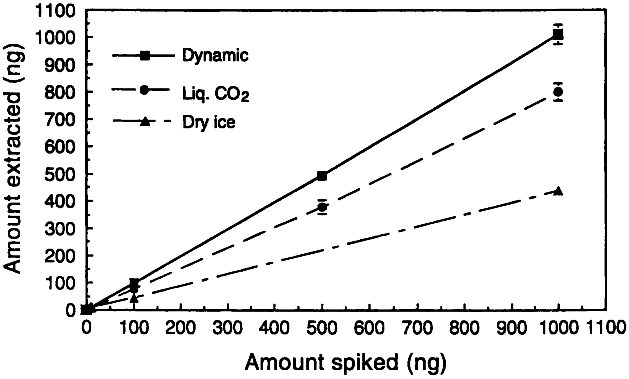

Figure 9. Extraction efficiency of alachlor from bovine liver as a function of fluid delivery system.

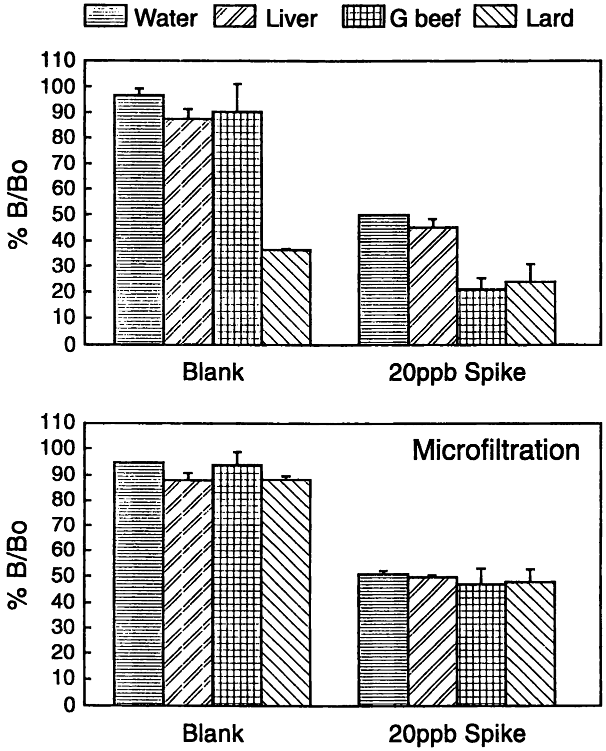

Figure 10. Effect of microfiltration of the extract from SFE on the EIA response for alachlor on different types of samples.

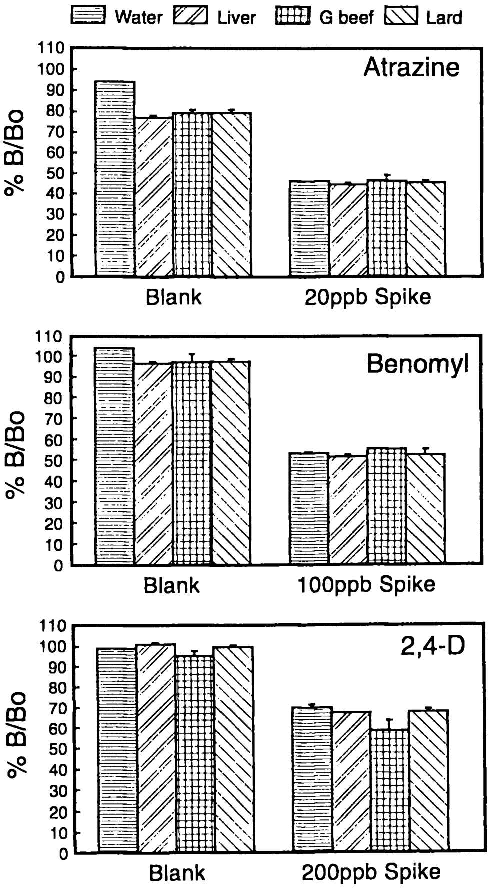

Figure 11. Results for the determination of atrazine, benomyl, and 2,4-D by EIA using dynamic SFE and microfiltration of the extract.

The above research has shown that both pumpless and conventional commercial-based dynamic SFE systems have value in residue analysis. Whereas, pumpless units appear to be limited to qualitative screening of toxicant residues in various sample matrices, the recent appearance of a commercial unit based on a thermal pump principle (*31*) opens the way for a pumpless, continuous extractor system with a dimension and size that should encourage portable use in the field.

Some sense of the impact of an SFE/EIA method on time savings and associated labor expense compared to a conventional existing chemical-based assay is provided in Figure 12. Here the key steps are listed in sequence for the determination of carbamate pesticides at trace levels in meat samples by both the proposed SFE/EIA method versus FSIS's conventional chemical protocol. The SFE/EIA assay consists of a relative simple and short extraction sequence, followed by extract filtration and centrifugation, dilution, membrane filtration, and final determination by EIA. On the other hand, the FSIS method involves sample homogenization in methylene chloride (a harmful solvent), several sequential sample treatment steps before gel permeation chromatography cleanup, which uses an eluent consisting of methylene chloride/

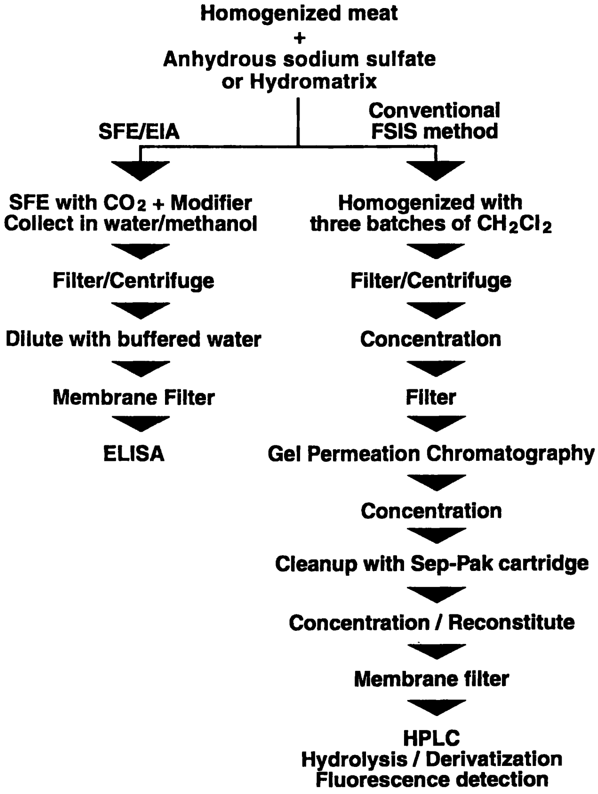
Homogenized meat
+
Anhydrous sodium sulfate
or Hydromatrix
SFE/EIA
Conventional
FSIS method
SFE with CO_2 + Modifier
Collect in water/methanol
Homogenized with
three batches of CH_2Cl_2
Filter/Centrifuge
Filter/Centrifuge
Dilute with buffered water
Concentration
Membrane Filter
Filter
ELISA
Gel Permeation Chromatography
Concentration
Cleanup with Sep-Pak cartridge
Concentration / Reconstitute
Membrane filter
HPLC
Hydrolysis / Derivatization
Fluorescence detection

Figure 12. Comparison of SFE/EIA and conventional chemical analysis methods for the determination of carbamates in meat samples.

cyclohexane. The appropriate fraction must then be isolated, concentrated, cleaned up again on a solid phase extraction cartridge, concentrated and reconstituted, filtered through a membrane filter, before the final analytical assay is performed using a classical carbamate HPLC analysis involving analyte hydrolysis followed by derivatization for fluorescence detection (*32*).

Although the above cited example may be extreme, it illustrates that SFE/EIA is inherently a simpler method for screening food matrices and other sample types for toxicant residues, and that it has considerable potential as a quantitative technique to support and confirm the results obtained from other analytical measurements.

Acknowledgments/Disclaimer

The assistance and loan of equipment by Ohmicron and Dionex Corporations for these studies is gratefully acknowledged.

Names are necessary to report factually on available data; however the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the products to the exclusion of others that may also be suitable.

Literature Cited

1. Vanderlaan, M.; Stanker, L. H.; Watkins, B. E.; Roberts, D. W. *Immunoassays for Trace Chemical Analysis*; American Chemical Society: Washington, DC, 1990.
2. Van Emon, J. M.; Lopez-Avila, V. *Anal. Chem.* **1992**, *64*, 79A–88A.
3. Vanderlaan, M.; Watkins, B. E.; Stanker, L. *Environ. Sci. Technol.* **1988**, *22*, 247–254.
4. Ellis, R. L. *J. Assoc. Off. Anal. Chem.* **1989**, *72*, 521–524.
5. Allen, J. C.; Smith, C. J. *Trends Biotech.* **1987**, *5*, 193–198.
6. Kaufman, B. M.; Clower, M., Jr. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 239–247.
7. King, J. W.; Hopper, M. L. *J. AOAC Int.* **1992**, *75*, 375–378.
8. Richter, B. L. In *Emerging Strategies for Pesticide Analysis*; Cairns, T.; Sherma, J., eds.; CRC Press: Boca Raton, FL, 1992; pp 51–68.
9. Hopper, M. L.; King, J. W. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 1013–1016.
10. Holcomb, M.; Thompson, H. C., Jr.; Hopper, M. L. In *Proceedings of the 3rd International Symposium on Supercritical Fluids*; Strasbourg, France, October 17–19, 1994, Vol. 3; pp 453–457.
11. Hawthorne, S. B. *Anal. Chem.* **1990**, *62*, 633A–642A.
12. McNally, M. E. P. *Anal. Chem.* **1995**, *67*, 308A–315A.
13. France, J. E.; King, J. W. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 1013–1016.
14. Nam, K.; King, J. W. *J. Agric. Food Chem.* **1994**, *42*, 1469–1474.
15. Wong, J. M.; Li, Q. X.; Hammock, B. D.; Seiber, J. N. *J. Agric. Food Chem.* **1991**, *39*, 1802–1807.
16. Lopez-Avila, V.; Charan, C.; Van Emon, J. *Environ. Lab.* **1993**, *5*(4), 30–33, 85.
17. Lopez-Avila, V.; Charan, C.; Van Emon, J. *Environ. Lab.* **1994**, *6*(3), 34–39.

18. Lopez-Avila, V.; Charan, C; Van Emon, J. Abstracts of the 209th ACS Meeting, Anaheim, CA, April 2–6, 1995, Vol. 1, AGFD Abstract No. 126.
19. Wright, B. W.; Wright, C. W.; Fruchter, J. S. In *Waste Testing and Quality Assurance*; Friedman, D., ed.; American Society for Testing Materials: Philadelphia, PA, 1991, Vol. 3; pp 3–14.
20. Pawliszyn, J. *J. High Resolut. Chromatogr.* **1990**, *13*, 199–202.
21. Myer, L.; Tehnrani, J. *Environ. Lab.* **1991**, *3*(6), 30–31, 46.
22. McHugh, M.; Krukonis, V. *Supercritical Fluid Extraction*; Butterworths: Boston, MA, 1986; pp 237–239.
23. Taylor, L. T. *Anal. Chem.* **1995**, *67*, 364A–370A.
24. Stahl, E.; Quirin, K.-W.; Gerard, D. *Dense Gases for Extraction and Refining;* Springer-Verlag: Berlin, Germany, 1988; pp 85–89.
25. Benville, P. E.; Tindle, R. C. *J. Agric. Food Chem.* **1970**, *18*, 948–949.
26. King, J. W.; Snyder, J. M.; Taylor, S. L.; Johnson, J. H.; Rowe, L. D. *J. Chromatogr. Sci.* **1993**, *31*, 1–5.
27. France, J. E.; King, J. W.; Snyder, J. M. *J. Agric. Food Chem.* **1991**, *39*, 1871–1874.
28. Hopper, M. L.; King, J. W. U.S. Patent 5,151,188; September 29, 1992.
29 Horwitz, W.; Kamps, L. R.; Boyer, K. W. *J. Assoc. Off. Anal. Chem.* **1980**, *63*, 1344–1354.
30. Brown, J. *Compound Evaluation and Analytical Capability, 1991 National Residue Program Plan*; U.S. Dept. of Agriculture Food Safety and Inspection Service: Washington, DC, 1991; pp 2.1–3.52.
31. Adams, M. A.; Otu, E. O.; Kozliner, M.; Szubra, J.; Pawliszyn, J. *Anal. Chem.* **1995**, *34*, 212–219.
32. Sher Ali, M. *J. Assoc. Off. Anal. Chem.* **1989**, *72*, 586–592.

RECEIVED October 3, 1995

Chapter 35

Supercritical Fluid Extraction–Enzyme-Linked Immunosorbent Assay Applications for Determination of Pesticides in Soil and Food

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This paper describes the use of off-line supercritical fluid extraction (SFE) and enzyme-linked immunosorbent assay (ELISA) for the determination of nine pesticides (alachlor, aldicarb, atrazine, carbaryl, carbendazim, carbofuran, cyanazine, 2,4-D, and metolachlor) in soil and food matrices. Soil samples (freshly spiked or spiked and aged soils) were extracted with supercritical carbon dioxide containing 10 percent methanol (as modifier) at a flow rate of approximately 2 mL/min. The extraction conditions were: pressure, 450 atm; temperature, 80 °C; and extraction time, 30 min (dynamic). Food samples consisting of baby food and Food and Drug Administration (FDA) Total Diet Study (TDS) samples were extracted at lower pressures and temperatures (150 atm, 70 °C) but for a longer period of time (15 min static and 60 min dynamic) and without the modifier to avoid extraction of the fat present in these samples. Acetonitrile was used as matrix modifier in the food extractions. In both cases, the extracted material was collected in reagent water. The benefits of SFE-ELISA include replacement of harmful organic solvents used in extraction, quick extractions with a relatively inexpensive extractant, reduced number of steps in the determination of the target compounds, and sensitive and relatively inexpensive assays.

The U. S. Environmental Protection Agency (EPA), National Exposure Research Laboratory (formerly U. S. EPA Environmental Monitoring Systems Laboratory) in Las Vegas, NV, is involved in an ongoing program that addresses sample preparation and instrumental analysis techniques that are faster and less expensive than the current methodologies and that prevent or minimize pollution from analytical laboratories. Two such techniques that have been evaluated in this study include supercritical fluid extraction (SFE) and enzyme-linked immunosorbent assay (ELISA). The potential

0097–6156/96/0621–0439$15.00/0

benefits of using SFE and ELISA have been described before by Nam and King (*1*, See chapter by King and Nam, this volume) and Lopez-Avila *et al.* (*2,3*). The SFE technique has been successfully applied to the extraction of pesticides from various environmental samples (*1–7*) as well as food matrices (*8–10*). A multiresidue method for the determination of 46 pesticides in foods and vegetables by SFE and gas chromatography/mass spectrometry has been recently published by Lehotay and coworkers (*10*). Despite the wide applicability of SFE to the extraction of pesticides from various matrices, only a few studies (*1–3*) actually considered ELISA for quantitative analysis of the target analytes. However, immunoassays have been recognized as alternative techniques to chromatographic methods for analyzing environmental contaminants, and are being used in field studies (*11*). This paper describes the use of SFE-ELISA for the determination of nine pesticides in soil and 10 pesticides in food matrices.

Experimental

Reagents. Nine ELISA test kits (Ohmicron Corporation, Newtown, PA) including those for alachlor, aldicarb, atrazine, carbaryl, carbendazim, carbofuran, cyanazine, 2,4-D, and metolachlor were tested for soil and the TDS samples. In addition, we used the chlorpyrifos ELISA kit from Ohmicron to analyze SFE extracts of baby food (Gerber beef vegetable, Gerber Products, Fremont, MI) spiked with chlorpyrifos. All immunological reagents used in this study, including paramagnetic particles coated with anti-pesticide antibody (suspended in buffered saline with preservatives and stabilizers), pesticide enzyme conjugate (horseradish peroxidase labeled pesticide analog), phosphate buffer, hydrogen peroxide solution (0.02% in a citric buffer), chromogen solution (3,3',5,5'-tetramethylbenzidine 0.4 g/L in an organic base), stopping solution (2 M sulfuric acid), and washing solution (preserved deionized water) were obtained from Ohmicron Corporation.

Materials. The soil samples used in this study are identified in Table I. The soil containing humic acid was prepared by mixing 3 g topsoil with 60 mg of humic acid sodium salt (Aldrich lot No. 00186HH, Aldrich Chemical Co., Milwaukee, WI). The topsoil and clay soil were obtained from Sandoz Crop Protection Co. (Gilroy, CA). The three soils identified as RT-801, RT-802, and RT-803 were obtained from RT Corporation (Laramie, WY). These samples contained various chlorophenoxy acid herbicides at levels ranging from 700 to 70,000 μg/kg. These samples were spiked only with the other eight pesticides since 2,4-D was already present in the samples at either 700, 7000, or 70,000 μg/kg.

Several composite solutions containing the nine pesticides in methanol were used for spiking. For example, to prepare the 10-μg/kg spiked soil sample, 300 μL of a 100-ng/mL solution was added to the 3-g soil sample. The 50- and 500-μg/kg spiked soil samples were prepared using 150 μL of a 1-μg/mL or a 10-μg/mL solution, respectively; the 700- and 7000-μg/kg spiked soil samples were prepared using 210 μL of a 10-μg/mL or a 100-μg/mL solution, respectively; and the 25,000-μg/kg spiked soil samples were prepared using 750 μL of a 100-μg/mL solution.

For spiking the soil matrix, 3-g portions of the soil matrix were weighed into an aluminum cup, and a concentrated solution containing the pesticides in methanol was added with a syringe. Losses were minimized by ensuring that the solution did not contact the aluminum cup. Mixing was performed by gently shaking the aluminum cup by hand. The spiked samples were then loaded into the extraction vessel within 100 min of spiking and sandwiched between two plugs of silanized glass wool.

The TDS sample used in this study is identified as sample RTP92A-007-DFPH (fatty); this is a composite solid sample from a diet pilot study that was conducted by Research Triangle Institute (Research Triangle Park, NC). The sample was kept frozen at -10 °C for approximately 5 months. Immediately prior to extraction, it was allowed to thaw at room temperature; 4-g portions were removed after thoroughly mixing the contents of the glass container, in which the sample was kept, with a glass rod. Each portion was spiked with a composite solution of the nine pesticides in methanol (spike level was 2 ng/g), and was then mixed with 1.5 g Hydromatrix, a pelletized diatomaceous earth (Varian, Harbor City, CA), and placed in the extraction vessel between two 2-g plugs of basic alumina.

The baby food was Gerber beef vegetable (Gerber Products); it was spiked with the nine pesticides or chlorpyrifos and then mixed with Hydromatrix, as indicated above, for the TDS sample.

SFE Procedure. The SFE operating conditions for soil samples are summarized in Table II. Soil samples (freshly spiked or spiked and aged soils) were extracted with a Dionex Model 703M SFE system. This system consisted of two pumps; one pump delivered the carbon dioxide; the second pump delivered the methanol at a constant volume ratio. The extracted material was collected in reagent water (pH 4.5) and was diluted with phosphate buffer immediately prior to analysis by ELISA. The dilution factors were chosen in such a way that the analyte concentration would fall within the linear range of the ELISA test kit.

The SFE operating conditions for food samples are summarized in Table III. TDS samples were extracted with an Isco SFX 2–10 extractor and the spiked baby food samples were extracted with the Dionex Lee Scientific SFE system. In this case, different conditions were used to minimize the extraction of fat from the food matrix. Acetonitrile (300 µL) was used as a matrix modifier.

All SFE experiments were performed with SFE/SFC-grade carbon dioxide (Air Products, Allentown, PA).

ELISA Procedure. For the ELISA, 100–250 µL of the soil extract (diluted with pH 4.5 reagent water to 10 mL and subsequently with phosphate buffer as indicated above), 250 µL of pesticide enzyme conjugate, and 500 µL of the anti-pesticide antibody coated paramagnetic particle solution was combined in a test tube. After vortexing for 1 to 2 s, the test tube was incubated at room temperature for 15–30 min. The mixture was separated using a magnetic separation rack, and was washed twice with the washing solution. The rack containing the tubes was removed from the magnet, and 500 µL of a freshly prepared chromogenic solution (hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine) was added to each test tube and allowed to develop color. The reaction was stopped after 20 min with 500 µL stopping solution (2 M sulfuric acid), and the color intensity in each test tube was determined at 450 nm using an RPA-1 photometric analyzer (Ohmicron Corporation).

Table I. Physico-chemical properties of the soil samples used in this study.

