Development of Monoclonal ELISAs for Azinphos-methyl. 2. Assay Optimization and Water Sample Analysis

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Two enzyme-linked immunosorbent assays (ELISA) for the insecticide azinphos-methyl have been optimized and characterized. Both ELISAs are based on monoclonal antibodies produced from an immunogen with a hapten containing a phthalimido moiety and on protein conjugates of heterologous ligands containing a 1,2,3-benzotriazine group. Assay I was performed in the conjugate-coated ELISA format and assay II in the antibody-coated format. Several physicochemical factors (ionic strength, pH, incubation times, and Tween 20 and BSA concentrations) that influence assay performance were studied and optimized. Regarding specificity, both monoclonal immunoassays highly cross-reacted with azinphos-ethyl and phosmet. Finally, both assays were applied to the analysis of azinphos-methyl in spiked real water samples. For assay I the sensitivity, estimated as the I_{50} value, was 0.40 nM, with a practical working range between 0.10 and 1.75 ng/mL and a limit of detection of 0.05 ng/mL. For assay II the sensitivity was 1.01 nM, with a practical working range between 0.32 and 2.54 ng/mL and a limit of detection of 0.08 ng/mL.

Keywords: *Azinphos-methyl; azinphos-ethyl; phosmet; immunoassay; ELISA*

INTRODUCTION

The presence of contaminants of both industrial and agricultural origin in food and the environment has led to increased public concern expressed in established regulations and monitoring programs. Much effort is, therefore, being made to improve the sensitivity and reliability of existing methods of analysis and to develop alternative or complementary analytical technology. Studies dealing with the improvement of classical methods of analysis are constantly being published (Tauler et al., 1996; Berger, 1997). Complementary methodology has also been developed in recent years, for example, gas chromatography together with mass spectrometry, solid phase extraction procedures fitted with chromatography analysis, and flow injection analysis (Hogendoorn et al., 1996; Lacorte and Barceló, 1996). Immunoassays (IAs) have recently been introduced in food, agricultural, and environmental analysis for both screening and quantitative purposes due to their high sensitivity, speed, high sample throughput, simplicity, and low cost.

Pesticides are important contaminants on which attention has been focused. Enzyme-linked immunosorbent assay (ELISA) is the most accepted method based on immunological reactions for pesticide analysis. Many ELISAs for pesticide analysis have been developed in the past two decades, as recently reviewed (Meulenberg et al., 1995; Szurdoki et al., 1996). Moreover, rapid advances have occurred in the development and validation of IAs for pesticide analysis, and a growing interest in IAs has been perceived (Aga and Thurman, 1997). Nevertheless, as immunological analysis of pesticides is relatively new in the field, its acceptance by analytical chemists is not complete yet. A broader spectrum of IAs for the detection of pesticides and further characterization and optimization of the product is still required. Moreover, expectations for analytical performance and adequate design of analytical strategies by chemists as well as by IA developing laboratories should be realistic. Guidelines for the adequate promotion and use of immunochemical methods as analytical tools have been suggested by the Analytical Environmental Immunochemical Consortium and others (Mihaliak and Berberich, 1995; Reeves et al., 1996; Williams et al., 1996). The key to proper ELISA development is the understanding of the properties of the method itself. Factors affecting assay performance need to be studied and optimized to achieve this knowledge and to produce sensitivite assays (Lucas et al., 1995; Hayes et al., 1995).

Azinphos-methyl (AM) is a common insecticide used for pest control. Residues of this pesticide have been found in apples, tomatoes, oranges, and even water (Neidert and Saschenbrecker, 1996; Hernández et al., 1996; Roy et al., 1997). Their analysis is generally performed by liquid−solid extraction followed by gas chromatographic techniques, either using nitrogen phosphorus or mass spectrometric detection. However, attempts to improve the extraction procedures, the stability of the pesticide, and the sensitivity of the method are currently being published (Lacorte et al., 1995; Ahmad et al., 1995). The purpose of this study was to develop, optimize, and characterize sensitive ELISAs based on monoclonal antibodies (MAbs) for the detection and quantification of AM in water samples.

MATERIALS AND METHODS

Reagents and Instruments. Pesticide standards, Pestanal grade, were purchased from Riedel-de-Haën AG (Seelze, Germany). Hapten−protein conjugate preparation and LIB-MFH14 and LIB-MFH110 MAbs production are described in

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the preceding paper (Mercader and Montoya, 1999). Bovine serum albumin (BSA) fraction V was from Boehringer Mannheim (Barcelona, Spain). Peroxidase-labeled rabbit anti-mouse immunoglobulins were from Dako (Glostrup, Denmark). Tween 20, *o*-phenylenediamine (OPD), and anhydrous *N*,*N*-dimethylformamide (DMF) were from Sigma-Aldrich-Fluka Química (Madrid, Spain). All other chemicals and organic solvents used were of reagent grade or better. Polystyrene high-binding ELISA plates were from Costar (Cambridge, MA) and Pyrex borosilicate glass disposable culture tubes from Corning Inc. (Corning, NY). Plates were washed in a 96 PW washer from SLT-Labintruments GesmbH (Salzburg, Austria), and absorbance values were read in dual-wavelength mode (490−650 nm) with an Emax microplate reader controlled under a SOFTmax PRO software package, version 1.2.0, from Molecular Devices Corp. (Sunnyvale, CA).

ELISAs. Two different ELISAs were chosen for optimization. The first ELISA, called assay I, used the conjugate-coated ELISA format with LIB-MFH14 MAb and OVA−HBA as coating conjugate. The second one, called assay II, used the antibody-coated format with LIB-MFH110 MAb and HRP-MBH as enzyme tracer. Optimum concentrations were determined by bidimensional titration before and after optimization of physicochemical assay parameters. The optimum reagent concentrations were defined as those which gave maximum absorbance around 1.0 in the absence of analyte with minimum reagent expenses. Usually, several combinations of immunoreagents were evaluated to select the most sensitive assay. A final volume of 100 μL/well was used in all steps, and incubations were performed at room temperature to avoid well-to-well temperature variability. Serial dilutions of standards were prepared, using borosilicate glass tubes to prevent adsorption of the analytes to the vials, from a stock solution in DMF 200-fold concentrated from the most concentrated value of the standard curve. Plates were washed four times between steps with washing solution (0.15 M NaCl with 0.05% Tween 20) and, finally, peroxidase activity was revealed with freshly prepared 2 mg/mL OPD and 0.012% H_2O_2 in 25 mM citrate/62 mM sodium phosphate buffer, pH 5.4. The enzymatic reaction was stopped after 10 min by adding 100 μL/well of 2.5 M sulfuric acid. The absorbance was immediately read at 490 nm with a reference wavelength at 650 nm.

