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IV.4.1
Biomonitors based on immunological principles

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IV.4.1.1. Introduction

Modern analytical chemistry is playing an increasingly greater role in the field of environmental monitoring, including solid waste, solid waste disposal and contaminated sites. Often critics charge that only a few chemicals are monitored, that the number of samples is inadequate to ensure detection of contamination, and that there is too long a delay between sample collection and communication of results back to the site. This is caused because required methods generally involve sample extraction, cleanup and determination by techniques such as gas chromatography (GC), liquid chromatography (LC), mass spectrometry (MS), thin-layer chromatography (TLC) or other methods. These analyses require well-equipped laboratories and trained personnel, and are often laborious and time consuming. Even with the best analytical methods, problems can arise when hundreds or thousands of samples must be handled. Concern over increasing sample load and rising costs, therefore, motivate the search for rapid, low-cost, simple and reliable tests that could also be automated or carried out on-site (field-portable assays). Besides others such as laser techniques and chemical sensors, attention has focused on the development of immunological techniques. Immunochemical procedures are widely used in clinical chemistry and endocrinology for the routine analysis of hormones, proteins, microorganisms and drugs (Gosling, 1990). In this chapter the basic principle of the immunoassay approach will be outlined and the applicability of this method for environmental monitoring discussed. It includes a general account of efforts to develop immunoassays for identification and quantification of pesticides and industrial chemicals and the development of official guidelines for the adoption of assay kits for contaminant detection in environmental samples, and also describes current commercially available assay kits. For further details on immunoassay methodology, the authors refer to excellent monographs published elsewhere (Tijssen, 1985; Price and Newman, 1991; Masseyeff et al., 1993; Crowther, 1995; Law, 1996) and to a comprehensive review of bioassay/biomarker technology for screening dioxins and dioxin-like compounds by Behnisch et al. (2001).

IV.4.1.2. Immunoassay technology

Immunochemical analytical methods are generally based on the principle of competition between an analyte and a labeled form of the analyte (the tracer) for a limited amount of

a specific binding protein (antibody, immunoglobulin) that was elicited in an animal in response to the injection of a suitable form of the target analyte. The extent of tracer binding will depend on the amount of analyte in the sample. From a calibration graph the amount of analyte can be interpolated.

IV.4.1.2.1. Antibody production

The most important reagent because it determines to a great extent sensitivity and selectivity in an immunoassay is the antibody, which can recognize and bind to the foreign substance. Normally, they are produced only in response to a large molecule (antigen). Small compounds of less than about 1000 Da (haptens) are unable by themselves to elicit an immune response since they are metabolized in the animal. But, after covalent coupling of the hapten to a large carrier protein (the resulting conjugate is termed the immunogen), the immunized animal will produce antibodies also against the attached small molecule. Therefore, the initial task in method development is to prepare an immunogen.

IV.4.1.2.1.1. Immunogen synthesis

If the target analyte contains a suitable group such like a hydroxyl-, sulfhydryl-, amino- or carboxyl function, it can be used for coupling. In the case that these groups should be preserved because they are considered to be of importance for the analyte recognition or if such groups are not present, an analog (analyte derivative) has to be synthesized. For the conjugation to the carrier protein several well-established methods originating from peptide chemistry are available (Hermanson, 1996). With several analytes the introduction of a 4–6-carbon spacer (bridge) between the analyte and the protein was proven to be of advantage for the production of useful antibodies, obviously owing to the better recognition of the attached analytes on the protein surface by the immune competent cells. As carriers a lot of proteins have been described such as serum albumins and gamma-globulins of various species (bovine, human), ovalbumin and thyroglobulin. In accordance with other investigators, we found high immune response using different hemocyanins (keyhole limpet hemocyanine, KLH; helix pomatia hemocyanine, HPH). Optimum coupling density (number of loaded analyte groups) depends on the selected carrier protein, although there is no consensus as to which ratio is best.

Depending on immunoassay format, additional synthesis of a coating antigen which is a heterologous analyte–protein conjugate (different protein compared to the immunogen), or an analyte–enzyme conjugate (the tracer compound in enzyme immunoassays) is required. Both make use of chemical coupling reactions as applied for immunogen synthesis.

