UDC 57.083.3:632.95
ISSN 1330-9862

review

Immunoassays for Pesticide Analysis in Environmental and Food Matrices*

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Received: January 16, 1998
Accepted: March 13, 1998

Summary

Immunochemical methods have increased considerably in the past years, and many examples of small and large scale studies have demonstrated the reliability of the immunotechniques for control and monitoring of contaminant residues in different kinds of samples. Application of the immunoassay (IA) methods in pesticide residue control is an area with enormous potential for growth. The most extensively studied IA is the enzyme-linked absorbent assay (ELISA), but several other approaches, that include radioimmunoassay and immunoaffinity chromatography, have been also developed recently. In comparison with classical analytical methods, IA methods offer the possibility of highly sensitive, relatively rapid, and cost-effective measurements. This paper introduces the general IAs used until now, focusing on their use in pesticide analysis, and discussing briefly the effects of interferences from solvent residues or matrix components on the IA performance. Numerous immunochemical methods commonly used for pesticide determination in different samples such as food, crop and environmental samples are presented.

Keywords: immunoassay (IA) methods, ELISA, pesticide residues, food, crops, environmental samples, matrix effect

Introduction

Despite the necessity of the use of pesticides as agricultural defensives, their use can sometimes cause several public health and environmental problems. Most environmental risks and ecological damage from pesticide use result from toxic effects of pesticides on various living organisms. In some investigations it was found that insecticides are the most toxic class of pesticides, followed by herbicides, acaricides (mite killers) and fungicides *(1)*. Impacts on non-target organisms depend on how the pesticide degrades and moves through the hydrological cycle, the soil and food chains. Adverse impacts on beneficial organisms tend to be greatest where several different pesticides, especially insecticides, are applied routinely. Today it is already known that about 500 insect pests, 270 weed species and 150 plant diseases are now resistant to one or more pesticides. Evidently, this increasing tolerance has occurred due to the inadequate use of these compounds. Inherently, they have shown a certain degree of toxicity for the mammalian, specially the less degradable and more persistent products. Although many pesticides can be analysed using conventional (and sometimes highly sophisticated) analytical techniques, there are many more than cannot be analysed or can only be analysed at levels that are too insensitive. For this reason, development of simple, rapid, sensible and selective methods for pesticide residue determination is highly desirable, because the necessity for monitoring of these residues is evident.

Most pesticide residue analyses have been performed using chromatographic techniques. In this respect,

* Paper submitted for the special issue of this journal on Immunochemical Techniques and Methods (B)

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one of the main decisions to be taken at the beginning of an analysis for pesticides is whether to use gas chromatography (GC) or liquid chromatography (LC). The choice of the analytical technique depends mainly on the class of pesticide to be studied, its stability and best detection forms. Applications of the analytical techniques in determination of different classes of pesticides have been described extensively in the literature *(2–5)*. As usual, papers devoted to herbicide analytical methodology dominated the applications literature during the last years. In fact, these compounds correspond today to 70–80% of the total amount of pesticides used on crops in developed countries. Chromatographic methods for determination of herbicides residues in crops, food and environmental samples were reviewed by Tekel and Kovacicova *(6)*. The chromatographic techniques usually used for herbicide determination are GC-NPD and GC--MS after solvent extraction *(7)*. Some multiresidue protocols also employ ECD detection for these compounds *(8)*.

In general, the conventional procedures for sample preparation before chromatographic analysis are more expensive and time-consuming than in the other analytical procedures. The chromatographic methods usually involve extensive purification and often derivatization of the target compounds; moreover, experienced technicians and expensive equipment are required. Although such methods continue to predominate, as exemplified by the widely used Luke multiresidue method, new and improved methods and technologies for the analysis of pesticides residues continued to evolve rapidly during the past ten years. In this respect, the immunotechniques and biosensors have been extensively explored; the detection and the quantification limits of most of these new methods are at low parts per billion (ppb) to parts per trillion (ppt) for water analysis, and low parts per million (ppm) to ppb for other types of samples, such as crops, feeds, soils and biological matrices *(2,9)*. The lower limit of residue measurement in foods for these methods was usually below the tolerance levels, which, depending on the pesticide used for crop protection, generally range from 0.1 to 50 ppm.

In this paper the determination of pesticides, that include the products used on a worldwide scale, such as organophosphorus, organochlorine and carbamate insecticides, triazine, phenylurea or chlorophynoxy acid herbicides, employing different assay formats will be reviewed. We will focus this review on different kinds of immunoassay (IA) methodologies developed for pesticide analysis as well as in crop and food, in environmental matrices.

Immunoassay Techniques for Pesticide Analysis

Immunochemical methods were first developed to substitute the expensive, time-consuming and laborious traditional analytical techniques. They involve the use of antibodies, which are polypeptide molecules produced by the immune-system cells when they are exposed to an antigenic substance. Antibodies are the key components of all immunochemical methods and are characterized by specific recognition sites in their structures which enable highly specific interactions with the antigens. The product of the binding of antigen to antibody is called an immune-complex, and the fact that this can take the form of a visible precipitate is of use in some analytical techniques, although for the majority of immunochemical techniques, it is simply the act of binding that is important.

