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review

Immunochemical Detection of Environmental and Food Contaminants: Development, Validation and Application

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Summary

Immunochemical methods, in particular immunoassays are very suitable for the detection of contaminants in environmental and food samples. The principles of immunoassay, components required and possible variants are discussed. Further, strategies for their designing, optimalizing and validating are given. Immunoassays are useful tools in screening and monitoring programmes due to the fact that they allow rapid analysis of target compounds in several matrices at a very low level. An updated overview is given of target substances of natural and synthetic origin for which immunoassays have been developed. Their applicability is illustrated by examples from field studies. Consideration is given to recent developments in in vitro antibody production and the establishment of norms.

Keywords: immunoassay, antibody production, food and environmental contaminants

Introduction

Immunochemistry is the discipline where immunological and analytical-chemical techniques are combined. The best-known immunochemical method is the immunoassay which has since long been used in clinical chemistry for diagnostic and therapeutic purposes. In environmental analysis the potential of immunoassay for the detection of contaminants has been recognized and an increasing number of assays has been developed. The main component in immunoassays is the antibody that specifically binds a target compound. Quantitation is generally performed by including a signal-generating component. Useful antibodies have been produced against various substances of natural and synthetic origin; for example, against pesticides, toxins, PAH, PCBs, viruses, bacteria, etc. Immunoassays are particularly useful for screening purposes because in a very short time many samples can be analyzed simultaneously and at a very low level.

In the present report, the general principles, design, optimization and validation of immunoassays will be explained, followed by examples of assays developed for environmental and food analysis. The applicability will be illustrated by the results of some particular studies. Variant immunochemical methods such as immunoaffinity chromatography and immunosensor are beyond the scope of this report and will not be discussed.

Principles

Molecular biology

Antibodies are proteins consisting of four polypeptides, two heavy and two light chains that are linked together by covalent disulfide bridges and form the characteristic Y-structure (Fig. 1). Amino acid analysis has revealed that the C-terminal end is highly conserved and exerts species-specific effector functions, whereas the N-terminal end is variable and responsible for antigen binding. Digestion studies have shown that the Fv fragment, $V_L + V_H$, is the smallest portion of an antibody required for antigen binding. At genomic level the constant region is encoded by a single-copy gene and multiple genes code for different variable segments. The diversity in antibody populations is caused in several ways. Naturally, mutations occur randomly at DNA level. In the genes for the variable region, particularly those parts encoding the complementary determining regions (CDR1, CDR2, CDR3) are hypervariable and highly susceptible to mutations. Further, the assembly of the chains may be inaccurate leading to deleted or altered amino acids in the final antibody *(1)*.

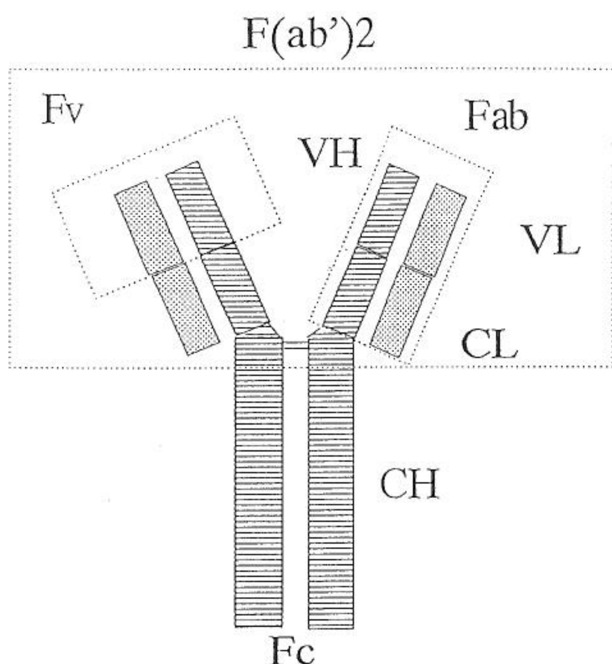

Fig. 1. Schematic representation of an antibody and its fragments Fc = constant fragment exerting effector functions; Fv = variable domain fragment; $F(ab')_2$, Fab = antigen-binding fragment; VH and VL = heavy and light chain variable domains; CH and CL = heavy and light chain constant domains. CDR1,2,3 are contained in the VH and VL regions.

Antibodies

For the performance of an immunoassay antibodies have to be produced. There are several ways to achieve this: polyclonal, monoclonal and recombinant antibodies. The first two types are raised in animals by immunizing them with a suitable substance that elicits an immune reaction. The last type is an *in vitro* method. Each type will be illustrated below.

Polyclonal antibodies

In nature, the immune system of animals, as well as of humans, produces circulating antibodies in response to an intruding foreign substance, the so-called immunogen. In practice, to raise antibodies for use in immunochemical methods an animal (rat, rabbit, sheep, goat) is immunized with a particular immunogen with repeated injections; after 2–3 months the serum is collected and the antibody isolated. Because several cells are involved in the immune response, the serum will contain a population of antibodies having differing affinities and specificities and is therefore designated a polyclonal antiserum. Most environmental contaminants, however, are low-molecular compounds that will not elicit an immune response. To raise antibodies against low-molecular compounds (haptens), they are generally conjugated to a carrier protein such as BSA (bovine serum albumin) or KLH (keyhole limpet hemocyanine) in order to make them immunogenic. Several protocols for the coupling of carrier proteins to haptens have been described. An overview of the principles, immunization and fusion protocols, selection procedures and quality control for the production of polyclonal antibodies is given by Catty and Raykundalia *(2)*. Additionally, guidelines for production and quality control are described herein.