Parameter	Units	Topsoil	Clay Soil	RT-801	RT-802	RT-803
pH		7.5	7.4	8.1	8.0	7.4
Cation exchange capacity	mequiv/100 g	14.6	21.3	3.8	10.0	10.5
Organic carbon content	%	0.1	1.8	0.76	0.4	2.48
Sand	%	57.6	33.6	66.0	29.0	63.0
Silt	%	21.8	35.4	26.0	50.0	19.0
Clay	%	20.6	31.0	8.0	21.0	18.0

Table II. SFE operating conditions for the soil samples.

Parameter	
Instrument	Dionex-Lee Scientific Model 703M with Cosolvent Addition Module; vessel size 10 mL
Operating conditions	
Fluid	Carbon dioxide with 10 % methanol
Pressure	450 atm
Temperature	80 °C
Flow rate	1.6–2.2 mL/min
Extraction time	30 min (dynamic)
Restrictor temperature	100 °C
Collection solvent	Reagent water at pH 4.5 (5 mL)[a]
Temperature of collection vial	2 °C
Sample size	3 g

[a] The SFE extract was first diluted to a 10-mL final volume with reagent water (pH 4.5). The diluted extract was subsequently diluted prior to ELISA to bring the analyte concentration within the linear range of the ELISA test kit.

Table III. SFE operating conditions for food samples.[a]

Parameter	
Instrument	Isco SFX 2-10 equipped with 260-mL syringe pump and extraction module for simultaneous extraction of two samples; restrictor 24-cm length x 50-μm ID fused-silica capillary; vessel size 10 mL
Operating conditions	
Fluid	Carbon dioxide
Pressure	150 atm
Temperature	70 °C
Flow rate	1–1.3 mL/min
Extraction time	15 min (static), 60 min (dynamic)
Restrictor temperature	100 °C
Collection solvent	Reagent water (5 mL)
Temperature of collection vial	Room temperature
Sample size	4 g food sample was dispersed with 1.5 g Hydromatrix and placed between two 2-g portions of basic alumina
Matrix modifier	Acetonitrile (300 μL)

[a] A Dionex-Lee Scientific extractor was used for the extraction of chlorpyrifos from spiked baby food samples. The extraction conditions for chlorpyrifos are as specified above except that the extraction time was 30 min (dynamic).

Concentrations of the nine pesticides were determined by comparing the results to a linear regression line using a ln/logit standard curve of the particular pesticide obtained at different concentrations as follows: 0, 0.1, 1.0, and 5.0 ng/mL for alachlor, atrazine, carbofuran, and metolachlor; 0, 0.25, 1.0, and 5.0 ng/mL for carbendazim; 0, 0.4, 1.5, and 5.0 ng/mL for carbaryl; 0, 0.1, 1.0, and 3.0 ng/mL for cyanazine; 0, 1, 10, and 100 ng/mL for aldicarb; and 0, 1, 10, and 50 ng/mL for 2,4-D.

Results and Discussion

Despite the fact that supercritical carbon dioxide can dissolve a wide range of nonpolar and moderately polar compounds, preliminary experiments indicated that the nine compounds listed in Table IV were not extracted from freshly spiked soil with supercritical carbon dioxide. Therefore, we used supercritical carbon dioxide modified with 10% methanol and operated the SFE system at 450 atm; the temperature was set at 80 °C resulting in a supercritical fluid density of 0.86 g/mL. In addition, we verified the potential for cross-reactivity of each antibody with the nine pesticides in the spiking mixture and present the data in Table IV.

Recoveries greater than 80% (Table V) were achieved for alachlor, atrazine, carbofuran, cyanazine (except for topsoil spiked at 10 μg/kg and soil mixed with humic acid spiked at 50 μg/kg), and metolachlor. The other four compounds exhibited recoveries ranging from 55.0 to 227% for aldicarb, 50.0 to 99.3% for carbaryl, 34.8 to 96.7% for carbendazim, and 40.3 to 99.3% for 2,4-D. Recoveries in excess of 130% were likely to be due to interferences from the matrix and not to quantification errors since we have repeated these experiments with separate portions of the spiked soil and obtained the same results. With the exception of carbendazim, recoveries of the target compounds from the spiked and aged soils were higher than those from the freshly spiked soils and also were less variable. This may be due to the fact that two of the aged soils were sandy-loam type soils that are less adsorptive than the clay-type soils.

Overall, the precision of the SFE-ELISA technique, as established from the percent relative standard deviations (RSDs) of triplicate or quadruplicate determinations, is 20% or better for 56 of the 81 RSD values (or 69% of the total determinations) given in Table V and 67 of the 74 RSD values (or 91% of the total determinations) given in Table VI.

For the food matrix, the approach taken in this study was quite different than that for the soil matrix. In the latter, we performed the extraction with supercritical fluid at relatively high pressure and high methanol content of the supercritical carbon dioxide and somewhat moderate temperature. For the food matrix, we knew that if we performed the extraction under these conditions, we would extract the fatty acids and triglycerides that would interfere with the ELISA determination. To minimize the amount of fat extracted by SFE, we chose to perform the extraction at 150 atm and 70 °C (density 0.51 g/mL). Under these conditions, only six of the target compounds (Table VII) were recovered from spiked Hydromatrix (recoveries were at least 70% with the exception of aldicarb at 69.3%). Carbaryl recovery was 49.4%, carbendazim was poorly recovered and 2,4-D was not recovered at all. The Hydromatrix was

Table IV. ELISA results for composite standards.

Compound	True Concentration (ng/mL)	Measured Concentration (ng/mL)
Alachlor	0.1	0.1
	1.0	1.2
	3.0	3.4
	5.0	5.8
Aldicarb	0.1	0.1
	1.0	0.7
	3.0	2.1
	5.0	5.5
Atrazine	0.1	0.2
	1.0	1.4
	3.0	2.4
	5.0	4.5
Carbaryl	0.1	0.1
	1.0	0.8
	3.0	2.5
	5.0	4.3
Carbendazim	0.1	0.1
	1.0	1.2
	3.0	3.4
	5.0	5.5
Carbofuran	0.1	0.1
	1.0	1.1
	3.0	2.9
	5.0	5.1
Cyanazine	0.1	0.1
	1.0	1.4
	3.0	3.0
2,4-D	0.1	ND[a]
	1.0	1.7
	3.0	2.5
	5.0	6.4
Metolachlor	0.1	0.1
	1.0	1.3
	3.0	3.0
	5.0	6.8

[a] ND = not detected.

Table V. SFE recoveries of the target pesticides from freshly spiked topsoil, clay soil, and soil with humic acid.[a]

		% Average Recovery			% RSD		
Compound	Spike Level (µg/kg)	Topsoil	Clay Soil	Soil Mixed with Humic Acid	Topsoil	Clay Soil	Soil Mixed with Humic Acid
Alachlor	10	86.0	96.7	95.6	15	16	8.3
	50	80.9	79.3	81.2	7.9	24	10
	500	103	112	87.7	5.2	11	11
Aldicarb	10	193[b]	187[b]	225[b]	36	26	29
	50	78.0	55.0	96.7	34	30	33
	500	61.5	69.0	85.1	9.3	26	32
Atrazine	10	106	104	119	8.4	1.9	8.4
	50	82.1	86.0	104	8.5	10	6.8
	500	89.1	107	124	5.0	8.6	12
Carbaryl	10	50.0	64.0	73.0	11	17	21
	50	56.5	60.0	99.3	17	21	8.5
	500	71.9	73.3	93.9	22	11	12
Carbendazim	10	37.8	75.6	96.7	12	35	18
	50	40.0	56.3	55.7	12	24	27
	500	34.8	52.5	61.3	24	17	29
Carbofuran	10	99.4	108	119	11	12	21
	50	94.5	90.0	113	25	7.9	12
	500	85.0	88.5	116	8.8	8.7	6.5
Cyanazine	10	75.6	84.4	121	20	9.8	18
	50	92.5	105	76.0	12	11	9.8
	500	86.0	89.0	103	8.0	17	12
2,4-D	10	61.3	99.3	81.3	20	34	27
	50	40.3	60.0	59.0	15	26	34
	500	43.3	69.0	50.4	30	34	6.8
Metolachlor	10	109	155[b]	189[b]	19	17	6.1
	50	85.0	85.3	132[b]	15	5.5	12
	500	101	102	136[b]	12	4.8	17

[a] The number of determinations was four. The sample size was 3 g. The SFE conditions are given in Table II.

[b] Cannot explain high recovery.

Table VI. SFE recoveries of the target pesticides from spiked and aged soil from RT corporation.[a]

Compound	Spike Level (µg/kg)	% Average Recovery			% RSD		
		RT-802	RT-801	RT-803	RT-802	RT-801	RT-803
Alachlor	700	115	80.5	110	23	16	2
	7000	101	89.3	86.0	11	11	5.8
	25000	94.7	100	117	29	3.8	9.6
Aldicarb	700	149	122	120	15	11	33
	7000	111	116	110	6.2	4.4	6.7
	25000	116	126	117	7.6	11	–[b]
Atrazine	700	116	106	105	5.5	13.5	8.2
	7000	103	96.5	115	0.4	6.8	6.4
	25000	128	119	136	5.8	13	22
Carbaryl	700	75.9	109	158	12	19	11
	7000	75.2	88.5	89.1	8.8	10	15
	25000	99.1	102	122	14	29	20
Carbendazim	210	76.3	37.7	44.9	6.6	13	13
	2100	81.6	36.7	30.9	8.5	13	10
	7500	89.7	60.5	31.3	24	14	15
Carbofuran	700	74.6	78.8	89.1	6.6	7.4	5.4
	7000	88.8	101	101	3.7	10	3.0
	25000	101	113	104	3.8	14	16
Cyanazine	700	88.3	84.4	87.1	7.4	4.2	16
	7000	75.9	82.4	78.8	7.8	1.2	6.2
	25000	90.1	106	105	25	14	17
2,4-D	700	69.9			14		
	7000		61.3			17	
	70000			46.2			16
Metolachlor	700	98.4	98.4	101	12	12	11
	7000	86.0	91.2	94.8	13	2.8	3.5
	25000	97.6	120	118	15	16	17

[a] The number of determinations was three. The sample size was 3 g. The SFE conditions are given in Table II.

[b] Duplicate determinations.

Table VII. Pesticide recovery from spiked TDS sample and hydromatrix.[a]

Compound	Spike Level (μg/kg)	TDS Sample[b] % Average Recovery	TDS Sample[b] %RSD	Hydromatrix[c] % Average Recovery
Alachlor	2	108.0	13.0	75.9
Aldicarb	2	86.5	29.0	69.3
Atrazine	2	74.6	14.0	118.0
Carbaryl	2	68.5	20.0	49.4
Carbendazim	2	10.5	14.0	16.7
Carbofuran	2	79.5	10.0	97.5
Cyanazine	2	30.4	24.0	70.0
2,4-D	2	ND[d]		ND
Metolachlor	2	72.6	8.7	76.9

[a] The extractions were performed by SFE using the conditions given in Table III. The extracts were diluted twofold prior to ELISA.
[b] Triplicate determinations.
[c] Duplicate determinations.
[d] ND = not detected.

added to the food matrix to disperse it and make it into a free-flowing powder. Recoveries of the target compounds from the spiked food matrix were comparable to those from the spiked Hydromatrix with the exception of cyanazine (Table VII). A manuscript describing the optimization of the SFE conditions using different baby foods is in preparation. Very encouraging results were obtained using SFE-ELISA to extract chlorpyrifos from spiked baby food (Table VIII).

The applications presented here indicate that SFE-ELISA is a promising technique for the determination of pesticides in soil as well as in food matrices. Use of multivessel SFE systems will result in increased sample throughput, and use of supercritical carbon dioxide in place of organic solvents will reduce pollution resulting from the laboratory. As the SFE technology matures and the various parameters that affect the extraction efficiency are better understood, then more SFE applications will be developed. Further developments in ELISA include multianalyte immunoassays and the automation of both the plate and tube assays.

Acknowledgments

The U.S. Environmental Protection Agency (EPA, through its Office of Research and Development (ORD), funded and collaborated in the research described here. It has been subjected to the Agency's peer review and has been approved as an EPA publication. Neither the EPA nor ORD endorses or recommends any trade names or

Table VIII. Chlorpyrifos recoveries from spiked baby food.[a]

Spike Level (µg/kg)	% Average Recovery	% RSD
1	134.0	14.0
10	98.3	7.4
1000	90.3	12.0

[a]Gerber beef vegetable. The number of determinations was three. The SFE conditions are given in Table III.

commercial products mentioned in this article; they are noted solely for the purpose of description and clarification.

Literature cited

1. Nam, K.-S.; King, J. W. *J. Agric. Food Chem.* **1994**, *42*, 1469–1474.
2. Lopez-Avila, V.; Charan, C.; Beckert, W. F. *Trends Anal. Chem.* **1994**, *13*, 118–126.
3. Lopez-Avila, V.; Charan, C.; Van Emon, J. M. *Environ. Testing Anal.* **1990**, *3*, 34–39.
4. Lopez-Avila, V., Dodhiwala, N. S.; Beckert, W. F. *J. Chromatogr. Sci.* **1990**, *28*, 468–476.
5. McNally, M. E. P.; Wheeler, J. R. *J. Chromatogr.* **1988**, *447*, 53–63.
6. Janda, V.; Steenbeke, G.; Sandra, P. *J. Chromatogr.* **1989**, *479*, 200–205.
7. Engelhardt, H.; Zapp, J.; Kolla, P. *Chromatographia* **1991**, *32*, 527–537.
8. Hopper, M. L.; King, J. W. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 661–666.
9. Lehotay, S. J.; Aharonson, N.; Pfeil, E.; Ibrahim, M. A. *J. AOAC Int.* **1995**, *78*, 831–840.
10. Lehotay, S. J.; Eller, K. I. *J. AOAC Int.* **1995**, *78*, 821–830.
11. Van Emon, J. M.; Lopez-Avila, V. *Anal. Chem.* **1992**, *64*, 78A–88A.

RECEIVED October 11, 1995

Chapter 36

Solid-Phase Fluorescence Immunoassay for the Detection of Antibiotic Residues in Milk

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We describe a rapid, inhibition immunoassay for several antibiotics found as residues in milk. The fluorophore is the recently developed compound, Cy-5™, and the excitation source is a semiconductor-diode laser. The solid-phase is the borosilicate glass surface (SiO_2) of a capillary tube that has been treated with a silanizing reagent. β-Lactam antibiotics (penicillin-G, ampicillin, cloxacillin, amoxicillin, ceftiofur and cephapirin) at the parts per billion level can be detected in a total assay time of less than three minutes. These assays were optimized to provide the greatest sensitivity at or near the regulatory safe/tolerance levels for the appropriate antibiotics. The potential for the use of this system for other types of assays will also be discussed.

Antibiotics are administered to cows for the prevention and treatment of infections, such as mastitis, and for the enhancement of animal growth and milk production (*1*). Antibiotics are also abused through off-label, illegal administration in an attempt to quickly bring a sick animal back into the producing herd. For the purpose of maintaining a safe and healthy food supply, considerable attention has been focused on identifying, monitoring and minimizing the existence of these antibiotic residues in milk and milk products. Since milk and milk products are widely consumed, antibiotic residues should be avoided for several reasons: (i) Some residues can cause allergic reactions in sensitive consumers. Approximately 5 to 10% of the population is hypersensitive to penicillin or other antibiotics (*2*). (ii) Small concentrations of antibiotic residues can aid in the selection of resistant strains of pathogens that are harmful to humans (*2*). (iii) Residues are often capable of interfering with starter cultures used in the production of processed milk products such as cheese and yogurt (*2*). Consequently, the United States Food and Drug Administration (US FDA) has established safe/tolerance levels for antibiotic residues in milk and milk products.

The antibiotics that are most commonly administered to lactating cows, and consequently those antibiotics that are usually found as contaminants in milk and milk-

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In Immunoassays for Residue Analysis; Beier, R., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

products, fall into the β-lactam group (penicillin G, ampicillin, cloxacillin, cephapirin, ceftiofur, and amoxicillin) (*3*). Several types of assays have been developed and marketed for the detection of β-lactam antibiotics in milk. These assays are based on one of a variety of biochemical phenomena. The most common are inhibition of bacterial growth by antibiotic residues (*4*), binding of antibiotic residues to appropriate cell-membrane receptors (*5*) and binding of the antibiotic residues to appropriately designed polyclonal and monoclonal antibodies (*6*). These assays have been developed to test milk samples in bulk tanks that contain the commingled milk from many cows or trucks which contain the milk from several farms.

Although they are generally useful, these assays involve many manipulations and/or long incubations. Most of these assays require a significant degree of sophistication and training of the operator. In many cases, the assays require visual inspection of a reaction that is transient. Another major problem is that virtually all commercially available assays produce an unacceptable number of false positives (samples with no or below the safe/tolerance level of antibiotics, that are incorrectly identified as having antibiotic residues above safe/tolerance levels) when used for individual cowside tests (*7*). Studies have shown that for individual cowside tests, for tests in samples of milk with high somatic cell counts, or samples of milk with high levels of bacteria, bacterial inhibition tests produced from 10 to 50% false positives. With receptor based assays, 48% of negative milk samples were perceived to be positive (*7*). Consequently, no commercially available test has been approved for use in testing samples of milk from individual cows, although studies sponsored by the US FDA are in progress. The high number of false positives is a concern for the dairy industry since they can lead to unwarranted waste of milk, can lead to the untimely slaughter of a potentially valuable dairy animal, and can result in a poor domestic and international perception of the US dairy industry by general and industrial consumers. No diagnostic procedure is infallible. Each procedure entails a probability of incorrectly identifying a negative sample as positive, however, the procedures that perform to specifications under the most stressful conditions are the most useful.

A rapid, inexpensive, single-assay method for testing milk samples, for residues of all six β-lactam antibiotics, that requires minimal manipulation, produces semi-quantitative and permanent results, and does not produce false positives would be very useful to the dairy industry. This paper describes preliminary studies aimed at developing an assay that fulfills these requirements and addresses the problems mentioned above. The assay, known as solid-phase fluorescence immunoassay (SPFIA) takes advantage of the specificity of antibody-antigen recognition, is conducted in a glass capillary tube and incorporates the use of a fluorescent label, Cy-5™, for detection.

Figure 1 diagrams the principle of the SPFIA. A milk sample is mixed with a known amount of a fluorescently-labeled antibody which is specific to a β-lactam antibiotic. An instantaneous binding reaction will occur between the antibody and the antibiotic in the milk sample. A solid support, the inside wall of a glass capillary tube in our format, with an antigen coating will then be exposed to the sample, the sample is removed, the capillary tube is washed, dried and examined with a fluorometer. Surfaces incubated with milk samples with high concentrations of an antibiotic will have fewer free antibody molecules to react with, thus yielding lower fluorescence signals, and surfaces exposed to milk samples with low concentrations of antibiotic

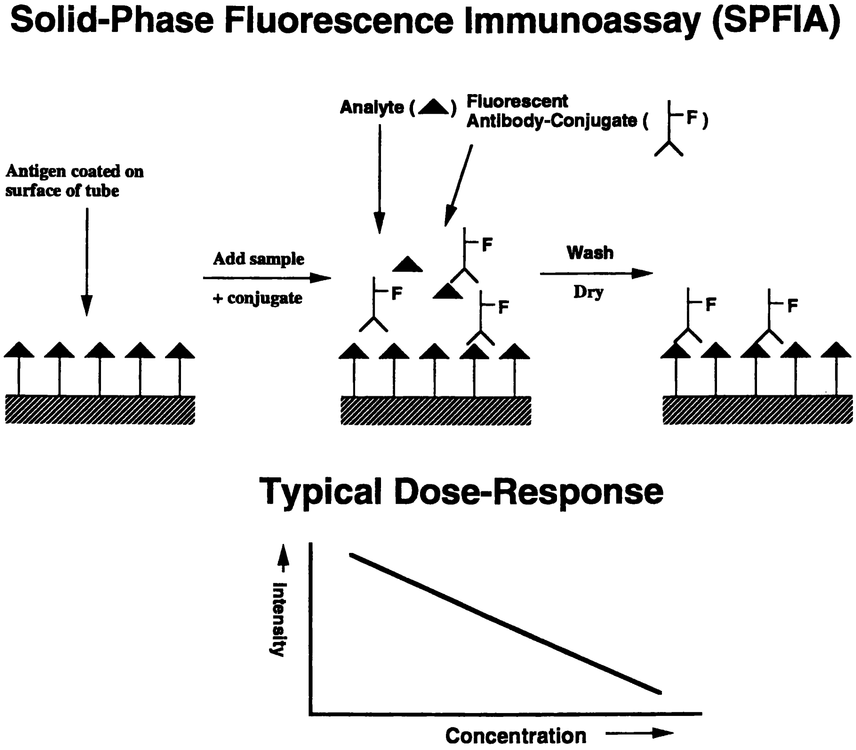
Solid-Phase Fluorescence Immunoassay (SPFIA)
Analyte (▲)
Fluorescent Antibody-Conjugate (⊢F)
Antigen coated on surface of tube
Add sample
+ conjugate
F
Wash
Dry
Typical Dose-Response
Intensity
Concentration

Figure 1. General Diagram of the Inhibition Assay. When the labeled antibody is added to the sample of milk, the free β-lactam drug in the sample will bind to a fractional number of antibody molecules. The concentration of unbound antibody will be inversely proportional to the amount of drug in the milk. When the solid-support is added to the sample, the remaining unbound antibody molecules are free to bind to the surface. The support is removed, washed, dried and examined by fluorimetry. The fluorescence intensity is inversely proportional to the initial amount of drug in the milk. Samples with low concentrations of drug will produce high fluorescence signals from the surface, and samples with high concentrations of drugs will produce low fluorescence signals from the surface. The plot at the bottom depicts and ideal, optimized dose-response. Compare this to the actual dose-responses observed in Figures 3 and 4.

will yield higher fluorescence signals. Mathematically, the measured fluorescence signal is inversely proportional to the concentration of analyte in the sample.