Conjugate-Coated ELISA (Assay I). Plates were coated with the appropriate concentration of the heterologous OVA−HBA conjugate in coating buffer (50 mM carbonate−bicarbonate, pH 9.6) by overnight incubation. The plates were then washed as described above, and 50 μL/well of standard or sample solution followed by 50 μL/well of LIB-MFH14 MAb solution at twice the desired assay concentrations was added. These solutions were prepared in different buffers depending on the experiment. Inhibition standard curves were prepared by serial dilutions from 200 to 0.002 nM competitor with a dilution factor of 5. Competitive immunological reaction was allowed to take place for 1 h, and then plates were washed as before. Afterward, a 1/2000 dilution of peroxidase-labeled rabbit anti-mouse immunoglobulins in PBS (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 0.05% Tween 20 was added, and the reaction was allowed for 30 min (nonoptimized conditions) or 1 h (optimized time). After washing, retained peroxidase activity was determined as indicated. Coating conjugate solutions were kept frozen at −20 °C in PBS, and concentrated MAb solutions were kept precipitated with 1 volume of saturated ammonium sulfate solution at 4 °C from which intermediate solutions in PBS were prepared and kept at 4 °C.

Antibody-Coated ELISA (Assay II). Plates were coated with LIB-MFH110 MAb at the appropriate concentration in coating buffer by overnight incubation. After washing, 50 μL/well of analyte (standards or samples) and 50 μL/well of the heterologous enzyme tracer HRP−MBH at twice the desired assay concentrations were added. Analyte and enzyme tracer were dissolved in different buffers depending on the experiment. Standard curves were prepared by serial dilutions from 2000 to 9.6×10^{-4} nM competitor with a dilution factor of 8. Competitive immunological reaction took place for 1 h, and then plates were washed as described. Retained HRP activity was developed and, afterward, plates were read as indicated. Stock solutions of HRP conjugates were diluted with 1 volume of a saturated ammonium sulfate solution, bubbled with pure argon air, and stored at 4 °C. An intermediate solution was prepared every one or two weeks in PBS containing 2% BSA and 0.01% sodium azide and kept at 4 °C in amber glass vials.

Data Analysis. Standards and samples were run in triplicate or quadruplicate wells, and the mean absorbance values were processed. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration and fitted to a four-parameter logistic equation using a Sigmaplot software package from Jandel Scientific (Erkrath, Germany)

$$y = \{(A - D)/[1 + (x/C)^B]\} + D \quad (1)$$

where A is the asymptotic maximum (absorbance in the absence of analyte, A_{max}), B is the curve slope at the inflection point, C is the x value at the inflection point (corresponding to the analyte concentration that reduces A_{max} to 50%), and D is the asymptotic minimum (background signal). When required, curves were normalized by expressing experimental absorbance values (y) as $(y/A_{max}) \times 100$. Assay sensitivity was defined as the analyte concentration that reduced the maximum absorbance signal in competitive assays by 50% (I_{50}) and corresponds to the C parameter of the sigmoidal curve.

Cross-Reactivity Studies. Inhibition curves with a set of pesticides or related compounds were executed in both optimized ELISAs and their I_{50} compared to that from a curve of AM run in the same plate. Cross-reactivity (CR) was calculated as follows:

$$CR = [I_{50}(AM)/I_{50}\,(compound)] \times 100 \quad (2)$$

Physicochemical Parameter Influence and Optimization. Several physicochemical factors influencing the immunological reaction were studied in both ELISAs. Modification of A_{max} and I_{50} parameters of the standard curves was evaluated under different conditions. Previously, immunoreagent concentrations used in these experiments were optimized by noncompetitive and competitive assays. Newly optimized assay conditions for each physicochemical parameter were used in subsequent experiments.

Tween 20 and BSA Concentrations. Requirement and optimum concentrations of these two common additives were studied. Competitive assays were performed using different concentrations of each separately. Briefly, standard analyte curves were prepared in PBS while MAb in assay I or enzyme tracer in assay II were added to serial dilutions of PBS/0.8% BSA (w/v) or PBS/0.4% Tween 20 (v/v) diluted in PBS (from 0.8 to 0% BSA or from 0.4 to 0% Tween 20). Thereafter, assays were executed as described. In these experiments, enzyme tracer stock solution used for assay II did not contain BSA.

Buffer Ionic Strength and pH. Competitive curves were performed with buffers of different ionic strength values but constant pH 7.4. Standard curves were prepared in bidistilled water, and a constant concentration of MAb in assay I or enzyme tracer in assay II was added to serial dilutions of a concentrated buffer (from 16× PBS to 0) in bidistilled water. Thereafter, assays were conducted as described. For assay II, buffers contained the optimized concentration of BSA. To evaluate the influence of pH, a set of buffers was prepared with constant ionic strength but with different pH values. First, a stock solution of 40 mM citrate, 40 mM hydrogen phosphate, and 40 mM Tris was prepared (final pH was 9.95). Different volumes of 5 N HCl were added to aliquots of the stock solution to reach the desired pH. The ionic strength of each solution was determined according to the formula

$$I = {}^1/_2 \sum c_i z_i^2 \quad (3)$$

where I is the ionic strength value, c is the concentration of each ion at equilibrium, and z is its charge. Afterward, the appropriate volume of 2 M NaCl was added to bring each

buffer to the same ionic strength value (I = 360 mM), and volumes were made equal with bidistilled water. For assay II, BSA was also added to each buffer from a concentrated solution to reach the optimized concentration. Competitive assays were subsequently performed as described by preparing standard curves of AM in bidistilled water and the MAb or enzyme tracer in the problem buffers.

Time. Competitive assays were conducted with different incubation times using the same standard and immunoreagent preparations, all of them in PBS. Enzyme tracer solution was prepared from the described concentrated stock containing the appropriate amount of BSA. When all of these conditions were established, bidimensional noncompetitive assays were performed again and immunoreagent concentrations readjusted to afford a maximum absorbance around 1.0 and the lowest I_{50}.

Studies of Solvent Effects. The assays' tolerance to methanol, ethanol, 2-propanol, acetonitrile, tetrahydrofuran, and acetone was evaluated between 0 and 10% solvent concentration (v/v). Standard curves of AM were prepared in PBS, and MAb or enzyme tracer was dissolved in PBS containing different amounts of solvent. Tolerance to DMF was also evaluated because standards were dissolved in this solvent. In this case, standard curves of AM were prepared by serial dilutions in PBS with several initial DMF concentrations.

Limits of the Working Range. AM recovery was determined in both optimized ELISAs from spiked buffer samples. Standards with several concentrations covering the complete inhibition range of the curves were prepared in DMF, and each one was diluted 10^5 times in PBS before assaying. Samples were dispensed in quadruplicate wells, and a standard curve prepared in PBS was run in each plate also in quadruplicate wells. MAb for assay I and enzyme tracer for assay II were prepared in PBS, the latter from a stock solution containing 2% BSA.

The limits of detection (LODs) of both IAs were also experimentally determined. Buffer solutions were spiked with AM at several concentrations inhibiting the ELISA maximum signal around 10% (I_{10}). The lowest value that always afforded positive results was taken into account as the putative LOD. Nevertheless, as suggested by Brady (1995), variability inherent to the measurement process should be considered, and a standardized approach is required. As proposed, this parameter may be calculated by inserting the response obtained for an unknown in the equation

$$y = y_0 - ts(1/n_0 + 1/n_1)^{1/2} \quad (4)$$

where y is the response corresponding to the LOD, y_0 is the mean response of the zero dose replicates, n_0 is the number of those replicates, and n_1 represents the number of replicates run of an unknown. The t statistic is the percentile of the Student's t distribution for a one-sided test at 95% probability with $n_1 - 2$ degrees of freedom, and s is the standard deviation of the response of the unknown.

Spiked Water Sample Analysis. Water samples were fortified with AM to evaluate potential matrix effects on both ELISAs. Final assay dilution of DMF was always 2×10^5. Mean absorbance values obtained from quadruplicate wells were interpolated in a standard curve run in the same plate also in quadruplicate wells. Standard curves and MAb or enzyme tracer were prepared in PBS, the latter from a stock containing 2% BSA. Inhibition curves covered the range between 1000 and 2×10^{-4} nM AM with a dilution factor of 4. Waters tested were Valencia tap water, a commercial bottled water, rainwater collected in a cistern, well water, and water from an irrigation channel of the agricultural area of Valencia. Turbid waters were filtered prior to use. Samples were spiked with several AM concentrations covering the assay working range and then conditioned, that is, buffered and roughly normalized for salt concentration, by adding 1 volume of 10× PBS to 9 volumes of water. A precipitate appeared in hard waters, which was removed by centrifugation. The conductivities of both the original water samples and the final assay solutions were measured.

RESULTS AND DISCUSSION

In the preceding paper (Mercader and Montoya, 1999) the synthesis of haptens and the production of high-affinity MAbs for AM are described. Because assay format can greatly affect sensitivity and specificity, different MAbs were used in three ELISA formats with all, heterologous and homologous, of the conjugates prepared. From this study, LIB-MFH14 with OVA−HBA in the conjugate-coated format (assay I) and LIB-MFH110 with HRP−MBH in the antibody-coated format (assay II) were chosen as the best combinations of immunoreagents and formats. For assay I, optimum reagent concentrations were 200 and 160 ng/mL of OVA−HBA and LIB-MFH14 MAb, respectively, and the I_{50} value obtained for AM was 0.8 nM. When assay II was used, optimum reagent concentrations were 1 μg/mL of LIB-MFH110 MAb and 25 ng/mL of HRP−MBH, with an I_{50} value for AM of 1.3 nM. These concentrations were used in subsequent experiments by which several factors affecting ELISA performance were studied.

Physicochemical Parameter Optimization. ELISA sensitivity and reliability can be enhanced by studying the influence of some physical and chemical parameters of the assay (Manclús and Montoya, 1996a). Buffer chemical properties as well as assay physical conditions are the most immediate parameters to be analyzed and optimized.

Effect of Additives Tween 20 and BSA. Tween 20, a nonionic surfactant, and BSA are the two most commonly used additives in ELISA to reduce nonspecific interactions. Nevertheless, both additives can greatly influence IA characteristics, and they may not be required (Stanker et al., 1989; Lee et al., 1995). The influence of different concentrations of Tween 20 and BSA on A_{max}, I_{50}, and the CVs at the inflection point of the AM standard curve was studied in assays I and II. It was observed that Tween 20 and BSA concentrations had similar effects on assay I. Concentrations >0.05% reduced A_{max}, whereas I_{50} values increased with higher additive concentrations. Tween 20 and BSA influenced assay II in a different way. Tween 20 concentrations >0.05% reduced A_{max} and increased I_{50} values. A_{max} was only slightly reduced with low amounts of BSA, whereas, under these conditions, I_{50} values did not change considerably. The effect of the concentration of any of these additives on CVs was not high in assay I, whereas some variation was observed in assay II.

When IA conditions are not fully optimized, the A_{max}/I_{50} ratio is a valuable estimate of the effect of a certain factor on the ELISA sensitivity, the highest ratio indicating the highest sensitivity (Manclús and Montoya, 1996b). Figure 1A shows the variation of this ratio as a factor of additive concentration. It is shown that Tween 20 similarly affected both assays: the lower its concentration, the higher their sensitivity. The same behavior was observed for BSA in assay I. Nevertheless, in assay II the maximum A_{max}/I_{50} ratio was achieved when some BSA was added. In conclusion, the use of Tween 20 should be avoided in these ELISAs, as well as BSA in assay I, whereas a concentration of BSA of 0.002% should be provided for assay II. Under these conditions, CVs remained below 4 and 8% in assays I and II, respectively.

Ionic Strength and pH Influence. It is important to know how changes in buffer conditions can affect the

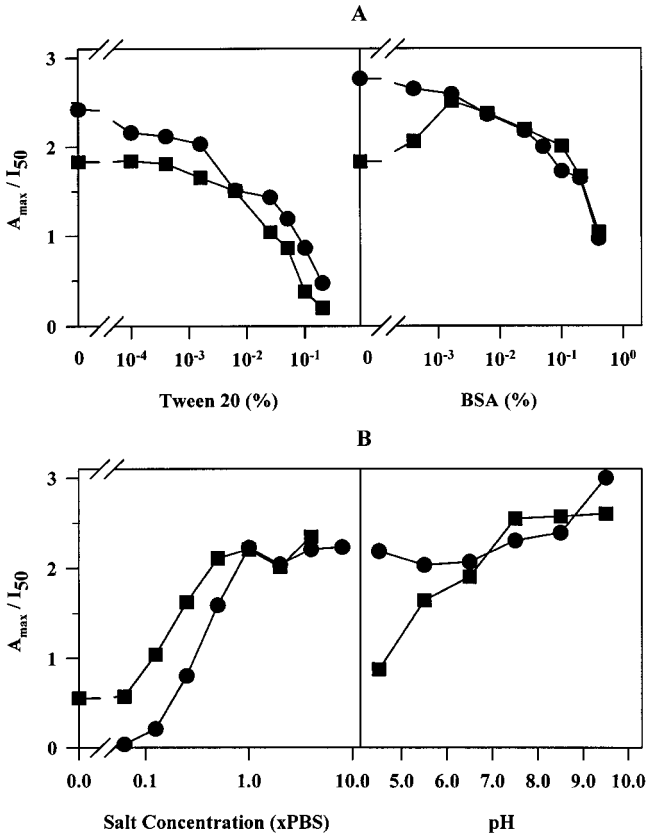


Figure 1. Representation of the influence of (A) Tween 20 and BSA and (B) salt concentration and pH on the A_{max}/I_{50} ratio for each ELISA: (●) assay I; (■) assay II.