IV.4.1.2.1.2. Polyclonal antiserum production

The rabbit is the most commonly used laboratory animal for antiserum production for small analytes. It offers the advantages of being easy to care for, and it produces a moderate amount of serum, often with high antibody amount (titer). However, also

chicken and larger animals such as goats, sheep and horses can be used if available. Only purified and well-characterized immunogens should be applied for the immunization. Together with an immunological adjuvant it is inoculated into the animals' body at reasonable dose and time-scale (immunization schedule) to get an optimum antibody response. While the number of substances with adjuvant activity (from the Latin, *adjuvare*/to help) and the literature describing their use has expanded enormously, their mode of action has remained largely a black box (Werner and Jolles, 1996; Cox and Coulter, 1997). As possible actions are under discussion both immunomodulation, antigen presentation, depot generation, targeting and induction of cytotoxic T-lymphocyte responses. In this laboratory, multiple intradermal injections using Freund's complete and incomplete adjuvants are preferred, similar as described by Vaitukaitis et al. (1971). However, there is no standard protocol, which guarantees success, and most approaches are largely empirical.

The blood is regularly checked for the presence of hapten antibodies and for its specificity and when the titer is satisfactory greater amounts of blood are taken from the ear vein or by cardiac puncture to obtain a stock of antiserum. The serum is separated, divided into small aliquots and in most cases stored at low temperatures (-20, -70°C). Laboratories without access to animal care facilities may provide contracting companies with immunizing conjugate and obtain antisera at specified intervals. Moreover, such companies increasingly provide chemical syntheses, antibody characterization and immunoassays development. However, the "all inclusive" option in most cases is very expensive and further, for the costumer supervision is hardly possible as well as early intervention into the procedure.

However, each polyclonal antiserum will vary in its composition and characteristics both from animal to animal and between the bleedings from the same animal. The characteristics of the antiserum reflect the composite properties of the mixture. Therefore, availability of a polyclonal antiserum is limited thus reducing commercial utilization. Despite these shortcomings in many cases, one animal will supply sufficient antiserum for a very large number of analyses, since the serum can be highly diluted for analysis. Still the majority of commercially available test kits for environmental contaminants is based on polyclonal antibodies.

IV.4.1.2.1.3. Monoclonal antibody production (hybridoma technology)

This technology, introduced by Köhler and Milstein in 1975, makes it possible to cultivate cell lines *in vitro* that can produce a single desired antibody essentially unlimited (Köhler and Milstein, 1975). It is based on the fusion of an antibody-producing cell (a B lymphocyte cell) with a long-lived cancer cell to produce a new hybrid cell with the phenotypic characteristics of both parents. It can grow indefinitely in culture and secrete antibodies uniform in terms of structure and function. Main steps are (1) immunization (generally mice), (2) cell fusion and screening for antibody producing hybridoma cell clones, (3) postfusion cell management and further selection and (4) expansion and scaled-up antibody production. The potentially unlimited supply over a long period of time (as long as the hybridoma cell line is maintained in culture or in storage) of a homogeneous well-characterized antibody reagent especially meets the

demands of regulatory authorities for standardized reagents and methods. The technology is now firmly established and numerous strategies are in use (Peters and Baumgarten, 1989; Stein, 1997). However, because of the high initial labor and costs needed laboratories often hesitate to establish the hybridoma technology.

IV.4.1.2.1.4. Recombinant antibodies (recombinant DNA technology)

In these days a third generation of antibody technology is going to enter the field of environmental immunology. It claims to overcome the disadvantages of hybridoma technology mentioned before by means of methods originating from molecular biology. Basic principle is to isolate genes that encode antibodies from an organism and purifying and reproducing them in another organism. Several different cloning and expression systems have been developed first of all in *E. coli* but also in insect cells, yeast, fungi and plants, all of them constitute easy to grow non-mammalian hosts (Huse et al., 1989; Lee and Morgan, 1993; Hayden et al., 1997). Types of antibody fragments produced are heavy (VH) and light (VL) variable domains, single-chain variable domains (scFv) or Fab fragments. Main advantage of the new technology is that molecular modeling and site-directed mutagenesis of cloned antibodies may provide a more cost-effective and efficient alternative to the tedious synthesis of haptens and accompanied numerous immunizations. It is an exciting new possibility directed on a more efficient manipulation of antibody binding sites to give desirable specificity, binding affinity, tolerance to physical parameters such as pH and temperature, and sensitivity to matrix constituents such as organic solvents and detergents. Type and number of antibodies normally obtained by conventional methods can be extended by several orders of magnitude. First reports on the production of recombinant antibodies, derived mainly from hybridomas, are promising (Karu et al., 1994; Bell et al., 1995; Kramer and Hock, 1996a,b; Hall et al., 1997, Scholthof et al., 1997). Still the method is far from being routine owing to its complexity.