Experimental

Capillary-Tube Surface Preparation. The surface of borosilicate, glass capillary tubes (Drummond Scientific, Broomall, PA) was treated with a silanizing reagent using a proprietary technique. The lengths of individual tubes used in the assay were typically 2.5 cm to 6 cm. The inner diameter was 0.65 mm and the outer diameter was 1 mm. The use of capillary tubes for the assay, instead of the wells of microtiter-plates or test tubes, was implemented to achieve a high surface area to volume ratio and to minimize the use of reagents. The high surface area to volume ratio allowed for a short two-minute incubation. In addition, our proprietary surface chemistry is designed for use on silicon dioxide (glass) surfaces. Tubes having other inner diameter dimensions were also examined, however, the 0.65 mm inner diameter tubing was found to be most useful with fresh, raw milk samples, which often contain fat globules that can be as large as several hundred micrometers (*8*). Such fat particles could potentially clog the capillary channels if tubes of smaller inner diameter were used. One additional point should be noted regarding assays in small diameter tubes. The transport of reactants to the surface, where the measurable binding reaction occurs, is strictly through diffusion. Therefore, potential problems due to irreproducible agitation are eliminated.

Synthesis of Antigen Conjugates for Coating Capillaries. All of the antigen conjugates, for coating onto the surface of the capillary tubes, were prepared by binding the appropriate β-lactam drug to either bovine serum albumin (BSA) or a polypeptide copolymer consisting of lysine and alanine subunits (Sigma Chemical Co., St. Louis, MO). The covalent linking of antigen to carrier protein was accomplished through the use of conventional homobifunctional or heterobifunctional linkers, such as Succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), *Bis*(sulfosuccinimidyl) suberate, and 1-Ethyl-3-(3-Dimethylaminopropyl)-carbodiimide Hydrochloride (Pierce Chemical Co., Rockford, IL) (*9*).

Procedure for Coating Antigen-Conjugate on Surface of Capillary Tubes. After the silanizing surface treatment the capillary tubes were incubated for 30 min to 24 h in a buffered solution of the antigen-conjugate (20–40 μg/mL). The incubation temperature was usually 4–7 °C, although occasionally room temperature incubation was used. The capillary tubes were removed, washed with distilled water, dried in a stream of compressed air, and then incubated in a solution of bovine-serum albumin (0.1% BSA in PBS-phosphate buffered saline-pH = 7.2, 0.05% Proclin 300-a biocide) for 1 h at room temperature. The purpose of this second incubation was to block any regions of the surface that were bare. The tubes were again removed, washed with distilled water and dried in a stream of compressed air (20–30 psi). They were stored in the dark, at room temperature in a sealed, foil pouch with an indicating desiccant package. Maintaining dry and dark conditions were important to maintain the stability of the coated tubes.

Antibody Production. Antibodies to the antibiotics in the penicillin family were produced by immunizing goats with a conjugate of keyhole limpet hemocyanin (KLH) and ampicillin. Ampicillin was used since it has an amino-group available for conjugation. The resulting bleeds were screened for cross-reactivity with penicillin G, ampicillin, cloxacillin and amoxicillin. The cross-reactivity of this antibody with penicillin G and ampicillin was comparable, while the cross-reactivity for cloxacillin and amoxicillin was approximately 50% less. This antibody also exhibited less than 1% cross-reactivity with ceftiofur and cephapirin. Antibodies to ceftiofur were developed by immunizing goats with KLH-ceftiofur. A monoclonal to cephapirin was developed using conventional techniques with KLH-cephapirin as the conjugate. Enzyme immunoassay studies indicated that the cross-reactivity of the ceftiofur antibody to the other five β-lactam drugs of interest was less than 1%. The cross-reactivity of the cephapirin antibody to the other five β-lactam antibiotics was less than 0.1%.

Antibody Conjugates. Monoclonal and polyclonal antibodies were prepared using conventional purification techniques. Cy-5-antibody conjugates were prepared using the protocols previously published and provided by the manufacturer (Biological Detection Systems, Pittsburgh, PA) (*10*). The Cy-5 fluorophore, available with an NHS ester functionality, was linked to amino groups of the antibody. Purification and isolation of the conjugated dye was performed through chromatography. Spectroscopic examination indicated that the number of Cy-5 dye molecules that were bound to each antibody molecule was usually between two and four. The ratio of antibody molecule to Cy-5 molecule could be controlled by modifying reaction conditions such as time of conjugation and/or ratio of Cy-5 concentration to antibody concentration in the reaction mixture. Batch to batch studies indicated that there was no significant increase in fluorescence intensity with increase in the number of Cy-5 molecules per antibody molecule. In fact, the fluorescence intensity often decreased when the fluorophore/antibody ratio was greater than four. This phenomenon was especially apparent when using monoclonal antibodies. It is unclear at this time whether this decrease was due to increased quenching with high loading of the fluorophore or due to decreased antibody binding affinity resulting from inactivation of the antibodies' recognition sites.

Fluorescence Measurements. Fluorescence signal intensities from individual capillary tubes were measured using a fluorometer that was designed and built in-house. The excitation source was a 3 mW semiconductor-diode laser (λ_{max} = 635 nm, TOLD 9521 (s), Toshiba, Japan). The fluorescence signal from the capillary tubes was collected and filtered through appropriate optics and measured using the current response of a silicon p-i-n junction diode. The resulting photocurrent was amplified, converted from an analog to a digital signal and processed on a 386-PC compatible.

Assay Protocol. The assay was begun by combining a measured amount of antibody-Cy-5 conjugate with the sample in a small container such as the well of a microtiter-plate. The antibody reagent was typically lyophilized and used as such in the assays. The raw milk sample and antibody reagent were mixed on a vibratory shaker. After mixing the antibody reagent and the sample for 10 s, the solution was sipped into and incubated in the appropriate capillary tube for 2 min. Sipping was performed through the use of a manifold device such as a modified pipettor or a cartridge (*vide infra*). All reagents were then washed out of the tubes with flowing distilled water, and they were dried with a stream of compressed air (20–30 psi). The fluorescence intensity of the tubes was then measured. Homemade cartridges were prepared to conduct multiple assays at once. These cartridges consisted of a common manifold and support structure for capillary tubes. The cartridge facilitated the removal of used tubes and insertion of new tubes. All of these procedures were conducted manually. An automated system to perform the assay has been developed and will be discussed in another publication (Kumar, A., Idetek Corp. Sunnyvale, CA, in progress).

Results and Discussion

Figure 2 shows the chemical structures of the six β-lactam antibiotics of interest (*11*). The structures of those in the penicillin family (penicillin-G, ampicillin, cloxacillin, amoxicillin) are quite similar. Consequently, one polyclonal antibody cross-reacts with all four drugs in that family. The structures of cephapirin and ceftiofur are sufficiently different from each other and from the antibiotics of the penicillin family that individual antibodies are required for each. For the studies discussed in this report, a monoclonal antibody was used for cephapirin and a polyclonal antibody for ceftiofur.

Table I lists the US FDA mandated safe/tolerance levels for the six β-lactam drugs (*12*).

Table I. Safe/tolerance levels for β-lactam drugs in milk. (Data from ref. 12)

Drug	Safe/Tolerance Level (ppb)[a]
Penicillin G	5
Amoxicillin	10
Ampicillin	10
Cloxacillin	10
Cephapirin	20
Ceftiofur	50

[a] ppb = parts per billion, 1 ppb is equal to 1 ng/mL.

A tremendous analytical challenge is posed by the these requirements. Since all of the safe/tolerance levels are different, the most efficient method for analysis is to conduct independent assays specific for each analyte. Consequently, the use of individual antibodies for each antibiotic or antibiotic family is necessary. The use of individual antibodies for each drug or drug-family also allows for the independent optimization of each assay.

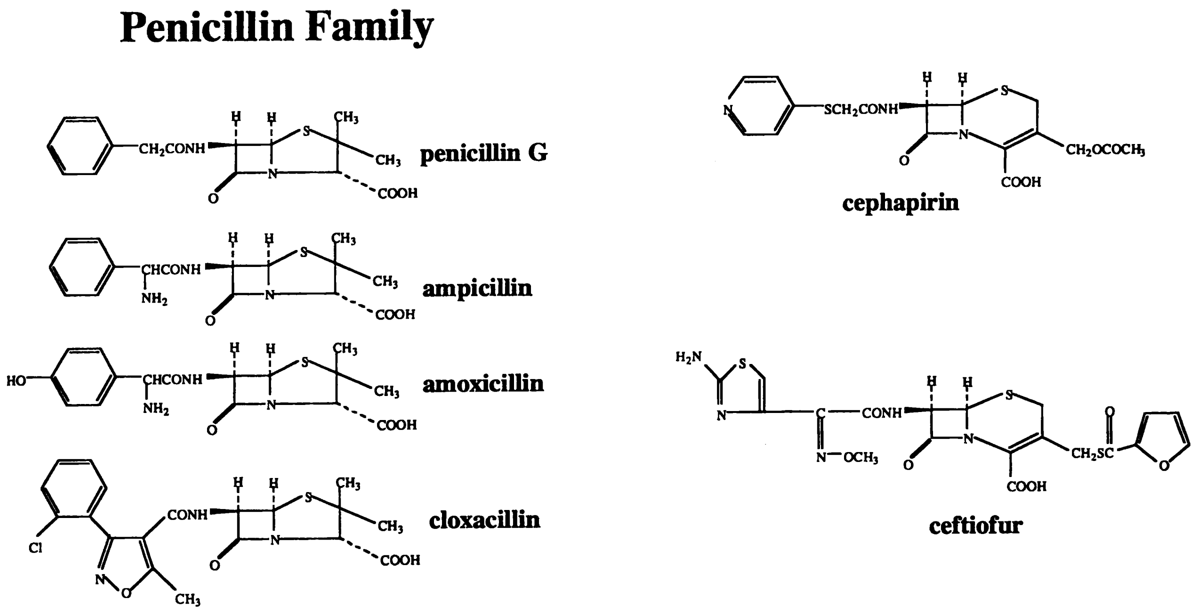
Penicillin Family
penicillin G
ampicillin
amoxicillin
cloxacillin
cephapirin
ceftiofur
CH₂CONH
CHCONH
NH₂
HO
CONH
Cl
CH₃
COOH
SCH₂CONH
CH₂OCOCH₃
H₂N
N—OCH₃
CH₂SC

Figure 2. Structures of β-Lactam Antibiotics. The structures of the penicillin family, are so similar that a single polyclonal antibody cross-reacts with each. The epitope is the β-lactam structure that is common to each. The affinity of the antibody to each is similar. Although, ceftiofur and cephapirin are in the β-lactam class, their structures are sufficiently different from the penicillin family and from each other to warrant the use of additional antibodies. An additional polyclonal antibody was used for ceftiofur and a monoclonal antibody was used for cephapirin.

Figures 3 and 4 display dose-response data for all six drugs of the β-lactam family that are regulated. Each point on the curve is an average of measurements made with between 30 and 120 individual samples of fresh, raw milk that were spiked with the appropriate drug concentration. These data were obtained after complete optimization of the assay protocol. The data depicted were also obtained in a blind study: one individual prepared the spiked samples and coded them randomly, while others ran the tests on the unknown samples. The data were collated after completion of the full study.

A few notable points can be made from the graphs of Figure 3 and 4. The goal was to optimize the assay so that the greatest response would be observed at or near the safe/tolerance level for the particular drug. This goal was achieved for each analyte: note that both axes are linear, not logarithmic. Normalized fluorescence values were calculated as the signal of interest divided by the signal for the sample containing no analyte, multiplied by 100%. The depicted error bars reflect one standard deviation, $\pm (\sigma/2)$.

The dose-response curves for all of the drugs in the penicillin family (Figure 3a-d) are similar, since the same antibody, which cross-reacts somewhat equally with each drug in the family, was used for all four drugs. The dose-response curve for ceftiofur begins to plateau for concentrations greater than 35 ppb (Figure 4b). The ceftiofur curve begins to lose sensitivity due to the nature of the antibody. However, this performance is still better than any other commercial assay system. The data indicate that coefficients of variation (CV = (signal/standard deviation) x 100) for each of the systems was less than 10% with few exceptions. In fact, the coefficients of variation of the cephapirin assay (Figure 4a), which uses a high affinity monoclonal antibody, was typically in the 1–2% range.

The data of Figures 3 and 4 are especially notable since all of the assays were conducted in fresh, raw milk. Milk is a complex mixture of fat, protein and numerous other constituents (*7,8*). Raw, unpasteurized and non-homogenized milk is especially complex(*7,8*), however, no dilution of the samples was required for excellent performance of these assays.

Figures 3 and 4 demonstrate the exceptional performance of this high sensitivity SPFIA. Although fluorescence assays are ubiquitous in the clinical diagnostics industry (*13*), solid-phase fluorescence formats have traditionally been difficult to develop (*14*). This difficulty is primarily due to a number of factors such as quenching of the fluorescent reagent when bound to the surface, background fluorescence from non-specifically adsorbed compounds from the sample matrix, background interference caused by scattering of the excitation light from a rough or porous surface, and others (*14*). We feel that through the combination of Cy-5, our proprietary surface chemistry on smooth glass surfaces, and high intensity laser excitation, many of the usual problems have been alleviated if not eliminated.

Cy-5 is a cyanine dye, that is provided by the manufacturer as a *N*-hydroxysuccinimide (NHS) ester (*10*). It has an absorbance maximum at 650 nm (extinction coefficient = 2×10^5 $M^{-1}cm^{-1}$), with an emission maximum at 667 nm (*10*). Such a small Stokes shift, 17 nm, requires a high degree of optimization of the filtering components on the measurement system. The quantum yield for Cy-5 in solution is 0.28 (*10*). Absorbance in the red region of the spectrum allows excitation using a helium–neon laser (He–Ne, λ = 632.8 nm) or some recently developed

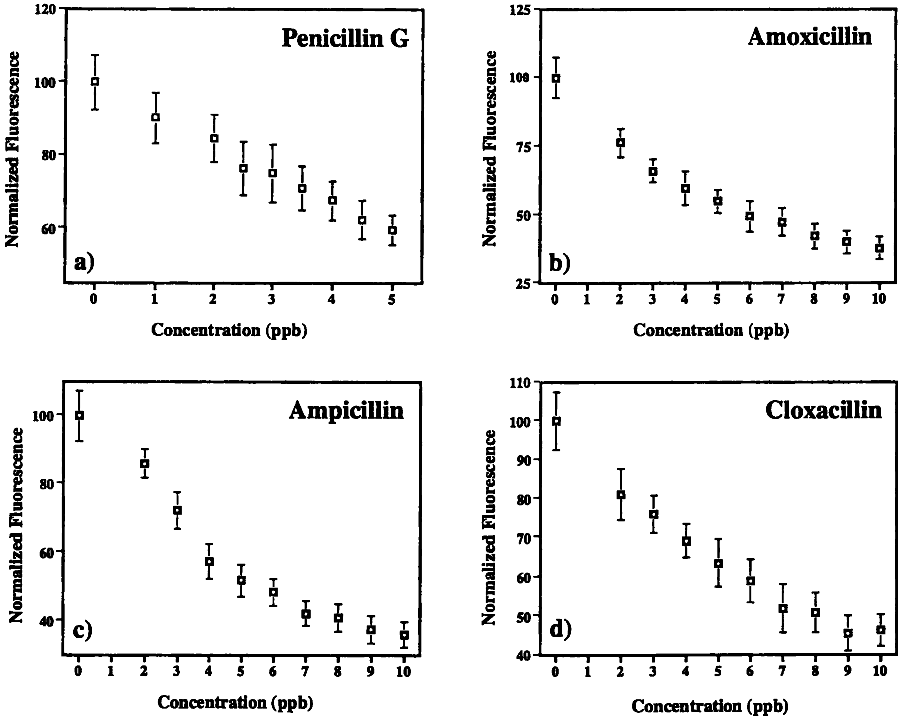

Figure 3. Dose-response data for penicillin family drugs. Note that all axes are linear. The error bars reflect one standard deviation. The normalized fluorescence was calculated by dividing the signal of interest by the signal for milk with no drug and multiplying by 100%. The response of all of the drugs is similar. Note that the assay for penicillin G was performed only to 5 ppb, since the USFDA safe/tolerance level is 5 ppb.

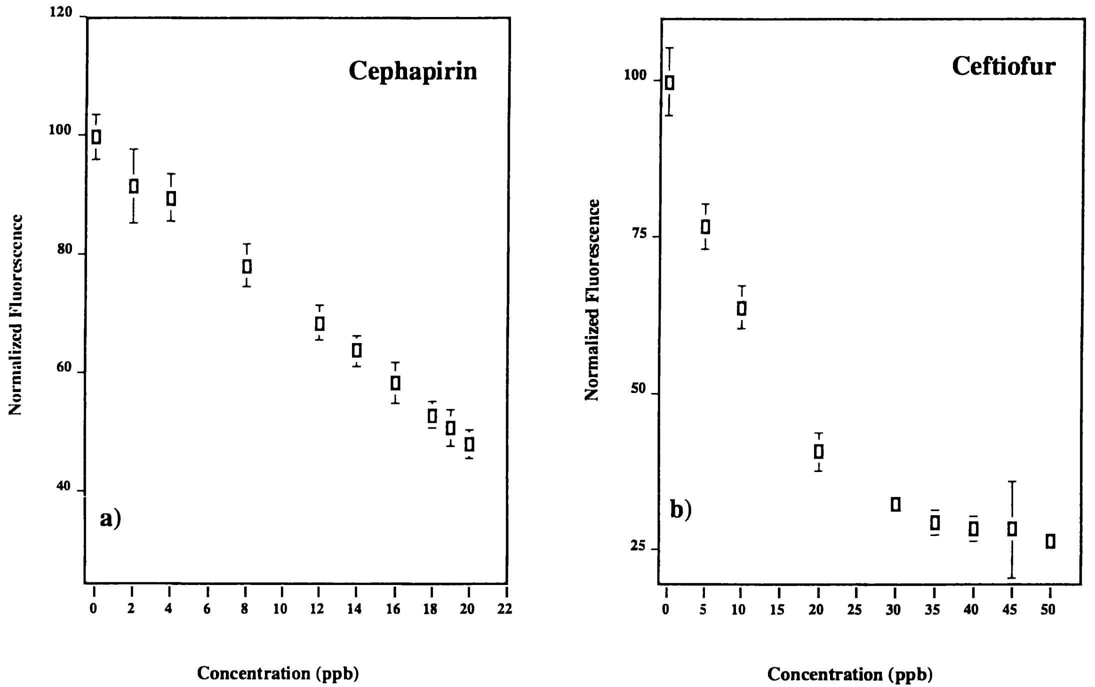

Figure 4. Dose-response data for Cephapirin and Ceftiofur. Note that all axes are linear. The error bars reflect one standard deviation. The normalized fluorescence was calculated by dividing the signal of interest by the signal for milk with no drug and multiplying by 100%. A monoclonal antibody was used for cephapirin and a polyclonal was used for ceftiofur.

semiconductor-diode lasers (630–650 nm). The optical properties of the dye make it useful for immunoassays, since very little background fluorescence is observed from biological fluids, such as milk, blood, and others in the spectral regions of interest (*10*). Although the quantum yield for the Cy-5-antibody complex bound to the surface is probably much less than 0.28, it is sufficient to provide the detection sensitivity required for the antibiotic assays.

In addition to providing assays for the β-lactam antibiotics, other potential assays can be conducted in the SPFIA format. To determine the applicability of this format to other systems, we conducted proof-of concept experiments for a number of analytes in addition to the β-lactam antibiotics, as shown in Table II.

Table II. Solid-phase fluorescence immunoassays (SPFIAs).