Monoclonal antibodies

Polyclonal antisera display several disadvantages. Animals have to be immunized and sacrified to obtain suitable antibodies. Especially in the case of rodents, the yield is ather limited and an identical polyclonal antiserum can never be reproduced. A further drawback is the presence of a population of different antibodies in the same serum, each with differing characteristics. The hybridoma technique for the production of monoclonal antibodies seems to have overcome the problems mentioned above. This technique has been developed by Köhler and Milstein *(3)* and comprises the steps of: *i.* immunization, usually mice; *ii.* fusion of spleen cells and myeloma cells; *iii.* primary selection of antibody producing cells; *iv.* culturing of individual cell clones; *v.* secondary selection of those cells that produce the antibody sought; and *vi.* scale-up of antibody production.

Such cell clones essentially present an infinite source of antibodies of defined characteristics. The production and particularly the selection step are very tedious and hence expensive. An overview of the principles, immunization protocols, fusion and selection procedures for murine monoclonal antibodies is given by Brown and Ling *(4)*. An example of the production of a monoclonal antibody against atrazine is given by Giersch and Kramer *(5)*.

Recombinant antibodies

The production of polyclonal as well as monoclonal antibodies depends on the immune response of the animal used. Despite its great potential, the usual immunization protocol may not yield a desired antibody. For example, in the case of haptens where hapten-conjugate molecules are used as immunogen, the immune system may not recognize the target compound or the immunogen is degraded by metabolic processes. Further, antibodies may be produced against the carrier protein or even the spacer moiety rather than the hapten portion. In the case of environmental or food contaminants, the immunogen may be toxic for the animal used. Molecular biology offers techniques for production *in vitro* and for manipulation of antibodies at the genetic level. Recently, several reports have been published on this subject *(1, 6–8)*. For the production of recombinant antibodies the genes coding for the heavy and light chains are isolated from antibody-producing cells and introduced into expression-vector systems. *E. coli* is the most widely used expression system because its genetics is well known, it is easy to use and there are many vectors available. Generally, antibody fragments containing the antigen binding site are expressed, such as VH, VL, Fv, Fab, $(Fab')_2$, rather than whole antibodies since correct protein folding is difficult to achieve in prokaryotic cells. The antibody fragments may be recovered from the cytoplasm, periplasm or the culture medium.

The process of producing recombinant antibodies involves the steps of: *a.* isolation of mRNA from hybridoma, spleen cells or lymphocytes; *b.* synthesis of cDNA by reverse transcriptase; *c.* amplification of the RNA-DNA

hybrid by PCR using suitable primers; *d.* ligation of dsDNA obtained into a bacterial plasmid vector; *e.* transformation of competent host cells; *f.* screening of transformed bacteria; *g.* scale-up; *h.* screening for desired antibody fragments.

Once antibody genes have been isolated and sequenced, they can be engineered to alter the specificity and/or affinity of the antibodies for target compounds. Especially the CDR regions that define antigen binding are candidate sites for mutagenesis.

A particular interesting development are phage display antibodies. Bacteriophages such as phage λ or fd contain single stranded DNA wherein foreign genes can be incorporated. The phage is used to infect competent bacterial cells wherein they can replicate and produce gene products. Both scFv and Fab fragments have been obtained in such a way. Especially useful are combinatorial libraries whereby genes for heavy and light chains can be combined at random. In the phage display technique, antibody genes are linked to phage coat protein genes g3p or g8p and expressed as fusion proteins along the outside or at the tip of the assembled phage. Selection of the desired antibody may be performed, for example, by using antigen-coated columns or (magnetic) beads.

Other expression systems may also be used such as the baculovirus system or eukaryotic host-vector systems (yeast, fungi, mammalian cell lines, transgenic animals and plants). Each system has its own advantages and disadvantages and especially with baculovirus and yeast good results have been obtained.

Tracers and formats

Detection and quantitation in immunoassay is achieved by incorporating a signal-generating component. Schematically, the reaction between antigen and antibody is given as:

$$Ag + Ab \rightleftharpoons Ag\text{-}Ab$$

The simplest format is the competitive immunoassay wherein as a detectable component a labeled antigen is added. The equation then becomes:

$$Ag + Ag^{*} + Ab \rightleftharpoons Ag\text{-}Ab + Ag^{*}\text{-}Ab$$

After equilibrium has been reached, the bound and the free phases are separated and usually the bound fraction is measured. The amount of analyte is thus inversely related to the signal level detected. Various separation techniques exist *(9)*, but most of them require a centrifugation step. For example, with relatively small antigens free components are removed by addition of charcoal, followed by centrifugation.

Initially, radioisotopes were used, but in recent years these have been replaced by enzyme, fluorescent and chemiluminescent labels. In such competitive immunoassays it is a prerequisite that the antibody recognizes the tracer and that the affinity of analyte and tracer are of comparable magnitude. When radio-labeled tracers are used, this generally poses no problems, especially with ^{3}H or ^{14}C, because of a high similarity between tracer and analyte. If larger groups are conjugated, the position where the tag is coupled should be properly chosen so that the antibody recognizes the tracer comparable to the analyte. In the case of enzyme tracers, the separation step is followed by adding substrate plus chromogen/fluorogen for subsequent measurement. While the first immunoassays were performed in the fluid phase, wherein antibody, tracer and sample antigen were mixed in a reaction vessel, the development of solid phase assays signified a great step forward. These can be divided into two types: those where the antigen and those where the antibody is linked to the solid phase. The first type is schematically illustrated in Fig. 2A. The antigen is coupled to a solid support, sample antigen and enzyme-labeled antibody are added and incubated; then the fluid phase is removed and the remaining amount of antibody is quantitated by performing an enzyme reaction and measuring the coloured product. Variant formats can be envisioned where the antigen-specific antibody is not labeled, but a second antibody is raised against the first antibody. Several other variants of such ELISA have been developed and described in reviews of the subject *(10)*. With regard to low-molecular compounds including pesticides and other contaminants, the coupling of antigen to a solid support may not be achieved. It may appear that due to the small size or conformational changes thereof the antibody is no longer able to bind or recognize it. To overcome these problems, the antigen may be linked through a spacer or first be coupled to a carrier protein.