IV.4.1.2.2. Types of immunoassays

Immunoassays can be classified in several directions: (1) type of tracer to quantify the analyte, (2) applied amount of antibodies and (3) separation of bound and free phases is required or not. Table IV.4.1.1 lists possible detection systems. Besides the very early reports on the preparation and characterization of antibodies for environmental contaminants applying hemagglutination (Haas and Guardia, 1968; Centeno et al., 1970), first immunoassays used isotope labels (radioimmunoassay, RIA).

However, practical limitations of RIA, for example, the potential hazards associated with the use of radioisotopes and the requirement of complex instrumentation, are arguments against its application in field test screening. After the introduction of the enzyme immunoassay technique (EIA), it was increasingly used and now is the overwhelming immunoassay type. In these assays an enzyme (most popular are horseradish peroxidase and alkaline phosphatase) is applied for labeling the target analyte or the antibody. If the assay is conducted in solution requiring no separation step the (homogeneous) assay is termed enzyme-multiplied immunoassay (EMIT, but

Table IV.4.1.1. Types of immunoassays classified according to the applied label.

Immunoassay	Marker
Radioimmunoassay	Radioisotope (^{125}I, ^{3}H, ^{14}C, etc.)
Enzyme immunoassay	Enzyme (horseradish peroxidase, alkaline phosphatase, β-D-galactosidase, etc.)
Fluorescence immunoassay	Fluorescein, coumarin derivatives, phycoerythrin, europium^{3+}, samarium^{3+}, terbium^{3+}
Luminescence immunoassay	Isoluminol derivatives, acridinium ester, etc.
Spin immunoassay	Free radical
Viroimmunoassay	Bacteriophages
Metal immunoassay	Metals
Particle immunoassay	Colloids, latex particles
Liposome immunoassay	Liposomes

sometimes also EIA). In contrast to that (heterogeneous) enzyme immunoassays involving the use of a solid phase for separation of bound and free phases before the enzymatic activity (end-point measurement) is determined are known as enzyme-linked immunosorbent assay (ELISA). Both EMIT and ELISA can be run as competitive or non-competitive formats.

IV.4.1.2.2.1. Enzyme-linked immunosorbent assay

Immunoassays for small environmental contaminants rely almost entirely on competitive ELISAs. Usually, plastic tubes, wells of microtiter plates or beads are used as solid phase. The two main principles are demonstrated in Figures IV.4.1.1a and IV.4.1.1b. In the direct competitive ELISA (capture assay) the antibody is immobilized on the solid phase (Fig. IV.4.1.1a). Sample and enzyme tracer (analyte–enzyme conjugate) are co-incubated with the antibody coated surface. Following this step, reagents are washed away and the amount of enzyme tracer bound to the immobilized antibodies is measured by its enzymatic activity after the addition of a chromogenic substrate causing the development of a color. In the indirect competitive format instead of the antibody a coating antigen is immobilized consisting of an analyte or analyte derivative coupled to a protein (this must be different from the protein in the immunogen!) on the solid phase (Fig. IV.4.1.1b). After the addition of sample and antibodies, target analyte from the sample and the coating antigen compete for the antibodies. Antibody binding to the solid phase occurs in inverse proportion to the amount of free analyte present. Quantification of bound antibody fraction by the addition of an enzyme-labeled secondary antibody (raised in a different animal species against the immunoglobulin fraction of the host species that was immunized with the target analyte) provides a measure of the amount of analyte initially present. With both direct and indirect competitive ELISAs, a decrease in