Analyte	Range of Detection	Total Assay Time (min.)
β-L Antibiotics		
Amoxicillin	1–10 ppb	3
Ampicillin	1–10 ppb	3
Cloxacillin	1–10 ppb	3
Penicillin G	1–5 ppb	3
Cephapirin	1–20 ppb	3
Ceftiofur	1–50 ppb	3
Other Antibiotics		
Tetracycline	1–30 ppb	3
Sulfamethazine	1–10 ppb	3
Hydrocarbons		
BTEX (benzene, toluene, ethyl-benzene, and xylenes)	1–10^3 ppb	3
Clinical Drugs		
Digoxin in Buffer	1–2 ppb	3
Pesticides/Herbicides	in progress	

For milk screening applications, the same format can be used to test for other antibiotics and contaminants. In addition, assays for hydrocarbon and pesticide wastes in water and soil, and assays for drugs of clinical significance should be possible. In principle, it should be possible to develop assays that can be used to screen any and all potential analytes for which an antibody of appropriate affinity exist. Certain matrices such as soil and solid food samples will require some sort of extraction protocol before conducting the assay.

The purpose of these demonstrations was not to develop optimized assays at this time, but to show the versatility of the system. In fact, most of the assays in Table II, except those for the β-lactam drugs, were not optimized at all.

One very important point to note has to do with the ability of this SPFIA to be formatted to operate multiple assays at once. Since each assay is conducted in its own capillary tube, a cartridge that allows the performance of several assays at once is possible. Such a system provides tremendous flexibility to the assay operator. Once specific assays have been optimized, they can be included in the cartridge in any combination desired. This type of commercial system will be discussed in another publication (Kumar, A., Idetek Inc. Sunnyvale, CA, in progress).

Conclusion

In addition to the versatility demonstrated by the number of analytes shown in Table II, there are numerous other factors that can make the SPFIA in capillary tubes even more versatile and superior. For example, the incubation time for all of the β-lactam assays was 2-min. This number was chosen due to market requirements. One of the requirements for an assay for antibiotics in milk is speed. The fastest current assay requires 7 min of time, therefore, our goal was to cut this time in half. With longer incubation times, several minutes or even hours, the assay would become correspondingly more sensitive and reproducible.

Also, the inner diameter of the capillary tube can control assay sensitivity. Since this assay was being developed for testing raw milk which can contain fat particles that can be several tenths of a millimeter in diameter, a relatively large inner diameter capillary tube was used (ID = 0.65 mm). For assays in matrices that do not have particulates that may plug the tube, smaller diameter tubes can be used. Smaller diameter tubes will produce tremendous increases in surface area to volume ratios resulting in faster and more sensitive assays.

In conclusion, the SPFIA discussed in this paper should provide for better and more efficient screening for β-lactam antibiotics in milk. It also is apparent that this assay format will support additional tests in many different areas, and the potential exists to improve the performance of this assay by controlling factors such as the geometries of tubes and incubation times.

Acknowledgments

Funding from the Small Business Innovation Research (SBIR) is acknowledged. Phase I grant # 1 R43 HD32266-01.

Literature Cited

1. Mitchell, M. J.; Yee, A. J. *Dairy, Food, Env. Sanit.* **1995**, *15*, 484–487.
2. Jones, G. M.; Seymour, E. H. *J. Dairy Sci.* **1988**, *71*, 1691–1699.
3. Brady, M. S.; Katz, S. E. *J. Food Prot.* **1988**, *51*, 8–11.
4. Gilbertson, T. J.; Mejeur, R. L.; Yein, F. S.; Jagian, P. S. *J. Dairy Sci.* **1995**, *78*, 1032–1038.

5. Tyler, J. W.; Cullor, J. S.; Erskine, R. J.; Smith, W. L.; Dellinger, J.; McClure, K. *J. Am. Vet. Med. Assoc.* **1992**, *201*, 1378–1384.
6. Van Eenennaam, A.; Cullor, J. S.; Perani, L. Gardner, I. A.; Smith, W.L.; Dellinger, J.; Guterbock, W. M.; Jensen, L. *J. Dairy Sci.* **1993**, *76*, 3041–3053.
7. Cullor, J. S. *Vet. Med.* **1992**, *87*, 1235–1242.
8. *Protein and Fat Globule Modifications by Heat Treatment, Homogenization and Other Technological Means for High Quality Dairy Products*; International Dairy Federation: Brussels, Belgium, 1993.
9. *Immunotechnology Handbook*; Pierce Chemical Co., Rockford, IL, 1992/3.
10. Mujumdar, R. B.; Ernst, L. A.; Mujumdar, R. S.; Lewis, C. J.; Waggoner, A. S. *Bioconjugate Chem.* **1994**, *4*, 105–111.
11. *The Merck Index, An Encyclopedia of Chemicals, Drugs and Biologicals;* Budavari, S., ed.; Merck & Co.: Rahway, NJ, 1989.
12. United States Code of Federal Regulations (CFR); Title 21, Part 556, April 1993; Grade "A" Pasteurized Milk Ordinance, 1993 Revision; U.S. Department of Health and Human Services, Public Health Service, Food and Drug Administration.
13. Hemmila, I. A. *Applications of Fluorescence in Immunoassays*; John Wiley and Sons: New York, NY, 1991.
14. Glad, C. *Appl. Biochem. Biotechnol.* **1982**, *7*, 75–89.

RECEIVED October 11, 1995

Chapter 37

Analysis of Sulfamethazine in Milk by an Immunosensor Assay Based on Surface Plasmon Resonance

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A recently developed immunosensor assay was applied in the analysis of sulfamethazine residues in milk from individual cows, herds and car tankers. Previously frozen milk samples were defatted by centrifugation whereas fresh milk required no sample preparations. The average relative standard deviation and the limit of detection of the assay were less than 2% and 1 µg/kg, respectively. The correlation with a conventional HPLC method was found to be high in analysis of incurred samples from a sulfamethazine-treated cow. In addition, the method was used to analyse 330 tanker milk samples, of which one was found to contain sulfamethazine at the low ppb level by both the immunosensor assay and HPLC.

Residues of antibiotics and chemotherapeutics occur in all types of food of animal origin as a consequence of their widespread use in animal husbandry. To comply with the dairy farmers wish for profitability, the veterinarian often resorts to antimicrobial substances to maintain or restore good health of an animal. In many infections the response to therapy is poor, and new, improved antimicrobial products are continuously introduced for treatment of resistant organisms. Meanwhile, public concern over food safety has never been stronger and consumers are increasingly aware of the health consequences of the food they eat.

Although most industrial countries already have control systems for antimicrobials in milk, higher standards of quality assessment are being introduced. In the European Community (EC), Commission Regulations No. 675/92, 3093/93 and 3426/93 (*1–3*) indicate maximum residue levels (MRLs) for a variety of pharmacologically active substances in animal derived foods. The MRLs that concern antibiotic substances in milk are shown in Table I.

There is still no common control system for residues in milk in the EC, but an integrated system, where two levels of control can be distinguished, has been discussed. For screening of farm milk samples at milk grading laboratories, microbial

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inhibitor assays are likely to remain the methods of choice. Positive results may be confirmed to various degrees, and for detection of certain substances more specific methods can be applied. For a number of substances, however, the methods currently in use have too low sensitivities to comply with new regulations. The inhibitor control is therefore insufficient and a complementary control, likely to be performed by national food authorities, will be required.

Table I. Maximum Residue Levels (MRLs) of Antibiotic Substances in Milk

Pharmacologically active substance	*MRL µg/kg*	*Status*
Antibiotics		
Penicillins		
Benzyl penicillin	4	Final
Ampicillin	4	Final
Amoxicillin	4	Final
Oxacillin	30	Final
Cloxacillin	30	Final
Dicloxacillin	30	Final
Tetracyclines[a]	100	Temporary until January 1996
Macrolides		
Spiramycin	150	Temporary until July 1995
Tylosin	50	Temporary until July 1995
Chemotherapeutics		
Sulfonamides[a]	100	Temporary until January 1996
Diaminopyrimidine derivate		
Trimethoprim	50	Temporary until January 1996
Other chemotherapeutics		
Dapson	–	Use of prohibited

[a] Sum of all substances belonging to the family

In the development of effective, future control strategies, fast screening procedures suitable for automation are of great importance. For many years biosensors have been applied to a range of biological systems. The technology has, however, been slow to penetrate the area of food analysis. Due to recent advances in both biotechnology and electronics, the development of biosensors that enable direct measurement of chemical components has accelerated (*5*). This paper describes the development of an immunosensor assay for detection of sulfamethazine (SMZ), a sulpha drug used in dairy cows.

Classification of Biosensors

A biosensor may generally be defined as an analytical device composed of two parts: a biological or biologically-derived element that detects the analyte integrated with a physicochemical element (transducer) which converts this detection event into an electronic signal. The biological component usually consists either of a biocatalyst or a bioreceptor. For biocatalysts, e.g. enzymes, recognition and binding of a substance is followed by a chemical reaction (*6*). Bioreceptors, e.g. antibodies, differ in that the binding is non-catalytic and essentially irreversible. Based on the mode of signal transduction, almost all biosensors fall into four categories, i.e. optical, mass, electrical and thermal (*5*). The merits of each system depend on the application, and have to be evaluated in comparison with other techniques currently on the market. Compared with biocatalyst sensors, bioreceptor sensors were slower to find acceptance (*6*), possibly because of the initial limited availability of receptor molecules and difficulties in monitoring binding events. Today, the detection of substances such as hormones and drugs can be achieved at very low concentrations and the development of suitable monitoring methods for immunosensors has proceeded rapidly. Optical biosensors are receiving considerable attention and are said to hold considerable potential for on-line quality and safety monitoring in the food industry. Surface plasmon resonance (SPR) is the principle for detection in many optical biosensors, such as the BIAcore®, an immunosensor instrument developed by Pharmacia Biosensor AB, Uppsala, Sweden. This paper describes the application of BIA (Biospecific Interaction Analysis) in real-time measurements of SMZ in milk.

Surface Plasmon Resonance Detection

At an interface between two transparent media of different refractive index, e.g. glass and water, light coming from the side of the higher refractive index will be partly reflected and partly refracted. Above a specific angle of incidence no light will be refracted and total internal reflection is observed. Although the light is totally reflected, an electromagnetic field component of the light called the evanescent wave will penetrate into the medium with the lower refractive index. If the interface between the two media is coated with a thin metal film and the light is monochromatic and plane polarised, the evanescent wave will interact with free oscillating electrons, plasmons, in the metal film surface. Light energy will thereby be lost to the metal film and the reflected light intensity will decrease. This phenomenon is called surface plasmon resonance (SPR) and occurs only at a sharply defined angle of incidence. The SPR angle is dependent on the refractive index in the close vicinity of the surface and the refractive index is, in turn, a linear function of the mass concentration. When a macromolecule binds to the surface, the mass, and thereby the refractive index on the sensor surface, changes, causing a shift in the SPR angle that can be used for biosensing purposes (*7*). By continuously monitoring the SPR angle, expressed as resonance units (RU) by the BIAcore software, and plotting the value against time, a sensorgram is obtained.

Sensor Surface

The sensor chip consists of a glass substrate on to which a thin gold film has been deposited. The gold film is covered with a carboxymethylated dextran hydrogel covalently attached through a linker layer (*8*). This dextrangel provides a hydrophilic environment suitable for studies of biospecific interactions and enhances the immobilisation capacity of the surface. There are four independent flow cells on each sensor chip surface, thereby enabling four different measurements to be performed, using different immobilised ligands if desired.

Assay Principle

To observe biomolecular interactions in the BIAcore instrument, one interaction partner is immobilised on a sensor chip and the other is injected over the sensor surface. The detector response is proportional to the mass of bound analyte, and for macromolecular analytes the response arising from the analyte can be measured directly. For detection of low molecular weight components, e.g. antibiotic substances, a different approach is required. In our assay, SMZ is immobilised to the sensor surface (*9*). Polyclonal anti-SMZ antibodies are allowed to react with free antigen in the milk sample. The milk sample (35 µL) is then injected over the sensor surface and free antibodies bind to immobilised SMZ and produce a signal which is in inverse proportion to the concentration of free antigen. After each measurement, the surface is regenerated by injection of dilute NaOH and HCl.

Determination of Sulfamethazine in Milk

To evaluate the above-described assay, SMZ has been analysed in spiked milk samples and samples from a SMZ treated cow. The method also has been used to analyse different categories of milk samples, i.e. samples from individual cows, herds and tankers, to study variations in response due to variations in milk composition. within In addition, 330 tanker milk samples from the Schleswig-Holstein area in Germany were screened for SMZ residues by the biosensor assay.

The precision and limit of detection (LOD) of the assay was determined by analysis of SMZ standards in both raw and defatted milk. Standard curves were constructed by analysis of samples spiked with known concentrations of SMZ. Using the recommended value of 3 standard deviations, the LOD was graphically determined to be 0.3–0.4 ppb (Figure 1). The average relative standard deviation in skim and raw milk were 1.9% and 3.4%, respectively. The relative standard deviations between defatted milk samples from individual cows (n=24), herds (n=39) and tankers (n=40), were 4.4%, 2.4% and 2.2%, respectively.

Milk samples from a SMZ treated cow (Selectavet sulfadimidine 33%) were collected up to nine days after the last injection (Institute for Hygiene, Federal Dairy Research Centre, Kiel, Germany). For a validation of the BIA assay, the milk samples also were analysed by liquid chromatography (*10*). The high correlation between the two methods is illustrated in Figure 2.

Because SMZ is no longer registered for use in lactating cows in Sweden, tanker milk samples were randomly collected in Schleswig-Holstein (Institute for Hygiene, Federal Dairy Research Centre, Kiel, Germany), frozen and sent to Uppsala for analysis by the biosensor assay. In total 330 samples were analysed, of which one sample was positive. The sample was returned to Kiel, where HPLC analysis confirmed the presence of very low concentrations of SMZ (0.9-2.1 ppb). Figure 3 shows the typical response for a limited number of analyzed samples.

Discussion and Conclusion

The aim of the present study was to investigate the applicability of BIA in determination of veterinary drug residues in milk. The developed assay detects SMZ

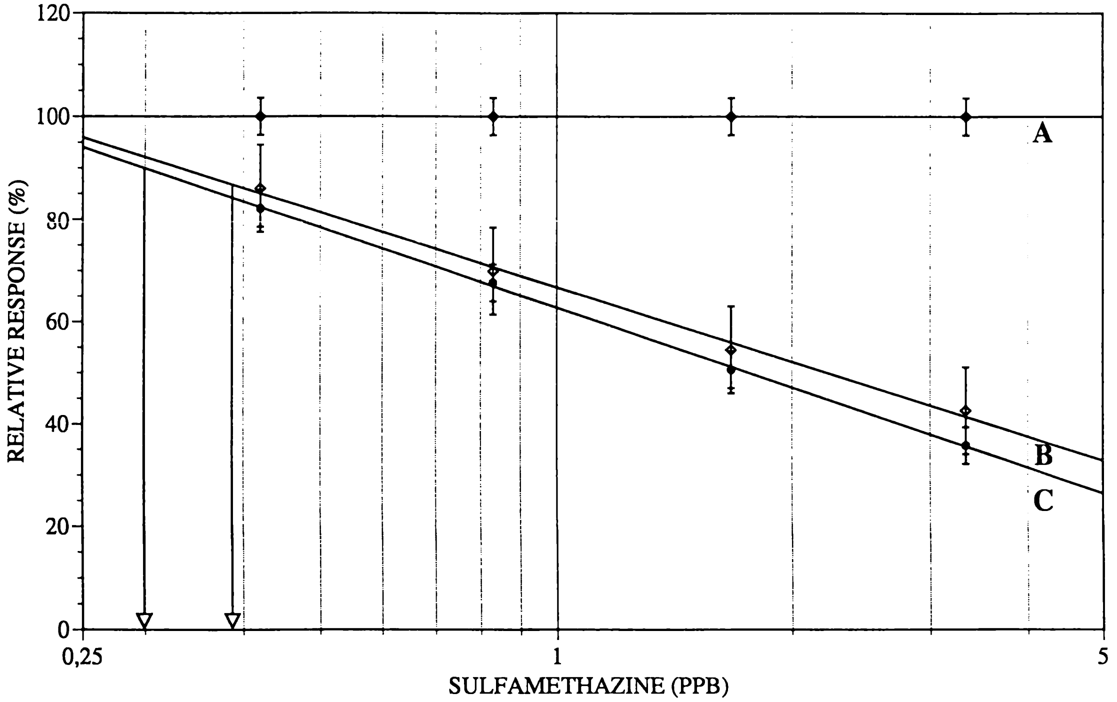

Figure 1. Standard curve for SMZ in raw and defatted milk. The response is expressed as percentage of the response of a negative control sample and the error bars indicate three average standard deviations (n=10). A= negative control milk (defatted), B= positive control milk (raw), C= positive control milk (defatted). The arrows indicate the limit of detection (LOD) in raw and defatted milk respectively. Reproduced with permission from reference 9.

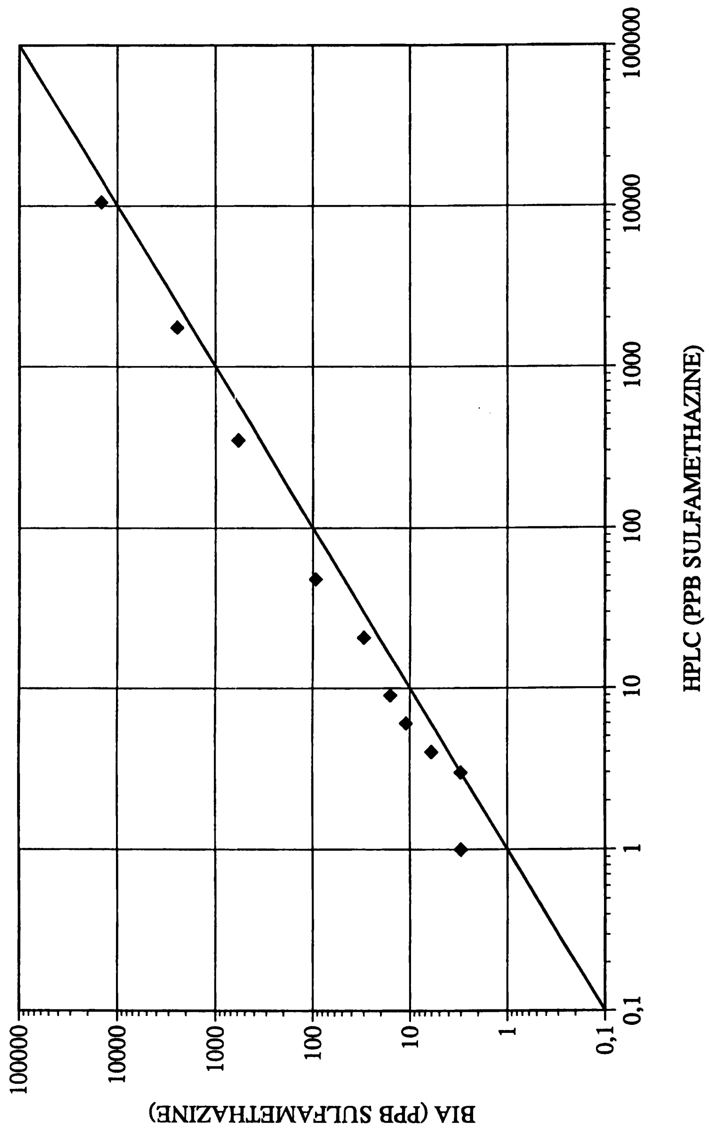

Figure 2. Correlation between results in HPLC and BIA in the analysis of incurred milk samples from a sulfamethazine-treated cow.

