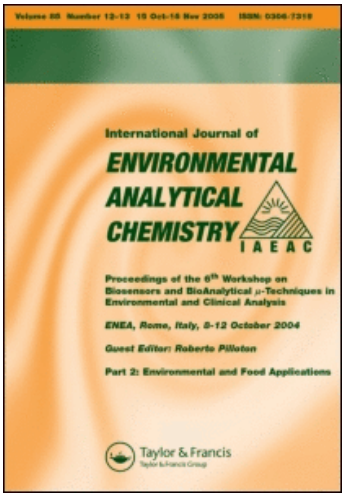

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Immunoassays in Environmental Analytical Chemistry†

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A review is presented which summarizes the recent developments of immunoassays in environmental analytical chemistry.

The basic principle of the method and the following steps in the development of an immunoassay procedure are discussed in detail: Synthesis of the immunogen, immunization procedure, synthesis of the labelled antigen (tracer), advantages and drawbacks of radioimmunoassay, fluoroimmunoassay and enzyme immunoassay. A special emphasis is put on approved methods which can be applied in almost any analytical laboratory.

Numerous examples of immunoassays for different pesticides and pollutants (dioxins, polychlorinated biphenyls, mycotoxins) are presented. Besides the respective test features, their applicability for residue determinations in biological samples is kept in the foreground. The advantages and drawbacks of the immunoassays are discussed in comparison with conventional analytical methods.

An outlook to future application fields for immunoassays and new trends (e.g. the utilization of monoclonal antibodies) is presented.

KEY WORDS: Immunoassays, pesticides, pollutants.

INTRODUCTION

Since its introduction in 1960, the immunoassay has become a broadly accepted analytical method, mainly in the field of clinical chemistry. It is used for the analysis of drugs, proteins, and viruses

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either for diagnostic or for therapeutic purposes. The great success of immunoassays in clinical chemistry induced several groups of researchers to investigate their potential for other analytical applications.

This review presents a survey of immunoassays in the field of environmental analytical chemistry and includes pollutants as well as pesticides. Advantages and limitations will be discussed with the emphasis on the special requirements in environmental analytical chemistry. Moreover, an outlook for future trends and developments is given.

PRINCIPLE OF IMMUNOASSAYS

All immunoassays are based on the specific reaction between an antigen and its corresponding antibody. As this reaction cannot be measured directly, a labelled antigen—the so-called tracer—is used as an auxiliary reagent.

Both the non-labelled antigen and the tracer tend to bind to the specific antibody binding sites. This reaction is based on the law of mass action. The more antigen is present in the sample, the less tracer is bound to the antibodies.

If the amounts of antibodies and tracer are kept constant, the only variable in the assay is the antigen concentration. Measuring either the free or the bound tracer concentration, consequently allows the indirect quantification of the antigen concentration. Although immunoassay makes use of the antiserum as a reagent of biological origin, it should be stressed that it is not a biological method but rather one based on a physico-chemical competition.

STEPS IN THE DEVELOPMENT OF IMMUNOASSAYS

Most compounds of environmental interest are of low molecular weight. Antibodies, however, can only be raised against compounds of molecular weights higher than 5000.

The low-molecular antigens—the so-called haptens—are therefore conjugated to a high molecular protein as for example bovine serum albumin (BSA). The entire conjugate is then used for the immunization.[1,2,3] The animals—mostly rabbits—produce anti-

bodies specific against the conjugate as well as against the hapten and the protein.

The basic requirement for the coupling reaction is the presence of a reactive group in the hapten.

If necessary, the reactive group can be introduced into the original antigen by chemical modification. A careful choice of the coupling site in the hapten is very important, because the specificity of the later antiserum can be dramatically influenced. Highest specificity of antibodies is usually obtained against those structures that are farthest away from the coupling site.

Two examples of immunoassays for 2.4-dichlorophenoxyacetic acid, abbreviated as 2.4-D, will elucidate this fact:

Rinder and Fleeker[4] immunized rabbits with 5-amino-2.4-D coupled via the 5-position to BSA. They obtained an antiserum, the affinity of which was about fivefold higher to 2.4.5-trichlorophenoxyacetic acid (2.4.5-T) than to 2.4-D. Specificity against the acid structure, however, was very good; the antibodies clearly discriminated between 2.4.5-T and the propionic acid analogue Silvex (2-(2.4.5-trichlorophenoxy)-propionic acid).

Knopp and Co-workers[5] conjugated 2.4-D via its free carboxylic group to the proteins for immunization. They obtained an antiserum, which showed tenfold higher affinity to 2.4-D than to 2.4.5-T. It could however not at all discriminate between 2.4-D and its methyl ester.

Both approaches can be valuable depending on the problem to be solved.

It should again be stressed that the desired properties of the antiserum can largely be determined by the careful choice of the immunogen structure.

The immunogen is subsequently used for the immunization of the test animals, mostly rabbits. The literature gives many examples for the successful performance of this procedure.[1,2,6] Normally, small aliquots of the conjugate are injected into the rabbits repeatedly during the course of several weeks or months. Blood samples from the animals can be taken some days after the so-called booster injections. This serum can already be used for the assay, either directly or after appropriate purification procedures as for example affinity chromatography.[2] Normally, two to five rabbits produce

enough antiserum for development of the immunoassay and its application.

As already mentioned at the beginning, an immunoassay is only practicable when an appropriate tracer is available. This means that the antigen must be labelled either with a radiolabel or with enzymes or fluorescent groups. Depending on the kind of label, the assay is called radioimmunoassay, enzyme immunoassay or fluoro-immunoassay. Each of these assays has its specific advantages and disadvantages.

In radioimmunoassay, for example, the use of radioactive tracers requires special permission from recognised authorities and rather expensive equipment. Moreover, the practicability of an assay is limited by the half-life of the isotopes. Only a few isotopes are suitable for radioimmunoassay. Normally, ^{125}I or ^{3}H are the only radiotracers which can be measured with adequate sensitivity.[5] ^{14}C-labelled antigens which would be easily available for pesticides, have unfortunately too low specific radioactivities for radioimmunoassays. Enzyme and fluoroimmunoassays were developed to overcome the problems with the radiotracers. In the beginning, enzyme immuno-assays were often not as sensitive as the respective radioimmuno-assays, but nowadays this no longer holds. Fluoroimmunoassays are only seldom described for pesticides,[1,7] although their feasibility has clearly been demonstrated. They are very cheap and have tracers with excellent stabilities, but are normally more sensitive to back-ground interferences of the samples.

On the basis of the published literature, it can be stated that up to 1981/82, radioimmunoassay was the method of choice for pesticides and pollutants. In the last three years the trend towards enzyme immunoassays has become obvious; especially ELISA—the enzyme-linked immunosorbent assay—is now the predominant method.[2,3,8,9] This test is based on the fact, that high-molecular antigens or antibodies can be adsorbed to a solid-phase support without loosing their immunological activity. Two different appro-aches to ELISA have been described: Huber and Hock[2] developed an ELISA for the determination of terbutryn, a triazine herbicide. They coupled terbutryn to alkaline phosphatase. The microtiter plates were first incubated with the antiserum solution, next with the sample or standard and finally with the enzyme-labelled antigen. After several washing steps the enzyme substrate was added. The

measured enzyme activity is inversely proportional to the antigen concentration in the sample, because only the free antibody binding sites can react with the enzyme-labelled antigen. A modified ELISA procedure does not make use of enzyme-labelled antigens but of specific enzyme-labelled second antibodies. Several published ELISAs are based on that principle.[3,9,10]

For the coating of the solid phase, a conjugate of antigen and protein is used. It is essential that this conjugate differs from the immunogen in order to exclude antibodies which recognize the carrier protein; for example: if the antigen-BSA-conjugate is used for the immunization, the coating antigen can be prepared from the antigen and ovalbumin.[9] The high-molecular character of the conjugate is needed in order to achieve adsorption to the solid phases. Wie and Hammock[10] extensively discussed the influence of coating and immunizing antigen structure on the sensitivity and specificity of immunoassays for benzoylphenylurea insecticides. After the coating and several washing steps, antiserum and sample, which are often preincubated, can be added. Only free antibody binding sites still bind to the coating antigen. Consequently, the more free antigen is present in the sample, the less antibody can be bound to the solid phase. After washing, the enzyme-labelled second antibody—e.g. horseradish peroxidase coupled to antirabbit immunoglobulin G of goats[9]—is added followed by the respective enzyme substrate. The colour development then occurs in direct proportion to the amount of antibody bound to the coating antigen. It is hence again in indirect proportion to the original concentration of free antigen in the sample.

One advantage of this procedure is the fact that a commercially available enzyme-labelled conjugate can be used. This makes the coupling of the enzyme to the antigen unnecessary.

IMMUNOASSAYS FOR PESTICIDES AND THEIR APPLICATION

Table I summarizes the pesticides for which immunoassays have been published in the past. It can be easily seen that immunoassays are not restricted to a certain class of compounds.

Numerous examples demonstrate the possibility of raising anti-

TABLE I
Immunoassays for pesticides

Pesticide	Class	Type of immunoassay	References
Diflubenzuron and derivatives	Insecticides	ELISA	8, 10, 11
Parathion, Paraoxon	Insecticides	RIA, ELISA	6, 12, 13
Paraquat	Herbicide	RIA, ELISA	14–18
S-Bioallethrin	Insecticide	ELISA	19, 20
Dieldrin, Aldrin	Insecticide	RIA	21
Benomyl and metabolites	Fungicide	RIA, FIA	7, 22
2,4-D, 2,4,5-T	Herbicide	RIA	4, 5
Diclofop-methyl	Herbicide	FIA, EIA	1
Terbutryn	Herbicide	ELISA	2
Metalaxyl	Fungicide	ELISA	9
Chlorsulfuron	Herbicide	ELISA	3
Triadimefon	Fungicide	ELISA	23

bodies against herbicides, insecticides, and fungicides once a suitable protein-conjugate for immunization has been synthesized. Moreover most of the authors do not only describe the successful development of a quantitative immunochemical method, but also present examples for their application in residue analysis.[1,2,9]

Two main application areas can be distinguished. One important field is the analysis of the different body fluids such as serum or plasma, urine, and milk. The other large field of application is the analysis of environmental samples such as water, soil, and plants.

On principle, all immunoassays have to be performed in aqueous buffer solutions, in order to retain the immunological reactivity of the antibodies. Consequently, aqueous samples can be used directly for the test performance without any further partition step. This feature makes the immunoassay the method of choice for all aqueous samples such as urine, serum, and water.[1,2,17]

Huber and Hock[2] used an ELISA for the determination of the triazine herbicide terbutryn in pond water. The natural water sample had only to be filtered and adjusted to a pH of 7.5 prior to its use in the assay. Sample concentrations between 25 ppb and 1.5 ppm laid in the measuring range without the need of any concentration step. Wie and Hammock[10] even described the direct analysis of milk by their ELISA for diflubenzuron without any sample extraction procedure.

This example elucidates perfectly one important advantage of immunoassays which is the minimum of required sample clean-up before analysis.

The minimum demand for sample clean-up also becomes obvious when solid environmental samples such as soil or plants are analysed. Crude extracts are prepared by homogenization or extraction of the sample with appropriate solvents.[1,3,9,12] These crude extracts are concentrated and taken up either in methanol or buffer solution. Normally, no other clean-up steps are necessary before the performance of the immunoassay.

This procedure works well for different commodities such as fruits[9,23] (apples, lemons, grapes, etc.), crops[1] (wheat, sugar-beets, potatoes) and soil.[1,3]

Newsome[9,23] described two different ELISA procedures for the quantification of two fungicides, Metalaxyl and Triadimefon. He spiked various food commodities for example apples, peas, and pineapples with 0.1–2.0 ppm of the fungicides and determined the recoveries by ELISA after extraction with methanol. The crude extracts were used in the test without any further purification other than filtration.

Some authors[1,9,23] compared their immunoassays with the approved conventional analytical method for the respective pesticide, as for example GC or HPLC. They found comparable recoveries and coefficients of variation which indicates that conventional methods are not superior to immunoassays in respect to accuracy and reproducibility.

Wie and Hammock[8] gave an extensive comparison of classical and immunochemical methods for the analysis of diflubenzuron residues in milk and water. The most striking features in this comparison are the number of assays per man-day and the approximate reagent cost per sample. With immunoassays, the sample throughput can be increased by a factor of ten or even more compared to routine GC or HPLC methods. At the same time, reagent costs are lower by a factor of at least twenty.

These advantages are also pointed out by other authors.[1,18]

On the base of all these results, it can be stated that immunological methods are always advantageous if large series of samples have to be analysed. Semi- or fully-automated immunoassay systems are already available in clinical chemistry and should also be applicable in environmental analysis as well.

As far as the limit of detection is concerned, immunoassays also compete favourably with conventional methods. Detection limits in the low ppb-range are normally obtained without difficulties.[2,3,8,17] Routine clean-up steps would even improve sensitivity further. This sensitivity is at the same time related to a high specificity for a single compound or a group of structurally related compounds depending on the assay design. Immunoassays are most likely to find application to those pesticides where analysis by classical procedures is very difficult or expensive.

One example is the group of benzoylphenylurea insecticides. As these compounds are nonvolatile, GC methods are only applicable after lengthy derivatization steps; HPLC analysis on the other side requires intensive clean-up procedures. In this case, the ELISA procedure described by Wie and Hammock[8,10] represents an appropriate alternative.

IMMUNOASSAYS FOR THE ANALYSIS OF POLLUTANTS

Two further classes of compounds that are of interest in environmental analytical chemistry have been successfully determined by immunoassays.

The first group is that of the polychlorinated pollutants such as biphenyls,[24,25] dioxins,[26] and dibenzofurans.[27] Radioimmunoassays using the respective ^{125}I-labelled tracers have been described for these compounds. Although these compounds are extremely hydrophobic, they can be determined in the aqueous immunoassay systems either after addition of a nonionic detergent (Cutscum, Triton)[24,26,27] or by utilization of 25% dimethyl sulfoxide in the assay medium.[25]

Specificity against the various isomers was influenced by the immunogen structure. Albro *et al.*,[26] for example, used 1-amino-3,7,8-trichlorodibenzo-*p*-dioxin as hapten for immunogen synthesis. They obtained antisera, the affinity of which was highest for the 2.3.7.8-TCDD and some pentachlorodibenzo-*p*-dioxins. The reactivity of the respective dibenzofurans was considerably lower; tetrachlorobiphenyls did not cross-react at all. The radioimmunoassays were used for the determination of the various compounds in adipose

tissue, liver, blood, urine, milk, air, and soil. All results were compared with those obtained by conventional GC-analysis and showed good agreement. Newsome and Shields[25] found detection limits of 20 ppb for milk and 2 ppb for blood. The immunoassays are thus applicable to screening samples in order to minimize the demand for mass spectrometric analysis and could be used supplementary to the conventional analytical methods. This is also true for the screening of a second important group of pollutants, namely the mycotoxins. During the past few years, several laboratories have developed immunoassay procedures for the determination of different mycotoxins, as for example aflatoxins[28] and ochratoxins.[29] They used either radioimmunoassay or ELISA for the screening of food and feed. Morgan *et al.*[29] developed an ELISA for the determination of ochratoxin A in barley. They obtained excellent recoveries at the low ppb-level and a detection limit of 60 ppt. At the same time, their antiserum was highly specific for ochratoxin A and did not significantly cross-react with related mycotoxins.

OUTLOOK

At the moment, the development of immunoassays for environmental analytical chemistry is still rather a field of research than a routine procedure. Although their applicability has been proved, it is unlikely that immunochemical methods will completely replace current, established analytical methods. But as they provide distinct advantages over conventional procedures, it is likely that they will open new application fields in many types of exposure and monitoring situations. Screening foods for pesticide residues, for example, can easily be cheapened and speeded up if a set of appropriate immunoassays is available. Moreover, portable field kits could be developed which make the analytical work independent from the laboratory and allow a first screening on the spot.

So far, little work has been done to investigate the possibility of raising antisera in different laboratories "on demand". The fact that an antiserum is unique and may not be exactly reproduced as far as affinity and specificity is concerned it is still taken as a drawback to immunoassays. Possibly, this could be overcome by the use of monoclonal antibodies as an immunologically homogeneous reagent of virtually unlimited supply.

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