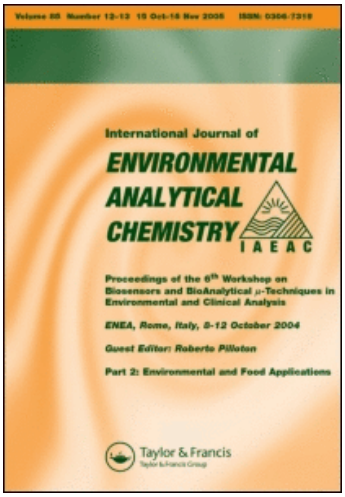

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Novel Concepts for the Immunological Detection of Bound Residues

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NOVEL CONCEPTS FOR THE IMMUNOLOGICAL DETECTION OF BOUND RESIDUES

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Immunoassays could be applied successfully to the determination of bound residues in soil and other complex matrices. Nevertheless, there was some doubt whether these assays could be regarded as quantitative and selective. We present some results, which imply that several approaches are quite promising. One approach is based on a non-competitive saturation immunoassay, which evens out the different cross-reactivities of the bound species. This may lead to a true molar sum value, which is not an equivalent concentration. For competitive assays, a method for the determination of the affinity constant(s) of the bound species is discussed. This would enable a correction for the cross-reactivity. The selectivity problem could be diminished very much, too. The application of special blocking reagents and the use of inhibition tests essentially solved the problem of non-specific binding (NSB). In addition, it could be proven experimentally that adsorbed analytes do not disturb the non-competitive assays, which have been suspected to be highly selective for covalently bound residues.

Keywords: Immunoassays; bound residues; non-extractable residues; cross-reactivity

INTRODUCTION

Although there are some definitions available for the term *bound residues*, it seems to be useful to specify and refine the main points to avoid confusion. Bound residues are often understood as the fraction of pesticides, which cannot be extracted from soil by non-destructive solvents. This can be considered as an empirical definition, similar to the meaning of *non-extractable residues*. Today there seems to be an increasing consent that not only pesticides, but also other, even not necessarily anthropogenic contaminants, should be included. In addi-

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tion, bound residues cannot only occur in soil but also in other complex (to some extent polymeric) matrices, like samples of biological origin or humic compounds in water. Solid surfaces of any kind or particles and colloids should be included, too. That the analyte cannot be isolated as a molecularly homogeneous species, is one of the main characteristics of such matrices. The advantage of this definition is its universality, but the drawback remains that there is a lack of mechanistic selectivity. Therefore, we think that a modification of the definition based on binding mechanisms would be helpful. At least three kinds of bound residues should be differentiated (Figure 1).

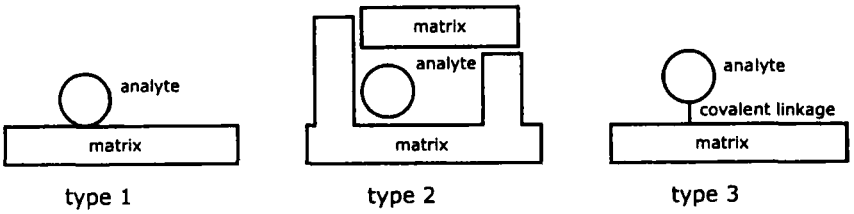

FIGURE 1 Schematic representation of the main types of bound residues. Type 1: Adsorbed residues, type 2: Entrapped residues, type 3: Covalently bound residues

Not included in this diagram are those covalent conjugates, which are considered to be secondary metabolites (e.g., glutathion conjugates) as they are chemically defined species. In addition, reassimilated ^{14}C should not be regarded as a bound residue as this is clearly not a contaminant any more, and can be seen as an analytical artifact of the ^{14}C-method. Included in this definition would be DNA-, cellulose-, lignin-, and protein-conjugates as they are structurally heterogeneous in most cases. Furthermore, we abandoned the phrase of many definitions that only residues formed by "good agricultural practice" are considered to be bound residues. This differentiation does not seem to be justified by scientific reasons and leads to logical contradictions.