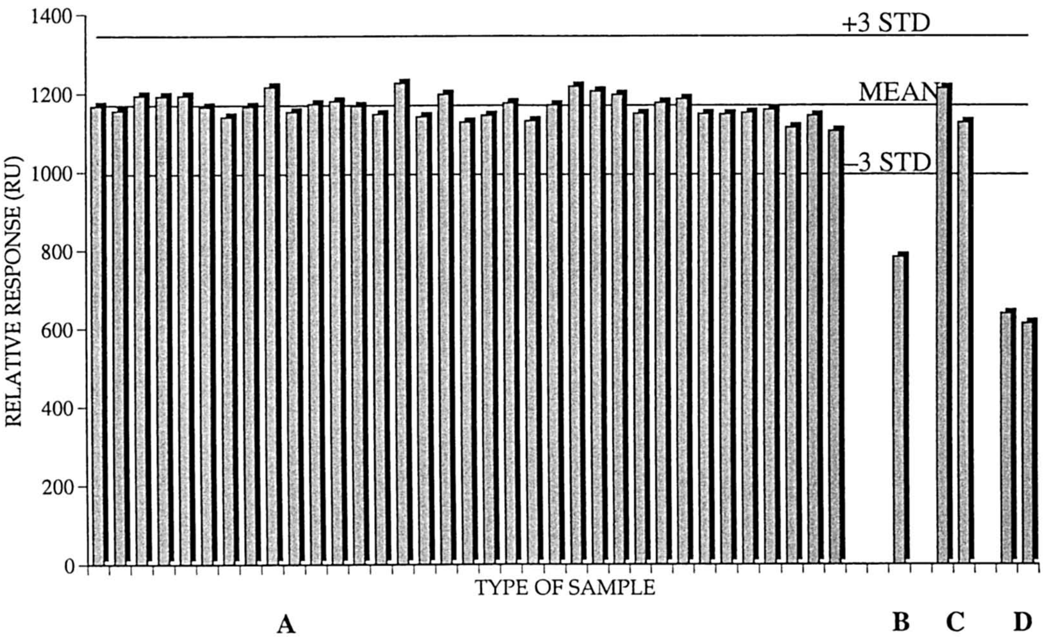

Figure 3. Typical response in the analysis of tanker milk samples from Schleswig-Holstein. Included in the graph are two positive (1.4 ppb SMZ) and two negative (0 ppb SMZ) control milk samples as well as one incurred tanker milk sample. A= negative tanker milk samples, B= incurred tanker milk sample, C= negative control milk samples, D= positive control milk samples.

concentrations of less than 1 ppb in raw milk with a high repeatability on the individual cow, herd and tanker milk levels. Frozen and thawed milk samples were centrifuged to remove the milk fat in order to avoid the risk that it would interfere with the assay. The small sample volume required, and the possibility to perform the analysis of fresh milk without any preparative steps, makes the technique most promising for future milk quality control. In addition, the assay has been used to analyze SMZ in other matrixes than milk, i.e. pork and beef. Preliminary results indicate the potential of the assay, once the extraction procedures have been optimised.

It is foreseen that assays for other veterinary drug residues can be developed in analogy with the BIA assay presented here. At present, a method for determination of enrofloxacin, a quinolone derivate used for treatment of Gram negative pathogens in mastitis therapy in Sweden, is under evaluation. This assay has approximately the same sensitivity as the SMZ assay, and requires no preparation of the milk sample. There also is great interest in looking at other types of substances, e.g. hormones and viruses. Because the method is based on general principles, provided that specific antibodies or other receptors are available, BIA technology may be applied in determination of a number of foreign substances of interest in food.

Acknowledgments

The authors would like to thank everyone involved in the project at the Pharmacia Biosensor AB, Uppsala, Sweden, for their support, and Drs. Gertraud Suhren and Philipp Hammer, Institute for Hygiene, Federal Dairy Research Centre, Kiel, Germany, for collaboration and for supplying antibodies. The Swedish Dairies Association is gratefully acknowledged for providing financial support.

Literature cited

1. Commission Regulation (EEC) No 675/92. *Off. J. Eur. Communities* **1992**, L73/8 of 19 March 1992.
2. Commission Regulation (EEC) No 3093/92. *Off. J. Eur. Communities* **1992**, L311/18 of 28 October 1992.
3. Commission Regulation (EEC) No 3426/93. Off. *J. Eur. Communities* **1993**, L312/15 of 15 December 1993.
4. Suhren, G.; Hammer, P.; Heeschen, W. Kiel. *Milchwirtsch. Forschungsber.* **1994**, 46, 237–248.
5. Deshpande, S. S.; Rocco, R. M. *Food Technol.* **1994**, 48, 146–150.
6. Brooks, S. L.; Turner, A. P. F. *Meas. Control* **1987**, 20, 37–43.
7. Liedberg, B.; Nylander, C.; Lundström, I. *Sens. Actuators* **1983**, 4, 299–304.
8. Löfås, S.; Johnsson, B. *J. Chem. Soc. Chem. Commun.* **1990**, 21, 1526–1528.
9. Sternesjö, Å.; Mellgren, C.; Björck, L. *Anal. Biochem.* **1995**, 175–181.
10. Suhren, G.; Heeschen, W. *Anal. Chim. Acta* **1993**, 275, 329–333.

RECEIVED October 11, 1995

Chapter 38

Recombinant Antibodies for Pesticide Immunoanalysis

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Standardized immunochemical methods for food analysis and environmental monitoring have profited from the use of monoclonal antibodies. However, this approach is restricted by the length of time required for antibody production to new analytes. The recombinant antibody technology is expected to compensate for these limitations. After a discussion of recombinant antibody state of the art, two approaches are shown based on the herbicidal *s*-triazines. Single-chain Fvs directed against different *s*-triazines were expressed as fusion proteins on the surface of M13 phages. In addition, a recombinant Fab for atrazine was produced in the pASK85-based expression system, which exhibited similar binding and displacement kinetics as the original monoclonal antibodies. The feasibility to obtain mutant antibodies may eventually replace the circumstantial approach to obtain new antibodies by new immunizations.

Immunochemical analysis has become an important tool for the detection of toxicants and pesticides in foods, water and other environmental samples. A major factor has been the use of hybridoma technology. The ability to produce monoclonal antibodies (Mab) means that techniques are available for the unlimited production of antibodies of the same isotype exhibiting constant properties. Although this technique is expected to remain in the future, a technology revolution has taken place during the last few years, which utilizes bacteria to display a diverse library of antibodies. This approach is equivalent to the mammalian immune repertoire and thereby avoids time-consuming immunizations and the use of live animals. Novel protein engineering strategies are used that rely on bacterial expression systems rather than mice for the rapid selection of antibodies. This topic has been reviewed intensively, e.g. by Winter and Milstein (*1*) and Irving and Hudson (*2*).

A comparison of the hybridoma and the recombinant approach shows interesting parallels. Both strategies use immortilization processes as central methods. The

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hybridoma method is based on the immortilization of B-lymphocytes, the antibody producing cells, whereas the recombinant antibody (Rab) approach relies on the immortilization of antibody genes. Both strategies require screening processes, although they are carried out at different levels during the production of antibodies.

The recombinant antibody approach

The basic technology of Rab production has been developed in the medical field and is now mainly targeting medical areas including cancer and HIV research. The principle of Rab production in bacteria was pioneered in 1988 by Better *et al.* (*3*) and Skerra and Plückthun (*4*). Further improvements were introduced by the groups of Winter (*5*), Lerner (*6*), Borrebaeck (*7*), Plückthun (*8*) and Skerra (*9*). These improvements included the isolation of light and heavy chain encoding DNA sequences by the polymerase chain reaction (PCR) technique, better screening and purification procedures, as well as the modification of Rab. A broadening of the field of Rab was observed during the last few years. It resulted in refined strategies for cloning and expression of antibody genes. Table I summarizes some of the important steps of this development.

Figure 1 (right part) gives a scheme for the production of Rab. It outlines a few general steps shared by different protocols. First, mRNA is isolated from hybridomas or B-lymphocytes. Following the synthesis of the complementary DNA-strand (cDNA), the desired immunoglobulin sequences are amplified by the PCR, which represents a central element in Rab production. Since one PCR cycle duplicates the included DNA sequence, up to 2^{25}–2^{35} copies are obtained after 25–35 repetitions of the temperature regime. The primer mixtures, deduced from data bases (*14*), facilitate the synthesis of any antibody encoding sequence without requiring knowledge of the specific nucleotide sequence. The primers are designed to hybridize with conserved frame regions of the variable domains and the constant domains, respectively, due to species-specific sequence homology. After insertion of restriction sites at the 3'- and 5'-end, the PCR amplificates are ligated with an appropriate vector and introduced in the host culture, most commonly *E. coli*. Each individual transformed bacterium containing an antibody encoding moiety propagates the foreign DNA by replication and transfers it to its descendants by cell division. These represent a recombinant bacterial clone.

If a hybridoma cell line is used as starting material, the bacteria exclusively contain the sequence of the corresponding Mab. However, if B-lymphocytes (which represent a heterogeneous cell population) are used as the source for the mRNA, the whole repertoire of antibody encoding DNA sequences is distributed to different clones and thus constitute a gene library. Subsequently, the individual heavy and light chain fragments can associate by random combination forming heterodimers that are expressed as functional Rab. These can be purified from culture supernatants or cell lysates.

Recombinant antibodies for environmental analysis

A few groups are already involved in Rab synthesis for pesticide and toxicant analysis, among them the laboratories of Hammock, Karu, and Stanker (See chapter by

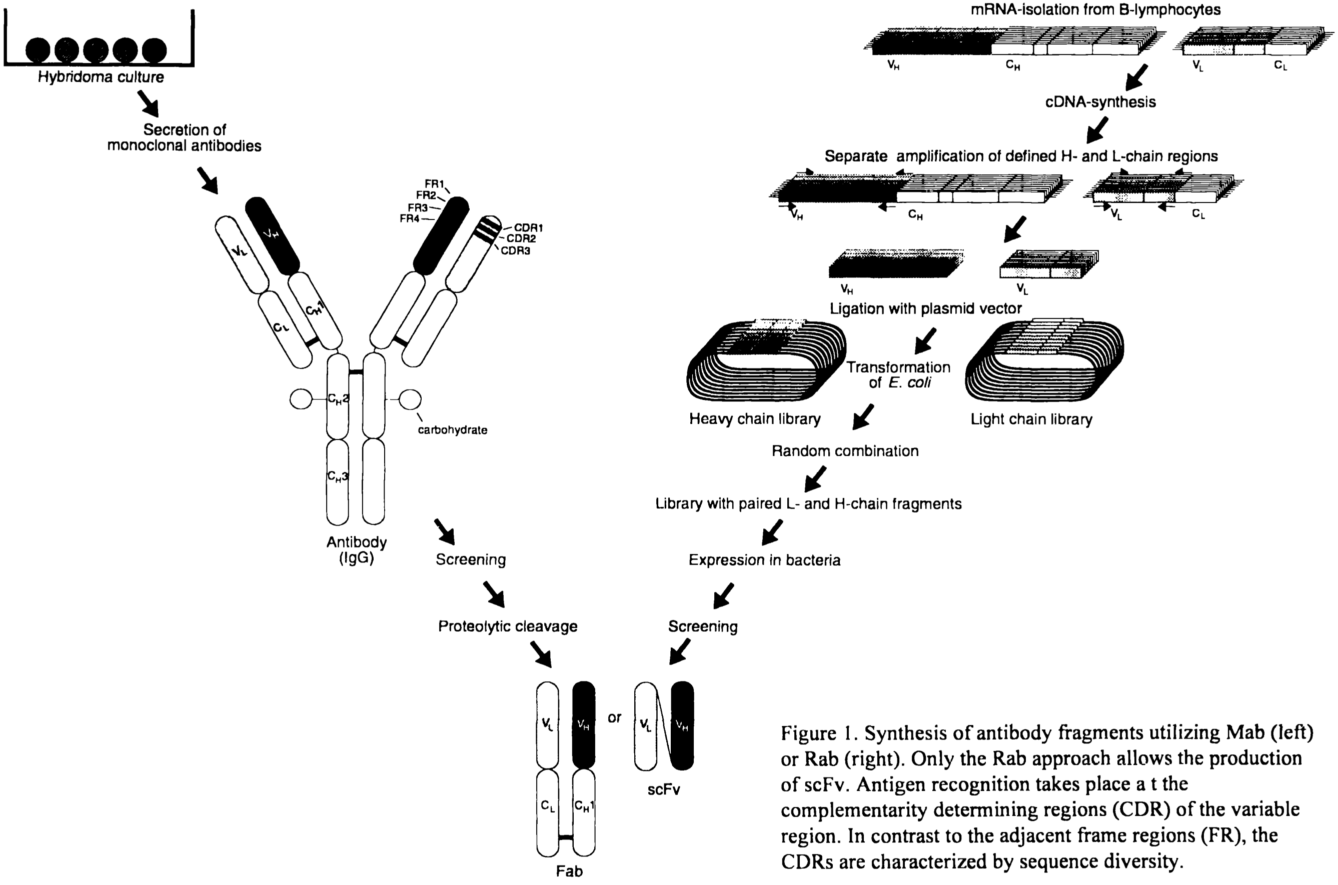
Hybridoma culture
Secretion of monoclonal antibodies
FR1
FR2
FR3
FR4
CDR1
CDR2
CDR3
V_L
V_H
C_L
C_H1
C_H2
C_H3
carbohydrate
Antibody (IgG)
Screening
Proteolytic cleavage
Fab
or
scFv
mRNA-isolation from B-lymphocytes
V_H
C_H
V_L
C_L
cDNA-synthesis
Separate amplification of defined H- and L-chain regions
Ligation with plasmid vector
Transformation of E. coli
Heavy chain library
Light chain library
Random combination
Library with paired L- and H-chain fragments
Expression in bacteria
Screening

Figure 1. Synthesis of antibody fragments utilizing Mab (left) or Rab (right). Only the Rab approach allows the production of scFv. Antigen recognition takes place a t the complementarity determining regions (CDR) of the variable region. In contrast to the adjacent frame regions (FR), the CDRs are characterized by sequence diversity.

Table I: Landmarks in recombinant antibody technology.

1984	chimeric antibodies	Neuberger *et al.* (*10*)
1986	humanized antibodies	Jones *et al.* (*11*)
1988	Rab in bacteria	Better *et al.* (*3*)
1988	Fv-fragments in bacteria	Skerra and Plückthun (*4*)
1992	combinatorial library	Barbas *et al.* (*12*)
1992	affinity maturation by chain shuffling	Marks *et al.* (*13*)

Kamps-Holtzapple and Stanker, this volume) in the USA, Harris in Scotland, Morgan in England and our group in Germany (Table II). It can be foreseen that Rab will become a common tool in environmental monitoring.

Hammock's group is focused on Rab expression in baculovirus. This eukaryotic expression system is well suited for the synthesis of larger proteins, e.g. complete antibodies as they are secreted by B-lymphocytes. In contrast to prokaryotic expression in bacteria, the baculovirus system enables the glycosylation of folded antibodies as it occurs in mammalians. This goal was achieved by starting with the synthesis of the appropriate PCR primers for the isolation of antibody DNA sequences and the development of a suitable expression vector (*15*). This part was restricted to *E. coli*, because of the vast knowledge available for Rab synthesis in this expression system. On the basis of pGEM5fZ(-) (Promega Corp.) the plasmid vector pGEMEB was constructed to allow separate cloning of H- and L-chain PCR amplificates derived from the hybridoma cell line AM7B2.1 (*20*). AM7B2.1 secretes Mab against the herbicide atrazine. In order to express the Rab derived from this Mab, the positive H- and L-chain clones were subcloned into the expression vector pET3d (*21*) to form a functional Fab fragment after the induction of bacteria. Although the light chain exhibits sequence deviations from the original Mab AM7B2.1, the recombinant Fab binds to an atrazine-phosphatase conjugate used as a tracer in a dot blot assay in like manner to the original antibody. Most importantly, binding did not take place to the recombinant Fab if the antibodies were desensitized by atrazine.

The screening used in this approach is based on the classical white/blue selection, which depends on a substrate turnover initiated by transformed bacterial clones. However, if screening of libraries with a large repertoire of different Rab is intended, this screening method is limited. Therefore, more efficient selection strategies were developed in recent years, most notably the phage display of Rab (*22–24*). This system allows the discrimination of desired Rab clones at an early state of production (Figure 2). Amplified antibody sequences are inserted into a phage or a phagemid vector. The vector includes the gene coding for the major (g8p) or minor (g3p) phage coat protein, which is placed after the Rab sequence. Phages bearing Rab sequences display the corresponding Rab as a fusion product with the coat protein on their surface. This enables the selection of desired clones in panning devices with immobilized target molecules. The primary advantage of the phage display system consists in the idea that the affinity and specificity of the immunoreactants is the dominating selection criterion.

Karu (*16*) utilized a phage display system developed by Lerner to express recombinant Fab fragments against the phenylurea herbicide diuron. The Fab encoding sequences were isolated from hybridoma cells and subsequently amplified by PCR. H- and L-chains were combined in the phagemid vector pComb3. After the transformation of *E. coli* and infection by helper phages, complete phage particles were synthesized presenting specific Fab fragments on their surfaces. These positive phages were enriched by diuron-BSA conjugates immobilized to a solid phase. After four rounds of selection, desorption of bound phages at pH 2.2 and reinfection of bacteria, four out of several hundred enriched clones reacted specifically with an alkaline phosphatase-labeled diuron tracer. Soluble Fab were derived from these positive phages by excising the vector sequence, which codes for the phage coat

Table II. Recombinant antibodies in pesticide and toxicant analysis.

1993	recombinant atrazine Fab	Ward *et al.* (*15*)
1994	recombinant diuron Fab	Karu *et al.* (*16*)
1994	scFv against organic pollutants	Shelton *et al.* (*17*)
1994	scFv against aflatoxin M_1, diacetoxyscirpenol, and parathion	Morgan [a]
1995	recombinant scFv for s-triazines	Hock *et al.* (*18*)
1995	recombinant scFv for glycoalkaloids	Kamps-Holtzapple and Stanker (*19*)

[a] (Morgan, R. A. M., Norwich Laboratory, Norwich, UK, personal communication, 1994)

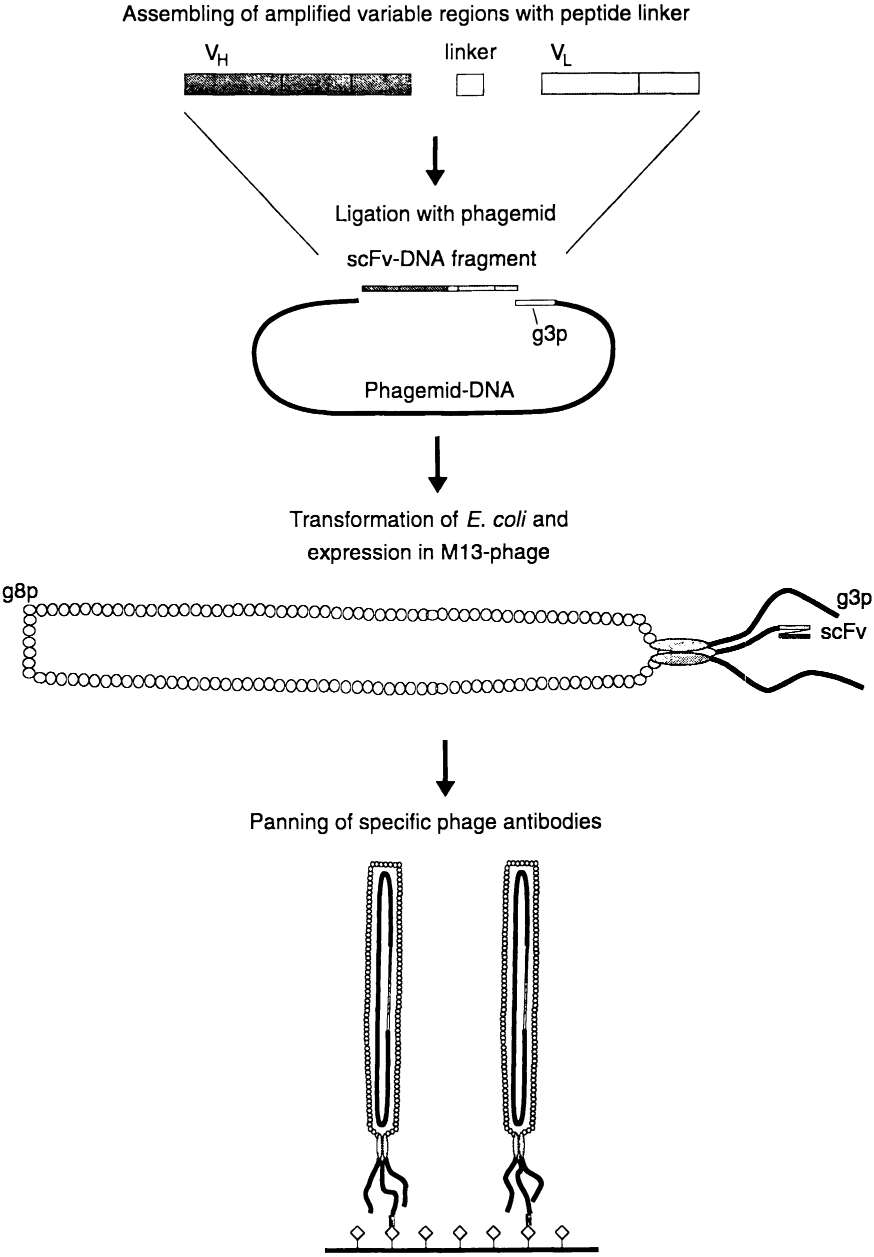
Assembling of amplified variable regions with peptide linker
V_H
linker
V_L
Ligation with phagemid
scFv-DNA fragment
g3p
Phagemid-DNA
Transformation of *E. coli* and expression in M13-phage
g8p
g3p
scFv
Panning of specific phage antibodies

Figure 2. Phage display of recombinant antibodies. Due to technical reasons, the scheme gives an abstracted image of *in vivo* conditions. The length of filamentous M13-phage depends on the size of enclosed DNA (estimated size of scFv presenting M13 phage: 1 µm length, 15–20 Å diameter).

protein. The recombinant Fab exhibited the same binding properties as the Fab fragments obtained by papain digestion from the corresponding Mab 481. The Rab as well as the Fab prepared by proteolytic cleavage showed increased sensitivities by almost two orders of magnitude in the competitive, indirect enzyme-linked immunosorbent assay (ELISA) for the hapten diuron.