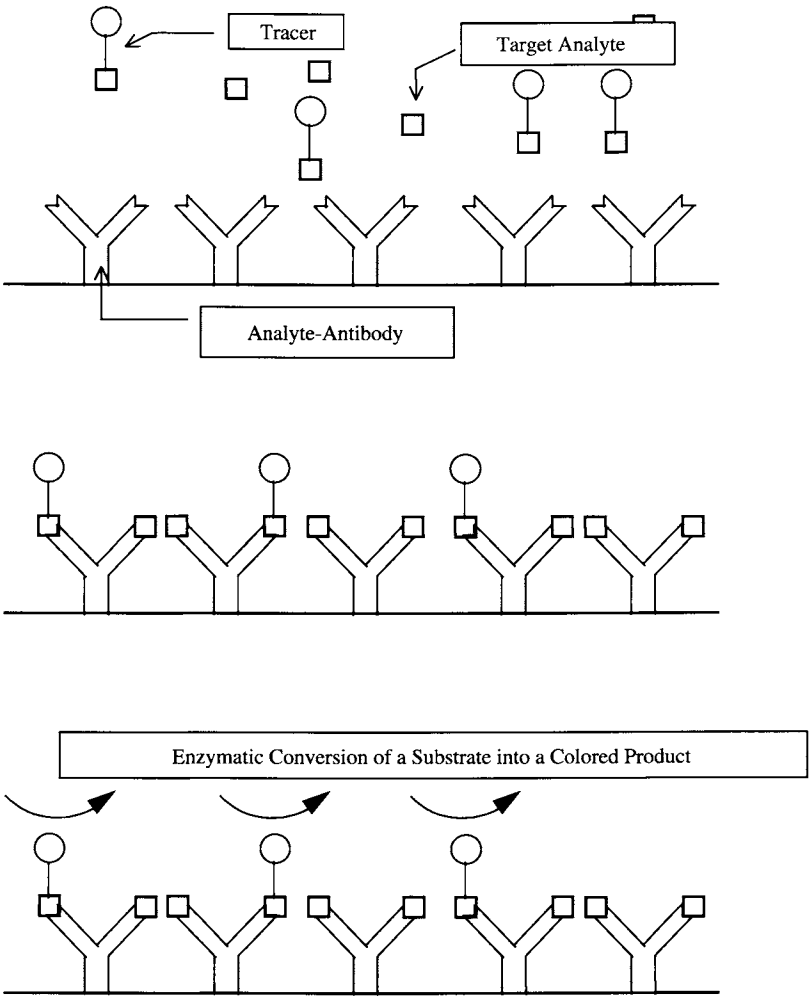

Figure IV.4.1.1a. Principle of a direct competitive ELISA.

enzyme activity is indirect proportional to the amount of analyte in the sample, i.e. the lighter the color produced at the end of the assay the greater is the amount of analyte in the sample. Absorbance can be measured in the laboratory by a special spectrophotometer designed to accommodate a 96-well microtiter plate or in the field by portable equipment or estimated visually.

Both formats have been proven to work with environmental samples. The direct format needs less incubation steps than the indirect one but has the drawback that for each target analyte a new tracer has to be synthesized. Moreover, tracer is exposed to sample matrix that could lead to interferences with enzyme activity by harmful sample components. In contrast, the indirect assay makes use of tracers (enzyme-labeled