Classification of immunoassays

An understanding of the forces holding antigen and antibody molecules together is major of importance in these techniques. In practice antigen-antibody reactions occur spontaneously without the aid of enzymes and only weaker forces (*e.g.* hydrogen bonds, hydrophobic and van der Waals' forces) are involved. There are undoubtedly a number of such interactions between a particular determinant on an antigen molecule and the complementary binding site on an antibody *(10)*. Also, the influence and control of environmental factors such as pH, and of the nature and concentration of other solutes have important implications in the design and execution of the experimental procedures employed in immunotechniques *(10,11)*. Principal IA formats commonly used in clinical and environmental applications are also presented and briefly discussed.

Competitive or non-competitive immunoassays. A competitive IA may be described by the immunochemical reaction:

$$2Ab + Ag + Ag^* \rightleftharpoons AbAg + AbAg^*$$

A free antigen (Ag) competes with a labelled antigen (Ag*) for a fixed and limited number of specific binding sites on the antibody (Ab) molecules. The extent of the binding of labelled antigen by the antibody depends on the concentration of unlabelled antigen and this allows the determination of the concentration of the unknown analyte (free antigen). A calibration curve is constructed from known amounts of antigen to determine the concentration of the unknown analyte. The competitive assays can be also divided into two groups: *i)* direct competitive, where the antigen-specific antibody is labelled and used to bind directly to the antigen. This kind of AI is intensively used in clinical analysis *(12,13)*; and *ii)* indirect detection where the antigen-specific antibody is unlabelled and its binding to the antigen is detected by a secondary reagent.

Non-competitive formats are used for the assay of large molecules with more than one epitope. This IA is a sandwich ELISA consisting of the adsorbing unlabelled antibody to a solid surface followed by the antigen. The antigen-antibody (Ag-Ab) complex is quantified by adding enzyme-linked antibody directed against the antigen, *i.e.* labelled antibody of the same specificity as the unlabelled antibody already adsorbed to the surface, not an anti-Ig. This approach requires that the antigen have at least two accessible binding sites for antibody because two antibody molecules must be bound to the same antigen molecule. The kind of antibody-binding IA depends mainly on two factors: the target compound and the immunoreagens used. Non-competitive assays are generally more sensitive than the competitive ones.

However, this type of assay is not suited for small molecules, such as most of the environmental contaminants, because the analyte must have multiple epitopes to allow simultaneous binding of two antibodies. Fig. 1 schematizes a sandwich ELISA format initially containing a coated antigen competing for the places on antibody *(14)*. This format has been extensively used for pesticide analysis *(15)*.

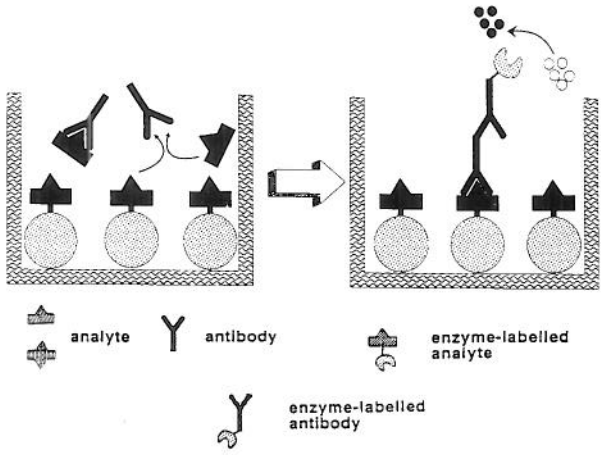

Fig. 1. Competitive »sandwich« enzyme-linked immunosorbent assay format with immobilised antigens and addition of both analytes and antibodies (limiting antibody concentration) to the solution; a labelled second antibody directed against the first antigen-specific antibody is added; after a wash step, detection is performed by adding substrate and chromophore. (From ref. *14*).

Homogeneous or heterogeneous IAs. Homogeneous IAs, which do not require a separation step, use signal modulation for detection where the activity of the antigen-enzyme conjugate is inhibited after binding to the antibody. The absence of enzyme activity results in a colorimetric change which can be used for quantification. This kind of assay offers the advantage of shorter analysis time relative to its equivalent heterogeneous assay, but it is less commonly used due to its inherent limitations, such as matrix effects and lower sensitivity.

In heterogeneous assays, physical separation of the bound and free reagent is required. Although this assay involves more steps due to the sequential rinsing required, removal of the unreacted components results in a less complex matrix for signal detection. Thus, sensitivity and detection limits are usually improved. An example of this assay is ELISA, the most popular format used for clinical and pesticide analysis.

Enzyme-linked immunosorbent assay (ELISA). It is a solid-phase assay that uses enzyme-linked techniques. The test is usually based on the fact that one of the reactants, usually antigen, is adsorbed onto the surface of a test tube or microtiter well. In order to determine the quantity of antibody in a sample, an aliquot of antiserum is reacted with the adsorbed antigen. The unreacted molecules are washed away, and an enzyme-linked anti-immunoglobuline (anti-Ig) is added. Finally, the substrate is added and the amount of developed color is determined. The content of antibody present can be quantified from standard curves, because the amount of color is proportional to the amount of enzyme-linked second reacted antibody. Variations of this basic technique have been described extensively in the literature *(16–21)* and in handbooks *(12,22–25)*.

In ELISA format with a known amount of antibodies linked to a solid support and a fixed concentration of enzyme conjugates, the photometric determination of the enzyme activity by absorption is related to the analyte concentration *via* a dose-response curve, such as that represented in Fig. 2. Calibration curves are constructed with standard antigen contents and have a sigmoidal shape with a linear portion – usually the format working range, which gives the first indication of the sensitivity of the test. On the *y*-axis, the variation of the absorbance *(A)* with the concentration of the target-compound (on the *x*-axis) is shown. In some cases, the absorbance values can be normalized between 100%, which corresponds to the absorption of a zero control (A_0), and 0%, which corresponds to the absorbance of a standard excess (A_{excess}). The transformation is performed according to the $(B/B_0)/\%$ values, calculated as follows:

$$(B/B_0)/\% = 100\,(A - A_{excess})/(A_0/A_{excess})$$

where A is the absorbance of the sample or standard. The standard curves, obtained by reporting A or $(B/B_0)/\%$ as a function of the logarithm of the analyte concentration, are convenient to work with, because the raw data can easily be reported on a graph, especially when a standard spectrophotometer is used.

Other immunochemical techniques and approaches. Less commonly used IA methods involve the use of fluorescence immunoassay (FIA), radioimmunoassay (RIA), chemiluminescence immunoassays, bioluminescent immunoassays, immunoaffinity chromatography (IAC), and flow-injection immunoassays (FIIA). FIA combines the specificity of antibodies with the sensitivity of fluorimetric assays by using antibodies coupled to a fluorescent chromophore. The first application to pesticides was developed by Colbert and Coxon *(26)* for the determination of paraquat in serum samples. FIAs have been developed in both competitive and non-competitive formats, and recently optimized for the rapid detection of 2,4-di and 2,4,5-trichlorophenoxyacetic acids (2,4-D and 2,4,5-T) and of simazine and atrazine *(27)*. Despite their simplicity, precision and possible automation, the use of FIA is still limited by the tendency of natural organic compounds to interfere with the signal detection. Chemiluminescent labels such as luminol can be used as tracers and in many cases, this type of assay is as sensitive as radioimmunoassay. Their use, however, is restricted, because there are no efficient detectors and due to the tendency of samples components to cause errors in the signal detection. Bioluminescence IAs are comparable in sensitivity to RIA and are based in the covalent bound between antigens and luciferase, as show in the following equation:

$$\text{luciferin} + \text{ATP} + O_2 \xrightarrow{\textit{luciferase}} \text{oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \textit{light}$$

IAC is a separation method based on the specific and reversible interactions between the antigen and the antibody. In IAC an immunosorbent (IS) is prepared by covalently immobilized antibody on the surface of a support. IS is then used for the selective extraction of a single analyte or a group of analytes. IAC is especially recommended for the isolation of polar ionic compounds *(26,28)*. On-site, automated FIIA monitors are a low cost option for obtaining continuous and quantitative data on dissolved aquatic chemical parameters *(29)*. Many recently reviewed papers *(30)* have been devoted to the automation of immunoassays through the use of continuous-flow systems. Attempts have been made in the development of immunosensors, that today represent an active area of study and begin to move from the laboratory stage to field testing and commercialization *(31)*. Thus, multianalyte assays for the environmental applications are emerging as new technologies in IA field.

Immunochemical methods for pesticide analysis

The potential of immunochemical techniques for pesticide residue analysis in various matrices such as soil, water, fruits, plants and biological fluids has been recognized *(32–34)*. Since the first work with IA development was published for the measurement of insuline in blood *(35)*, uncountable IA methods for screening, monitoring and regulatory analyses in the laboratory and field are being developed for residue pesticide control, and numerous commercial kits became available for particular single and multiresidue analyses *(36)*. The sensitivity of IAs is in the range of ng/mL, and the analyses have been carried out in varous formats, such as ELISA and magnetic particles enzyme immunoassay. Accordingly, we have found in literature several coupling of IAs not only with classical extraction and clean-up procedures, but also with modern techniques such as microbore HPLC, GC/MS and supercritical fluid extraction (SFE) *(37,38)*. Developmental work has focused mostly on pesticides used in crop agriculture, and a selection of field-portable and laboratory kits are commercially available. Moreover, IAs have been extensively used in water-quality surveys of surface and ground water and in studies of the fate and transport of pesticides in rivers, reservoirs, and the atmosphere *(39–41)*. In 1994 the EPA issued a *Guide to Environmental Immunochemical Analysis* with the aim of helping the users with information on the new immunotechniques and kits for pesticide analysis *(42)*. Table 1 presents EPA recommended methods used for determination of some environmental contaminants. ISO/CD No. 15 089 *(43)* supplies guidelines for selective immunoassays for the determination of plant treatment and pesticide agents. The principal goal of this EPA document is the assurance of the drinking, ground and surface water quality through standardized analytical procedures for pesticide determination (including their metabolites) using IA techniques.

IAs for pesticide analysis in environmental samples

Numerous works applying immunotechniques for detection of pesticides in environmental samples, such as water, soil and air, have been developed in the last years, and several kits have been used for this purpose. Table 2 lists some of the most commonly used types of commercial kits for pesticide analysis.

Several IA methods showed good correlation with the results obtained using conventional chromatograph-

Table 1. EPA immunoassay methods useful for the separation, detection and quantitation of organic contaminants in diverse environmental and waste matrices

Method number	Immunoassay
4010A	Screening for pentachlorophenol
4015	Screening for 2,4-D-dichlorophenoxyacetic acid (2,4-D)
4020	Screening for polychlorinated biphenyls
4030	Soil screening for petroleum hydrocarbons
4035	Soil screening for polynuclear aromatic hydrocarbons (PAHs)
4040	Soil screening for toxaphene
4041	Soil screening for chlordane
4042	Soil screening for DDT
4050	TNT explosives in water and soils
4051	Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in soil and water

Table 2. Some of the most commonly used commercial kits for pesticide determination and its manifacturers

Analyte	Idetek	Millipore			Ohmicron	
	Quantix	Envirogard Tube	Envirogard Quantitube	Envirogard Plate	RaPID Assay	InQuest
Aldicarb		x		x	x	x
Atrazine	x	x	x	x	x	
Carbofuran		x			x	x
Chlorothalonil	x					
2,4-D		x	x	x	x	
Diazinon				x		x
Fenitrothion		x		x		x
Metolachlor	x	x			x	
Paraquat				x	x	
Pentachlorophenol				x	x	
Triazines	x	x	x	x		
Urea herbicides				x		

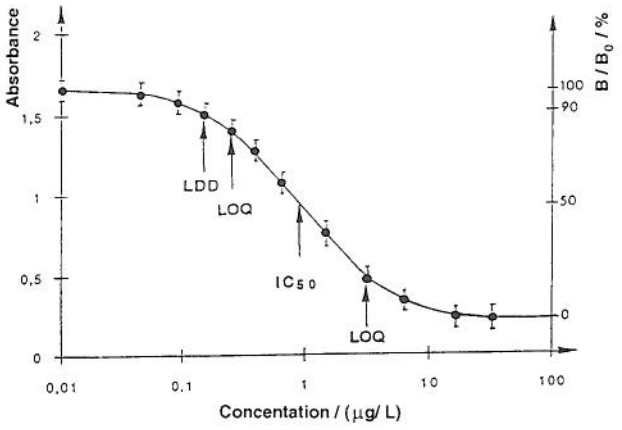

Fig. 2. Typical sigmoidal dose reponse of an immunoassay. Usual analytical parameters are detached, and defined as: LDD – limit of detection at least detectable dose; LOQ – lower and upper limit of quantification; IC_{50} – inhibition concentration at 50%. A_0 and A_{excess} are the absorbances of the zero control and of the standard excess solution. The IA sensitivity is often characterized by the LDD value that can be defined as the dose which inhibits 10% of the binding of the antibody with the enzyme tracer at 90% B/B_0 (From. ref. *14*).

ic methods, and no matrix interferences on the assay performance were observed. Bushway *et al. (44)* described an IA for determination of atrazine in water and soil samples by commercial test tubes method. The results showed a linear relationship from 0.5 to 1.0 ng mL. The IA and HPLC methods showed good reproducibility with percent coefficients of variation (CV) ranging from 23.8 to 4.1. In spite of the fact that these results were obtained from different water and soil sources samples that varied greatly in type and quality, no significant matrix effects were observed. Comparing the efficiency of the ELISA with methods such as GC and HPLC for determination of atrazine human exposure in the field Reed *et al. (45)* concluded that ELISA method was adaptable to field use with excellent sensibility, due to the elimination of clean-up steps and rapid detection of potential exposure hazards. Thurman and co-workers *(46)* examined the accuracy, precision and cross-reactivity of the triazine herbicides determination by comparing the results obtained by ELISA and GC/MS. The correlation between these methods was 0.99 for spiked distilled water containing a mixture of herbicides. ELISA was checked also for interference by naturally occurring humic and fulvic acids, and no difference between the immunoassay response was noted. These results suggest that dissolved organic matter in natural water does not affect the ELISA analysis. Similar results were found by Ulrich *et al. (47)* applying a developed sandwich-immunoassay for triazine residues linked to soil humic substances. A good correlation between GC and ELISA was observed by other authors *(48,49)* in the detection of atrazine, 2,4-D and aldicarb sulphone in water samples. The ELISA results were slightly higher than those obtained by chromatography.

Many applications of IAs to complex matrices are limited to screening of samples due to the potential bias of analysis toward false-positives results or overestimation of the analyte concentration. Some IA performances may be affected significantly by matrix components that interfere with the assay detection system and the antibody-antigen interactions. In order to detect alachlor in water samples, Feng *et al. (50)* developed an inhibition ELISA method, and in order to test the IA 208 water samples from rivers and water treatment plants were analyzed. The results showed that ELISA was generally less accurate and less precise than traditional GC/MS method. The authors suggested that since ELISA was conducted without any pre-treatment, the assay was more susceptible to sample matrix effect, and they concluded that the most effective use of this ELISA would be as primary screenning of alachlor in water showing a detection range from 0.2 to 8.0 ppb.

The kind of antibody can highly influence the IA performance by defining its specificity to certain compound on other structurally similar compounds. Monitoring atrazine residue in soil samples by ELISA, using specific monoclonal antibodies (Mabs), Schalaeppi *et al. (51)* observed no interferences in the assay and obtained a good correlation between IA and GC and HPLC analysis. The anti-atrazine Mabs cross-reacted with propazine and, to a much lower extent, with a few other *s*-trazines and hidroxy-*s*-triazines. On the other hand, interferences during UV monitoring of hydroxyatrazine by LC was observed. Goh and collaborators *(52,53)* adapted a previously developed monoclonal antibody-based ELISA to analyse simazine in Californian soils and studied the influence of the extraction procedure on the IA. They reported the non-specificity of the ELISAs for the *s*-triazine herbicide family. In 1993 Goh and his group *(54)* used a competitive inhibition IA to atrazine and simazine residue analysis in soils according to ELISA format proposed by Schneider and Hammock *(55)*. A competitive ELISA for the quantitation of cyanazine in water and soil was developed by Lawruk *et al. (56)* using a novel magnetic particles solid phase. Cyanazine was covalently attached to a bovine serum albumin (BSA) carrier, and the resulting herbicide-protein conjugate was used in rabbits to produce polyclonal antibodies specific for cyanazine. The limit of detection was 0.035 ng/mL in water and 3.5 ng/mL in soil.

Gascón *et al. (57,58)* have noticed the influence of the matrix in ELISA kit performance for atrazine, total chlorotriazines and alachlor, especially in the case of the estuarine water from Ebro delta (Tarragona, Spain). They found a good correlation between ELISA and GC-MS, but the cross-reactivity values for estuarine water were lower than for distilled water, probably due to the presence of the dissolved humic substances. The same effect was observed by Dankwardt and co-workers *(59)* during the determination of non-extractable atrazine residues in the soil samples. The presence of soil humic substances produced a decrease in the absorbance values mainly at low atrazine concentrations. Addition of bovine serum albumin (1%) to the enzyme tracer eliminated the non-specific reactions observed in this assay. Toscano *et al. (60)* observed a strong influence of the pH and concentrations of humic substances on the ELISA for atrazine in water samples. This effect was evident at low atrazine concentration and low pH values, leading

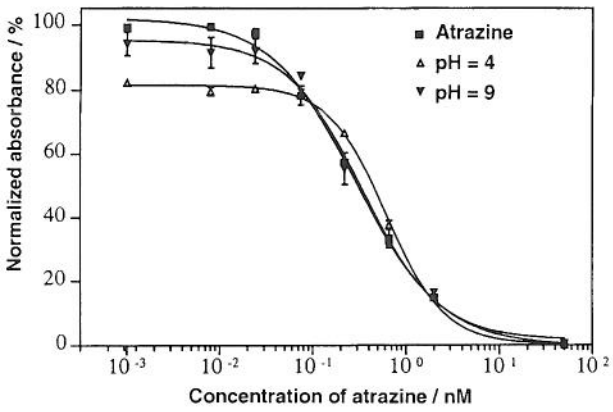

Fig. 3. Effect of the pH on the performance of the atrazine determination by ELISA. (a) Atrazine in phosphate buffer-Tween (pH = 7.0); (b) natural pH of the tropical water sample (40 mg L^{-1} of dissolved organic matter); (c) adjusted pH with NaOH solution. (From ref. *60*).

to an overestimation of the analyte concentration, as shown in Fig. 3.

Frequently, antibodies can cross-react with compounds that present similar structures as the target analyte, producing false-positive results or overestimation of concentrations compared with the results obtained by conventional methods. Thus, various IAs have been evaluated for cross-reactivities since a single analyte is not expected in a real environmental situation. Sometimes, the cross-reactivity can be advantageous and can be used as a screening tool for a class of related compounds. For example, a commercial ELISA applied by Aga *et al. (61)* for alachlor determination uses antibodies which cross-react with the ethane sulfonic acid (ESA), a major degradation product of alachlor. Alachlor and ESA were separated by sequential elution using solid phase extraction and were quantitatively measured by ELISA, showing a detection limit of 0.01 ppb for alachlor and 0.05 ppb for ESA with a precision of ±10%. An urinary study of occupational alachlor contamination in commercial applicators was reported by Biagini and collaborators *(62)*. For this, a commercially available IA kit was used and the obtained results were statistically different from those obtained with HPLC. The deviation was attributed to the matrix effect.

The development of new antibodies and technologies may allow identification and quantification of cross-reacting analytes and solution of the problem of overestimation due to cross-reactivity *(63)*. Abad and Montoya *(64)* have produced monoclonal antibodies for carbaryl from a hapten preserving the carbamate group characteristic of *N*-methyl-carbamate (NMC) pesticides. The conjugate showed a high affinity for carbaryl (IC_{50} = 3.6 nM) and minimum cross-reactivities for several NMCs and 1-naphthol, the main carbaryl metabolite. Krämer and co-workers *(65)* developed a highly selective ELISA for 1-naphthol. They have not observed cross-reactivity with carbaryl and tested the assay for residue determination of the degradation product in undiluted human urine and in soil extract. The assay was also tested the presence of organic solvents and showed a tolerance of at least 10% solvent (acetone, acetonitrile, methanol). As noticed in several works, not only cross-reacting compounds interfere in the IAs performance, but also organic solvents from the extraction procedures affect the assays by disrupting the antibody-antigen interactions. This effect was observed by Muldoon *et al. (66)* when they applied an ELISA method to analyze pesticides from materials generated during agricultural operations. Pesticide waste and rinsate samples were diluted 1:2 in acetonitrile and were analyzed by HPLC. The acetonitrile-diluted samples were further diluted in PBST and analyzed by ELISA. Total *s*-triazine content estimated by ELISA was highly correlated with HPLC results resulting in a slope of 0.83. Absence of interferences was probably due to a minimum 100-fold dilution required for these sample type which contain high concentrations of the analyte. This was effective in diluting out any potential sample matrix interference if present. Bushway *et al. (67)* employed commercially available competitive inhibition enzyme IA kits to study the degradation of benomyl to carbendazime in soil samples. Different detection limits varying from 1 to 3 ng/mL for tube IA, plate IA and HPLC analysis were achieved.

An improved IA method using specific rabbit polyclonal antibodies was developed by Del Valle and co-workers *(68)* to analyze chlodiamino-*s*-triazine in environmental samples. The assay was more sensitive and specific than previously reported mouse polyclonal antibody-based ELISAs *(69–71)*. Rabbit antibodies have been considered to be generally of higher affinity than mouse antibodies *(72,73)*. Two sensitive tests were developed by Hill *et al. (74)* for monitoring the environmental dissipation of chlorpyrifos, using a high-affinity polyclonal antibody immobilized onto microwells and polystyrene tubes. The assays were sufficiently sensitive for direct analysis of the pesticide in irrigation drainage water samples, after addition of phosphate buffer to the sample to slow its hydrolysis.

The classical analyses of molinate involve extraction with toluene or dichloromethane-ether. With solvent exchange Gee *et al. (75)* obtained acceptable results using ELISA for thiocarbamate herbicides. They also observed that the matrix effect decreased when the water samples were buffered by the addition of 10 times concentrated PBS-Tween. The within-run and between-run variability was less than 10% and the limit of detectability was 3 ppb. Also Xiao *et al. (76)* tested various polar and water-miscible solvents to extract molinate. They observed that at volume fractions higher than 10% of acetonitrile-propylene glycol (1:1) or higher than 5% of methanol in ELISA buffer the standard curves were significantly different from buffer alone. A strong evidence for a matrix-derived interference was noticed by authors. Goh *et al. (77)* used a commercial EIA kit to quantify atrazine residues in soil, to compare the extraction efficiency of four solvents, and to compare the results obtained with GC analysis. Methanol (>10%) and acetonitrile (>4%) levels in the final dilution interfered with the EIA system. In addition this kit was only suitable for a single known triazine due to cross-reactivity for some of the metabolites of atrazine.

Linde *et al. (78)* carried out a regulatory application of an ELISA for monitoring bromacil herbicide residues

in soil. Good reproducibility and exact results were obtained, and the modified method agreed well with the HPLC analysis.

IAs for pesticide analysis in crop and food samples

Although in the last years ELISA methods for determination of pesticides have been appearing increasingly, there is still a lack of IAs for pesticide residue quantitation in food and crop samples. Since the end of the '80s, several publications have emerged with the goal of focusing on this theme. The increased regulation of foodstuffs in modern society requires analytical methods which are easy to perform, sensitive, specific and relatively inexpensive. IAs techniques can provide complementary and/or alternate approaches in reducing the use of costly, sophisticated equipment and analysis time, but still maintaining reliability and improving sensitivity.

The utility and applicability of an analytical method depends in great part on the absence of matrix interferences. In this regard, ELISA is not different from the other detection techniques, because this question must be addressed by running appropriate blanks and controls. In 1987, Newsome and Collins *(79)* developed IAs for determination of benomyl and thiabendazole in 3 crops, but low sensibilities, with limits of quantification (LOQ) about 0.35 ppm for benomyl and 0.3 ppm for thiabendazole, were obtained. No control was carried out to improve the pesticide detectabilities and the low sensibilities were attributed to the matrix effect. Van Emon and co-workers *(80)* applied an ELISA method to determine paraquat residues in milk, beef and potatoes. In order to assess the reagents and matrix interferences, the reactionary medium was slightly acidified and a limit of detection (LOD) of 1.4 ppb was achieved.

Although the application of IA techniques has contributed tremendously to the quality control and safety of food supply, specially because, in their most simple forms, these techniques provide excellent screening tools to detect adulteration and contaminations qualitatively. Cochrane *(81)* evaluated a number of IA diagnostic kits to determine their usefulness in a regulatory analytical laboratory environmental in the food, feed and pesticide areas. Four rapid enzyme immunoassays were tested and some modification in the analytical protocols were incorporated in order to reduce the matrix effects. Bushway *et al. (82)* developed a polyclonal enzyme immunoassay method to determine atrazine in processed milk including skim, low-fat, whole, chocolate, evaporated and non-fat dry milk. Atrazine concentration was linear from 0.2 to 6.4 ng/mL and cross-reactivity was such that the method was used to determine other triazine pesticides in milk samples.

Many risk assessment models project risk based on an assumption of the presence of the pesticide residues at these tolerance levels or at a level equivalent to the limit of analytical detectability. This fact has already been under discussion, and data which demonstrate the absence of detectable levels of pesticides in infant formula have been presented *(38)*. In this respect, it is evident that the effect matrices is one of the numerous factors which have made it difficult to obtain the sensibility of the most conventional analytical methods. In fact, IAs compete with the other analytical techniques due to their selectivity. Indubitably, the development and application of specific antibodies in IAs is the key to further increase of the sensibility.