ELISA characteristics (Krämer et al., 1994; Abad and Montoya, 1997). The variation of A_{max}, I_{50}, and CVs at the inflection point of the AM inhibition curve was studied for assays I and II with different ionic strength values and pH conditions. For assay I, high salt concentrations deeply reduced A_{max} and raised CVs, whereas I_{50} values quickly rose with low salt concentrations. No assay could be performed in this ELISA in bidistilled water. The lowest salt concentration tested that gave a competitive curve was 0.06× PBS. At extreme tested pH values, A_{max} and I_{50} values were lowered. A reverse effect was observed when the ionic strength influence was studied in assay II; that is, A_{max} was deeply reduced with low salt concentrations. I_{50} values and CVs did not vary significantly in this case. Low pH values reduced A_{max} and raised CVs, whereas no remarkable effect was observed on I_{50} values.

Figure 1B shows the dependence of the A_{max}/I_{50} ratio on salt concentration and pH. The opposite effect of ionic strength on A_{max} in assays I and II was counterbalanced by variation in the I_{50} values in such a way that the A_{max}/I_{50} ratio followed nearly the same pattern in both assays. A plateau was reached, so the lowest salt concentration affording the highest A_{max}/I_{50} ratio was selected (1× PBS). pH variation had little effect on this ratio in assay I, although it seemed to be higher at pH 9.5. Nevertheless, a pH of 7.4 was mantained because AM is not stable at basic pH. In the case of assay II, the A_{max}/I_{50} ratio was lowered at pH values below 7.0, and for the same reason as before, the pH chosen as optimum was 7.4. In any of the selected conditions, CVs remained below 5 and 8% in assays I and II, respectively. The marked effect of the ionic strength on ELISA parameters can be related to the hydrophobic properties of the analyte and had direct implications when real samples were to be analyzed. Similar results were obtained by Manclús and Montoya (1996b) for chlorpyrifos immunoassays in two different ELISA formats.

Optimum Incubation Times. Usually, longer incubation times in ELISA give higher absorbance values, and therefore sensitivities can be improved. Nevertheless, short periods are recommended for ELISA applicability. An experiment was performed with incubation times of 0.5, 1.0, and 2.0 h for the competitive immunological reactions and 0.5 and 1.0 h for the secondary reaction in assay I. The enzymatic reaction was kept for 10 min. Again, the highest A_{max}/I_{50} ratio indicated the most sensitive option. In both assays, longer incubation times afforded higher absorbance values but similar I_{50} values. Curve slopes were not very much affected. The highest sensitivity for assay I was obtained when both immunological reactions were kept for 1 h. For assay II, 1 h of incubation also afforded the highest A_{max}/I_{50} ratio.

Organic Solvent Tolerance. Six organic solvents typically used in AM residue extractions were included in competitive assays of both optimized ELISAs to evaluate their tolerance from 1.25 to 10% solvent contents. A_{max} and I_{50} changes were recorded, and results are shown in Figure 2 for assay I and in Figure 3 for assay II. It can be observed that organic solvent tolerance was greater for the latter assay. Acetonitrile was an exception: although a concentration of 2.5% was tolerated in assay I, amounts >1.25% were not tolerated in assay II. Methanol was the best tolerated solvent in both cases, followed by 2-propanol, although high concentrations of the latter greatly reduced sensitivity in assay I. A 10% concentration of both solvents doubled I_{50} values in assay II. On the contrary, acetone was the worst tolerated solvent in both cases. Also, in assay I, ethanol and tetrahydrofuran were not tolerated, because A_{max} was so greatly reduced that I_{50} values could not be determined. Therefore, solvent contents have to be avoided or controlled because ELISA parameters may be altered.

DMF tolerance was further studied. For assay I, a 50% reduction of A_{max} was observed with 0.02% DMF, whereas no absorbance reduction was observed if the solvent content was reduced to 0.001%. For assay II, A_{max} was reduced to 50% with 0.1% DMF, whereas no reduction was observed if solvent was reduced to 0.001%. Competitive IAs were usually performed with 0.5% DMF in the most concentrated point of the dilution series and with dilution factors of 5 and 8 for assays I and II, respectively. Now, a set of curves was prepared with 0.5, 0.05, 0.005, and 0.0005% DMF in the most concentrated standard value. No difference was observed between any of the curves in three independent experiments using the same dilution factors (data not shown). Moreover, competitive curves performed using standards prepared in methanol presented the same parameters as those typically prepared from stocks in DMF with 0.5% solvent in the most concentrated standard point.

Specificity of the AM Immunoassays. Competitive curves performed with the two finally selected MAbs in their respective optimized assay formats revealed that these MAbs also recognized phosmet, to an even higher extent than AM (Table 1). In both ELISAs, AM and azinphos-ethyl were similarly recognized, as expected, because these molecules share the same ring system. CRs with other pesticides were very low or

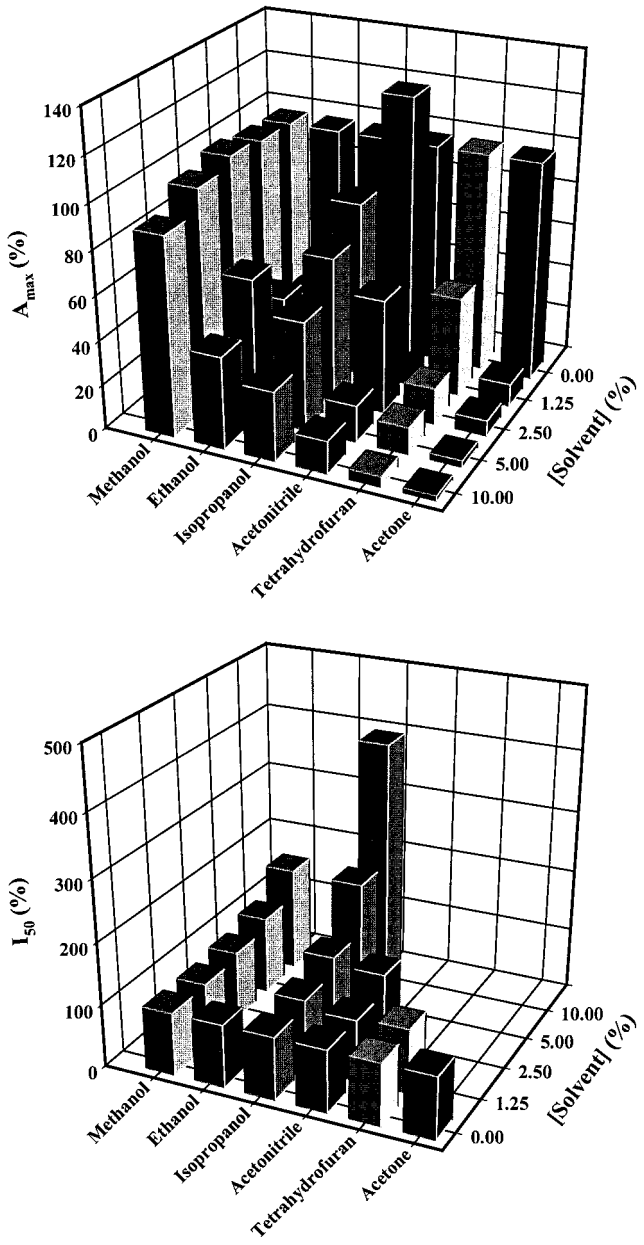


Figure 2. Influence of different organic solvents on the analytical parameters of the azinphos-methyl competitive standard curve in assay I. Results are the mean of three independent experiments with four replicate wells per plate.

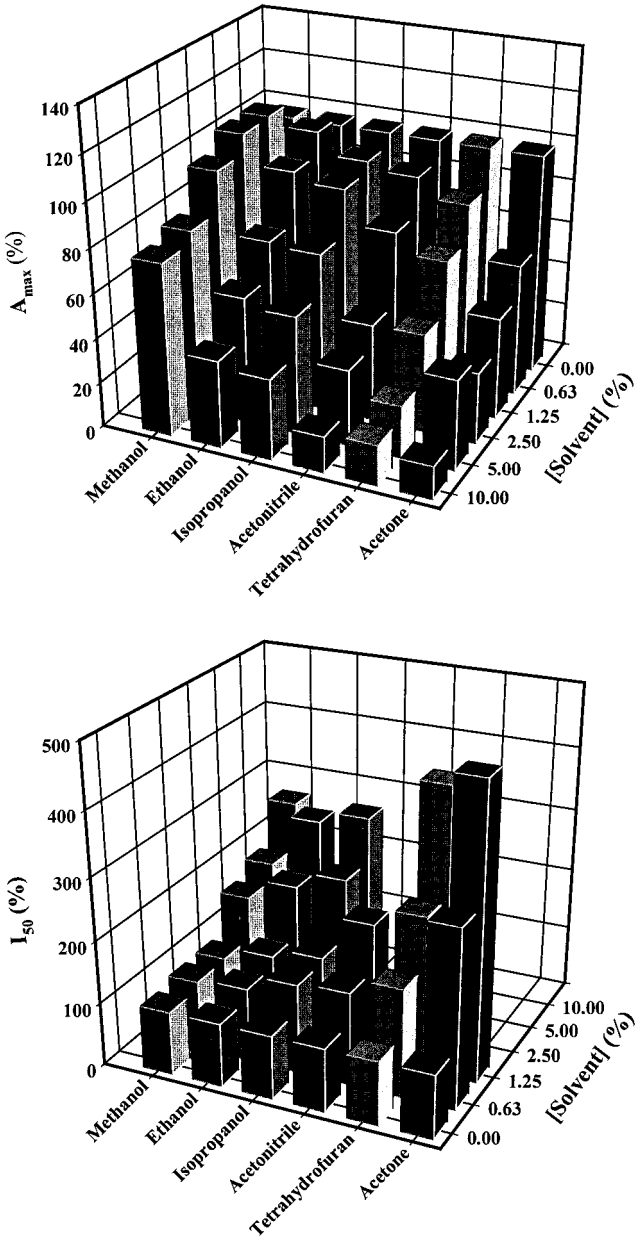
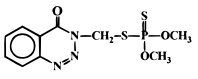
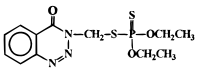
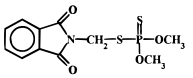
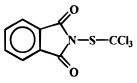
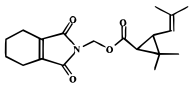
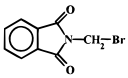
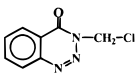


Figure 3. Influence of different organic solvents on the analytical parameters of the azinphos-methyl competitive standard curve in assay II. Results are the mean of three independent experiments with four replicate wells per plate.

nonexistent: only folpet and tetramethrin had CRs between 1 and 5%. Besides relative CRs shown in Table 1, corresponding to pesticides structurally related to the AM or phosmet ring systems, other pesticides were tested for CR. Most of them are common organophosphates such as dialifos, fosalone, methidathion, quinalphos, diazinon, parathion, chlorfenvinphos, bentazone, chlorpyrifos, and chlorpyrifos-methyl. Aldicarb, captan, captafol, dimethoate, demeton-*S*-methyl sulfone, and carbaryl were also tested. In all cases, CRs observed were <0.2% in both assays. Apart from pesticides, compounds used in the synthesis of haptens or that are degradation products of AM or phosmet were included in the CR study. Only *N*-(chloromethyl)benzazimide, used in the synthesis of MBX-type haptens (Mercader and Montoya, 1999), was recognized significantly. The corresponding molecule for MFX-type haptens, *N*-(bromomethyl)phthalimide, was also tested, but it was not recognized probably due to steric hindrance of the bromine atom. Other molecules tested but not recognized were *N*-(hydroxymethyl)benzazimide, benzazimide, *N*-hydroxybenzazimide, *N*-hydroxyphthalimide, phthaloylglycine (FA), anthranilic acid, and phthalimide. LIB-MFH110 MAb (assay II) did not recognize FA, either in the free form or conjugated to HRP. On the contrary, although LIB-MFH14 MAb (assay I) did not recognize free FA, the OVA−FA conjugate provided enough signal to perform competitive assays. This result suggests an electronic configuration change in the hapten when it was conjugated. It is likely that these MAbs interact with the *N*-(mercaptomethyl)benzazimide or phthalimide ring systems, thus demonstrating the usefulness of *fragmentary haptens* as immunogens to produce high-affinity MAbs. Nevertheless, high-affinity MAbs discriminating between AM and phosmet were not obtained, although MAbs specific to phosmet could be produced from MFX-type haptens as suggested in the previous article (Mercader and Montoya, 1999). Lack

Table 1. Specificity of the Optimized AM Immunoassays

compound	structure	CR[a] (%) *Assay I* (LIB-MFH14 MAb)	CR[a] (%) *Assay II* (LIB-MFH110 MAb)
Azinphos-methyl		100.0	100.0
Azinphos-ethyl		82.7[b]	111.7[b]
Phosmet		136.9[b]	169.4[b]
Folpet		1.3	1.4
Tetramethrin		3.9	5.5
N-(Bromo methyl) phthalimide		1.4	1.8
N-(Chloro methyl) benzazimide		76.6	57.9

[a] Percentage of cross-reactivity was calculated from four curves run in triplicate wells. [b] Values were calculated from 10 curves run quadruplicate wells.

of antibody specificity is sometimes considered a drawback for residue analysis by IAs. However, this may be taken as an advantage, as careful choice of antibodies together with an appropriate statistical analysis may turn IAs showing several cross-reactivities into potential multiresidue systems (MELISA; Jones et al., 1994; Wortberg et al., 1996).

Analytical Parameters of the Optimized AM Immunoassays. After physicochemical parameters of the assays were optimized, reagent concentrations were readjusted to reach a maximum absorbance around 1.0 in the absence of analyte by noncompetitive bidimensional titration. Final assay conditions are summarized in Table 2. Sensitivities, estimated as I_{50} values for AM, were 0.40 and 1.01 nM, with curve slopes of 1.26 and 0.81 for assays I and II, respectively. Typical competitive curves for AM in both ELISAs are shown in Figure 4. The working range was experimentally established by spiking assay buffer with different amounts of AM in the 10−90% inhibition range and determining the recovery in both assays. Considering as accurate those values between 80 and 120% recovery with CVs below 20%, the assay limits of quantification were 0.15−2.75 nM (20−90% inhibition) and 0.50−4.00 nM (35−75% inhibition) for assays I and II, respectively (Table 3). In assay I, the practical working range for samples is between 0.10 and 1.75 ng/mL, taking into account a dilution factor of 2 inherent to sample measurement in ELISA. For assay II, the experimental working range was significantly narrower than the theoretical one (20−80% inhibition), samples being accurately measured between 0.32 and 2.54 ng/mL. In both assays, CVs were higher when lower concentrations were determined.

Table 2. Summary of the Parameters of the Optimized AM Immunoassays

	ELISA assay I	ELISA assay II
format	conjugate-coated	antibody-coated
immunoreagent concentrations		
[OVA−HBA] (ng/mL)	200	
[HRP−MBH] (ng/mL)		20
[LIB-MFH14] (ng/mL)	60	
[LIB-MFH110] (ng/mL)		1000
incubation time	1 h +1 h	1 h
optimum buffer conditions		
Tween 20 (%)	0	0
BSA (%)	0	0.002
salt concentration	1× PBS	1× PBS
pH	7.4	7.4
best tolerated solvent (%)	methanol (5)	methanol (1.3)
other recognized pesticides	azinphos-ethyl, phosmet	azinphos-ethyl, phosmet
analytical characteristics[a]		
A_{max}	1.26	1.12
A_{min}	0.02	0.00
I_{50} (nM)	0.40	1.01
20−80% inhibition (nM)	0.14−1.32	0.18−5.65
10% inhibition (nM)	0.07	0.07
operational limits[b]		
working range (ng/mL)	0.10−1.75	0.32−2.54
LOD (ng/mL)	0.05	0.08

[a] Data are the average of 10 independent standard curves. [b] Data were obtained from six independent experiments spiking assay buffer.

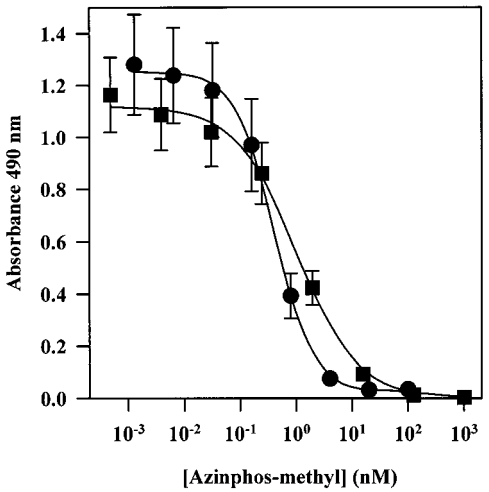


Figure 4. Representative standard curves for azinphos-methyl under optimized assay conditions using assay I (●) and assay II (■). Each point represents the mean ± SD of 10 independent curves run in quadruplicate wells. Competitive curves are fitted to experimental points by the four-parameter logistic equation.

LODs were also experimentally determined as described under Materials and Methods. The lowest AM concentrations tested that always afforded positive results were 0.08 and 0.13 nM for assays I and II, respectively. When these values were inserted into the equation proposed by Brady (1995), lower concentrations were obtained, but, as suggested, the highest values were considered. Therefore, LODs for AM were established at 0.05 and 0.08 ng/mL for assays I and II, respectively.

Analysis of Spiked Water Samples. Analytical

Table 3. Recovery of AM from Spiked Buffer

assay I				assay II			
AM (nM) in assay	AM recovered[a] (nM)	recovery (%)	CV (%)	AM (nM) in assay	AM recovered[a] (nM)	recovery (%)	CV (%)
2.75[b]	3.15	114.5	10.7	16.00	24.40	152.5	19.9
1.76	2.12	120.2	4.7	8.00	10.32	129.0	15.6
1.41	1.59	112.9	8.3	**4.00**[b]	4.76	119.0	13.4
0.19	0.17	88.4	17.9	2.00	2.16	108.0	11.5
0.15[b]	0.16	104.0	17.8	1.00	0.92	92.0	13.6
0.12	0.08	66.9	26.2	0.80	0.79	98.8	10.7
0.10	0.08	86.6	26.8	**0.50**[b]	0.44	88.0	20.6
0.08	0.06	74.0	39.4	0.25	0.16	64.0	32.8
0.04	0.02	55.0	63.3	0.13	0.05	38.5	63.9
0.02	0.03	125.0	89.8	0.06	0.02	33.3	116.2

[a] Data are the mean of six independent determinations run in quadruplicate wells. Data were obtained by interpolation in a standard curve run in quadruplicate wells in the same plate. [b] Limits of quantitation are shown in boldface characters.

Table 4. Recovery of AM from Spiked Water Samples

	assay I				assay II			
water sample[a]	AM added (ng/mL)	AM recovered[b] (ng/mL)	recovery (%)	CV (%)	AM added (ng/mL)	AM recovered[b] (ng/mL)	recovery (%)	CV (%)
bottled	0.10	0.07	70.0	78.5	0.10	0.04	40.0	57.5
	0.25	0.13	52.0	32.1	0.50	0.41	82.0	24.9
	0.50	0.48	96.0	25.1	1.00	0.82	82.0	19.7
	1.00	1.12	112.0	8.8	2.00	1.92	96.0	10.4
cistern	0.10	0.10	100.0	26.7	0.10	0.12	120.0	33.6
	0.25	0.20	80.0	23.2	0.50	0.54	108.0	11.8
	0.50	0.43	86.0	8.0	1.00	1.00	100.0	7.1
	1.00	1.15	115.0	5.2	2.00	2.22	111.0	12.2
well	0.10	0.14	140.0	21.9	0.10	0.08	80.0	54.1
	0.25	0.21	84.0	25.7	0.50	0.46	92.0	18.6
	0.50	0.41	82.0	9.0	1.00	1.00	100.0	27.8
	1.00	1.23	123.0	6.9	2.00	1.81	90.5	11.6
tap	0.10	0.17	170.0	27.4	0.10	0.24	240.0	36.1
	0.25	0.27	108.0	17.9	0.50	0.75	150.0	14.7
	0.50	0.50	100.0	9.2	1.00	1.12	112.0	17.2
	1.00	1.25	125.0	9.6	2.00	2.39	119.5	10.6
channel	0.10	0.29	290.0	24.2	0.10	0.37	370.0	16.1
	0.25	0.39	156.0	12.9	0.50	0.75	150.0	7.9
	0.50	0.72	144.0	8.6	1.00	1.16	116.0	13.2
	1.00	1.61	161.0	6.0	2.00	2.44	122.0	11.5

[a] Water samples were treated for pH and ionic strength conditioning with 1 volume of 10× PBS to 9 volumes of sample. [b] Data listed are the mean of eight independent determinations. Samples were run in quadruplicate wells and interpolated in a standard curve run in quadruplicate wells in the same plate.

ELISA reliability is commonly evaluated by spiking matrix samples with the target analyte. Several water samples from a variety of origins were analyzed. Because the ionic strength of the medium was shown to influence both ELISAs, final assay conditions were studied according to this factor. Important differences in sample ionic strengths were observed. Therefore, water samples fortified with different amounts of AM, to final concentrations within the experimental working range of each assay, were conditioned as described (Manclús and Montoya, 1996b) to make their conductivities equal to that of PBS. Results of the analysis, expressed as the percentage of recovery, are shown in Table 4. Added AM was accurately recovered with assay I at concentrations between 0.25 and 1.00 ng/mL in all waters but channel water. For this sample, positive results were obtained when a blank was assayed, suggesting important matrix effects or the presence in the sample of unidentified cross-reacting compounds. As another exception, bottled water sample spiked with 0.25 ng/mL was not accurately determined. With this assay, CVs were generally acceptable, being higher at concentrations <0.50 ng/mL. The determined limit of quantitation of 0.10 ng/mL was accurately measured only in cistern water. With assay II, recoveries were good for all water samples at 1.00 and 2.00 ng/mL. The 0.10 ng/mL sample was outside the determined working range but near the LOD, and values below this limit were obtained in bottled and well water, with high CVs.

CONCLUSIONS

In the preceding paper (Mercader and Montoya, 1999) the production of a set of MAbs for azinphos-methyl was described. Afterward, two IAs using those MAbs were selected, one in the conjugate-coated and the other in the antibody-coated ELISA format. Both ELISAs use heterologous conjugates that improved the sensitivity of the homologous assay. Physicochemical factors known to influence assay performance were optimized in both ELISAs. The withdrawal of Tween 20 was demonstrated as an important improvement in assay performance, whereas BSA was required in the antibody-coated assay. Furthermore, the dependence of the analytical parameters of these ELISAs on ionic strength and pH was extensively evaluated. Usually, polar environments favor interactions between antibodies and hydrophobic analytes. In our assays, salt concentrations lower than 1× PBS reduced assay properties, whereas salt concentrations up to 8× PBS changed curve parameters but did not improve assay sensitivity. Incubation times were also optimized to afford the most sensitive assays. After optimization, assay sensitivities were enhanced and reagent concentrations were significantly lowered. When

organic solvent tolerance was investigated, the antibody-coated ELISA was revealed to tolerate organic solvents better. Methanol has been demonstrated to be the best tolerated solvent, whereas other solvents tested showed little or no tolerance. Both optimized IAs showed the same specificity. High cross-reactivity was observed only with the analogue pesticide azinphos-ethyl and with phosmet, as could be expected from the chemical structure of the hapten used to produce the MAbs employed in these assays. Nevertheless, cross-reactivity should not be taken as a drawback. In fact, the development of multiresidue IAs is actually being investigated by many authors, and the use of cross-reacting MAbs together with a proper mathematical analysis may be a suitable approach.

Assay precision and accuracy in the absence of matrix effects from real samples were investigated. A narrower working range than the theoretical 20−80% inhibition was observed in the antibody-coated assay. Furthermore, a standardized method to LOD determination was applied by considering the assays variabilities. Nevertheless, applicability of IAs is usually estimated on real samples previously spiked with the target analyte. This way, matrix effects of a limited number of samples were evaluated, and in some cases important deviations were observed. In these cases, results would need to be confirmed with another analytical method to ascertain the possible presence of any of the recognized pesticides. Further studies would also be required to identify other factors potentially affecting assay reliability in real water samples. In summary, two highly sensitive IAs for AM based on MAbs have been developed in two different ELISA formats. These IAs have the appropriate sensitivity, precision, and accuracy to determine azinphos-methyl in environmental water samples at levels near the maximum residue limits established by European legislation.

ABBREVIATIONS USED

A_{max}, absorbance in the absence of competing analyte; AM, azinphos-methyl; BSA, bovine serum albumin; CR, cross-reactivity; CV, coefficients of variation; DMF, *N*,*N*-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; FA, phthaloylglycine; I_{50}, concentration of analyte giving 50% inhibition of the maximum absorbance; IA, immunoassay; LOD, limit of detection; MAb, monoclonal antibody; PBS, 10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4.

LITERATURE CITED

Abad, A.; Montoya, A. Development of an enzyme-linked immunosorbent assay to carbaryl. 2. Assay optimization and application to the analysis of water samples. *J. Agric. Food Chem*. **1997**, *45*, 1495−1501.

Aga, D. S.; Thurman, E. M. Environmental immunoassays: Alternative techniques for soil and water analysis. In *Immunochemical Technology for Environmental Applications*; Aga, D. S., Thurman, E. M., Eds.; ACS Symposium Series 657; American Chemical Society: Washington, DC, 1997; pp 1−20.

Ahmad, N.; Guo, L.; Mandarakas, P.; Appleby, S.; Bugueno, G. Passive diffusion through polymeric membranes: A novel cleanup procedure for analysis of azinphos-methyl and azinphos-ethyl residues in fruits and vegetables. *J. AOAC Int*. **1995**, *78*, 1450−1454.

Berger, B. Improved chromatographic method for the simultaneous determination of ten phenylurea herbicides and some of their degradation products in soil. *J. Chromatogr. A* **1997**, *769*, 338−342.

Brady, J. F. Interpretation of immunoassay data. In *Immunoanalysis of Agrochemicals. Emerging Technologies*; Nelson, J. O., Karu, A. E., Wong, R. B., Eds.; ACS Symposium Series 586; American Chemical Society: Washington, DC, 1995; pp 266−287.

Hayes, M. C.; Dautlick, J. X.; Herzog, D. P. Quality control of immunoassays for pesticide residues. In *New Frontiers in Agrochemical Immunoassay*; Kurtz, D. A., Skerritt, J. H., Stanker, L., Eds.; AOAC International: Arlington, VA, 1995; pp 237−250.

Hernández, F.; Serrano, R.; Miralles, M. C.; Font, N. Gas and liquid chromatography and enzyme linked immunosorbent assay in pesticide monitoring of surface water from the western Mediterranean (Comunidad Valenciana, Spain). *Chromatographia* **1996**, *42*, 151−158.

Hogendoorn, E. A.; Hoogerbrugge, R.; Baumann, R. A.; Meiring, H. D.; de Jong, A. P. J. M.; van Zoonen, P. Screening and analysis of polar pesticides in environmental monitoring programmes by coupled-column liquid chromatography and gas chromatography−mass spectrometry. *J. Chromatogr. A* **1996**, *754*, 49−60.

Jones, G.; Wortberg, M.; Kreissig, S. B.; Bunch, D. S.; Gee, S. J.; Hammock, B. D.; Rocke, D. M. Extension of the four-parameter logistic model for ELISA to multianalyte analysis. *J. Immunol. Methods* **1994**, *177*, 1−7.

Krämer, P. M.; Marco, M. P.; Hammock, B. D. Development of a selective enzyme-linked immunosorbent assay for 1-naphthol: The major metabolite of carbaryl (1-naphthyl *N*-methylcarbamate). *J. Agric. Food Chem*. **1994**, *42*, 934−943.

Lacorte, S.; Barceló, D. Improvements in the determination of organophosphorus pesticides in ground- and wastewater samples from interlaboratory studies by automated on-line liquid−solid extraction followed by liquid chromatography-diode array detection. *J. Chromatogr. A* **1996**, *725*, 85−92.

Lacorte, S.; Ehresmann, N.; Barceló, D. Stability of organophosphorus pesticides on disposable solid-phase extraction precolumns. *Environ. Sci. Technol.* **1995**, *29*, 2834−2841.

Lee, N.; Skerritt, J. H.; McAdam, D. P. Hapten synthesis and development of ELISAs for detection of endosulfan in water and soil. *J. Agric. Food Chem*. **1995**, *43*, 1730−1739.

Lucas, A. D.; Goodrow, M. H.; Seiber, J. N.; Hammock, B. D. Development of an ELISA for the N-dealkylated s-triazines: Application to environmental and biological samples. *Food Agric. Immunol.* **1995**, *7*, 227−241.

Manclús, J. J.; Montoya, A. Development of an enzyme-linked immunosorbent assay for 3,5,6-trichloro-2-pyridinol. 2. Assay optimization and application to environmental water samples. *J. Agric. Food Chem*. **1996a**, *44*, 3710−3716.

Manclús, J. J.; Montoya, A. Development of enzyme-linked immunosorbent assays for the insecticide chlorpyrifos. 2. Assay optimization and application to environmental waters. *J. Agric. Food Chem*. **1996b**, *44*, 4063−4070.

Mercader, J. V.; Montoya, A. Development of monoclonal ELISAs for azinphos-methyl. 1. Hapten synthesis and antibody production. *J. Agric. Food Chem*. **1999**, *47*, 1276−1284.

Meulenberg, E. P.; Mulder, W. H.; Stoks, P. G. Immunoassays for pesticides. *Environ. Sci. Technol.* **1995**, *29*, 553−561.

Mihaliak, C. A.; Berberich, S. A. Guidelines to the validation and use of immunochemical methods for generating data in support of pesticide registration. In *Immunoanalysis of Agrochemicals. Emerging Technologies*; Nelson, J. O., Karu, A. E., Wong, R. B., Eds.; ACS Symposium Series 586; American Chemical Society: Washington, DC, 1995; pp 288−300.

Neidert, E.; Saschenbrecker, P. W. Occurrence of pesticide residues in selected agricultural food commodities available in Canada. *J. AOAC Int*. **1996**, *79*, 549−566.

Reeves, S. G.; Berberich, S.; Dautlick, J.; Mihaliak, C. The Analytical Environmental Immunochemical Consortium. *Food Agric. Immunol.* **1996**, *8*, 137−139.

Roy, R. R.; Wilson, P.; Laski, R. R.; Roberts, J. I.; Weishaar, J. A.; Bong, R. L.; Yess, N. J. Monitoring of domestic and imported apples and rice by the U.S. Food and Drug Administration pesticide program. *J. AOAC Int.* **1997**, *80*, 883−894.

Stanker, L. H.; Bigbee, C.; Van Emon, J.; Watkins, B.; Jensen, R. H.; Morris, C.; Vanderlaan, M. An immunoassay for pyrethroids: Detection of permethrin in meat. *J. Agric. Food Chem*. **1989**, *37*, 834−839.

Szurdoki, F.; Jaeger, L.; Harris, A.; Kido, H.; Wengatz, I.; Goodrow, M. H.; Székács, A.; Wortberg, M.; Zheng, J.; Stoutamire, D. W.; Sanborn, J. R.; Gilman, S. D.; Jones, A. D.; Gee, S. J.; Choudary, P. V.; Hammnock, B. D. Rapid assays for environmental and biological monitoring. *J. Environ. Sci. Health* **1996**, *B31*, 451−458.

Tauler, R.; Lacorte, S.; Barceló, D. Application of multivariate self-modeling curve resolution to the quantitation of trace levels of organophosphorous pesticides in natural waters from interlaboratory studies. *J. Chromatogr. A* **1996**, *730*, 177−183.

Williams, K. J.; Thorpe, S. A.; Reynolds, S. L. The use of ELISA for the determination of pesticide residues in food. *Int. J. Environ. Chem*. **1996**, *65*, 149−152.

Wortberg, M.; Goodrow, M. H.; Gee, S. J.; Hammock, B. D. Immunoassays for simazine and atrazine with low cross-reactivity for propazine. *J. Agric. Food Chem*. **1996**, *44*, 2210−2219.

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