In addition, Karu (*16*) investigated the semi-synthetic combinatorial library approach to select Rab with significant affinity and specificity towards diuron. The libraries, which were provided by Barbas (*25,26*), differed only in their CDR3 domains. After five rounds of panning on diuron carrier conjugates, 600 colonies in the positive fraction were tested in the direct ELISA. Nine percent of these clones bound diuron-alkaline phosphatase tracer with low affinity and a single clone showed weak competition with free diuron.

We used two different approaches for the expression of Rab, recombinant single chain Fv (scFv) and recombinant Fab. The phage display of scFv was based on the work of Winter (*27*). mRNA from our hybridoma cell lines K1E4, K4E7 and P6A7 (*28–30*) was isolated in order to synthesize scFvs against the *s*-triazine herbicides terbutryn, atrazine and terbuthylazine, respectively. The scFv molecule consists of the heavy and light chain variable region connected by a $(Gly_4Ser)_3$ peptide linker (Figure 1). Covalent binding prevents dissociation of the paired V_H and V_L domains caused by the varying stability of the heterodimer due to the structure-dependent differences of the binding energy between the heavy and light chain moiety in individual antibodies. scFvs are expressed as g3p coat protein conjugates on the surface of the phage particle.

Our efforts have been focused on providing an efficient screening procedure, which facilitates the rapid isolation of appropriate clones. Since selection by panning suffers from significant contamination of positive clones with unspecific ones, repeated cycles of selection are required, especially when large libraries are handled. Using immunomagnetic screening for phage particles derived from the hybridoma cell line K1F4, we obtained 184 out of 1.48×10^6 transformed clones in the positive fraction by an unique selection step using the solid phase of the triazine-coated paramagnetic beads. Almost 80% of the positive fraction (143 clones) reacted specifically with the corresponding terbutryn conjugate.

Comparison of the phage-conjugated scFv with the corresponding Mab showed similar concentration-dependent binding to the immobilized triazine-ovalbumine conjugate in the non-competitive ELISA (Figure 3). Unspecific binding was excluded because incubation with uncoupled ovalbumine resulted in very low absorbance measurements. However, phage-associated scFv bound to the immobilized analyte could not be replaced by the free analyte. This common effect may be due to the stickiness of tagged phage particles on the microtiter plate surface after binding to the coat-conjugate. Soluble scFv are presently being tested by ELISA and surface plasmone resonance. Initial results indicate that soluble scFv are replaced by free analytes.

The scFv contains an artificial linker sequence to connect the variable domains. For certain applications, e.g. structure analysis imaging the binding pockets of natural antibodies, Fab fragments are the molecules of choice. Dissociation of the heterodimer is avoided by the stabilizing disulfide bonds between the heavy and light chain constant domains. Once this Fab fragment is obtained it can be fused into

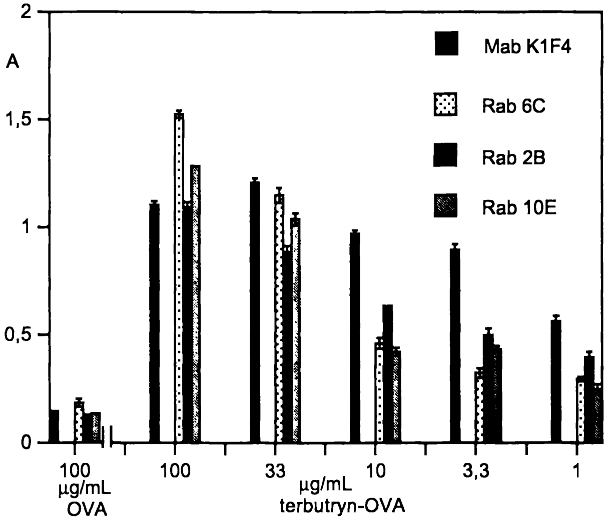

Figure 3. Mab and Rab in the non-competitive, indirect ELISA.

vectors to religate an Fc region to generate full antibody sequence. In our second approach we used a Fab expression system developed by Skerra (*31*). The principle of Fab synthesis is shown in Figure 4. It is based on the vector pASK85, which contains the constant domains C_H1 and C_L of a murine antibody. Therefore, PCR amplification of the antibody genes is again reduced to the variable regions of the antibody of interest. This strategy minimizes sequence deviations generated during PCR because the accuracy of PCR is inversely proportional to the length of the amplified DNA product. After separate cloning of the H and L variable regions into the vector, the complete light chain is excised and cloned into the corresponding heavy chain vector. Following transformation of *E. coli* and induction of expression, the chains are secreted into the periplasm of the transformants. In this bacterial compartment protein folding, association of a functional heterodimer and formation of disulfide bonds is accomplished. Expressed Fab fragments are purified from cell lysates by affinity chromatography using immobilized Zn^{2+}. This strategy takes advantage by a histidine hexamer extension at the C-terminus of the heavy chain exhibiting an affinity for Zn^{2+} ions. Calibration curves obtained with competitive ELISAs indicate the concentration-dependent displacement of the recombinant Fab by the analyte similar to the original Mab K4E7 (Figure 5). The absorption values ranged between 1.5 for the zero control and 0.08 for the analyte excess.

Perspectives

It can be foreseen that the Rab technology will be readily established in environmental research. This approach is considered as an improvement in comparison with the Mab technology. A significant difference between Mab and Rab is that the latter is accessible for selection and modification of antibody properties without requiring new immunization. Methods are now provided to rapidly isolate desired clones in antibody libraries and to manipulate individual Rab by genetic engineering. Thereby, the specificity and affinity of Rab can be designed to match the specific demands of food and environmental analysis. Two different sources of antibody genes, natural and synthetic ones, could be used to reach this goal.

Natural antibody genes are isolated from nonimmunized or immunized donors to synthesize antibody libraries. The Rab of the former library are not biased toward a particular antigen, thus providing a highly diverse antibody repertoire. Since this library type corresponds to an early stage of lymphocyte development, the affinity of the Rab obtained is very low. Utilizing the antibody genes derived from immunized donors for the production of a library, the antibody repertoire is shifted towards the immunogen. However, these Rab are characterized by increased association constants. This is due to the affinity maturation of stimulated B-lymphocytes, which accompany the immunization process.

Libraries on the basis of synthetic antibody genes are commonly using an existent antibody as a backbone. Diversity and refinement of the library is accomplished by varying the CDRs (Figure 1). CDR sequences can be randomized by means of PCR. Synthetic primers are applied, which generate the whole repertoire of possible sequence variations. This repertoire is theoretically limited only by the length of the targeted sequence.

The engineering of a single CDR region was first applied by Barbas *et al.* (*12*). The complete heavy chain CDR3 was randomized in a length of 48 nucleotides to

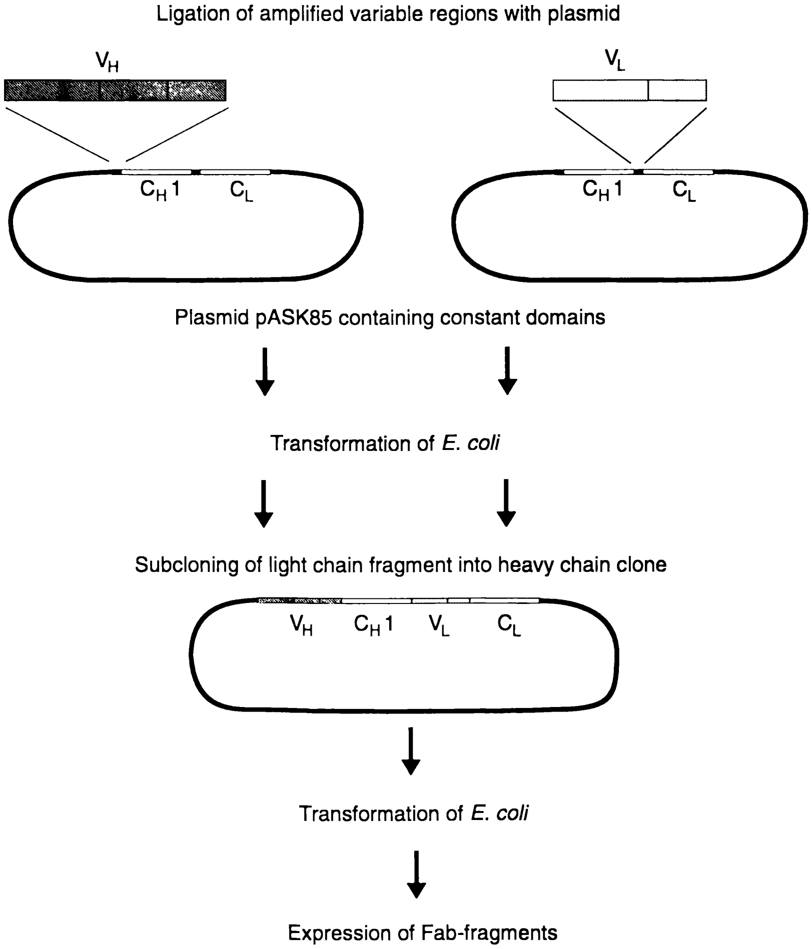
Ligation of amplified variable regions with plasmid
V_H
V_L
C_H1
C_L
C_H1
C_L
Plasmid pASK85 containing constant domains
Transformation of *E. coli*
Subcloning of light chain fragment into heavy chain clone
V_H
C_H1
V_L
C_L
Transformation of *E. coli*
Expression of Fab-fragments

Figure 4. Recombinant Fab synthesis using the approach of Skerra (9).

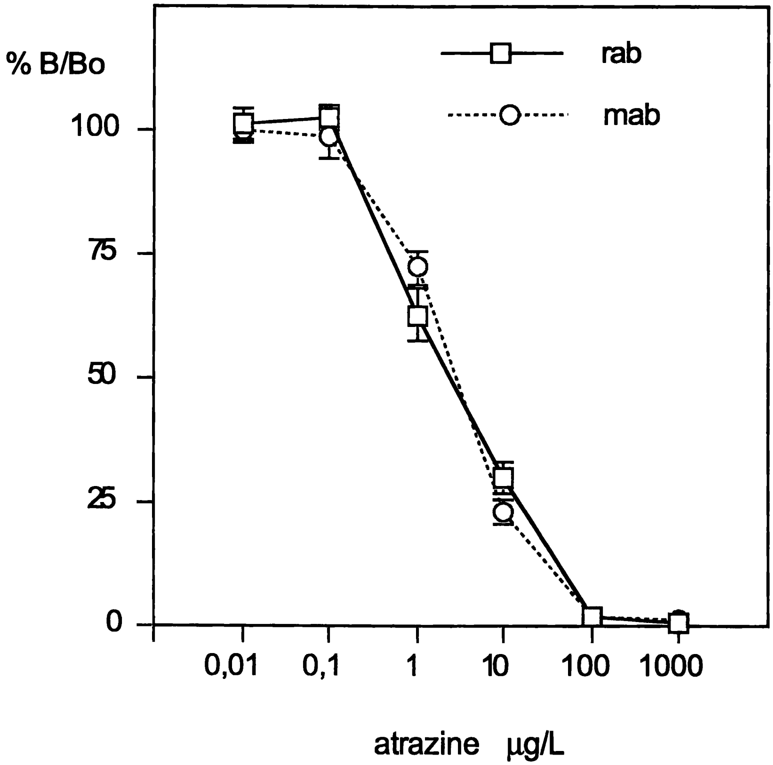

Figure 5. Mab and recombinant Fab in the competitive, indirect ELISA.

yield a library of 5x10^7 clones. Since the representation of all possible sequence variations would necessitate more than 10^{20} clones in this experiment, it is obvious that the potential size of the synthetic library exceeds the number of bacterial clones in the generated library by several orders of magnitude. This is because the achievable transformation rates in bacteria are limited by the available techniques, e.g. electroporation. If this restriction can be overcome, the extent of sequence variations included in a library will always surpass the possible variations of Rab specificities and affinities toward a defined target analyte. This is due to the fact that not every sequence variation generates structural changes, which are relevant for the immune reaction. Therefore, molecular modelling studies of structural binding sites will become an indespensible tool in antibody engineering. However, the above mentioned libraries provide a pool of antibody diversity from which particular clones are isolated for further affinity improvement by advanced genetic manipulation, e.g. chain shuffling (*13*) or CDR walking (*32*). Work is in progress to apply this strategy for the improvement and diversification of our Rab against *s*-triazines. This approach takes advantage of the high level of optimization already reached by the first generation of our Rab.

Acknowledgments

The authors wish to thank Dr. Arne Skerra for his advice in recombinant Fab production and for generously providing the pASK85-based Fab system. We thank the Deutsche Forschungsgemeinschaft for a grant (Ho 383/29-1). We are indebted to Dr. R. Beier and Dr. B. Hammock for reading the manuscript.

Literature Cited

1. Winter, G.; Milstein, C. *Nature* **1991**, *349*, 293–299.
2. Irving, R.; Hudson, P. In *Monoclonal antibodies. The second generation;* Zola, H., ed.; BIOS Scientific Publishers Ltd.: Oxford, UK, 1995; pp 119–139.
3. Better, M.; Chang, C. P.; Robinson, R. R.; Horwitz, A. H. *Science* **1988**, *240*, 1041–1043.
4. Skerra, A.; Plückthun, A. *Science* **1988**, *240*, 1038–1041.
5. Orlandi, R.; Gussow, D. H.; Jones, P. T.; Winter, G. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 3833–3837.
6. Sastry, L.; Alting-Mees, M.; Huse, W. D.; Short, J. M.; Sorge, J. A.; Hay, B. N.; Janda, K. D.; Benkovic, S. J.; Lerner, R. A. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 5728–5732.
7. Larrick, J. W.; Danielsson, L.; Brenner, C. A.; Abrahamson, M.; Fry, K. E.; Borrebaeck, C. A. K. *Biochem. Biophys. Res. Commun.* **1989**, *160*, 1250–1256.
8. Pack, P.; Kujau, M.; Schroeckh, V.; Knüpfer, U.; Wenderoth, R.; Riesenberg, D.; Plückthun, A. *Bio/Technology* **1993**, 1271–1277.
9. Skerra, A. *Gene* **1994**, *141*, 79–84.
10. Neuberger, M.. S.; Williams, G. T.; Fox, R. O. *Nature* **1984**, *312*, 604–608.
11. Jones, P. T.; Dear, J. H.; Foote, J.; Neuberger, M. S.; Winter, G. *Nature* **1986**, *321*, 522–525.

12. Barbas III, C. F.; Bain, J. D.; Hoekstra, D. M.; Lerner, R. A. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 4457–4461.
13. Marks, J. D.; Griffiths, A. D.; Malmqvist, M.; Clarkson, T. P.; Bye, J. M.; Winter, G. *Bio/Technol.* **1992**, *10*, 779–783.
14. Kabat, E. A.; Wu, T. T.; Perry, H.; Gottesman, K. S.; Foeller, C. In *Sequences of Proteins of Immunological Interest*; US Department of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda, MD, 1991.
15. Ward, V. K.; Schneider, P. G.; Kreißig, S. B.; Hammock, B. D.; Choudary, P. V. *Prot. Eng.* **1993**, *6*, 981–988.
16. Karu, A. E.; Scholthof, K.-B. G.; Zhang, G.; Bell, C. W. *Food Agric. Immunol.* **1994**, *6*, 277–286.
17. Shelton, A.; Graham, B.; Byrne, F.; Learmonth, D.; Porter, A.; Harris, W. *ICHEME Industrial Immunology*, Biotechnology '94: Brighton, UK, 1994; pp 8–10.
18. Hock, B.; Dankwardt, A.; Kramer, K.; Marx, A. *Anal. Chim. Acta* **1995**, in press.
19. Kamps-Holtzapple, C; Stanker, L. H., 209th ACS Meeting & Exposition: Anaheim, CA, 1995.
20. Karu, E. A.; Harrison, R. O.; Schmidt, D. J.; Clarkson, C. E.; Grassman, J.; Goodrow, M. H.; Lucas, A.; Hammock, B. D.; Van Emon, J. M.; White, R. J. In *Immunoassays for Trace Chemical Analysis*; Vanderlaan, M.; Stanker, L. H.; Watkins, B. E.; Roberts, D. W., eds.; ACS Symposium Series 451, American Chemical Society: Washington, DC, 1991; pp 59–77.
21. Studier, F. W.; Rosenberg, A. H.; Dunn, J. J.; Dubendorf, J. W. *Methods Enzymol.* **1990**, *185*, 60–89.
22. Smith, G. P. *Science* **1985**, *228*, 1315–1316.
23. Huse, W. D.; Sashy, L.; Iverson, S. A.; Kang, A. S.; Alting-Mees, H.; Burton, D. R.; Benkovic, J. S.; Lerner, R. A. *Science* **1989**, *246*, 1275–1281.
24. Hoogenboom, H. R.; Griffiths, A. D.; Johnson, K. S.; Chiewell, D. J.; Hudson, P.; Winter, G. *Nucl. Acids Res.* **1991**, *19*, 4133–4137.
25. Barbas III, C. F.; Lerner, R. A. In *Methods: A Companion to Methods in Enzymology*; Lerner, R. A.; Burton, D. R., eds.; Academic Press: Orlando, FL, 1991; pp 119–124.
26. Barbas III, C. F.; Rosenblum, J. S.; Lerner, R. A. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 6385–6389.
27. Winter, G.; Griffiths, A. D.; Hawkins, R. E.; Hoogenboom, H. R. *Annual Rev. Immunol.* **1994**, *12*, 433–455.
28. Giersch, T.; Hock, B. *Food Agric. Immunol.* **1990**, *2*, 85–97.
29. Giersch, T. *J. Agric. Food Chem.* **1993**, *41*, 1006–1011.
30. Giersch, T.; Kramer, K.; Weller, M. G.; Hock, B. *Acta Hydrochim. Hydrobiol.* **1993**, *21*, 312–315.
31. Skerra, A. *Gene* **1994**, *151*, 131–135.
32. Barbas III, C. F.; Hu, D.; Dunlop, N.; Sawyer, L.; Cababa, D.; Hendry, R. M.; Nara, P. L.; Burton, D. R. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 3809–3813.

RECEIVED July 31, 1995

Chapter 39

Development of Recombinant Single-Chain Variable Portion Recognizing Potato Glycoalkaloids

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The variable portion (Fv) of a monoclonal antibody (Mab), consisting of the variable heavy and light chains (VH and VL) may be synthetically linked to form a single-chain Fv (scFv). Recombinant scFvs recognizing potato glycoalkaloids were developed from hybridoma cell lines expressing Mabs against solanidine. Following mRNA extraction and first-strand cDNA synthesis, both the VH and VL gene sequences were amplified by the polymerase chain reaction (PCR) using specific primers. The VH and VL DNA fragments were then joined with a neutral DNA linker and cloned into phagemid pCANTAB 5E (Pharmacia). Phage displaying the recombinant antibody as a fusion protein with phage tip protein g3p were isolated. Soluble antibodies were then obtained from two strains of *E. coli* infected with antigen-positive phage by incubation of the bacteria in the presence of isopropyl-1-thio-β-D-galactopyranoside (IPTG).

The development of monoclonal antibody (Mab) methods (*1*) has made it possible to derive individual antibodies of invariant specificity and selectivity and to immortalize the cells that produce these antibodies, so that an infinite supply is available (*2*). However, the disadvantages inherent in monoclonal antibody production include the use of animals, the extensive commitment of time, labor and expense, the requirement of technical expertise in screening a large number of hybridomas, and most importantly, the inability to alter antibodies produced by hybridomas. The recent development of the PCR technology has made it possible to clone antibody gene fragments, insert these fragments into phagemid cloning vectors and express the antibody protein encoded by the gene fragment on the surface of filamentous phage grown in *E. coli*.