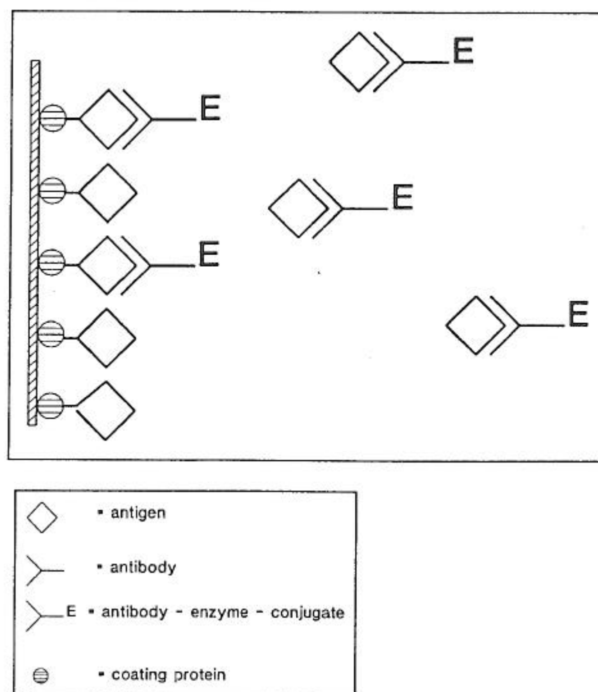

Fig. 2A. Schematic representation of solid phase enzyme immunoassay with antigen coated *via* coating protein
Antigen-carrier conjugate is coated onto solid support; sample antigen and labeled antibody are added and after incubation the free phase is removed; substrate and chromogen are added and the enzyme reaction product is measured.

The alternative type of immunoassay where the antibody is linked to a solid support has been widely used for environmental analysis. This format is schematically depicted in Fig. 2B. Specific antibody is coated onto the solid support, sample antigen and tracer are added and incubated; then the free phase is washed away and on the remaining bound phase an enzyme reaction is performed and the coloured product is measured. Again numerous variants can be designed. For example, when an antigen contains two or more epitopes, e.g. in the case of peptides or proteins, a first antibody is coated onto the solid phase, incubated with sample antigen and quantitated by adding a labeled second antibody against a different epitope (sandwich immunoassay). As an alternative, after addition of unlabeled second antibody, a labeled third antibody is added. For more details and different embodiments such as homogeneous immunoassays (EMIT, polarization fluorimmunoassay, etc.) reference is made to reviews published *(11–13)*. The choice of a particular format depends a great deal on the presence of functional groups on the analyte where labels can be covalently coupled and on the size of the analyte molecule. Several reports have been published wherein various approaches were described. For example, with regard to the enzyme tracer in an immunoassay for tracing atrazine levels in water, seven different tracers were followed and tested *(14)*. By selection of one particular conjugate a sensitivity of 15 ppt of atrazine in the assay could be achieved.

Particular forms of solid-phase immunoassays developed for rapid qualitative detection are the dipstick, the immunofiltration and the cloth enzyme immunoassay embodiments. In these formats the antibody is coated onto a strip, membrane or cloth. After incubation with sample antigen and enzyme-tracer the response is visualized by a colorigenic reaction.

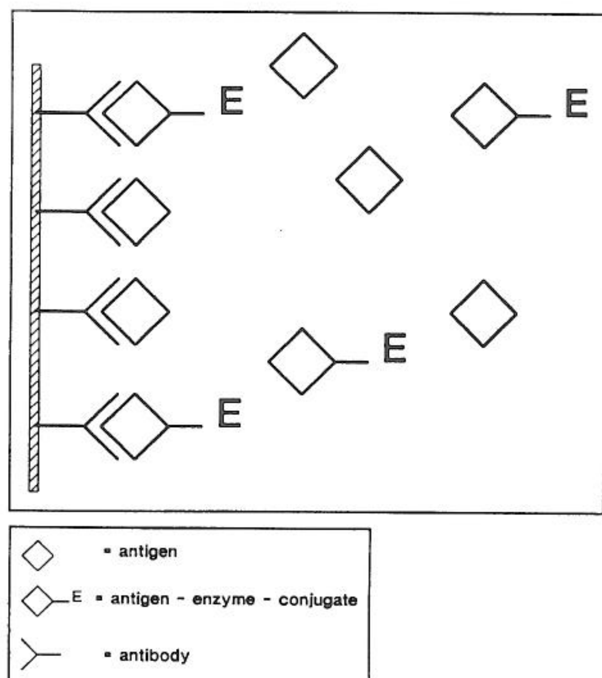

Fig. 2B. Schematic representation of solid phase enzyme immunoassay with coated antibody
Antibody is coated onto solid support; sample antigen and enzyme tracer are added and after incubation the free phase is removed; substrate and chromogen are added and the enzyme reaction product is measured.

A variant method is flow-injection immunoassay (FIIA) wherein the reaction between antibody and antigen/tracer is performed on-column and the reaction product is passed to a detector system. FIIA can be readily automated and is expected to improve the speed (less than two minutes) and quality of immunoassays. In this type of assay, the binding component is coupled to a solid support and the remaining components of the immunochemical reaction are added sequentially. The final product of the reaction is passed to a detector for quantitation. Usually enzyme tracers are used in combination with chromophores or fluorophores for UV, fluorescent or chemiluminescent detection. Several applications in drug detection, environmental analysis and bioprocess monitoring have been reviewed *(15)*.

Developmental strategies

For the development of a suitable immunoassay a number of objectives should be defined: target compound, immunogen, type of antibody, specificity, format of the assay, tracer to be used. Subsequently, each immunoassay should be optimized with regard to several parameters comparable to any other analytical method. The different steps to be followed in designing an immunoassay will be explained hereinafter.

Immunogen

The size of the target compound is essential in the immunization step. Relatively large substances (> 5000 Da) may be used as an immunogen. As mentioned above, haptens have to be made immunogenic by coupling to a carrier molecule. The design and synthesis of haptens and their coupling to macromolecular carriers strongly determine the affinity and specificity of the resulting antibody. Recently, important reviews concerning the general principles of hapten design for ELISA have been published *(16–20)*. Generally, functional groups on the protein and the hapten are used for covalent linking. In the case of small molecules, the site of linkage should be chosen so that the resulting conjugate closely mimics the target compound. If no functional groups are present on the antigen, these may be introduced by derivatization. Moreover, it might be necessary to include a spacer between the antigen and the carrier molecule to freely expose the antigenic determinants of the antigen.

Using the proper immunogen animals are immunized to raise antibodies (polyclonal, monoclonal, recombinant).

Choice of format

Once an antibody has been obtained, the format to be used defines the remaining components of the assay. Because it is beyond the scope of this report to discuss all variants, only the competitive ELISA will be exemplified.

Enzymes are the most used labelling substances and they are detectable, especially after amplification, at a very low level. Various enzymes may be used as immu-

noassay labels *(21)*. They can be combined with various substrates and chromogenic, fluorescent or luminescent substances, which are all commercially available. In the format depicted in Figure 2A, antigen is coupled to the solid phase and the labeled antibody is used for quantitation. Antibody-enzyme conjugates should retain both immunological and enzymatic activities and several methods of conjugation have been described and applied successfully. The coating antigen used in such an ELISA should be able to compete with sample antigen. In the case of haptens the same or a similar hapten-protein conjugate as used as an immunogen may be used for coating.

In the alternative ELISA format (Fig. 2B) antibody is coated onto the solid phase, usually by simple adsorption. Now the antigen is enzyme-labeled as competitor in the assay. Procedures for the labeling of protein antigens are comparable to those for antibodies. Non-protein antigens and haptens should contain a functional group, usually an amino or carboxyl group, or may be derivatized to contain one. Sometimes a spacer is built in between hapten and enzyme. To allow adequate immunological recognition as well as enzyme activity the site of conjugation is very important and strongly defines the sensitivity and specificity of an enzyme immunoassay. Several procedures for the preparation of hapten-enzyme conjugates have been described.

Specification/Optimization

The next step in designing an immunoassay is to specify its characteristics. Several parameters have to be determined: specificity, sensitivity, precision, linearity, accuracy, recovery, interferences, matrix effects.

The specificity is a crucial aspect in an immunoassay and is dependent on the antibody as well as on the enzyme-tracer in the case of labeled antigen or on the coated antigen in the case of labeled antibody. The specificity is assessed by determining the cross-reactivity of related and unrelated substances. The sensitivity depends on the concentrations of the components in the assay, buffers used, pH, ionic strength, temperature, incubation time, etc. Further, the matrix wherein the analyte has to be measured may influence the results. Aqueous samples may be analyzed without pretreatment; other types of samples such as soil, sediment, food products normally have to be subjected to an extraction step. Organic solvents, however, negatively affect the performance of an immunoassay and should be kept at a low level. If an extraction step is to be included, it is recommended to prepare calibration standards in the same matrix as the analyte.

Additionally, the precision of an assay is assessed by determining intra- and inter-assay variation. Advantageously, control samples with known concentrations (high, medium and low) are included in each assay. Linearity is determined by measuring samples with high levels of analyte in serial dilution series. The accuracy and recovery of an assay are determined by measuring spiked and incurred samples and comparing the results with established reference methods. Finally, possible interferences may be included in the assay to assess their influence, e.g. salts, heavy metals, humic acids, substances from plant and animal tissue, etc. Each of the above mentioned parameters may optionally be optimized by varying assay conditions and concentrations.

Validation

Further, an immunoassay once developed should be validated. Norms for environmental immunoassays have been established by the EPA in the USA and the DIN Institute in Germany. Within a short period of time ISO norms will also be available. These norms combined with interlaboratory studies will stimulate acceptance of the immunoassay as a reliable method for the detection of contaminants in all kinds of samples. Recently, reports have been published with regard to the quality control of immunoassays for pesticides, interpretation of immunochemical data, guidelines for validation of immunochemical methods and quality standards for immunoassay kits *(22–25)*.

Immunoassays for Environmental Analysis

World-wide the presence of toxic residues in water, soil, air, and food is recognized as a threat to ecosystems and human health. Thousands of different compounds and organisms may be present as potentially toxic contaminants. Many chemicals derive from industrial activities and transportation. Pesticides are used world-wide in enormous amounts in agriculture, horticulture, animal husbandry, food preservation, hygiene, etc. Toxins are produced by several microorganisms and may be present in the environment as naturally occurring substances, or in crops, food and food products and decrease the quality of these products. Several books and reviews have been published about environmental pollutants, their properties, and analytical methods for their detection if any. An elucidating review about the presence and determination of pesticides has been given by Torres *et al. (26)*.

Immunochemical methods have proven to be rapid and relatively inexpensive means for the detection and optional quantitation of contaminants in various matrices. They are particularly useful for those substances that pose problems in conventional methodologies such as highly polar compounds that are difficult to extract from aqueous samples. The strengths of immunoassay are its rapidity, specificity, sensitivity and cost-effectiveness; samples can be measured directly or after extraction and many samples can be analyzed simultaneously. As drawbacks of immunoassay are mentioned possible matrix effects or other interferences and the cross-reactivity of antibodies. However, this latter feature may be used advantageously in those situations where monitoring for groups of related compounds is to be conducted. Several reviews dealing with immunoassays for environmental and food contaminants have been published. Table 1 gives an updated listing of target substances for which assays have been developed. It has to be noted that a series of immunoassay kits is commercially available *(27,28)*.

Most of the immunoassays listed were developed for pesticides in aqueous samples. With some modifications it may also be possible to develop or adapt immunoassays for detection in extracts of soil, food, animal

Table 1. Immunoassays for pesticides and other contaminants

The information in this table is a compilation from several review articles *(27–34)* supplemented with data from the latest articles. Only the lowest detection limit reported is given for each target compound.

Format = design of the immunoassay; the lowest detection limit found is listed in ppb in order to include detection in aqueous and solid samples; occasionally, e. g. for organisms, other units may be used; – = data not available; EIA = Enzyme Immunoassay; RIA = Radio Immunoassay; IRMA = Immuno Radio Metric Assay; FIA = Fluorescence Immunoassay; CEI = Chemiluminescence Immunoassay; LIA = Luminescence Immunoassay; FIIA = Flow Injection Immunoassay; prec. = precipitation assay; aggl. = agglutination assay.

Compound	Format	Detection limit
Pesticides		
Alachlor	EIA/FIIA	0.03
Aldicarb	LIA	0.25
Aldrin	EIA/RIA	1
Amidochlor	EIA	0.25
Amitrole	EIA	1.7
Anilides	EIA	0.25
Atrazine	EIA/FIA	0.0055
Azinphos-methyl	EIA	9
BAY SIR	EIA	0.5
Benomyl (MBC)	RIA	0.1
Bentazon	EIA	-
Bio-allethrin	Prec./RIA	0.45
Bioresmethrin	EIA/RIA	2
Bromacil	EIA	0.01
Butachlor	EIA	0.5
Captan	EIA	1.5
Carbaryl	EIA	0.05
Carbofuran	EIA	0.056
Chlordane	EIA	25
Chlorothalonil	EIA	0.07
Chlorotoluron	EIA	6
Chlorpyrifos	EIA	0.05
Chlorpyrifos-methyl	EIA	0.6
Chlorpyrifos-ethyl	EIA	0.2
Chlorsulfuron	EIA	0.4
Clomazone	EIA	1
Cyanazine	EIA/RIA	0.035
Cypermethrin	EIA	50
2,4-D	RIA/EIA	0.005
DDA	EIA	10
Dichlorprop	FIA	0.01
Dichlorprop-methyl ester	EIA/FIA	0.11
Diclofopmethyl	EIA/FIA	23
Didecyldimethyl-ammonium chloride	EIA	8000
Dieldrin	EIA/RIA	2
Difenzoquat	EIA	0.8
Diflubenzuron	EIA	0.5
Diuron	EIA	0.01
Endosulfan	EIA	0.2
Enrofloxacin	EIA	1.6
Fenitrothion	EIA	1
Fenoxycarb	EIA	4
Fenpropimorf	EIA	0.013
Fluoxypyr	EIA	0.1
Fluoroxypyr	EIA	0.1
Halofuginone	EIA	0.52
Hepatochlor	EIA	4
Hexaconazole	EIA	0.1
Hexazinone	EIA	0.13
Imazamethabenz	EIA	0.5
Imazaquin	EIA	0.45
Imazethapyr	FIIA	0.1
Iprodion	EIA	30
Isoproturon	EIA	0.009
Ivermectin	EIA	0.1
Lindane	EIA	20
Linuron	EIA	3
Maleic hydrazide	EIA	0.11
MBC	EIA/RIA	0.1
Metalaxyl	EIA	0.1
Metazochlor	EIA	0.03
Methabenz-thiazuron	EIA	0.05
Methomyl	EIA	0.45
Methoprene	EIA	30.4
Metolachlor	EIA	0.05
Molinate	EIA	1
Pesticides		
Myclobutanil	EIA	0.3
Nisin	EIA	5
Norflurazone	EIA	1
Oxfendazole	RIA	3
Paraquat	RIA/EIA/FIA	0.03
Parathion	RIA/EIA	0.2
Penfluron	EIA	0.5
Pentachlorophenol	EIA	0.06
Permethrin	EIA	1.5
Phenothrin	EIA	1.5
Picloram	EIA/RIA	5
Pirimiphos-methyl	EIA	0.4
Procymidone	EIA	0.8
Propazine	EIA	0.014
Prometryn	EIA	1
Pymetrozine	EIA	50
Simazine	EIA	0.05
Sulfathiazole	EIA	12
2,4,5-T	EIA/RIA	3
Terbuthylazine	FIA/EIA	0.01
Terbutryn	EIA/FIA/RIA	0.05
Thiabendazole	EIA	0.05
Thiobencarb	EIA	1
Triadimefon	EIA	2
Triasulfuron	EIA	0.01
Triazoles	EIA	12
Triclopyr	EIA	0.1
Trifluralin	EIA	100
Trimethopim	EIA	50
Urea herbicides	EIA	0.08
Warfarin	RIA	25
Metabolites		
15-Acetoxynivalenol	EIA	50
Chlorodiamino-s-triazine	EIA	1
De-ethylatrazine	EIA	0.01
De-isopropyl-atrazine	EIA	0.01
OH-atrazine	EIA	0.01
OH-simazine	EIA	–
Naphthalene-metabolite	EIA	0.005
1-Naphtol	EIA	10
1,2-Naphthoquinone	EIA	–
4-Nitrophenol	EIA	0.2
Paraoxon	EIA/RIA	1
3-Phenoxybenzoic acid	EIA	50
THPI	EIA	0.3
3,5,6-Trichloro-2-pyridinol	EIA	0.04
Toxins		
3-Acetyl deoxynivalenol	EIA	1
Acetyldeoxynivalenol	EIA	0.001
Aflatoxins	EIA/RIA	0.1
B1	EIA/RIA	0.025
B1, B2	EIA	2
B1, B2, G1	EIA	1
B1, B2, G1, G2	EIA	0.001
M1	EIA/RIA	0.005
Q1	EIA	2
B. cereus toxin	EIA	1
B. thuringiensis toxin	EIA	0.03
C. botulinum toxin-A	RIA	1
C. perfringens enterotoxin E	Aggl.	10^5
C. perfringens enterotoxin A	RIA/EIA /Aggl.	0.2
Citrinin	EIA	0.4
CryA (b)	IRMA	0.1
Cyclopiazonic acid	EIA	7 ng
Deoxynivalenol	EIA/RIA	50

Table 1. continued

Compound	Format	Detection limit
Toxins		
Deoxyverrucarol	RIA	25 ng
Diacetoxyscirpenol	EIA	16
Fumonisin	EIA	5
Fusarochromanone	EIA	5
Group A Trichothecenes	RIA/EIA	0.05 ng
HT-2 toxin	RIA	0.1 ng
Maitotoxin	EIA	45
Microcystins	EIA	0.05
Neurosaxitoxin	RIA/EIA	0.1
Nivalenol	EIA	0.1
Ochratoxins	EIA	0.02
A	EIA/RIA	0.04
3'-OH-T-2 toxin	RIA	0.1 ng
Polyether toxins	EIA/RIA	1 ng
PR toxin	RIA	50
Rubratoxin	RIA	0.1 µg
B	RIA	0.1 µg
Roridin A	EIA/RIA	5
Saxitoxin	EIA	2
ST toxin *(E. coli)*	EIA	1
Staphylococcus		
enterotoxins	EIA	0.1
A	RIA/EIA	0.025
A, B, C, D	EIA	0.1
A, B, C, E	EIA	5
B	RIA/EIA	0.63
C	RIA	1.3
D	RIA	0.67
E	RIA	1
Sterigmatocystin	EIA	0.01 pg
Tetrodoxin	EIA	5 pg
T-2 tetraoltetracetate	RIA	0.5 ng
T-2 toxin	RIA/EIA	0.04
Zearalenone	EIA	500
Antibiotics/Anabolica/ /Hormones		
β-Agonists	EIA	0.5
Cephalexin	EIA	30
Chloramphenicol	RIA	0.002
Clenbuterol	FIA	0.01 ng
Colistin	EIA	30
Dexamethasone	FIA	174
Diethylstilbestrol	RIA	0.030
Dihydrostreptomycin	EIA	5
17β-Estradiol	CEI/EIA/RIA	0.2
Estrone	RIA	200 pg
Gentamycin	EIA	0.1
Gibberellin A3	EIA	30
Hexoestrol	RIA	0.0006
Hygromycin B	EIA/RIA	13,600
β-Lactam antibiotics	Aggl./EIA	5
MPA	RIA	0.010
17-O-Methyltestosterone	CIA	8 pg
Antibiotics/Anabolica/ /Hormones		
Monensin	EIA	2
Neomycin	EIA	10
Oxytetracycline	EIA	0.2
Progesterone	RIA	5
Streptomycin	EIA	2
Sulfadimethoxine	EIA	100
Sulfamethazine	EIA	1
Sulfonamides	EIA	5
Testosterone	RIA	10 pg
Trenbolone	RIA	0.1
Trenbolone acetate	RIA	0.024
17-O-Trenbolone	RIA	0.005
17β-OH-Trenbolone	RIA	0.046
Zeranol	RIA/EIA/FIA	1
Organisms		
Bacillus	EIA	10^3 org.
Campylobacter	Aggl.	–
ChMl virus	EIA	10
E. coli 0157:H7	EIA	10 org./mL
Flaviviruses	EIA	0.1
IB virus	EIA	100 org.
Listeria monocytogenes	EIA	10^6 org./mL
Moulds	EIA	10^3 CFU/g
Mycosphaerella pinodes	EIA	5 ng protein/mL
Nitrosococcus oceanus	EIA	–
Salmonella	RIA/EIA	0.4 CFU/mL
Organic chemicals and others		
Alkaloids	EIA	23 pg
4-Acetamidobiphenyl	RIA	5.7 pg
Benzo[a]pyrene	RIA	0.003
Benzene/nitrobenzene	EIA	500
Cyclobutanones	EIA	0.064
p,p'-DDT	EIA	100
p,p-DDE	EIA	180
N,N'-diacetylbenzidine	RIA	–
Ergot alkaloid	EIA	10
Furanocoumarins	EIA	0.1
Glycoalkaloids/aglycons	EIA	1
4-nitrophenol & mono subst. 4-NPs	EIA	0.2 pbb
MATP	LIA/EIA	3,500
PAH	EIA	100
PCBs	FIIA/RIA/EIA	0.1
PCDDs (2,3,7,8-TCDD)	RIA/EIA	0.067
PCDFs	RIA	20 pg
PCP	EIA	1
Phycocyanin	EIA	10.000
Soman	EIA	0.3
Taxol	EIA	0.5
2,4,6-TNT	EIA	0.05

and food samples. Additionally, assays were designed for industrial pollutants (PCBs, BTEX), heavy metals (In(III), Hg(II)), toxins (several mycotoxins, polyether toxins, microcystins), organisms (viruses, bacteria, fungi/moulds), natural products (alkaloids, plant hormones) and antibiotics (sulfonamides, gentamycin, tetracycline, β-agonists, etc.).

The screening of pesticides has been largely aimed at the detection of active parent compounds. Depending on the specificity of the antibodies used assays can be considered as single-compound specific or group-specific. Recently, interest was also directed at specific metabolites such as hydroxylated, dealkylated or chlorodiamino triazines; THPI, a captan metabolite; 3,5,6-trichloro-2-pyridol, a chlorpyrifos and triclopyr metabolite; 15-acetoxynivalenol, derived from the mycotoxin deoxynivalenol.