The prior sample preparation is still a critical point for pesticide residue analysis by IA methods. As for chromatographic techniques, extraction of more polar compounds is usually more complicated. A competitive ELISA was developed by Bushway *et al. (83)* for quantitation of methyl 2-benzimidazolecarbamate in fruit juices. They minimised the matrix effects by diluting the samples before immunoanalysis. In most ELISA investigations, the initial, more expensive and time-consuming experimental part is precisely the solvent extraction before the assay. Rapid methods for water or acetonitrile extraction were evaluated by Lehotay and Argauer *(84)* to screen for the presence of carbofuran and aldicarb sulfone in meat and liver or near their regulatory tolerance levels using a commercial ELISA kit for analysis. The final extract must be diluted in order to eliminate the solvent effect that in some cases can be more expressive than the natural compound effects. In some cases by simple pre-dilution of the sample, if it is in the liquid form, the matrix effect can be avoided. Nunes *et al. (85)* have minimized the matrix and organic solvent effects in the ELISA for carbaryl by diluting the methanolic extracts in the assay buffer. In Fig. 4. a comparison of the calibration curves of some diluted and non-diluted extracts is shown. In most cases a factor of dilution of 1:20 was sufficient to eliminate undesired effects without loosing the method sensibility.

In general, ELISA methods for pesticide analysis in complex matrices are still accompanied by sample pre-treatments in order to eliminate the interferences and to minimise the cross-reactivities. But in some cases the method recoveries are lower when compared with particular methods which do not employ previous sample treatment. If either cross-reactivities matrix interferences are not observed, the application of the IA directly in the untreated sample is still preferable. In addition, the final methodology could be considerably simplified. Fruit juices without prior clean-up were analysed by Itak *et al. (86)* for the determination of benomyl (as carbendazim) and carbendazim pesticides. The estimated sensitivity of the method was 30 ppb based on the dilution factor and sensitivity estimates in water. Bushway *(87)* quantified the fungicide thiabendazole in fruit juices and their concentrates (bulk and store bought) without clean-up by simply injecting 50 microliters of dissolved sample into an HPLC. The same extracts were shown to be thiabendazole positive by an ELISA method.

Pre-treatment procedures in food and crop samples still limiting the use of ELISA as a screening method for pesticide analysis, as was observed by Bushway *et al. (88)*. They optimised a complete sample preparation procedure for determination of MBC in blueberries by a competitive enzyme IA. The sample treatment consisted of a previous solvent extraction, liquid-liquid partition and a clean-up step before analysis. Lehotay and Miller *(89)* evaluated three commercial IA kits of different manufacturers for detection of alachlor in cow milk, and one of them was chosen for assay of chicken eggs and li-

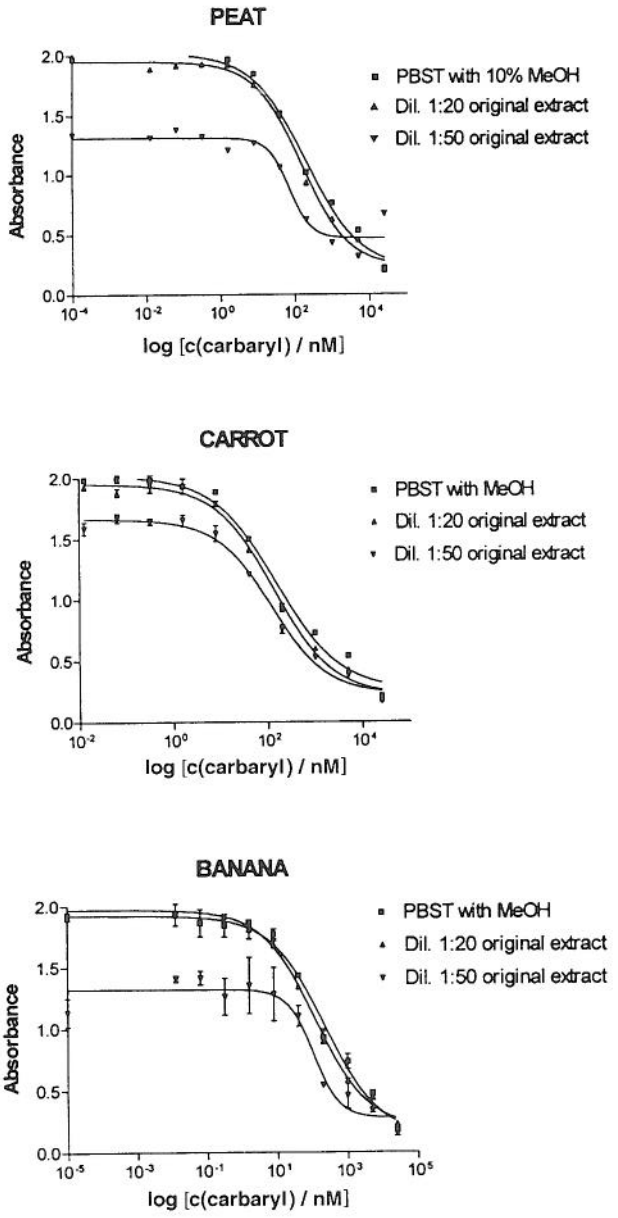

Fig. 4. Effect of the extract dilution on the ELISA for determination of the carbary insecticide in vegetable and crop samples. Standard curves obtained by dissolving the methanolic vegetable extracts in phosphate buffer-Tween (pH=7.0). (From ref. *85*).

vers. Brandon *et al. (90)* prepared a monoclonal antibody and used aqueous extraction. The sulfoxide and sulfone metabolites of albendazole were readily extractable and quantificable by the method, and multiple benzimidazole drug and pesticide residues were detected and quantified at concentrations levels between 1 and 8 ppb.