Adsorbed residues (type 1) are very much a function of the extraction method, as with a very exhaustive method with several solvents and repetitive treatments it should be possible to extract essentially all of the type 1. Furthermore, there should be an equilibrium between the adsorbed and free contaminant which should lead to complete extraction after a sufficiently long time. Especially ionic compounds like paraquat might be counted to type 1. It is well known that paraquat, binding very firmly to soil, can be extracted with concentrated acid [1].

Entrapped residues (type 2) should not be extractable without destruction or alteration of the matrix. Hence, these bound residues are a true reservoir of the

parent compound and its metabolites, respectively. They might be released, for instance after a swelling of the soil. It also seems reasonable that entrapped residues are very well protected from degradation and should have an extremely long half-life [2]. Recently it could be shown [3] that the silylation of soil leads to an extensive alteration of the structure of humic acids following an effective release of bound residues (probably of type 2).

Covalently bound residues (type 3) cannot be extracted from the matrix by nondestructive methods at all. Therefore, they should show very long half-lives, too. The risk of a release seems to be low, if the covalent linkage is stable. This might not always be the case, considering the relatively labile bonds in secondary metabolites and other conjugates, which can be postulated to be intermediates for the formation of covalently bound residues. It has to be mentioned that it is often possible to extract covalently bound residues together *with* the matrix, at least partially, without cleaving the bond between contaminant and matrix molecule.

In this paper preferentially the type 3 in soil is discussed, which is mainly associated with humic material.

Several applications of an immunological determination of bound residues in soil and water have been reported [4–14]. The main field has been the measurement of bound triazine herbicides, for example atrazine. Furthermore, polycylic aromatic hydrocarbons (PAHs) and nitroaromatic compounds, like trinitrotoluene (TNT) have been determined in a bound state.

One difficult problem are the cross-reactivities of immunoassays. Therefore, most results are given as "equivalents" in relation to a standard substance. Up to this point, the analysis of bound residues is not more complicated as other immunoassays. The main point is that an acknowledged reference method is lacking in most cases and the structure of the bound residues is unknown *a priori*. In addition, cross-reactivities are not the same in competitive or non-competitive formats. Interestingly, only a few studies have been performed to examine the cross-reactivities of non-competitive immunoassays [15].

Perhaps the most troublesome characteristic of immunoassays for the determination of bound residues is the occurrence of non-specific binding (NSB). This results in relatively high signals, independent of the concentration of bound contaminants. This leads to a high number of false positives or even false negatives, if blank values are subtracted. The main reason for this peculiarity seems to be the heterogeneity of the humic material, and that humics tend to adsorb to proteins (the main reagents of all immunoassays) very effectively. In practice, there is an additional factor, which restricts the application of immunoassays in environmental analysis. This is the very limited availability of antibodies of high affinity and selectivity, especially of monoclonal ones.

Up to now, these impeding factors prevented the application of immunoassays for the routine analysis of bound residues. In this paper we would like to show that there exist several ways to overcome these limitations.

EXPERIMENTAL

N-(2,4,6-Trinitrophenyl)-6-aminocaproic acid was synthesized as previously described [16]. N-(2,4-Dinitrophenyl)-6-aminocaproic acid can be purchased from Sigma, Deisenhofen, Germany. "Atrazine-mercaptopropionic acid" was synthesized according to a procedure described in [5] from atrazine and 3-mercaptopropionic acid. The monoclonal antibody AM7B2.1 directed to triazine herbicides was kindly supplied by Dr. A.E. Karu, Immunochemistry Facility, Berkeley, USA. The monoclonal antibody TNT A/1.1.1 was donated by SDI, Inc., Newark, USA. The sources of antibodies, enzyme conjugates and other chemicals and procedures, unless not described here, have been published previously [5,7,8,9,12,13,14].

RESULTS AND DISCUSSION

Suppression of non-specific binding (NSB)

Several methods have been tried to suppress the non-specific binding in immunoassays for the detection of bound residues. The most important method is the use of special blocking agents. In a recent paper [7] we have shown that the severe problems with humic acids are mainly correlated with ionic interactions. This could be used for a new non-competitive test design (Figure 2) based on a coating of positively charged proteins (e.g., histons).

This shows that the isoelectric point (IP) of the components of an assay is an important parameter for non-specific interactions. As proteins have hydrophobic and hydrophilic, negatively and positively charged areas, they are quite prone to NSB. In most immunoassay procedures Tween 20 is an important reagent, for example for washing buffers. This non-ionic surfactant is useful to suppress hydrophobic interactions — but it is ineffective against ionic NSB. This is mechanistically obvious, as Tween 20 has no charged groups and consists of hydrophobic parts and hydrophilic oligo-ethyleneglycol chains.

Nevertheless, the NSB even in optimized assays cannot be considered as zero, and what is even worse, a NSB subtraction is not possible as samples can show different background values. However, besides the usual blocking agents there is another approach, which solved this problem. A selective inhibition of the signal

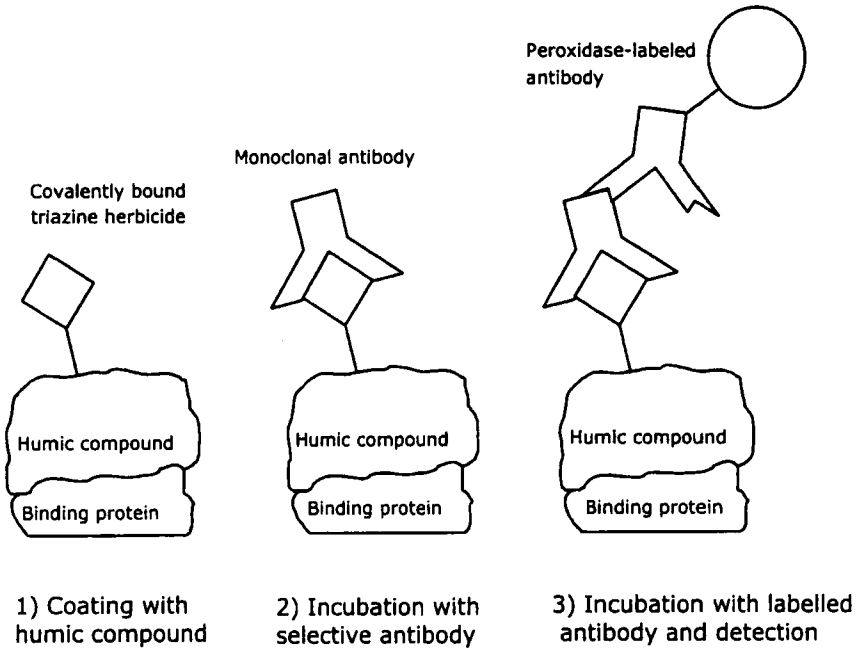

FIGURE 2 Non-competitive immunoassay for the detection of bound residues in humic compounds

would differentiate very effectively between the signal caused by the analyte and the signal caused by NSB. Each sample has to be measured twice: Once in a normal non-competitive immunoassay, and once in the presence of a surplus of a *soluble analyte*. In the latter case the specific signal should be suppressed completely. The remaining signal can be regarded as the NSB signal of the sample (Figure 3). As each sample gets its own NSB value, the variation of the NSB does not harm any more. It can be shown that the inhibition method works very selectively, is not concentration dependent, and leads to a blank value which is not significantly different from the real blank sample. The detection limit for "atrazine" can be determined to a typical value of 35 µg/kg soil. As for bound residues no molecular formula is known, quantitative or semiquantitative results should be preferentially given in mol/kg soil and not in a mass concentration.

Preparation and characterization of standard materials

In synthetic conjugates analyte concentrations of more than 1% (of the humic acid weight) could be reached, in highly contaminated real samples only 0.01%

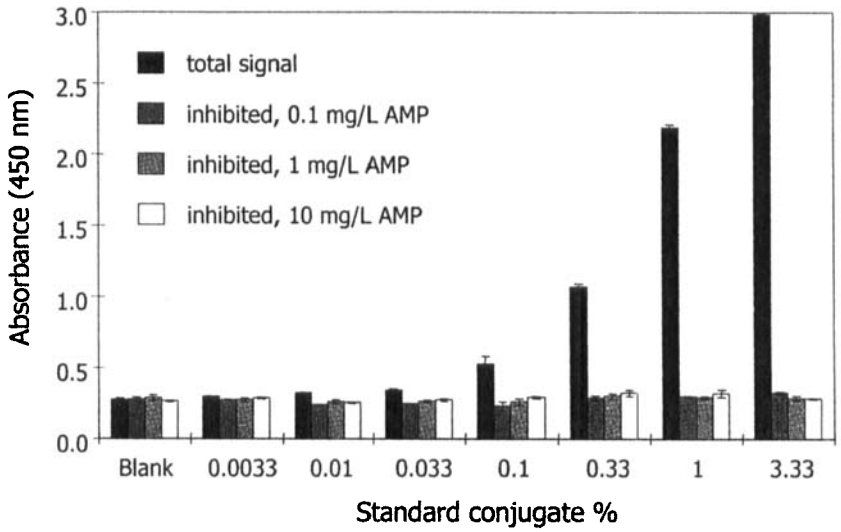

FIGURE 3 Inhibition test for the determination of the NSB

might be realistic. Although quite a lot different techniques for the characterization of such conjugates might be useful, we prefer cleavage methods in conjunction with a chromatographic quantification of the free cleavage product. The use of elemental analysis proved to be also very successful – if the hapten contains some "rare" elements. In Figure 4 the synthesis of a triazine-humic acid conjugate is shown. In Table I the coupling density of such a conjugate is listed, determined by three independent methods. The cleavage method gives significantly lower values as the two methods based on elemental analysis. This might be caused by the fact that the used cleavage method was structurally selective.

TABLE I Coupling density of an AMP-humic acid conjugate determined by different methods

Oxidative cleavage	17 ± 3 µmol triazine/g humic acid
Elemental analysis by combustion[a]	50 ± 3 µmol triazine/g humic acid
Elemental analysis by TXRF[ab]	48 ± 2 µmol triazine/g humic acid

a. *Calculated from sulfur content.*
b. *Total Reflection X-ray Fluorescence. The internal standard was gallium and a Mo x-ray tube was used.*

Based on a mean molecular weight of 3000 Da of the humic acid, the coupling density of the synthetic conjugate can be estimated to be between 1:7 and 1:20 (triazine:humic acid). This means that multivalent conjugates are not very likely.

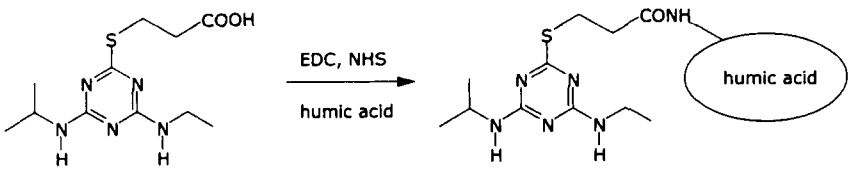

FIGURE 4 Synthesis of a standard conjugate for the determination of bound triazine herbicides [5]. The AMP conjugate should imitate a humified glutathion-bound triazine

The oxidative cleavage method was developed recently for the analysis of triazine humic acid conjugates [9]. It relies on the oxidation of the sulfur next to the triazine ring to a sulfoxide (or sulfone). These excellent leaving groups enable a reaction with nucleophiles. The mixture methoxyethanol/methoxyethanolate has proven to be particularly suited as the humic acid is reasonably soluble in this solvent and the product, "atrazine-methoxyethanolate" (AME), can be extracted with satisfactory recoveries and analyzed by GC.

Cross-reactivity

The cross-reactivity of an immunoassay is difficult to be eliminated. Although the principle of multidimensional analysis with the help of antibody arrays has been proposed quite early, the realization of this obvious concept is still in an early stage and will not be discussed in detail here.

One trivial "solution" of the problem is accepting the cross-reactivities as a fact, which leads to the use of equivalents, which give you a relative information, if the general composition of a sample does not vary too much. For many applications this information is absolutely sufficient, especially if a few positive samples have to be identified in many negative ones (screening). As detection limits heavily depend on the cross-reactivity of the analyte, cross-reactivity information is nevertheless very useful. Therefore immunological test kits are nearly always delivered with a list of important cross-reactants. Although a thorough cross-reactivity study has been published in this field recently [6], the measurement of model compounds still has not solved the problem as no reliable structural information is available for bound residues.

Beatty et al. [18] published an interesting method for the determination of the affinity constant, which is essentially equivalent with the cross-reactivity in competitive assays. This method is based on the immobilization of antigen and a dilution series of the antibody. The resulting sigmoidal curves lead to the affinity constant, without any knowledge of the antigen concentration. We would like to propose that this could open a possibility of quantifying covalently bound resi-

dues by competitive immunoassays as the cross-reactivity of the bound residue might be determined even from a real sample. Another advantage would be the high sensitivity of the method.

Another option is the independent determination of bound residues in real samples for instance with cleavage methods or ^{14}C methods to calibrate immunoassays. To our knowledge, this was not extensively tested so far. The problem may occur that these methods do not measure the same types of bound residues and therefore this approach only can be expected to work properly, if the relation between the different types does not vary too much, which may not be true in samples of different age.

Determination of the cross-reactivity in a non-competitive assay

As already mentioned, some papers have been published about the possibility of measuring affinities or cross-reactivities, respectively, with a non-competitive format, but this was not widely recognized. This kind of assay is especially useful, if no purified antigen/hapten is obtainable. Bound residues can be seen as a highly unpure analyte where the pure analyte cannot be isolated. Therefore, this approach should be applicable to bound residues, too. To facilitate the examination, a model system of BSA conjugates was used instead of bound residues based on humic material.

Model system of a saturation assay

To elucidate the mechanism of cross-reactivity in non-competitive assays (Figure 5) we constructed a model system on base of chemically modified bovine serum albumin. As haptens we selected a series of nitroaromatic compounds with 1 to 3 nitro groups, which had a significantly different affinity to the monoclonal TNT antibody (Table II).

TABLE II Competitive cross-reactivities of the haptens used for this study

compound	*midpoint [nmol/L]*	*molar CR [%]*	*coupling density*[a] *[mol/mol]*
2,4,6-trinitrotoluene	2.8 ± 0.2	100 (*per def.*)	–
N-(2,4,6-trinitrophenyl)-6-aminohexanoic acid	3.9 ± 0.1	71 ± 7	18
N-(2,4-dinitrophenyl)-6-aminohexanoic acid	87 ± 14	3.2 ± 0.7	7
N-(4-nitrophenyl)-6-aminohexanoic acid	130000 ± 30000	0.0022 ± 0.0007	11

a. BSA conjugates

Although Ekins [17] has discussed the mechanism of non-competitive assays in detail, the application of this knowledge is largely lacking in many fields. Therefore, we tried to apply saturation analysis to a model system of bound hapten residues.

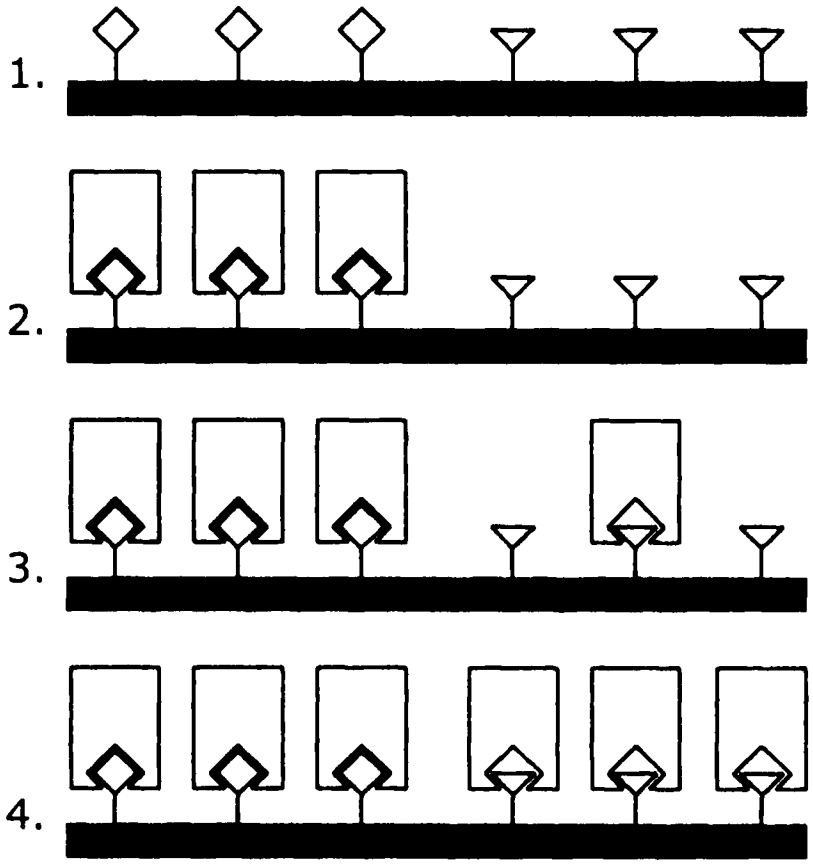

FIGURE 5 Schematic representation of the saturation effect in non-competitive immunoassays leading to an equalized cross-reactivity. 1. Haptens of different affinity on a surface. 2. Low concentration of antibody: structural selectivity, only high-affinity sites are detected. 3. Medium concentration of antibody: low-affinity sites will be partially filled. 4. High concentration of antibody: full saturation, no selectivity. Both haptens show 100% cross-reactivity. A true molar sum value will be obtained

The aim to obtain conjugates with very low, but equal coupling densities could not be achieved with bovine serum albumin (BSA), yet. Therefore, some of the experiments shown here may be hampered by the fact that the coupling density

was not equal and much greater than 1. The coupling densities have been determined by UV absorbance measurements. One problem was that the spectra are not additive and that peak maxima are changing after conjugation. Therefore, the measurements have not been performed at a constant wavelength, but at the respective peak maximum. For the quantification of the protein the absorbance at 278 nm was used. In Table II the coupling densities of three conjugates are shown.

From equilibrium theory one could expect the cross-reactivity of a non-competitive assay to depend very much on the concentration of the antibody and reaches saturation, even for haptens with different affinity constants. On the other hand, there has to be a cut-off limit where the affinity is too low to lead to a binding at all, otherwise all kinds of non-specific binding would also lead to a saturation signal. An equilibrium simulation was performed for different affinities and for different antibody concentrations. It could be shown that in this simulated system, the behavior is as expected.

One can conclude that these saturation assays might be well suited to get a quantitative result for covalently bound residues. It has to be kept in mind that the result would not be an equivalent any more (as in all cases when the cross-reactivity or the identity of the analyte is not known), but a result in mol/kg matrix would be obtained. This cannot be converted into a mass concentration as the molar mass of a covalently bound residue is not known in most cases and would be difficult to define as analyte and matrix form one molecule. In addition, different cross-reacting species (of different mass) might be included in the molar sum value.

In Figure 6 it can be realized that a cut-off affinity exists, which lies between the affinities of DNP (dinitrophenyl derivative) and MNP (mononitrophenyl derivative), the former being bound to saturation, the latter being not bound at all (similar to BSA negative control). It is striking that the maximum absorbance of the DNP conjugate is only about 50% of the TNP (trinitrophenyl) conjugate. We first assumed that the different coupling densities are the cause of this behavior. Considering the relatively high coupling density and the size of an antibody binding site, this seems to be unlikely. Only about one antibody binding site per BSA molecule may bind in a sterically controlled action. In addition, normalization to the coupling density does not lead to identical curves. As this result is reproducible, a more general mechanism may be the reason for this deviation.

Furthermore, we assumed that the weaker binding hapten DNP might lose a fraction of the bound antibodies during the washing procedures. This could be disproved as the length of the washing procedure did not affect the signal (data not shown). Finally, it might be suspected that the TNP conjugates are bound by a monovalent mechanism (one antibody binding site is left free) as the affinity is