As mentioned before, very short assay times in the order of minutes can be achieved with alternative formats: dipstick, polymycin cloth and immunofiltration enzyme immunoassays. Besides, such assays may be used as on-site field tests. An example of target substance is gentamycin, that can be measured in milk by a dipstick with MAb *(35)*. Similar dipsticks as well as immunofiltration assays were developed for sulfonamides in milk *(36)*; fumonisin B1 in corn-based foods *(37)*; streptomycin/dihydrostreptomycin in milk *(38)*; atrazine in water and foods *(39)*.

Schneider *et al.* *(40)* designed a multimycotoxin dipstick wherein different antibodies were sequentially coated onto an immunoaffinity membrane mounted onto a plastic support. By merely dipping the dipstick into the toxin-enzyme conjugate and then sample solution, the analytes and tracers were allowed to bind to their corresponding antibody. The amount of respective analytes was visualized by subsequently incubating the dipstick in the developing solution. Another target compound was *Fusarium* T-2 toxin that may be present in wheat and can be detected with monoclonal antibody coated dipstick EIA. The polymyxin-cloth EIA appears to be suitable to screen for *Salmonella* contamination in food products *(41)*.

Application of immunoassays

Among the immunoassays listed in Table 1, several have been tested and validated with real samples. However, there are relatively few reports about the actual application. Immunoassays can be used for direct measurement of target analytes in aqueous samples and this makes them extremely suitable for monitoring studies. For example, Thurman *et al.* *(42, 43)* made a survey in the US Corn and Soybean Belt area to assess the impact of herbicide usage and the persistence of triazines. In the periods of pre-planting, post-planting and harvest water samples were collected from rivers and streams. The samples were screened with immunoassay and evaluated for compliance of EPA maximum contaminant levels for drinking water. GC-MS was used to identify individual compounds. It appeared that during all periods in response to rainfall large amounts of herbicides were flushed from cropland and transported to the surface water system. Furthermore, several herbicides including their metabolites exceeded MCL and were found to be rather persistent.

In Wisconsin a comparable survey was conducted *(44)*. Similarly, atrazine residues were analyzed with commercial immunoassays kits in more than 2000 well water samples for a first screening. Positive wells (> 35 ppb) were resampled and assayed with immunoassay and GC for confirmation. In this study it appeared to be possible to determine the location of drinking water wells contaminated with atrazine residues in a large area.

Immunoassay of contaminants in soil samples is usually performed in extracts. Here the sampling and pretreatment protocol is very important to obtain reliable results. An ELISA kit for PCBs in soil samples was used in a screening programme of contaminated sites *(45)*. The kit was able to effectively screen out negative samples. Positive samples were confirmed by GC. It was mentioned that prescreening of soil samples with immunoassay could increase testing capability and provide substantial savings.

Microcystins are produced by cyanobacteria and are highly toxic. They are found in surface, well and drinking waters, especially in hot summer months. Ueno *et al.* *(46)* used an ELISA for microcystins that recognized the major microcystin derivatives. They performed a two-year epidemiological survey wherein more than thousand water samples were collected in the area of Haimen city in China. They concluded that drinking water is a major source of microcystins and consequently may explain the high incidence of primary liver cancer in this area.

A high quality of food and food products is important for human health. Contaminants may comprise naturally occurring substances (e.g. alkaloids), various types of toxins (produced by bacteria, fungi, moulds and other small organisms), pesticide residues (due to agricultural usage or as a result of application during storage and transport), and antibiotics and growth stimulating agents (used for meat production). It was shown that immunoassays are very suitable for the screening of food and food products. Raw fish and especially shell fish belong to the daily diet in Japan and large parts of Europe. However, fish may contain low-molecular weight toxins produced by microscopic dinoflagellates, which are generally non-detectable by simple chemical tests, whereas bioassays lack specificity. Park *(47)* described the results of several screening programmes for shell fish toxins. It was concluded that the Ciguatect test kit is an excellent tool applicable in harvesting areas as well as in the market-place.

Animals for meat and milk production are often treated with antibiotics and/or growth stimulators. To ensure safe human consumption, MRLs have been established for many residues and routine test programmes are being conducted. An example of the applicability of immunoassays in this field is given by Haasnoot *et al.* *(48)*. An immunoassay was developed for the screening of sulfonamides in swine plasma and urine. It appeared that the test could be used as in indicator for the edibility of animal tissue.

In our laboratory, we have used commercial kits for the detection of herbicides in river water (Rhine) in the scope of a monitoring programme. For three consecutive years, in the spring time, daily samples were collected about 80 km upstream from a drinking water production plant. Target compounds were phenyl urea herbicides and two different kits were used: a single-compound specific kit for isoproturon, and a group-specific kit for urea herbicides. The European Community norm for individual pesticides is 0.1 ppb and for the sum of pesticides 0.5 ppb. In the case of compliance of these norm levels the intake of river water for the production of drinking water has to be stopped. In 1995 we found a peak level of 0.8 ppb for urea herbicides (confirmed with HPLC) during a relatively short period. In 1997 the concentration of isoproturon remained very low. For the group of phenyl ureas it was found that after rainfall the concentration increased, but never exceeded 0.5 ppb. The results are depicted in Fig. 3.

To give an example of the applicability of a dipstick immunoassay, Mills *et al.* *(49)* developed and used a dipstick ELISA for the detection of conarachin, a peanut protein that may elicit an allergic reaction in sensitive individuals. The assay was designed as a sandwich ELISA using first antibody coated onto immunostick for capturing the antigen, and second antibody raised against a different epitope as detector component. Contamination of various food products with peanut was detected at a level of 0.1% after a simple extraction step.