Since the '80s various types of IAs developed for the use in cereal analysis. Detection and determination of numerous meat species, non-meat proteins, microorganisms and bacterial toxins, pesticides, mycotoxins and other contaminants in meat and meat products by the means of IA kits were carried out. Skerritt *et al. (91)* developed an IA method for quantification of three organophosphate pesticides in wheat grain and flour-milling fractions. They noticed the effect of solvent present in the final extracts on the ELISA performance. Baumann and Hart-De Kleijn *(92)* determined quinmerac in some cereals at concentrations below the established tolerance. Permethrin and phenotrin were determined in stored grain products by ELISA, using three different assay formats *(93)*. In order to reduce the solvent interferences and matrix effects the authors evaluated different extraction methods and discussed the effectiveness of clean-up procedures on ground grain. Organophosphate, pyretroid, and methoprene residues in wheat and products and milling fractions were quantified by IAs *(94)*.

In contrast to the conventional chromatographic techniques for pesticide analysis in foods, IAs have not been extensively characterised yet. At the moment, a few works have presented as principal objective the validation of the proposed ELISA by another already validated methodology. As for any analytical method, quality control and assessment of the stability of materials and equipment are required. In addition, IA evaluation involves defining working range, sensitivity, precision, accuracy, linearity, specificity and matrix effects. Complementary, analytical techniques for confirmation and comparison of the sensitivities, selectivities and efficiency, for validation of the IAs for food analysis have been little by little used by few work groups *(86,87,94, 95)*.

Conclusions and Perspectives

Immunoassay techniques provide a simple, powerful and inexpenssive screening method with enormous potential which includes the generation of quantitative data. The need for newer and cost-effective methods to detect small quantities of pesticide, its metabolites and its degradation products in the environmental samples has been recognised. Therefore, in the last years numerous immunoassays have been developed or improved for monitoring pesticide residue in the environment. A particular interesting application involves water quality control with regard to pesticides, for which immunoassays have been developed, including commercially available kits. In order to evaluate the ELISA performance, various researchers have analysed different kinds of environmental samples. Some studies reported no matrix interferences when the immunoassays were carried out in samples that did not suffer any pre-treatment. On the other hand, most of the assays presented negative effects in the direct measurements of the analyte in water and soil samples. In general, these effects are due to the presence of the extraction solvents, content of the dissolved organic matter, pH, salts and metals. These effects could be eliminated by employing sample clean-up procedures or by appropriate dilution of the extracts. In addition, it was reported that the different antibody-coating antigen combinations have also showed different responses in the sensitivity of the immunoassays commonly used for pesticide determinations in complex matrices.

As agricultural products and foods are very important for human health, the maintenance of their quality is essential. In this respect, several works that include

the use of different IAs have been developed for pesticide analysis in these kinds of matrices, but in general the previous sample treatment employs the established extraction/clean-up procedures used for the more conventional detection methods. Usually, simple indirect ELISA methods have been more extensively applied to determine pesticide residues in foodstuffs. Sometimes, a simple dilution of the extracts in the buffer assay before ELISA is appropriate to minimise the matrix interferences. Failure to adjust the buffer concentration and also the final pH can result in complete inhibition of antibody in the assay. These methods have shown to have potential for application in screening of large numbers of foodstuffs.

Immunochemical methods are gaining acceptance and confidence of the analytical chemists. They are competing successfully with traditional analytical methods because they are now evaluated by the same criteria according to well-defined quality assurance plans. However, there are still several limitations to their use, that include the lack of accuracy and precision for some analytes. This is the main reason why some of the methods are quantitative and others are not. These limitations are leading towards new discoveries, and new enzyme-based analytical techniques for pesticide residue monitoring in food and in environmental samples. Indubitably, IA methods and biosensors will be further developed in the near future as simple, cheap and efficient methods for monitoring of environmental contaminants.

Acknowledgements

I. A. Toscano and G. S. Nunes thank for the fellowship obtained from Conselho Nacional de Desenvolvimento Cientifico (CNPq-Brazilian Agency). This work was also supported by the Commission of the European Communities Environmental Programs, Contract number ENV4-CT97-0333.

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Imunološko određivanje pesticida u okolišu i u hrani

Sažetak

Posljednjih je godina porastao broj imunokemijskih postupaka i u mnogim je prikazima potvrđena njihova pouzdanost u kontroli i provjeri ostataka zagađivača u različitim uzorcima. Imunološki postupci imaju velike mogućnosti primjene u kontroli ostataka pesticida. ELISA je najintenzivnije istraženi postupak, a nedavno su usavršeni mnogi drugi pristupi kao što su radioimunološka određivanja i imunoafinitetna kromatografija. U usporedbi s klasičnim analitičkim postupcima, imunološki postupci omogućuju visokoosjetljiva te relativno brza i jeftina mjerenja. Ovaj pregled daje opće podatke o imunološkim postupcima, usredotočujući se na njihovu primjenu u analizi pesticida, te ukratko razmatra međudjelovanje ostataka otapala ili sastojaka matriksa na uspješnost imunološkog određivanja. Prikazani su mnogi imunokemijski postupci uobičajeni za određivanje pesticida u različitim uzorcima hrane, godišnjeg uroda i uzoraka okoliša.