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In Immunoassays for Residue Analysis; Beier, R., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

Due to the relative ease of bacterial fermentation, cloning and expression of antibody fragments in bacteria is an attractive addition or alternative to antibody production by hybridoma technology. Antibody-expressing hybridoma cells or splenocytes from immunized mice (*3*) can be used to produce antibody fragments. The affinity and/or specificity of the recombinant antibodies can be altered by mutagenesis (*4,5*) or by chain shuffling (*3,6*) to obtain antibodies with the desired characteristics without further use of laboratory animals. Alternatively, *in vitro* techniques can be used to build diverse semi-synthetic repertoires from a limited number of variable heavy and variable light genes (*7,8*). Recombinant antibodies offer several advantages over hybridoma-derived monoclonals in that they are more easily cloned and screened, they do not require large-scale cell culture, they offer a stable genetic source, and they can be genetically manipulated in order to alter the specificity and/or affinity of the antibody for certain antigens (*9*).

IgG antibodies consist of two identical heavy (H) chain polypeptides and two identical light (L) chain polypeptides held together by disulfide bridges and non-covalent bonds (Figure 1). At the N-terminus of each chain is a domain, called the variable (V) region, in which there is considerable amino acid variation. Each V-region consists of regions of hypervariability, referred to as complementarity determining regions (CDRs), separated by more conserved regions, referred to as framework regions (FRs) (*10*). The antibody combining site is formed by the CDRs of the V-heavy (VH) and V-light (VL) domains. These variable domains, which are devoid of interchain disulfide bonds, associate by noncovalent interactions to constitute the Fv fragment.

The Fv fragment (Figure 1) is the smallest antibody unit that retains the intact binding site (*11-14*); however, since in the Fv fragment, the VH and VL domains are not held together by covalent bonds, and since the hypervariable CDRs form about one-quarter of the contacts at the interface between the two domains, the stability of Fv heterodimers varies greatly and unpredictably (*15*). In a recombinant version of Fv, referred to as the single-chain variable fragment (scFv), the VH and VL polypeptides of the Fv heterodimer are synthetically linked together with a neutral linker and expressed as a single polypeptide chain (*13,15,16*). The linker must be able to span the 3.5-nm distance between its points of fusion to the VH and VL domains without distortion of the native Fv conformation (*15*). Although a linker of about 10 amino acid residues would be sufficient, a 15-residue linker is typically used in order to prevent conformational strain that could be induced by a short connection, while avoiding steric interference with antigen binding that could occur with a long connection. A commonly used linker consists of three units of $(Gly)_4Ser$ (*17*). The small Gly residues minimize steric hindrance and the Ser residues confer hydrophilicity, making the linker more soluble (*9*). Correct folding both *in vitro* (*17-21*) and *in vivo* (*22*) has been obtained with this linker.

The scFv may be either displayed (*23,24*) on the surface of filamentous bacteriophage by fusion to the gene 3 protein (g3p), a minor coat protein at the tip of the phage (*25*), or produced as a soluble antibody fragment. Phage-displaying scFv fragments also carry a copy of the gene. Therefore, antigen-positive phage

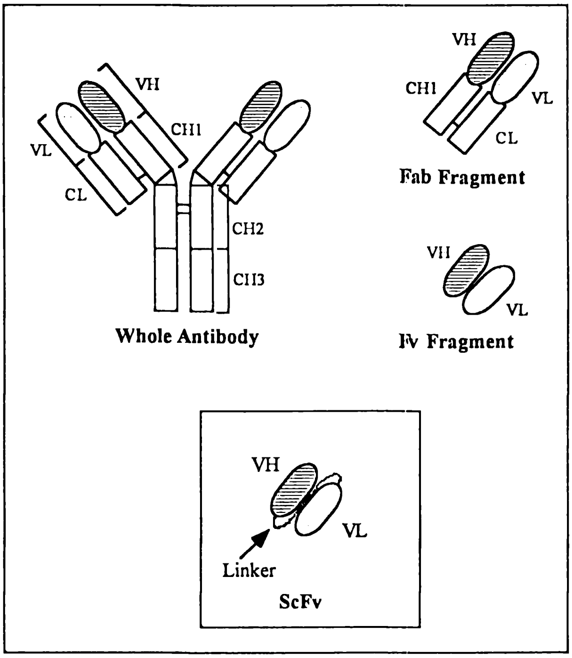

VH
CH1
VL
CL
CH2
CH3
Whole Antibody
VH
CH1
VL
CL
Fab Fragment
VH
VL
Fv Fragment
VH
VL
Linker
ScFv

Figure 1. Schematic diagram of the structure of the antibody molecule and various antibody fragments. VH and VL represent the variable regions of the heavy- and light-chains, respectively. CH1, CH2, and CH3 are the constant regions of the heavy chain. CL is the constant region of the light-chain.

can be enriched (panned) by capturing on antigen-coated plates, and bound phage can then be propagated on a fresh culture of *E. coli*. Soluble antibodies can be made from antigen-positive phage and used as reagents in enzyme-linked immunosorbent assay (ELISA) studies.

Glycoalkaloids are potentially toxic nitrogen-containing secondary plant metabolites found in a number of plant species, including potatoes and tomatoes (*27*). Commercial potatoes produce α-solanine and α-chaconine, both of which are glycosylated derivatives of the aglycon solanidine, whereas wild potatoes produce solasonine (Figure 2). Tomatoes produce the glycoalkaloid α-tomatine, which is the glycosylated derivative of tomatidine. Since high levels of these compounds are toxic, antibody-based tests that can monitor glycoalkaloid levels are required. The Mabs which we used to produce scFv fragments against glycoalkaloids were developed using solanidine-ovalbumin (OVA) as the immunogen (*26*).

In this chapter, we describe the development of solanidine-positive scFv antibody fragments recognizing the aglycon of commercial potato glycoalkaloids and discuss the difficulties that we encountered with this technology.

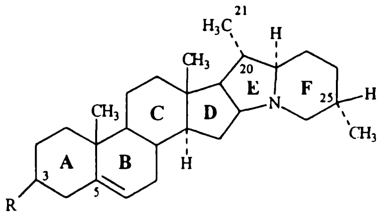
Materials and Methods

Monoclonal Antibody Production. Production of the hybridoma cell lines producing their respective Mabs and cross-reactivity studies using a competition ELISA were previously reported (*26*).

mRNA Isolation. Messenger RNA from 5 x 10^6 hybridoma cells was isolated using the MicroFast Track kit from Invitrogen Corp. (San Diego, CA).

ScFv Production. A schematic representation of the procedure used to produce recombinant scFv fragments is depicted in Figure 3. ScFvs were produced using the ScFv Module part of the Recombinant Phage Antibody System (RPAS) (*28*) from Pharmacia Biotech (Piscataway, NJ). Briefly, first-strand cDNA was prepared from mRNA primed with random hexamers. The antibody VH and VL genes were then amplified by PCR using primers designed to hybridize to opposite ends of the variable region of each chain. After gel purification and quantification of the primary PCR products, the purified VH and VL DNA products were assembled into a single gene using a DNA linker oligonucleotide which is complementary to both the 3' end of the VH gene and the 5' end of VL gene, and which encodes the linker polypeptide, $(Gly_4Ser)_3$. After PCR amplification of the assembled antibody scFv DNA, *Sfi* I and *Not* I restriction sites were added to the 5' and 3' ends, respectively, also by PCR.

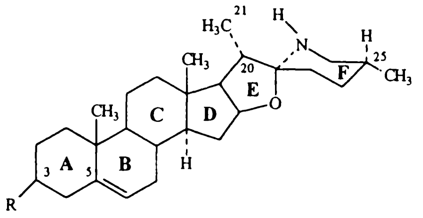
Phage Production. Recombinant phage were produced using the Expression Module part of the Recombinant Phage Antibody System. Briefly, the scFv product was inserted into the phagemid pCANTAB 5E and used to transform competent *E. coli* TG1 cells. TG1 is a suppressor strain that allows readthrough (suppression) of the stop codon present between the scFv and gene 3 sequences.


21
H3C
H
CH3
20
E
F
25
H
N
CH3
C
D
CH3
A
B
H
3
5
R

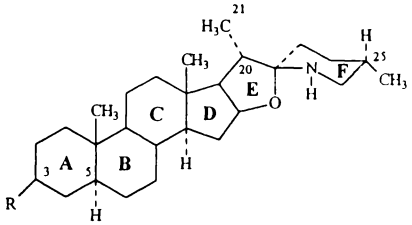
Solanidine R = OH

α-Solanine R = O-galactose - glucose
rhamnose

α-Chaconine R = O-glucose - rhamnose
rhamnose


21
H3C
H
N
H
25
CH3
CH3
20
F
E
O
CH3
C
D
3
A
5
B
H
R

Solasonine R = O-galactose - glucose
rhamnose


21
H3C
H
25
CH3
20
N
F
CH3
E
H
O
CH3
C
D
3
A
5
B
H
R
H

Tomatidine R = OH

α-Tomatine R = O-galactose - glucose - xylose
glucose

Figure 2. Structures of commercial potato (upper), wild potato (center), and tomato (lower) alkaloids.

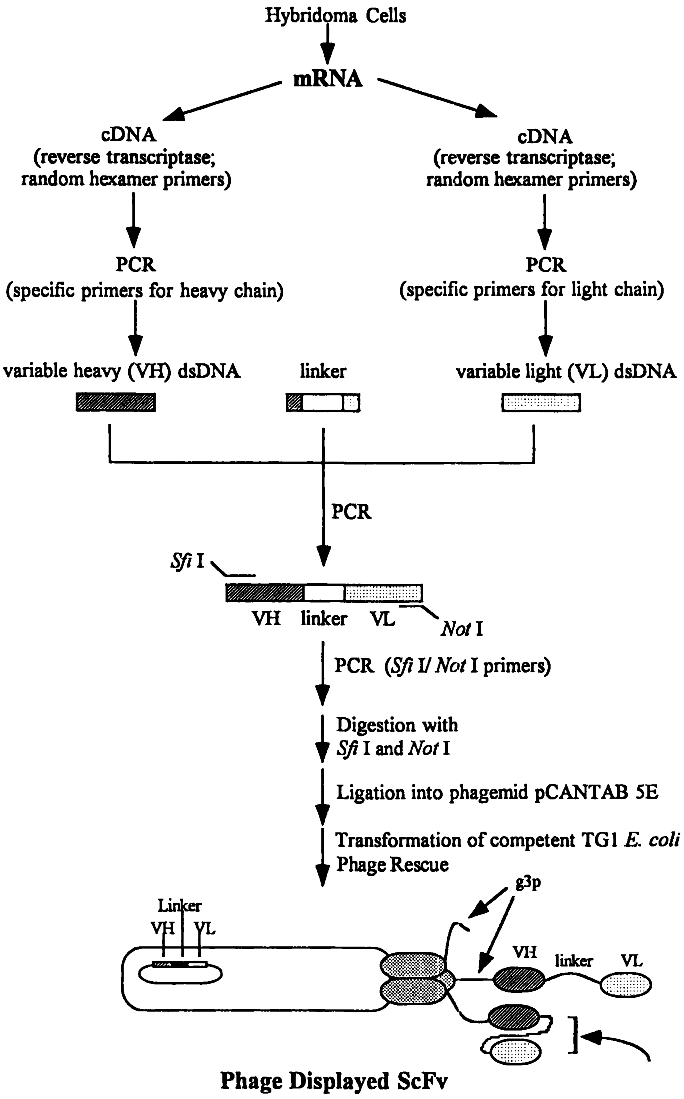

Hybridoma Cells
mRNA
cDNA
(reverse transcriptase;
random hexamer primers)
cDNA
(reverse transcriptase;
random hexamer primers)
PCR
(specific primers for heavy chain)
PCR
(specific primers for light chain)
variable heavy (VH) dsDNA
linker
variable light (VL) dsDNA
PCR
Sfi I
VH linker VL
Not I
PCR (Sfi I/ Not I primers)
Digestion with
Sfi I and Not I
Ligation into phagemid pCANTAB 5E
Transformation of competent TG1 E. coli
Phage Rescue
g3p
Linker
VH
VL
VH
linker
VL
Phage Displayed ScFv

Figure 3. Schematic protocol for the production of phage displayed scFvs. Protocol adapted from *(28)*.

Numerous transformants were obtained and 600 colonies were picked for phage rescue using M13KO7 helper phage. The resulting phage containing phage-displayed recombinant antibodies were panned in a 25-cm^2 cell culture flask coated with solanidine-BSA. (An immunoassay was used to verify that the solanidine-BSA conjugate bound to the surface of the flask.) After washing with phosphate buffered saline (PBS), antigen-binding phage were used to reinfect log-phase TG1 cells. After two additional rounds of panning, antigen-positive clones were determined by ELISA.

Soluble Antibody Production. A schematic representation of the procedure used to produce soluble antibodies is depicted in Figure 4. Antigen-positive phage were used to infect exponentially growing *E. coli* HB2151 or TG1 cells. Although TG1 is a suppressor strain, allowing production of phage-displayed scFvs, soluble antibodies also can be obtained from this strain because suppression of the amber stop codon is only 20% efficient (*28*). Infected cells were incubated first in the presence of 2% glucose (at 30 °C) and then in the presence of 1 mM IPTG for 24 h to induce production of soluble scFv antibodies. Soluble antibodies recognizing solanidine were detected by ELISA.

ELISA Protocol. Antigen-positive phage and soluble antibodies recognizing solanidine-BSA were detected by ELISA according to Pharmacia's protocol using solanidine-BSA as the plate coating antigen (1000 ng/well). Mouse anti-M13 antibody conjugated to horseradish peroxidase was used to detect bound phage. Mouse anti-E tag antibody (Pharmacia) was used to detect bound soluble scFv antibody, followed by goat anti-mouse IgG-peroxidase conjugate. The binding of phage-scFv or soluble scFv antibody to untreated plates or plates coated only with BSA was also determined.

Results and Discussion

ELISA Studies. Competition ELISA experiments (summarized in Table 1) using Mabs Sol-106 and Sol-129 demonstrate the differences in the specificities of these two antibodies toward the major glycoalkaloids found in potatoes and tomatoes. Although both monoclonal antibodies recognize the aglycon and glycoalkaloids found in commercial potatoes (solanidine, α-solanine, and α-chaconine), only Sol-129 recognizes the alkaloids found in wild potatoes (solasonine) and tomatoes (tomatidine and α-tomatine).

The immunogen consisted of succinylated solanidine conjugated to the carrier protein; therefore, both monoclonal antibodies must recognize the aglycon portions of the glycoalkaloid molecules and not the sugar moieties. As can be seen in Figure 2, the aglycon of the glycoalkaloids found in commercial potatoes (solanidine) is different from those found in wild potatoes or tomatoes. Since Mab Sol-106 recognizes only the glycoalkaloids (and aglycon) found in commercial potatoes, whereas Mab Sol-129 recognizes those found in tomatoes and in commercial and wild potatoes, we wanted to isolate the variable portions of both

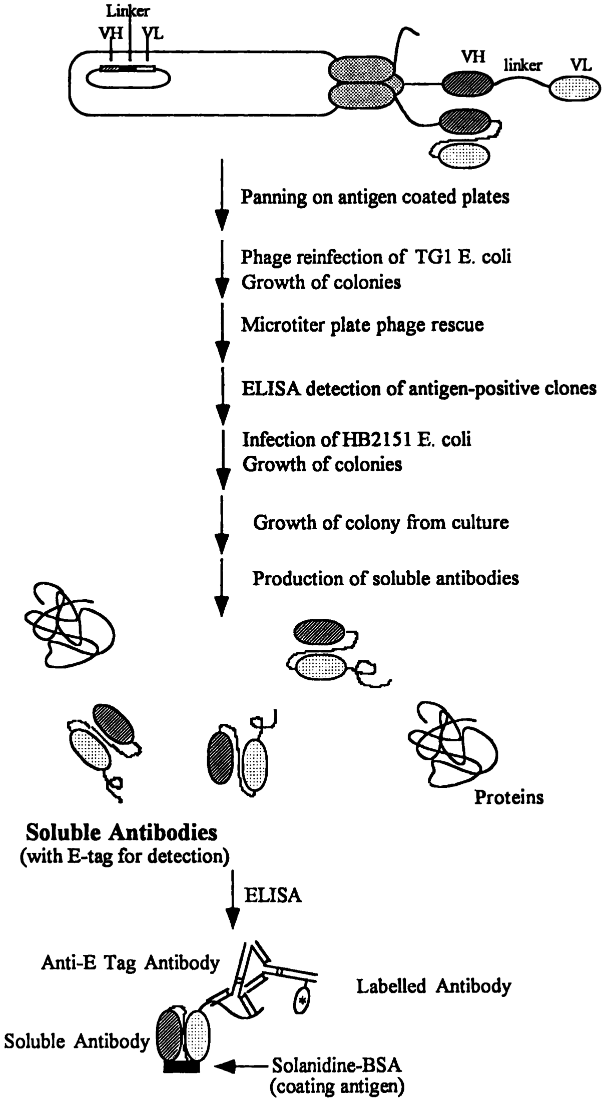

Linker
VH
VL
VH
linker
VL
Panning on antigen coated plates
Phage reinfection of TG1 E. coli
Growth of colonies
Microtiter plate phage rescue
ELISA detection of antigen-positive clones
Infection of HB2151 E. coli
Growth of colonies
Growth of colony from culture
Production of soluble antibodies
Proteins
Soluble Antibodies
(with E-tag for detection)
ELISA
Anti-E Tag Antibody
Labelled Antibody
Soluble Antibody
Solanidine-BSA
(coating antigen)

Figure 4. Schematic protocol for the production of soluble scFv antibodies. Protocol adapted from (*28*).

antibodies and develop scFvs in order to investigate reasons for the differences in their specificities.

Table I. Cross-reactivity of Mabs Sol-106 and Sol-129: IC_{50} Values[a]

Compound	Sol-106 IgG2a	Sol-129 IgG1
solanidine	14 ± 2.4	2.5 ± 0.35
α-solanine	49 ± 8	2.6 ± 0.25
α-chaconine	54 ± 13	2.8 ± 0.2
solasonine	NC	36.0 ± 3.2
α-tomatine	NC	5.4 ± 3.5
tomatidine	NC	10.4 ± 2.0

[a] IC_{50} values given in ppb ± one standard deviation.
NC, no competition at 10,000 ppb.
Data from Stanker *et al.* (*26*).

Development of ScFvs. The production of the scFv from Sol-106 proceeded according to the protocol supplied by Pharmacia. The ~350 bp VH and VL fragments were obtained using the specific primers, and attachment of the two fragments with the linker presented no problems. Gel electrophoretic analysis of the VH and VL fragments as well as the assembled product is shown in Figure 5. The ~750 bp assembled VH-linker-VL was directionally cloned into the phagemid pCANTAB 5E and antigen-positive phage were isolated on solanidine-BSA coated tissue culture flasks.

This scenario, however, was not always the case. We observed that the scFvs fell into one of three categories. (a) As described with one antibody (Sol-106), the protocol was followed exactly and no problems were encountered in producing an antigen-positive phage clone. (b) In other cases, the VH domain amplified well during the primary PCR, but the VL domain did not. In our experience, we did not observe the opposite situation. (c) In the third case (Hept-2, an antibody to the insecticide heptachlor), the VH gene amplified well following the protocol and the VL gene could be sufficiently amplified if 50 PCR cycles were used rather than 30, but the PCR joining reaction with the linker was problematic. Despite many attempts using varying ratios of the VH fragment, VL fragment and linker, we were unable to obtain the ~750 bp VH-linker-VL assembled product for this antibody even though sufficient VH and VL products were produced during the primary PCR amplification. It may be that the additional PCR cycles introduced errors in the amplified products that interfered with the assembly reaction. Also, the addition of adenosines to the ends of the

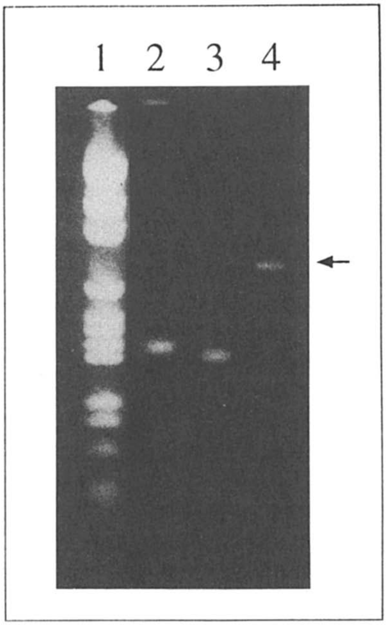


Figure 5. Electrophoretic analysis of recombinant antibody gene fragments. Lane 1, molecular weight markers; lane 2, VH gene PCR fragment (~350 bp); lane 3, VL gene PCR fragment (~340 bp); lane 4, VH-linker-VL scFv. Arrow points to this ~750 bp assembled product.

PCR products by Taq polymerase may have played a role in preventing assembly. Table II summarizes the results obtained using six different antibodies.

Table II. Summary of PCR Amplifications and Assembly Attempts

Mab	Isotype	Antigen	VH Amplification	VL Amplification	VH-VL-Linker Assembly
Sol-106[a]	IgG2a, κ	solanidine	yes	yes	yes
Sol-129	IgG1, κ	solanidine	yes	poor	no
Sol-48	IgG2a, κ	solanidine	yes	poor	no
Sol-59	IgG2a, κ	solanidine	yes	poor	no
Sol-67	IgG1, κ	solanidine	yes	poor	no
Hept-2[b]	IgG2a, κ	heptachlor	yes	yes [c]	no

[a] Solanidine Mab and isotype data from Stanker *et al.* (*26*).

[b] Hept-2 Mab and isotype data from Stanker *et al.* (*29*).

[c] The VL of Hept-2 was not produced in sufficient quantity when amplified for 30 PCR cycles. Upon amplification using 50 PCR cycles, a sufficient quantity of the VL gene was produced, but attempts to link the VH and VL gene fragments were unsuccessful.

Development of Soluble Antibodies. Soluble antibodies were obtained from Sol-106 since this was the only antibody that yielded an antigen-positive scFv phage clone. Once an antigen-positive phage has been isolated using phage-display, it would seem that production of soluble antibodies would be routine. However, we did not find this to be the case. In order to produce the soluble antibodies, the protocol recommends that the pCANTAB 5E vector containing the scFv gene be used to transform the nonsuppressor *E. coli* strain HB2151. This strain is expected to yield more soluble recombinant antibodies than TG1 cells in the presence of the *lac* inducer IPTG. In our hands, TG1 cells produced a better response than did HB2151 cells (Figure 6).

Cells from both *E. coli* strains were incubated approximately 20 h in the presence of 1 mM IPTG, the cells were sedimented, and the culture supernatants (50 mL) were analyzed for antigen-positive soluble ScFv antibodies by ELISA. These supernatants did not give positive results, so they were concentrated 20-fold using a 10,000 MWCO concentrator (Amicon) and then reanalyzed. As can be seen in Figure 4, the 20-fold concentrated supernatant from TG1 cells produced a greater response than did that of HB2151. Serial dilution of the concentrated supernatants diminished the response so that at a 20-fold dilution (corresponding to the concentration of the original culture supernatant), the HB2151 sample response corresponded to that of the background. The response of the TG1 sample at this dilution was weak but observable. It is unclear why the original TG1 sample did not give a response even though the concentrated sample, diluted on the ELISA

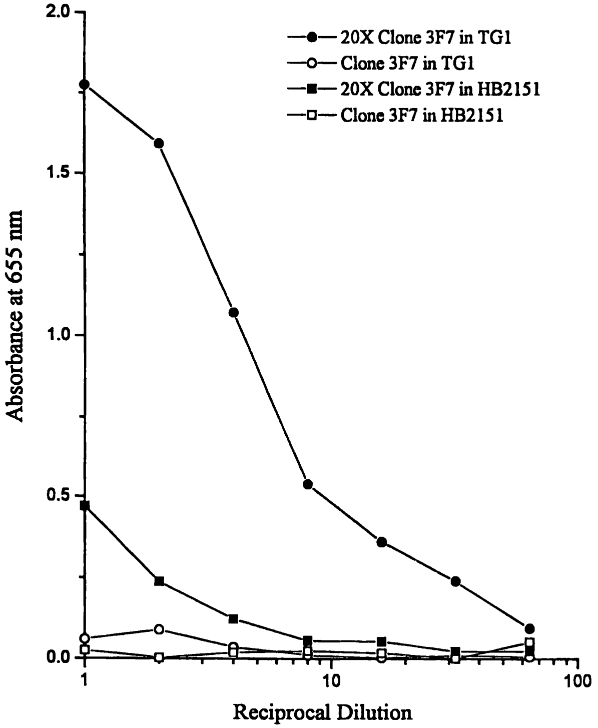


Figure 6. ELISA dilution analysis of unconcentrated and 20-fold concentrated bacterial supernatants containing soluble antibodies from Sol-106. (□) Unconcentrated and (■) 20-fold concentrated clone 1 soluble antibody from *E. coli* HB2151; (○) unconcentrated and (●) 20-fold concentrated clone 1 soluble antibody from *E. coli* TG1.

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plate 20-fold, gave a weak response. Most likely, the difference is due to the fact that the original sample contained 100 µL of bacterial supernatant when analyzed whereas the concentrated sample, at a 20-fold dilution, would have contained less than 5 µL of bacterial supernatant in approximately 95 µL of assay buffer (100 µL per well). Something in the bacterial supernatant may interfere with the assay such that samples that would give a weak response in assay buffer produce no response in bacterial supernatant.

In addition to difficulties with obtaining soluble antibodies, we found that the TG1 and HB2151 soluble antibody samples which produced a good response in the dilution analysis ELISA (Figure 6) did not recognize free solanidine in a competition ELISA. Since it has been reported previously (*30*) that soluble antibodies against aflatoxin M_1 exhibited weak inhibition of binding on day 1 and no inhibition on day 2, both the dilution and the competition ELISAs were performed on the same day. Despite this precaution, although the samples bound to immobilized hapten, they did not exhibit target recognition in solution.

In order to determine the cell fractions in which the soluble antibodies accumulate, the periplasmic and whole cell extracts as well as the 20-fold concentrated supernatant were analyzed by Western blot. Soluble antibodies were detected in both the whole cell extract and the 20-fold concentrated supernatant, but not in the periplasmic extract. This was the case for both TG1 and HB2151 *E. coli* strains. Since soluble antibodies located in the whole cell extract may not be biologically functional, and since soluble antibodies did not accumulate in the periplasm, it may be that the soluble antibodies detected in the supernatant leaked from a cellular compartment that did not allow correct antibody folding to occur.

Alternatively, the antibodies may not be functional due to errors that may have been introduced by Taq polymerase during PCR amplifications. DNA sequence analysis revealed that the VH domain belongs to subgroup IIB, and the VL domain belongs to subgroups IV (FR1 and CDR1) and IIB (FR2 through FR4). In the VH domain, there were no mismatches in 42 invariant residues whereas, in the VL domain, there were 9 mismatches in 59 invariant residues. One or more incorrect residues in either chain may have prevented the production of a functional antibody.

Conclusions

Recombinant antibody technology has the potential to produce scFv fragments against virtually any hapten using either hybridomas derived from spleens of immunized mice, splenocytes derived from immunized mice, or diverse libraries of immunoglobulin VH and VL gene fragments derived from immunologically naïve animals. This technology takes advantage of the relatively conserved nucleotide sequences at the 5' ends of FR1 and at the 3' ends of the J regions in both the VH and VL domains (*31*). Although it is clear that the primers we used are capable of producing VH and VL fragments from certain antibodies, additional and/or alternative primers may be necessary to develop functional scFv fragments from other antibodies. Since the sequences of all J segments in mouse are known (*10*), but the sequences of all of the 5' ends of VH and VL domains are not, the difficulties encountered with PCR cloning of variable domains from mouse hybridomas most likely is due to insufficient or tailed priming of the 5' portions (FR1) of the VH and VL domains.

Our future research will include using different primers and procedures to obtain functional scFvs or Fabs. A potential method of choice that would overcome the difficulties involved in priming the 5' portion of the cDNA is the one-side PCR procedure reported by Heinrichs *et al.* (*32*). This method allows the rapid cloning and direct sequencing of rearranged antibody variable domain genes from any species for which the constant domain sequences are known and does not require prior knowledge of the FR1 regions of the VH and VL domains. Use of higher fidelity DNA polymerases such as Vent or Pfu rather than Taq in PCR procedures may also aid in the production of functional antibodies.

As with any new technology, difficulties must be resolved before it will be widely used. These will most likely be overcome in the near future since recombinant DNA technology provides the potential for generating antibodies against an unlimited variety of haptens, and the expression systems that are available will allow large-scale production of these engineered antibodies.

Acknowledgments

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Literature Cited

1. Köhler, G.; Milstein, C. *Nature* 1975, *256*, 495–497.
2. Karu, A. E.; Scholthof, K.-B. G.; Zhang, G.; Bell, C. W. *Food Agric. Immunol.* **1994**, *6*, 277–286.
3. Ward, E. S. *Mol. Immunol.* **1995**, *32*, 147–156.
4. Gram, H.; Marconi, L.-A.; Barbas, C. F.; Collet, T. A.; Lerner, R. A.; Kang, A. S. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, 89, 3576–3580.
5. Hawkins, R. E.; Russell, S. J.; Winter, G. *J. Mol. Biol.*, **1992**, *226*, 889–896.
6. Marks, J. D.; Griffiths, A. D.; Malmquist, M.; Clackson, T. P.; Bye, J. M.; Winter, G. *Biotechnol.* **1992**, *10*, 779–773.
7. Hoogenboom, H. R.; Winter, G. *J. Mol. Biol.* **1992**, *227*, 381–388.
8. Barbas, C. F.; Bain, J. D.; Hoekstra, D. M.; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4457–4461.
9. Plückthun, A. *Methods: A Companion to Methods in Enzymology* **1991**, *2*, 88–96.
10. Kabat, E. A.; Wu, T. T.; Perry, H. M.; Gottesman, K. S.; Foeller, C. *Sequences of Proteins of Immunological Interest*, 5th edition, **1991**, U.S. Department of Health and Human Services, NIH: Washington, DC.
11. Plückthun, A.; Pfitzinger, I. *Ann. N.Y. Acad. Sci.* **1991**, *646*, 115–124.
12. Givöl, D. *Mol. Immunol.* **1991**, *28*, 1379–1386.
13. Whitlow, M.; Bell, B. A.; Feng, S.-L.; Filpula, D.; Hardman, K. D.; Hubert, S. L.; Rollence, M. L.; Wood, J. F.; Schott, M. E.; Milenic, D. E.; Yokota, T.; Schlom, J. *Protein Eng.* **1993**, *6*, 989–995.

14. Sandhu, J. S. *Crit. Rev. Biotechnol.* **1992**, *12*, 437–462.
15. Huston, J. S.; Mudgett-Hunter, M.; Tai, M.-S.; McCartney, J.; Warren, F.; Haber, E.; Oppermann, H. *Methods Enzymol.* **1991**, *203*, 46–88.
16. Bird, R. E.; Hardman, K. D.; Jacobson, J. W.; Johnson, S.; Kaufman, B. M.; Lee, S.-M.; Lee, T.; Pope, S. H.; Riordan, G. S.; Whitlow, M. *Science* **1988**, *242*, 423–426.
17. Huston, J. S.; Levinson, D.; Mudgett-Hunter, M.; Tai, M.-S.; Novotny, J.; Margolies, M. N.; Ridge, R. J.; Bruccoleri, R. E.; Haber, E.; Crea, R.; Oppermann, H. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5879–5883.
18. Chaudhary, V. K.; Queen, C.; Junghans, R. P.; Waldmann, T. A.; Fitzgerald, D. J.; Pastan, I. *Nature* **1989**, *339*, 394–397.
19. Batra, J. K.; Fitzgerald, D.; Gately, M.; Chaudhary, V. K.; Pastan, I. *J. Biol. Chem.* **1990**, *265*, 15198–15202.
20. Kreitman, R. J.; Chaudhary, B. K.; Waldmann, T.; Willingham, M. C.; Fitzgerald, D. J.; Pastan, I. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 8291–8295.
21. Tai, M.-S.; Mudgett-Hunter, M.; Levinson, D.; Wu, G.-M.; Haber, E.; Oppermann, H.; Huston, J. S. *Biochemistry* **1990**, *29*, 8024–8030.
22. Glockshuber, R.; Malia, M.; Pfitzinger, I.; Plückthun, A. *Biochemistry* **1990**, *29*, 1362–1367.
23. Parmley, S. F.; Smith, G. P. *Gene* **1988**, *73*, 305–318.
24. Smith, G. P. *Science* **1985**, *228*, 1315–1317.
25. McCafferty, J.; Griffiths, A. D.; Winter, G.; Chiswell, D. J. *Nature* **1990**, *348*, 552–554.
26. Stanker, L. H.; Kamps-Holtzapple, C.; Friedman, M. *J. Agric. Food Chem.* **1994**, *42*, 2360–2366.
27. Friedman, M. In *Supercritical Fluid Science and Technology*; Johnston, K.; Penninger, J.M.L., eds.; American Chemical Society: Washington, DC, 1992, ACS Symposium Series 406; 429–462.
28. *Recombinant Phage Antibody System: Overview*, Pharmacia Biotech Bulletin No. XY-038-00-02, **1993**.
29. Stanker, L. H.; Watkins, B.; Vanderlaan, M.; Ellis, R.; Rajan, J. In *Immunoassays for Trace Chemical Analysis: Monitoring Toxic Chemicals in Humans, Food, and the Environment*; Vanderlaan, M.; Stanker, L. H.; Watkins, B. E.; Roberts, D. W., eds.; American Chemical Society: Washington, DC, 1991, ACS Symposium Series 451; pp 108–123.
30. Lee, H. A.; Wyatt, G.; Garrett, S. D.; Yanguela, M. C.; Morgan M. R. A. In *Immunoanalysis of Agrochemicals: Emerging Technologies*; Nelson, J. O.; Karu, A. E.; Wong, R. B., eds.; American Chemical Society: Washington, DC, 1995, ACS Symposium Series 586; pp 22–30.
31. Orlandi, R.; Güssow, D. H.; Jones, P. T.; Winter, G. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 3833–3837.
32. Heinrichs, A.; Milstein, C.; Gherardi, E. *J. Immunol. Methods* **1995**, *178*, 241–251.

RECEIVED July 6, 1995

Appendix

The number of companies that handle assay kits is increasing exponentially. In large part this is because there is a need for fast, dependable and accurate assay methods for residues both in the laboratory and for on-site evaluation. For the convience of the reader, we have attempted to list companies, around the world, that handle assay kits. Authors from this volume have submitted the names, addresses and phone numbers of the various companies listed, and they have been compiled by the editors. The editors have not evaluated any products from these manufactures nor have they validated the compiled information. Finally, this list is undoubtely not a complete compilation of companies; however, it should serve as a usefull starting point for the reader interested in obtaining further information on the availability of particular immunoassay kits.

ABC Research Co., 3437 SW 24th Ave., Gainsville, FL 32607, USA
☎ 904–372–0436
Fax 904–378–6483

Accurate Chemical & Scientific Corporation, 300 Shames Dr., Westbury, NY 11590, USA
☎ 516–333–2221
Fax 516–997–4948

Alta Analytica, 5070 Robert J. Matthews Pkwy., Suite 2, El Dorado Hills, CA 95630, USA
☎ 916–933–1640
Fax 916–933–0940

Aquaria S.R.L., Palazzo Marco Polo, Il Girasole, 20084 Lacchiarella, ITALY
☎ 39–2–9009–1399
Fax 39–2–905–4861

Arachem, 85A, Jalan SS 21/60, Damansara Utama, 47400 Petaling Jaya, Selangor Darul Ehsan, MALAYSIA
☎ 603–719–6668
Fax 603–719–6673

Axel Serrau GmbH, Stefansbecke 42, 4322 Sprockhovel, GERMANY
☎ 49–2–339–6037
Fax 49–2–339–6030

Baker Chemikalien, H. S. Richardson Straße, 64521 Gross, Gerau, GERMANY
☎ 49–615–271–0556
Fax 49–615–271–0399

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Published 1996 American Chemical Society

In Immunoassays for Residue Analysis; Beier, R., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

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☎ 49–669–891–9250
Fax 49–669–891–9252

BASF Corporation, Agricultural Research Station, Environmental Chemistry, P.O. Box 220, 67117 Limburgerhof, GERMANY
☎ 49–623–6680
Fax 49–623–660690

Bioanalytica Biotechnology, 11 Laskareos Str., 11471 Athens, GREECE
☎ 301–643–6138
Fax 301–646–2748

Biocode Ltd., University Road, Heslington, York, ENGLAND, YO1 5DE, UK
☎ 44–190–443–0616
Fax 44–190–443–0495

BioDesign International, 105 York St., Kennebunk, ME 04043, USA
☎ 207–985–1944
Fax 207–985–6322

Bioenterprises Pty Ltd., 28 Barcoo St., Roseville, N.S.W. 2069, AUSTRALIA
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Fax 61–2–417–7858

BIOMAN Products Inc., 400 Matheson Blvd., Unit #4, Mississauga, Ontario, L4Z IN8, CANADA
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Fax 905–890–0370

Biomedica Gesellschaft, Divischgasse 4, 1210 Wien, AUSTRIA
☎ 43–1–292–3527
Fax 43–1–290–1361

bioMérieux S. A., 69280 Marcy l'Etoile, Lyon, FRANCE
☎ 33–7–887–2000
Fax 33–7–887–2090

bioMérieux Ltd., Grafton House, Grafton Way, Basingstoke, Hants, ENGLAND, UK
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Fax 44–125–681–6863

bioMérieux Vitek Inc., 595 Anglum Dr., Hazelwood, MO 63042–2395, USA
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Fax 314–731–8800

BioNebraska, Inc., 3820 NW 46th St., Lincoln, NE 68524, USA
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Fax 402–470–2345

Bio-Tek Instruments Inc., Highland Industrial Park, PO Box 998, Winooski, VT
05404–0998, USA
☎ 802–655–4040
Fax 802–655–7941

Biotic Chemical Co., 7F-2, No. 6, Lane 42, Sec 2, Yung-An S. Road, Lu-Chow,
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Fax 886–2–848–4512

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Centocor Ltd., Captia House, Broadford Park, Shalford, Guildford, Surrey,
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Fax 44–148–357–4822

Charm Sciences, Inc., 36 Franklin St., Malden, MA 02148–4120, USA
☎ 617–322–1523
Fax 617–322–3141

Chemopol Ltrd., Kodanska 46, 100 10 Prague-10, CZECH REPUBLIC
☎ 42–26–715–1111
Fax 42–26–731–1092

CIS Ltd., Unit 5, Lincoln Park, Business Centre, Lincoln Road, High Wycombe,
Bucks, ENGLAND, HP12 3RD, UK
☎ 44–149–453–5922
Fax 44–149–452–1785

Cortecs Diagnostics, Techbase 1, Newtech Square, Deeside Industrial Park,
Deeside, CLWYD, ENGLAND, CH5 2NT, UK
☎ 44–124–428–8781
Fax 44–124–428–0221

Dakila Trading Corp., 208 Pilar St., Mandaluyong, Rizal, Metro Manila,
PHILIPPINES
☎ 632–707511–15
Fax 632–721–0739

Diagnostic Products Corporation (DPC), 5700 West 96th Street, Los Angeles, CA 90045, USA
☎ 213–776–0180
Fax 213–642–0192

Diagnostix Inc., 5730 Coopers Ave. #27, Mississauga, Ontario, L4Z 2E9, CANADA
☎ 905–890–6023
Fax 905–890–6024

Digen Ltd., 65 High St., Wheatley, Oxford, ENGLAND, OX33 1XT, UK
☎ 44–186–587–2156
Fax 44–186–587–2413

Dr. Bommeli AG, Stadionstraße 12, CH-3097 Liebefeld-Bern, SWITZERLAND
☎ 31–972–3444
Fax 31–972–2901

DuPont Agricultual Products, Experimental Station 402, Wilmington, DE 19880-0402, USA
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Fax 302–695–4296

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Fax 606–266–9759

EM Science, 480 democrat Road, PO Box 70, Gibbstown, NJ 08027–0070, USA
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Fax 609–423–4389

Ensys Environmental Products, Inc., PO Box 14063, Research Triangle Park, NC 27709, USA
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Fax 919–941–5519

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Fax 31-55-425017

FLUROCHEM LTD., Wesley St., Old Glossop, Derbyshire, ENGLAND, Sk13 9RY, UK
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Fax 408/745-0243

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Fax 207-856-0474

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Fax 49-40-532-891-11

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Fax 65–779–1112

In Sung Hi-Tech Co., Ltd., In Sung Bldg. 89–111, Shinjung 2-Dong, Yangcheon-ku, Seoul, KOREA
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Fax 822–644–1996

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Pierce, PO Box 117, Rockford, IL 61105, USA
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Fax 32–3–454–1888

SCETI Company, Ltd., DF Bldg. 2–2–8 Minami-Aoyama, Minato-Ku, Akasaka PO Box 47, Tokyo 107, JAPAN
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Fax 81–3–3404–4472

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☎ 852–543–7442
Fax 852–541–4089

Scientific Certification Systems, The Ordway Building, One Kaiser Plaza, Suite 901, Oakland, CA 94612, USA
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Fax 302–456–6770

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☎ 49–89–6513–1350
☎ 49–89–6513–1353
Fax 49–89–6513–1398

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***Production:* Amie Jackowski**
***Indexing:* Deborah H. Steiner**
***Acquisition:* Michelle D. Althuis**
***Cover design:* Peggy Corrigan and Ross C. Beier**

Printed and bound by Maple Press, York, PA