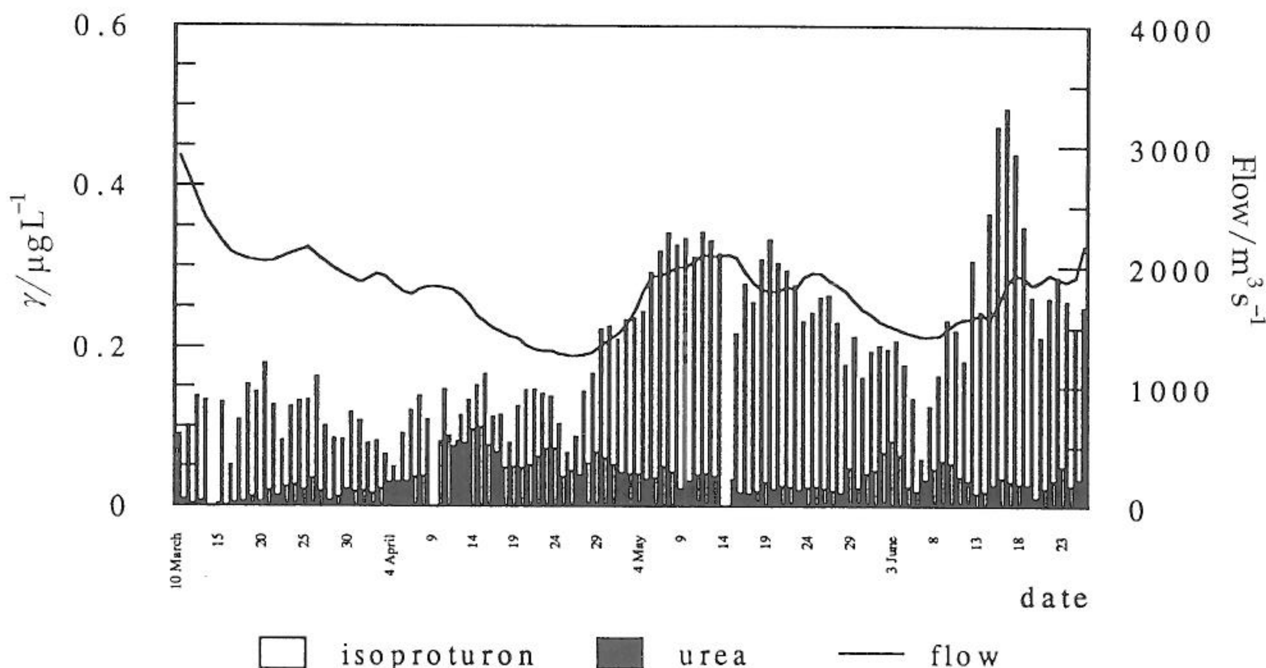

Fig. 3. Monitoring of phenylurea herbicides in the river Rhine
During spring time phenylurea herbicides were measured in the river Rhine with both an immunoassay kit specific for isoproturon and a group-specific kit in daily water samples. Concentrations (μg/L) and flow rates ($m^3 s^{-1}$) are plotted.

Although somewhat different from common immunoassays, the flow-injection immunoassay (FIIA) is a promising new development for screening purposes. Several FIIAs have been developed in different embodiments. Most are based on the competitive reaction between the sample analyte and tracer for antibody binding sites on a solid support. For example, atrazine can be measured with an antibody-bound silica capillary by sequentially injecting sample, atrazine-AP conjugate, wash solution and substrate. After the reaction the reaction product is measured electrochemically *(50)*, the total run time being about one hour due to relatively long incubation periods.

A much faster assay was developed for alachlor *(51)*. Here antibody was coupled to a column; as a tracer atrazine-tagged liposomes containing ferrocyanide were used. Sequentially, the sample and tracer were injected and after the reaction detergent was added to lyse the liposomes and release the marker for electrochemical detection. The total run time was 10 minutes with a detection limit of 5 ng.

A modified embodiment has been described by Irth *et al. (52)* for the measurement of digoxin and digoxigenin with fluorescent detection. The FIIA system consisted of a reaction coil for the immunochemical reaction between sample analyte and fluorescein labeled antibody; the reaction mix was passed through an antigen-bound column to remove unbound antibody, whereas the immunocomplex (antigen – labeled antibody) passed through and was quantitated by fluorescence detection. This FIIA system was coupled to an LC separation system so that the effluent from that system could automatically be used in the FIIA system.

Concluding remarks

Immunoassay has proven to be a useful technique for the screening of contaminating substances in environmental and food samples. Although it is not yet a fully accepted method, the increasing number of target substances for which assays have been developed and publications on the application of results will help introducing them in the analytical laboratory. ELISA is expected to remain the favorite embodiment, but other embodiments may soon be marketed such a fluorescent or chemi/bioluminescent immunoassays. Further promising developments are multi-analyte immunochemical systems wherein more than one compound or group of compounds can be detected simultaneously. The principles of such systems have already been described *(53–55)*. Additionally, as a result of more stringent rules for the use and handling of experimental animals conventional antibodies will increasingly be replaced by recombinant antibodies which can be produced with *in vitro* methods. In this respect, phage libraries present an infinite source of genes coding for antibody fragments which can be combined at random. Moreover, site directed mutagenesis offers a tool for producing tailor-made antibodies or their fragments.

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Imunokemijsko određivanje onečišćivača u okolišu i hrani: razvoj, provjera i primjena

Sažetak

Imunokemijske metode, osobito imunokemijski postupci, vrlo su pogodne za određivanje onečišćavača u okolišu i uzorcima hrane. Prikazana su načela imunokemijskog određivanja i mogućih varijacija te potrebni sastojci. Nadalje, opisan je način provedbe, optimiranja i provjere postupka. Imunokemijska su određivanja vrlo korisni postupci za provjeru i provođenje ispitivanja jer omogućuju brzu analizu vrlo malih udjela određenih spojeva u različitim uzorcima. Iznesen je suvremen pregled spojeva prirodnog i sintetskog porijekla određivanih imunokemijskim postupcima. Primjenjivost postupka ilustrirana je primjerima iz prakse. Navedeni su najnoviji postupci priprave antitijela in vitro i utvrđivanje normativa.