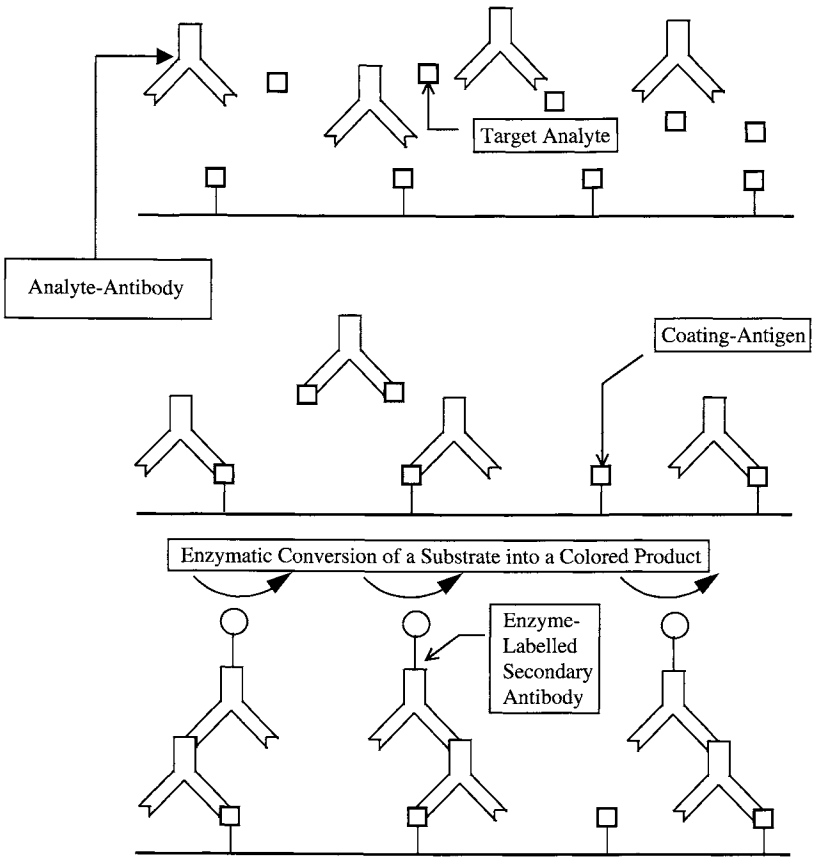

Figure IV.4.1.1b. Principle of a direct competitive ELISA.

secondary antibodies), which are available commercially and will not come into contact with the sample matrix. Although theoretically the indirect format should be more sensitive than its direct counterpart, this seems to be of secondary importance for environmental monitoring if at all.

Analyte amounts in unknown samples are interpolated from an assay calibration curve, which routinely is run on each microtiter plate. The exact shape of this curve is not important, and available curve-fitting programs can approximate this curve using either linearization by logit–log transformation of the data or a sigmoidal shape by four-parameter logistic equations. Mostly, the latter is used plotting logarithmic analyte concentration versus absorbance and taking into consideration only the linear part. In practice, ranges for quantification are most often restricted from 10 to 90% or from 20 to 80% inhibition. Absorbances on the upper or lower asymptotes of the curve can at best be specified as being below or above the corresponding thresholds.

IV.4.1.3. Optimization and validation of an immunoassay

IV.4.1.3.1. Cross-reactivity (CR)

The specificity of an antibody refers to the degree of CR that is the extent to which an antibody reacts with related compounds. For a user it is very important to know which analytes can be trapped in an assay, i.e. which compound could give a positive signal (analyte present!). CR is not limited to polyclonal antisera that constitute a mixture of many different antibodies derived from several lymphocyte clones, but is also an intrinsic property of monoclonal antibodies. Generally, CR is expected as the concentration of a compound needed to displace 50% of the antibody from bound target analyte or target analyte–protein conjugate (Abraham, 1969). It is calculated according to the formula:

$$\%\,\mathrm{CR} = \frac{\text{Target analyte concentration at 50\% antibody binding}}{\text{Concentration of the cross-reacting compound at 50\% antibody binding}} \times 100$$

Data are obtained running the ELISA successively with the target analyte and potential cross-reacting substances. Then, the concentrations at the center point of the calibration curves are compared. In another approach, the percentage CR is defined as the quotient of displacement of the antibody at different points of the calibration curve (DeLauzon et al., 1973). This takes into account that the degree of CR of a compound can vary over the concentration range of the calibration curve. However, at least for environmental immunoassays this approach did not prevail against the calculation of the 50% value. Specificity of antisera may vary strongly and is first of all the consequence of the synthesized immunogen and the applied tracer or coating antigen. In some cases aimed specificity should be as high as possible, for example, when samples should be screened for a single compound. For the determination of sum parameters such as the 16-EPA PAHs, BTX or highly toxic dioxins, lower selectivity is required to use the immunoassay as class specific test. Therefore, an antiserum or antibody should be characterized very early in assay development to find out if it meets the requirements of the assay.

IV.4.1.3.2. Assay sensitivity

Required sensitivity for environmental contaminants is mostly in the ppb or ppt range, depending on target analyte, sample matrix and set threshold limits. According to IUPAC, assays' limit of detection (LOD) is defined as the analyte concentration that will arise after extrapolation to the calibration curve of a zero dose (sample without analyte) minus its threefold standard deviation. For many immunoassays this LOD is still outside the linear part of a sigmoidal calibration curve and therefore is prone to experimental error. As mentioned earlier, the nominal working range should be limited to the linear part that is from 10 to 90% or 20 to 80% of inhibition as concentration estimates in this range are much more reproducible. With constant random error, the precision of an immunoassay increases as the slope of the dose–response curve increases and vice versa. But a steep

calibration curve will cover only a narrow concentration range. