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review

Enzyme Immunoassays for Analysis of Pesticides in Water and Food

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Summary

Enzyme immunoassays offer reliable tools for the analysis of pesticides in water and food. They profit from the unique properties of antibodies as binding proteins with respect to their selectivity and affinity towards the ligands such as pesticides. Both polyclonal and monoclonal antibodies can be used to develop sensitive immunoassays. The choice of antibody depends on the scope and intention of antibody application. The structure of the immunoconjugate is an important factor for the development of sensitive and selective antibodies. For the analysis of small molecules such as pesticides usually competitive immunoassays with phase separation are applied. Optimization of the assay can be achieved by varying the test system (e.g. heterologous instead of homologous tracer system) or applying amplification methods. Common error sources, especially cross-reactivities and matrix effects, are discussed. Representative examples are given for the application of enzyme immunoassays for the determination of pesticides in water and food samples. New techniques such as immunoafinity chromatography, coupling of liquid chromatography with immunoassays and immunosensors are discussed. An outlook is given on multianalyte detection and the potential of recombinant antibodies in water and food analysis.

Keywords: enzyme immunoassays, antibodies, pesticides, cross-reactivity, matrix effects, water analysis, food analysis

Introduction

The extensive use and the persistence of many pesticides have led to the widespread occurrence of pesticide residues in water, soil and agricultural products (e.g. *1–4*). For example atrazine, a s-triazine herbicide still used in many countries, was found in drinking water at concentrations of up to 3 μg/L *(5)*. In food mainly dithiocarbamates, organochlorine and organophosphorous compounds have been observed *(4,6–9)*. Aldrin and dieldrin, two organochlorine insecticides, were detected in milk and eggs from Egypt, at concentrations up to 1.2 mg/L in milk and up to 0.7 mg/L in eggs *(8,9)*.

Pesticide residues in water and food are commonly analysed by gas chromatography (GC) or high pressure liquid chromatography (HPLC). Both methods require rather expensive and sophisticated technical equipment. However, large scale screening can do with simpler and more inexpensive approaches. For this purpose immunochemical methods are valuable supplements. Originally developed in the medical field *(10)* they have become increasingly important for environmental analysis (e.g. *11–17*). Immunoassays offer distinct advantages for the detection of pollutants in water and food. Water and liquid food samples can often be analysed without clean-up procedures or solvent extraction. Although solid foods require extraction steps, analytes can be detected at very low concentrations even in low sample volumes. Many samples can be analysed in a short period of time.

Immunochemical Analysis

Antibody production

Immunochemical analysis is based upon the specific reaction between an antibody (Ab) and its corresponding antigen or hapten. Due to their small molecular masses pesticides have to be coupled to a carrier molecule, usually a protein, in order to induce an Ab response in the vertebrate immune system *(18)*. The site of coupling to the carrier, the coupling procedure as well as the number of haptens bound to the carrier can be of

major importance for the sensitivity and the selectivity of the resulting Ab *(18–20)*.

Antibody production is conveniently carried out in warm-blooded animals, e.g. rabbits, sheep, mice or chicken. Polyclonal antibodies (pAb) are obtained from the serum and comprise a mixture of different Ab populations. Monoclonal antibodies (mAb) consist of a single monospecific Ab population. These Ab are produced in cell culture by a single hybridoma cell derived from the fusion of B-lymphocytes with myeloma cells *(21)*. The advantages of mAb for the analysis of pesticides are well known (Table 1). The hybridoma technology guarantees the unlimited production of mAb with constant properties. The cultivation of cell lines in larger production systems such as minifermenters allows the supply of large amounts of Ab. Up to 100 μg of Ab per mL cell culture supernatant can be obtained *(22)*. However, the production of mAb needs special equipment, and the material and media for tissue culture are quite expensive. Therefore, the production of pAb will be sufficient for many applications and can be carried out in any laboratory with animal facilities at a fraction of the costs of mAb. In many cases pAb also show higher affinities towards the analyte in comparison to mAb *(23)*.

Optimization of immunoassays

Up to now, immunoassays for approximately 100 pesticides and their metabolites have been published *(16,24)*. Most of them are suitable for the detection of relevant concentrations of these substances in water and food. The EC Directive for Drinking Water *(25)* limits the pesticide concentration of a single substance to 0.1 μg/L and the sum of pesticides to 0.5 μg/L in drinking water. In Germany, the maximum concentration of atrazine allowed in food is for example 0.5 mg/kg in corn, 0.1 mg/kg in other plant foods, 10 mg/kg in spices, coffee, tea, products similar to tea, and oil seeds *(26)*.

In order to obtain highly sensitive enzyme immunoassay (EIA) optimization of the test system is indispensable. An appropriate assay format has to be chosen. For low molecular mass analytes (haptens) in solution, competitive tests have to be employed, using limiting Ab concentrations. Two different formats are available, with immobilized Ab (Fig. 1a), and with immobilized coating conjugate (Fig. 1b). In the first variant analyte and a labelled analyte (tracer) compete for the free Ab binding sites. After removal of unbound reactants the bound tracer yields a signal that is inversely proportional to the analyte concentration. The second variant employs an immobilized hapten-carrier conjugate on the solid phase to which analyte and Ab are added. The Ab binds to the free analyte or to the immobilized hapten according to the concentration of the reactants. If a labelled Ab is used, the amount of Ab bound to the solid phase can be directly determined after a washing step. Alternatively, a secondary labelled Ab may be used to detect the Ab

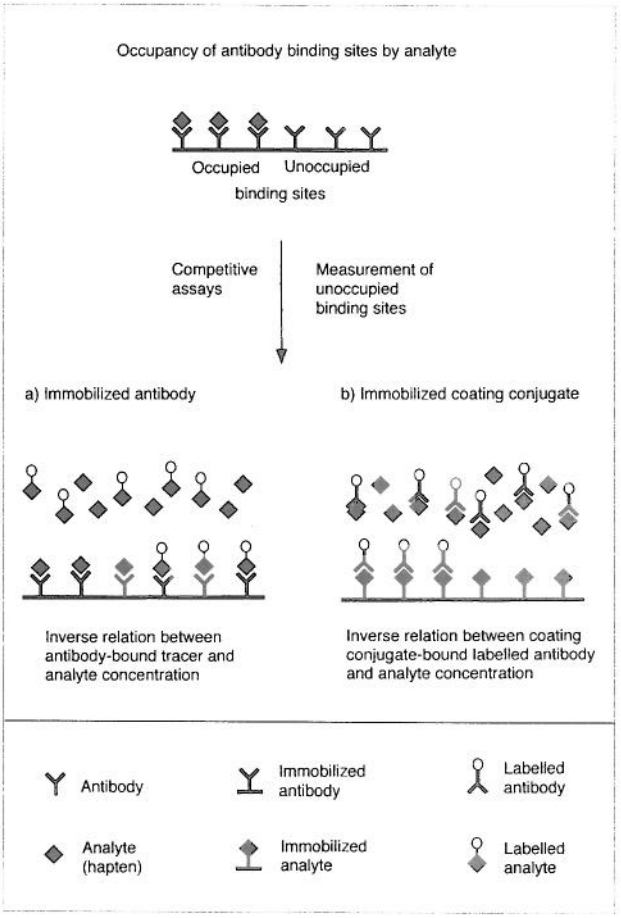

Fig. 1. Principle of the competitive enzyme immunoassay. In the first format with immobilized Ab (a) the plates are coated with antibodies. Analyte and enzyme labelled analyte compete for the antibody binding sites. In the second format a hapten-protein conjugate is immobilized to the solid phase (b). This protein conjugate and the free analyte compete for the binding sites of the antibody in solution.

Table 1. Properties of polyclonal and monoclonal antibodies

Properties	Polyclonal antibodies (pAb)	Monoclonal antibodies (mAb)
Supply	Limited and variable	Unlimited production possible
Uniformity	Changing properties with different sera and bleedings	Constant properties of a mAb
Affinity	Mixture of Ab with different affinities, affinity often higher with pAb	Uniformly high or low, can be selected by testing
Cross-reactivity	Results from different selectivities and low affinity interactions	Different, dependent upon the individual Ab
Classes and subclasses	Typical spectrum	One defined isotype
Demands on the antigen	High purity required for specific antisera	Impure antigens or mixture of antigens can be used for immunization, pure antigens necessary for screening
Costs	Low	High

which have bound to the solid phase. The signal is inversely proportional to the amount of free analyte in the sample. Very sensitive competitive immunoassays have been developed with detection limits between 1 and 50 ng/L, for example for triazines and urea herbicides *(27–29)*.

The binding of Ab to analyte can be favored by selecting an EIA hapten for which the chosen Ab has a lower affinity in comparison to the analyte, thus improving the assay sensitivity *(19)*. Therefore, very often the use of a heterologous hapten coupled to the enzyme tracer can lead to a greater sensitivity compared to a homologous system, where the hapten derivatives used for immunoconjugate and enzyme tracer synthesis are identical. The use of signal amplification techniques such as catalysed reported deposition *(30)*, enzyme cascades or (strept)avidin-biotin interactions *(31)* can also improve assay sensitivity. However, this is limited by the affinity constant of the Ab *(32)*.

Besides polystyrene microwell plates and tubes. Ab-coated magnetisable particles have been used as solid phase for EIA *(33,34)*. Another solid phase support for immunoassay (IA) are membranes. They can be used for dipsticks, which are incubated for a short time in the reagent solutions *(28,35)*, or for dot-blots and immunofiltration tests. Here the reactants are filtered through the membrane *(13,36)*. The test principle is the same as for the microwell plate tests but the reaction time is much shorter due to the high surface area of the membrane and the short distance between reaction partners. Application of remission measurements yields a proportional relationship between analyte and remitted light *(13,35)*. By using a pocket reflectometer this set-up is ideally suited for field-monitoring purposes.

Cross-reactivity

Depending on the immunoconjugate used for immunization and the class of chemicals under investigation, cross-reactivities of the Ab with haptens similar to the analyte are frequently observed (e.g. *19,23*). Therefore, it should be checked which compounds cross-react to which degree with a given Ab. This is usually done by comparing the standard curves of the analyte under investigation with similar haptens, using the IC_{50} value (analyte concentration that decreases the signal to 50% of the negative control) as the reference. However, it should be recognized, that the cross-reactivity of a polyclonal antibody to a substance may not be the same over the whole measuring range. Oubina *et al. (37)* investigated the cross-reactivities of an EIA for chlorpyriphos-ethyl in water by adding the cross-reactants at different concentrations while the concentration of chlorpyriphos-ethyl remained the same. This method gave much higher values compared to the conventional method when the IC_{50} of the target analyte and the cross-reacting reagents were used.

Sometimes unexpected pesticide metabolites may lead to false positive results for the parent compound. The strong cross-reactivity of an alachlor Ab to the sulfonic acid metabolite, for example, produced frequently false positive values when an alachlor screening kit was used *(38)*. This problem could be solved, however, by applying solid phase extraction prior to immunoassay and sequential elution of the two compounds with different organic solvents.

If an Ab is selective for a single compound, it is called monospecific *(23)*. Wittmann and Hock *(39)*, for example, obtained a pAb against hydroxyatrazine, which only reacted with hydroxyatrazine, while 18 s-triazines and 10 other herbicides showed no reaction up to concentrations of 10 mg/L. An Ab that recognizes several compounds to the same extent, e.g. a group of s-triazines, can be used for the screening of a class of herbicides (group-specific Ab) *(40)*. If cross-reacting compounds are not expected in the samples because the compounds are not licensed, such as propazine in most European countries, a group-specific Ab can also be used for quantitative measurement of one compound *(41)*.

Sample preparation

Sample preparation is usually not required for water samples except for removal of particulate material and pH adjustment. In some cases humic substance may interfere with the test *(42,43)*, but the concentration of humic substances in drinking water is usually very low. If an EIA is not sensitive enough for the direct measurement in the water sample, extraction of the analyte from the sample can be carried out by solid phase extraction using C_{18}-columns or immunoaffinity columns *(44,45)*. In some cases interfering metabolites are removed by solid phase extraction using sequential elution steps as described in the previous section for alachlor and alachlor sulfonic acid *(38)*.

No sample preparation except for pH adjustment was performed for liquid foods in most cases. Wittmann and Hock *(46)* spiked milk and apple juice samples with atrazine. The EIA was directly applied to the samples, obtaining values very close to the spiking concentration. In the next step milk, juice and canned corn samples were spiked again with atrazine and then extracted with methanol for HPLC measurements and water for EIA. HPLC and EIA results showed good agreements, however, only about 20–50% of the spiked atrazine was recovered by both methods. Franek *et al. (47)* also spiked juice and milk samples with atrazine and simazine. In this study matrix effects of the samples were observed, as the standard curves prepared in juice or milk showed a lower assay sensitivity compared to standard curves in water.

For the extraction of pesticides from solid foods a variety of solvents have been tested, such as acetone, ether, petroleum ether, methanol, acetonitrile, hexane *(48)*. Direct analysis of extracts by EIA requires the use of solvents that are miscible with water and (at low concentrations) non-denaturing to proteins such as Ab. EIA are to a certain degree tolerant of a variety of solvents, but each system must be tested to determine which solvent can be accepted and to what extent. Nugent *(49)* used 10% propanol for a chlorpyrifos EIA. An EIA using monoclonal antibodies against s-triazines tolerated up to 10% methanol *(50)*. Usually the extracts are further diluted with water prior to the EIA, but an EIA for parathion was developed, in which the analyte dissolved in hexane could be directly measured in the EIA without prior removal of the heane. This was achieved by using

Ab encapsulated in reverse micelles composed of Aerosol T whith aqueous centres *(51)*. However, a 10^4-fold decrease in sensitivity was observed.

Hill *et al. (48)* investigated wheat grain for the presence of the insect growth regulator methoprene. Either whole grain or ground grain was extracted using different solvents and sequential dilution with water. The best results were obtained with 5% methanol and 2.5% acetonitrile, both causing less than 10% inhibition of color development in a solvent-free control. However, the assay was 2.5–3 times more sensitive when the assay standard curve was performed in methanol compared to acetonitrile. Adding protein to the methoprene-methanol diluent (0.5 g L^{-1} final concentration of human serum albumin) increased the sensitivity of the test in 2.5% acetonitrile 5-fold, while no significant change with 5% methanol was observed. The effect of the proteins is assumed to shield the Ab-hapten complex from the denaturing effects of acetonitrile.

Matrix effects

Natural water samples and especially food extracts may contain substances in addition to the target analyte that may interfere with the test. Several groups investigated the influence of ions on the EIA *(52–54)*. Ruppert *et al. (52)* observed an inhibition by several anions like azide, which inhibits the peroxidase by binding to the heme group of the enzyme. Therefore, azide should not be added as antimicrobial agent to buffers used in EIA with a peroxidase tracer. Most cations did not interfere except for Ca^{2+}, which lead to an activation of the peroxidase. No interference by different ions such as nitrate, copper, magnesium etc. up to a concentration of 250 ppm was detected in an EIA for pentachlorphenol in water *(53)*. While ions may inhibit the enzyme used as a label or lead to precipitates by reacting with buffer components, humic substances may bind non-specifically to the Ab and thereby interfere with the specific binding of the analyte. These reactions usually lead to false positive values.

Some problems can be solved by changing the buffer system to avoid precipitation or by adding bovine serum albumin to the enzyme tracer to prevent unspecific binding of humic acids or other interfering substances *(43)*. The buffering capacity of the assay buffer should also be checked, as some water or food samples (orange juice, cola) may show low pH values. No effects were observed between the pH 3 and 10 by different investigators *(33,40,55)*.

Bushway *et al. (56)* investigated milk samples by EIA. For the quantification of atrazine in milk the standard curve has to be derived from the same type of non-triazine milk source as the sample because of a matrix effect due to the milk fat. Milk products (whole, chocolate, 2%, evaporated, half and half) with the highest fat content showed lower recoveries of spiked atrazine than the non-fat milk products (skim and non-fat dry milk). In the non-fat milk products the same concentrations for the different fortification levels of atrazine were observed as with water. The matrix effect could be removed by a 1:10 dilution of the milk samples. However, this lowered the detection limit from 0.2 µg/L to 2 µg/L.

The variability in immunoassay data due to matrix effects was decreased by the development of a standard addition ratio method *(57)*. This technique was used to develop a screen for 2,4-dichlorphenoxyacetic acid in fruits and potatoes using a commercial EIA. To compensate for matrix effects observed at 0.5 g produce/mL, the standard is added to an aliquot of each sample extract and run in parallel, without the need of a standard curve. The apparent response of each sample varies according to its matrix, but the ratio of spiked to blank sample is consistent (±4.6%) and characteristic of the quantity spiked.

We were able to reduce matrix effects caused by humic substances by adding 1% bovine serum albumin (BSA) to the enzyme tracer *(43)*. Therefore, this was also applied to food analysis. Different dilutions of orange juice and black tea were prepared and spiked with 1 ppb of atrazine. The samples had to be diluted at least 1:10 (juice) to 1:20 (tea) to correctly measure the spiking concentration. After adding BSA to the enzyme tracer, 1 ppb was detected in all orange juice samples including the undiluted juice (Fig. 2). The atrazine concentration in the black tea could be assayed correctly after a 1:5 dilution (Fig. 2). Therefore, by adding BSA to the assay reagents the limit of detection can be significantly lowered as the assay can be carried out in undiluted samples or in low dilutions.

Quality control of immunoassays

In spite of the simple handling of the assays, expert knowledge is required, especially to recognize and re-

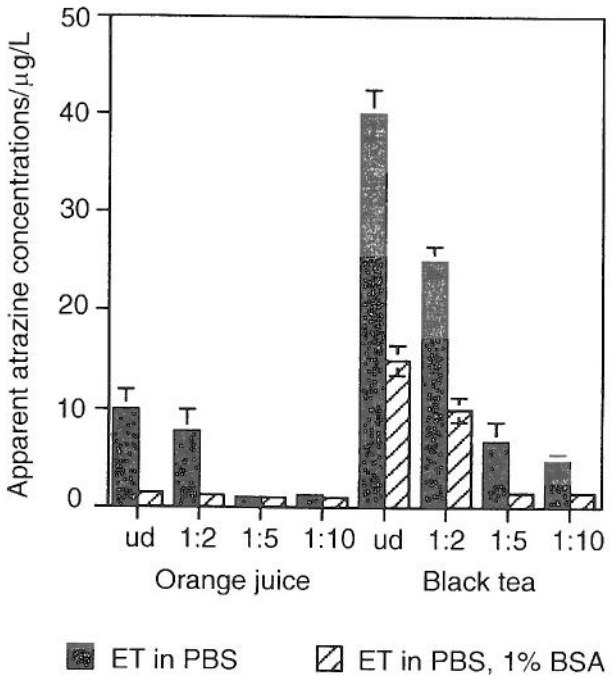

Fig. 2. Analysis of different dilutions of spiked liquid food samples with pAb S2 according to Dankwardt *et al.* (3). The samples were diluted first and then spiked with 1 µg/L simazine. The influence of bovine serum albumin (BSA) on the reduction of matrix effects was tested by addition of 1% BSA to the enzyme tracer (ET), ud = undiluted

move incident errors. Therefore, immunoassays should be performed by trained pesonnel. The developent of simple and rapid assays, e.g. dipstick assays or immunofiltration tests *(13,28,35,36)* reduce the requirement for trained users, but one has still to be aware of potential problems such as interferences by the sample matrix with test components.

Data on the variability of an EIA provide important information on the consistency of the test. Coefficients of variation (CV) of immunoassay measurements are usually between 10 and 20% (e.g. *56,58*) for an optimized assay, although more precise results can be obtained *(59, 60)*. Same-day and day-to-day CV should be determined *(56)*. Interlaboratory tests as carried out by Hock and the Immunoassay Study Group *(61)* for the investigation of triazines are important tools for testing the general applicability of an assay. However, several conditions like exact description of the assay including calibration curves, detection limits, cross-reactivities, a working range close to the middle of the test, enough parallel measurements etc. must be met. Recently, a prenorm has been established for a standardized procedure for immunoassays in water in Germany *(62)*.

A validation of the results obtained by EIA should be carried out besides statistical evaluation. To a limited extent this can be done by EIA itself. Dilution of samples as well as spiking of the authentic sample with known amounts of the contaminant can be used to check whether the matrix interferes with the EIA *(63)*. However, spiked samples do not completely mimic real unknown samples. They do not contain potential metabolites of the contaminant nor residues from other compounds which may be present in real samples. Therefore, an immunoassay should also be validated by a different established method like HPLC, GC or GC/MS. During the last years many groups have used this approach and usually obtained correlation coefficients of >0.9 *(2,47, 64–66)*. Often a slight overestimation of the immunoassay in comparison with HPLC or GC is observed due to the antibody cross-reactivity or matrix effects.

Immunoanalysis of water and food samples

EIA have intensively been used for the determination of pesticides in surface and rainwater *(2,5,38,55, 67–72)* as well as groundwater (e.g. *2,38,69,73,74)*. A substantial part of these studies was carried out for triazine herbicides *(2,5,55,67,69,71,73,74)*. This illustrates the widespread occurrence of these herbicides in the aquatic environment. Many investigators have used commercial test kits, which allow the investigation of samples without time-consuming Ab production. Thurman et al. *(2)*, for example, used a Res-I-Mune kit (ImmunoSystems) for the investigations of triazines in surface and groundwater. The EIA was compared to GC/MS results obtained from samples that were extracted by solid phase extraction (SPE). Correlation coefficients between 0.91 and 0.95 were obtained after introducing cross-reactivity factors for each of the triazines in order to calculate a sum parameter for the GC. The majority of the samples contained only atrazine (up to 3 μg/L). The EIA results corresponded well with the atrazine concentrations obtained by GC/MS.

Mouvet *et. al. (75)* compared four commercially available test kits and one in-house developed assay for the determination of triazines in surface and grounwater. Operational characteristics, cross-reactivity, sensitivity, CV and agreement with GC-LC measurements were investigated. Detection limits were determined between 0.003 and 0.07 μg/L. Intra-assay coefficients of variation were below 7% for all tests, inter-assay ones below 20%. Correlation studies between the EIA kits and GC-LC were carried out for samples from different water matrices. Depending on the water source different levels of significance were observed with different tests. The best results were obtained for surface water, while not all the kits showed a good agreement for lysimeter samples.

Besides the triazine some other pesticides were investigated in water samples, also using commercial test kits. Alachlor was determined in ground and surface water using commercial tests *(68)*. Solid phase extraction was carried out prior to EIA to remove interfering substances and to concentrate the analyte. Concentrations of up to 0.8 μg/L were observed, and a comparison with GC/MS showed a correlation coefficient of 0.95 with a slight underestimation by EIA. The occurrence of carbaryl was determined by Marco *et al. (76)* in well-water from Spain with their own assay and compared with a commercial test kit. Both IAs yielded a good agreement with conventional methods. Concentrations of 0.08–1.37 μg/L were observed. Two commercial test kits were used in the Netherlands to determine 2,4-D concentrations in the rivers Rhine and Meuse *(72)*. By diluting the kit standards with kit 0-buffer and calculating the detection limit on the basis of the error in the 0-standard the detection limit as originally indicated by the manufacturer was significantly lowered. The water matrix substantially affected the recovery of 2,4-D with one assay kit, yielding unexpectedly low recoveries in demineralized and tap water. However, similar results were obtained by EIA and GC/MS for spiked samples from the river Rhine (with a slope of about 1 and r = 0.99). Routine samples were also analysed and yielded analyte concentrations mostly below the detection limit (0.03–0.05 μg/L).

A variety of pesticides has been determined in food samples by EIA (Table 2). In many cases Ab against the target compound were in-house developed. Still, mainly pAb were used, but mAb were applied for the determination of atrazine, benzimidazoles, thiabendazoles and carbaryl *(35,78,79,89,90)*.

Peels of apples, potatoes, oranges, grapefruits and bananas were investigated for thiabendazole residues employing an EIA with mAb *(90)*. Residues were extracted by soaking peels overnight in 80% methanol and filtering the decanted supernatants. Most of the thiabendzole was extracted within 1 h, but an extraction time of 16 h was chosen as uniformly high recoveries from all matrices were obtained. A 20-fold dilution eliminated significant matrix effects. The EIA had a detection limit of 0.1 ppm in peel samples, corresponding to 10–40 ppb in the whole fruit or tuber. Results obtained by EIA were compared with HPLC analyses. Although the EIA values were higher compared with the HPLC results, the two sets of data were highly correlated. The higher values by EIA were attributed to the loss of thiabendazole

Table 2. Sample preparation and sensitivity of a selection of EIA for pesticides in food samples
DL = Detection limit, QL = Quantification limit, IC_{50} = analyte concentration required for 50% inhibition

Analyte	Format	Food type	Simple treatment	Detection limit (DL), test range	Ref.
Alachlor (Metolachlor)	EIA, pAb	Grain	Grinding, extraction with methanol-water	DL=20 ppb	*(77)*
Aldrin (Dieldrin)	EIA, pAb	Eggs	Removal of egg shells, homogenization, dilution	0.006–0.7 μg/mL	*(8)*
Aldrin (Dieldrin)	EIA, pAb	Milk	No treatment	DL=0.01 ppm	*(9)*
Atrazine	EIA, pAb	Milk, juices	No treatment	0.03–3 ppb	*(47)*
Atrazine	EIA, pAb	Milk	No treatment	DL=0.2 ng/mL	*(56)*
Atrazine	EIA, pAb	Juices, tea	No treatment	1 ng/L–10 μg/L	*(46)*
Atrazine (Cyanazine)	EIA, pAb	Grain	Grinding, extraction with methanol-water	DL=20 ppb	*(77)*
Atrazine	Dipstick EIA, mAb	Milk, juices, tea	No treatment	0.3–10 μg/L	*(35)*
Benzimidazoles	EIA, mAb	Bovine liver	Extraction with DMF, water or citric acid	0.3–30 ppb	*(78)*
Carbaryl	EIA, mAb	Apple and grape juices	No treatment, dilution	QL=2 ng/mL	*(79)*
Carbendazim (Thiabendazole)	EIA, pAb	Fruit, vegetables	Extraction with methanol	0.4–10 ng/mL DL=0.01 mg/kg	*(80)*
Captan	EIA, pAb	Fruits	Extraction with methanol, ether-hexane, evaporation, reconstitution in PBS	1–200 ng/mL	*(81)*
2,4-Dichlorpheno-xy-acetic acid	EIA, pAb	Grain	Grinding, extraction with methanol-water	DL=20 ppb	*(77)*
	EIA, pAb	Apples, grapes, potatoes, oranges	Extraction with acetonitrile	0.7–50 ppb	(57)
Difenzoquat	EIA, pAb	Wheat and barley products	Extraction with HCl (cereals), degassing and dilution with PBs (beer)	DL=16 ng/g (cereals) 0.8 ng/mL (beer)	(82)
Levamisole	EIA, pAb	Meat, milk	Homogenisation in PBS (meat), no treatment (milk)	0.1–30 μg/mL DL=1 μg/kg	*(83)*
Metalaxyl	EIA, pAb	Vegetables	Extraction with methanol	0.1–2 ppm	*(84)*
Methomyl	EIA, pAb	Grape leaves	Extraction with DFR wash (0.04% Aerosol-OT)	DL=0.45 ppb IC_{50} 4.15 ppb	*(85)*
Methoprene	EIA, pAb	Wheat grains, milling fractions	Extraction with methanol or acetonitrile	DL=60 ppb IC_{50} 0.75 ppm	*(48)*
Myclobutanil	EIA, pAb	Apples, grapes	Extraction with PBS	0.3–200 μg/mL	*(86)*
MBC (methyl-2-benzimidazole carbamate)	EIA, pAb	Wine	Evaporation, reconstitution with H_2O	IC_{50} 4 ppb	*(87)*
Procymidone	EIA, pAb	Wine	Dilution with BSA-Tween 20 diluent	IC_{50}=35 ppb (white wine), 75 ppb (red wine)	*(87)*
	EIA, pAb	Vegetables	Extraction with Na_2SO_4, ethylacetate, evaporation, reconstution in petroleum ether, solid phase extract (SPE)	DL < 20 μg/kg	*(88)*
Thiabendazole	EIA, mAb	Bovine liver	Extraction with 10% DMSO, water or PBS+Tween	0.3–30 ppb	*(89)*
	EIA, mAb	Fruits and vegetables (peels)	Extraction with 80% methanol	0.2–10 ppb	*(65)*
Triadimefon	EIA, pAb	Fruits	Extraction with methanol	IC_{50}=2.4 ng/mL	*(90)*

during sample work-up for HPLC and the high dilutions necessary for EIA.

Triadimefon was added to different food commodities such as apples, pears, pineapples and grapes. Fungicide residues were determined by EIA and gas chromatography after extraction with ethyl acetate or methanol *(90)*. Ethyl acetate was required to extract grapes for the EIA procedure, since methanol resulted in coextractives which gave too low values. Methanol, however, yielded good recoveries at 0.5 ppm and above for other commodities and was preferable to ethyl acetate since it avoided an evaporation step. Low recoveries were obtained at 0.1

ppm and were not improved using ethyl acetate. On the whole, the recoveries obtained by EIA correlated with those measured by gas chromatography. The middle of the test was observed at 2.4 μg/L.

A mAb-based EIA was applied to the determination of carbaryl in apple and grape juices *(79)*. The juices were used without any sample pretreatment and spiked with different carbaryl concentrations. The influence of the matrix dilution was investigated using different dilutions of the samples. For proper analysis the samples should be diluted at least 1:5 – 1:10. With a dilution of 1:100 the most accurate and precise results were obtained. Therefore 2–5 μg/L are considered the lowest cabaryl concentrations in juices that can be reliably measured with the EIA. Coefficients of variations ranged from 4 to 13%, with most of them below 8%.

A dipstick immunoassay using mAb immobilized on a membrane was used for the determination of atrazine in water and liquid food samples *(35)*. The measuring range was 0.3–10 μg/L using reflectance detection. The total assay time was 25 min using precoated dipsticks. The atrazine concentrations could be determined directly in spiked water, milk and juice samples yielding satisfactory agreement with the spiking concentrations. The black tea samples, however, showed an overestimation due to the unspecific binding of the tannins to the membrane.

Paraquat was determined in milk, beef, and potatoes using an EIA with pAb *(91)*. Potatoes were shredded with dry ice. The potato and the meat samples were extracted with HCl after spiking of the samples. The acid extracts were evaporated to dryness and reconstituted for the EIA. The milk was diluted with phosphate buffer. The EIA was able to detect less than 1 ppb of paraquat in whole milk and down to 2.5 ppb in beef. The efficiency of HCl for extracting potato and ground beef was determined by using methyl-^{14}C paraquat. Recoveries between 60 and 70% were obtained. Since paraquat is known to bind tightly to many matrices, recoveries for both matrices were determined after storage of the spiked samples for several days at 21 °C.

Ibrahim *et al. (9)* investigated eggs for aldrin and dieldrin residues. An EIA with pAb was applied. The EIA detected only dieldrin, but aldrin was metabolized to dieldrin. The eggs were homogenized after removal of the shell. This solution was diluted 1:2 with wash buffer containing 2% BSA. Egg samples were collected in Egypt and assayed by EIA. The standard curve for dieldrin was prepared in egg solution of non-contaminated eggs. The egg samples showed concentrations of aldrin and dieldrin in eggs up to 0.7 mg/L. These are concentrations above the World Health Organization average daily intake levels.

Integration of liquid chromatography with immunoassay

Up to now, EIA are not yet available for all compounds of interest. The most criticall ones are polar, because they are difficult to analyse with chromatographic methods.

If samples containing several contaminants are analysed by EIA, only the target substances are recognized. Multianalyte analysis, however, was carried out by combining different Ab against triazines (e.g. *92–94*). Quantitation of individual s-triazines in each sample was carried out by using the analyte reactivity coefficients for each Ab and solving three simultaneous equations (one per Ab) with three unknowns (one per analyte) by matrix inversion *(92)*. Also neuronal networks *(93)* or iterative procedures *(94)* have been used.

More recently, Ab have also been used in conjunction with liquid chromatography (LC), e.g. to preconcentrate an analyte from a large volume of sample and separate it from an interfering matrix before chromatographic separation *(45,95,96)*. This is especially interesting for food analysis, as many foods contain coloured substances (e.g. fruit juices, vegetabe extracts) or high fat content (e.g. cheese, milk). In this case an immunoadsorbent column is used before analysis by LC. The immunoadsorbent column contains immobilized specific Ab which bind the analyte, while interfering substances pass through *(97)*. Common immunoaffinity matrices are protein A or G immobilized on silica, cyanogen bromide-activated Sepharose, synthetic polymers or activated silica supports. Ab are coupled to the solid phase through affinity adsorption or covalent binding through their amino, carboxyl or carbohydrate groups *(97)*. The analyte can be eluted from the immunoadsorbent column by a pH gradient *(44)* or an organic solvent *(45)*. Therefore, large sample volumes with low concentrations of the analyte can be reduced to small volumes with sufficiently high concentrations without co-extracting interfering substances. This raises the effective sensitivity of the analysis. Hage *et al. (98)* used a high-performance immunoaffinity column, which contained immobilized Ab against atrazine, simazine, deethylatrazine and hydroxyatrazine. This was combined with a reversed-phase column for separation of the extracted compounds. This technique gave good correlations with GC methods and allowed for the direct analysis of samples in the ppt-ppb range within 12 min.

Ab mixtures can be used to bind substances from different compound classes, e.g. the phenyl urea herbicides and the triazines *(99)*. In this case, the eluted compounds were injected into the LC, yielding a detection limit of 0.03–0.5 μg/L from samples volumes as low as 25 or 50 mL.

When cross-reacting Ab are applied in EIA, the obtained signal is not only related to the analyte, but also to related compounds. This problem can be circumvented by the use of LC prior to the immunoassay (IA). LC-IA was applied by Krämer *et al. (95)* to determine 4-nitrophenols. The nitrophenols were separated with different LC-systems and determined by EIA. LC-IA was about 8–10 times more sensitive compared to LC with UV detection. Therefore, the integration of LC with IA combines the high separation quality of the LC and the sensitivity of an IA *(100,101)*.

Conclusion and Outlook

It has been shown that EIA can provide reliable tools for water and food analysis. The strength of the method lies in the possibility to screen a large number of samples within a short time at low costs. Therefore,

EIAs can be valuable supplements to conventional analytical methods.

Some restrictions are imposed by the fact that IA are de facto single analyte methods. However, new approaches are being undertaken, such as the integration of IA with LC. Furthermore, multi-analyte systems are under development. One concept is the microspot IA *(102)*, which uses many microspots with fluorescence-labelled Ab of different selectivity immobilized on a chip. After incubation with the analyte (antigen or hapten) a fluorescence-labelled tracer Ab is added. The tracer Ab is either directed against the antigen or consists of an anti-idiotype Ab directed against the binding site of the capture Ab. Sensor and tracer Ab carry different fluorescence labels. Therefore, it is possible to determine the amount of analyte bound to the sensor Ab with optical scanning methods by measuring the signal ratio (ratiometric assay). Lately, a variety of non-competitive and competitive microspot analyses systems have been developed, mainly related to the medical field *(103)*, but are clearly of particular importance in areas such as environmental monitoring. Another possibility is the use of cross-reacting Ab for multianalyte detection as described in the previous section.

Immunochemical analysis is a fast developing field with numerous possibilities for further improvements and developments. Two main directions can be observed. Much effort is put into the development of continuous measurements, such as flow injection immunoanalysis (FIIA) and immunosensors *(32)*. A quasi-continuous FIIA of pesticides was developed by Krämer and Schmid *(104)* on the basis of a competitive IA. Here, the Ab are immobilized on a membrane. The reaction takes place in the membrane reactor, the central part of the flow injection system. All reagents are sequentially added to the reactor and the product is assayed with the aid of a flow fluorimeter. The measuring range of the flow injection analysis almost equals that of the EIA. Wittmann and Schmid *(105)* used an Ab column reactor filled with polystyrene or glass beads with the Ab immobilized *via* the avidin/biotin system. This system showed a stable Ab activity for a minimum of 500 measuring cycles. Detection limits for atrazine of about 1 ng/L with pAb and 30 ng/L with mAb could be reached.

Some relatively simple sensing devices are immunoassay-based dipsticks *(13,28,35)*. The Ab are immobilized on a membrane and the dipstick is then introduced into the sample. If relfectance detection is used, a quantitative signal is produced. Immunofiltration, also a membrane-based test, was used to screen rain and surface water, but only visual detection was used *(36)*. In more complicated systems the immunological recognition system is immobilized in the direct vicinity of a transducer, an electrochemical, optical or gravimetric device. They respond to chemical compounds or ions and yield electrical signals which depend on the concentration of the analyte. Immunosensors with piezoelectric crystals as physical sensors are in a relatively advanced state of development *(106)*. They function as microbalances onto which Ab are immobilized. Other physical sensors use optical systems such as surface plasmon resonance (SPR), interferometry or grating couplers *(107,108)*. A biosensor employing SPR was used for the determination of atrazine *(109)*. A detection limit of 0.05 μg/L of atrazine in water was reached with an analysis time of 15 minutes. Bier and Schmit *(110)* used a grating coupler immunosensor for the determination of terbutryn, a triazine herbicide. A detection limit of 15 nmol/L (c. 3.6 μg/L) was established. Interesting developments are also to be expected from Ab electrodes *(111)*.

Also new strategies for Ab production are being developed. Genetically engineered Ab appear very attractive because their selectivity and affinity can be tailored by site directed mutations without requiring new immunizations *(112)*. Methods are now provided to rapidly isolate desired clones from Ab libraries and to manipulate individual recombinant Ab to match specific demands of environmental analysis. Binding proteins derived from Ab but consisting only of a part of their light or heavy chain (Fv) and recombinant Ab fragments (Fabs) directed against different s-triazines have been produced *(113–115)*. An EIA using a single chain Fv showed a measuring range from 3 to 30 ppb *(115)*. The cross-reactivity pattern of the recombinant Ab was very similar to the one from the mAb it was derived from. A promising goal is the completely synthetic production of binding proteins or other synthetic receptors which are fitted to the structure of the analyte by molecular design. The use of libraries guarantees to close the bottleneck Ab production. Also, Ab with special properties such as resistance to matrix effects or organic solvent stability can be selected from the libraries, providing an important contribution to the analysis of water and food samples.

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Analiza pesticida u vodi i hrani enzimsko imunokemijskim postupkom

Sažetak

Enzimski imunokemijski postupak je pouzdan način analitičkog određivanja pesticida u vodi i uzorcima hrane. Njegova je prednost u jedinstvenom svojstvu antitijela kao proteina koji se selektivno i s određenim afinitetom veže na ligande, kao što su pesticidi. Za provedbu osjetljivog imunokemijskog određivanja mogu se koristiti poliklonska i monoklonska antitijela. Izbor antitijela ovisi o svrsi i namjeni njihove primjene. Struktura imunokonjugata važan je činitelj u pripravi osjetljivih i selektivnih antitijela. Za analizu malih molekula, kao što su pesticidi, obično se primjenjuje kompetitivni imunokemijski postupak s odvajanjem faza. Optimiranje određivanja postiže se mijenjajući sustav testiranja (npr. heterologan umjesto homolognog sustava indikatora) ili primjenjujući postupke amplifikacije. Razmotreni su opći uzroci pogreške, osobito utjecaji unakrsne reaktivnosti te utjecaj matriksa. Prikazani su tipični primjerci primjene enzimskih imunokemijskih postupaka za određivanje pesticida u vodi i uzorcima hrane. Razmotreni su i novi postupci kao što su imunoafinitetna kromatografija i povezivanje tekućinske kromatografije s imunokemijskim određivanjem i imunosenzorima. Iznesene su mogućnosti multianalitičkog određivanja te potencijal rekombinantnih antitijela u vodi i pri analizi hrane.