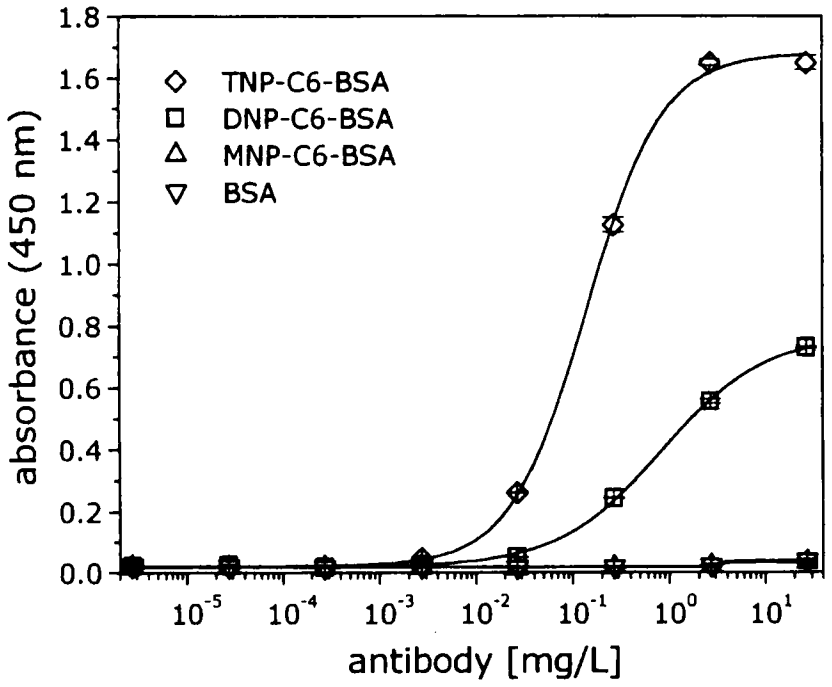

FIGURE 6 Antibody saturation curves of different immobilized haptens. The cross-reactivity of DNP (dinitrophenyl conjugate) reached 44% (of the trinitrophenyl conjugate, TNP) at the highest antibody concentration tested, which is about 10 times higher than the corresponding competitive value (4.5%). Different cross-reactivity definitions have been applied for competitive and non-competitive assays [12]

very high and rearrangement may be too slow. In contrast, the DNP hapten has a much lower affinity (about 20 times, see Table II) and therefore may rearrange to a very stable bivalent conformation. This mechanism would have the advantage to explain the approximate 2:1 relation of the signals.

Non-competitive determination of adsorbed haptens?

It has been questioned whether non-competitive immunoassays are selective for covalently bound residues. As adsorbed analyte (bound residue type 1) might be present in considerable amounts, this could not be ruled out completely. Considering the mechanism of a non-competitive immunoassay, it was suspected that adsorbed residues should not lead to any signal in the assay. Recently we performed some experiments, which showed that an influence of adsorbed species is

very unlikely. We spiked non-contaminated soil with 1 g/kg of TNT and analyzed the aged soil (5 days, room temperature). The result was completely negative. No significant signal could be detected [8]. Similar results have been obtained with atrazine in soil, even for an aging period of several months. By the way, these experiments emphasize the importance of natural conditions for the formation of covalently bound residues (type 3). It seems reasonable to assume a biological/biochemical mechanism for their formation in these cases. An exception might be chemically reactive pesticides, like anilazine [19].

Although the mechanism of non-competitive immunoassays makes is very unlikely that adsorbed haptens could be detected, a small number of publications just state this [20,21,22]. As free and adsorbed haptens might interfere with our sandwich-like immunoassays, we tested the effect of adsorbed hapten, too. It could be shown that haptens cannot be detected with normal concentrations of antibodies (about < 1 mg/L). With a very high antibody concentration, a significant difference between a TNP-N-C6 and a TNP-ε-lysine could be seen, where the lysine derivative bound stronger as the carboxylic acid derivative, similar to one of the mentioned publication [21]. As covalently bound haptens should be detectable at much lower antibody concentrations, it is not very likely that some of the haptens have been immobilized at chemically activated sites, although such a mechanism cannot be completely ruled out for irradiated or otherwise activated microtitration plates. Perhaps the "non-specific" binding of antibodies might be enhanced by the complexation of a hapten with a positively charged group, which interacts with negative groups at surface of the microtiter plate. Another paper stated the immunological measurement of 2,4-dinitrobenzene sulfonate adsorbed on brick chips [20]. By accident we found, that a similar compound, trinitrobenzenesulfonic acid (TNBS), showed a strange behavior, too. During an attempt to synthesize a TNBS-humic acid conjugate, it was found that we had good non-competitive calibration curves at the beginning, but after exhaustive purification of the humic acid, no signal could be obtained any more. We explain this result, and perhaps the one of the mentioned paper, with a chemical modification of the blocking proteins as the reported cases dealt with protein-reactive analytes. During the incubation of the assay the coating proteins or the antibodies on the surface might have been modified by the contaminating soluble reagent. In the second step, this chemically immobilized hapten could be detected quite easily.

Use of several antibodies for structure elucidation

As mentioned in a section before, the use of several antibodies would enable the examination of the molecular structure of bound residues. The cross-reactivity

patterns of each antibody can be determined with soluble haptens or even with conjugates [23]. Only a limited number of general possibilities for the structure of bound residues of a compound are chemically possible. Therefore, the decision, which possibilities occur, might be relatively straightforward. The mathematical treatment of data obtained by several antibodies of different selectivity has not been examined, but it is likely that an approach similar to the one used by Schneider et al. [24] might be applicable. A first effort has been published for a 3-antibody system, whereas two of the antibodies were non-reactive towards bound triazine residues. This allowed a tentative assignment of the covalent binding orientation of atrazine [10], which is consistent with our hypothesis of a glutathion pathway. Recently a work was reported concerning the formation of bound residues of the BASF fungicide kresoxim-methyl [11]. The author used three different antisera against three metabolites of kresoxim-methyl and could show that only one (bound) metabolite could be found in real samples. For the quantification it was assumed that the synthetic conjugate shows the same cross-reactivity as the natural one.

Examination of soil samples

Several soil samples with different levels of triazine contamination have been tested. In Table III the concentration of extractable triazines (the sum of parent compound and metabolites determined by HPLC) is compared with the concentration of covalently bound residues determined by immunoassay and by oxidative cleavage/gas chromatography [9]. It can be seen that especially the cleavage data and the immunoassay values correlate fairly well, which supports the assumption that these methods determine the same type of bound residues. On the other hand, soluble and covalently bound data do not need to be correlated.

Sensitivity considerations

In general, non-competitive immunoassays are regarded as more sensitive as competitive ones, which can be shown on a theoretical and experimental basis. Unfortunately, this is not the case with the shown non-competitive assays for bound residues as the first immobilization step of the humic compound is essentially non-selective. As only for instance one of a thousand humic acid molecules carries a covalently bound residue, in this case, 99.9% of the surface is wasted. Up to now it was not possible to invert the assay and to begin with the more selective step. This mechanism leads to a loss of sensitivity, which means detection limits for soil in the lower µg/kg range. Hence, competitive assays should be

preferred, if even lower concentrations have to be analyzed, irrespective their drawbacks concerning cross-reactivity and interference by soluble analytes.

TABLE III Soil samples contaminated with atrazine and its degradation products

Soil sample No.[a]	*Sum of extractable triazines by HPLC* [mg/kg]*	*Covalently bound triazines by non-competitive immunoassay [mg/kg]*[b]	*Covalently bound triazines by cleavage/gas chromatography [mg/kg]*[b]
B1	0.012	< d.l.	–
95224	0.018	< d.l.	< d.l.
B50	0.025	< d.l.	–
B25	0.029	< d.l.	–
B100	0.037	< d.l.	–
95223	0.051	< d.l.	< d.l.
8707	0.10	< d.l.	–
8643	0.13	0.090	–
8609	0.48	0.070	–
8591	0.70	0.098	–
9159	4.0	0.27	0.43
95222	5.2	0.13	0.054
8551	11.3	1.51	1.73

a. Soil samples and HPLC data have been kindly supplied by G. Henkelmann, LBP, Munich.
b. Calculated as atrazine, detection limit (d.l.) for immunoassay about 0.035 mg "atrazine" per kg dry soil.

CONCLUSIONS

Although it seemed almost impossible to achieve any quantification with immunoassays for bound residues some years ago, recent work could show that there are several suitable approaches, which might be useful for the measurement of unknown samples. For the analysis of synthetic conjugates, cleavage techniques and elemental analysis proved to be very useful. Competitive assays might be improved considerably by the determination of the affinity constant of the real residue. Quantification and structure elucidation would be facilitated. For non-competitive assays the application of inhibition assays and blending calibration procedures lead to considerable improvements. Theoretical and experimen-

tal work showed that non-competitive assays can overcome the cross-reactivity problem by using saturation conditions.

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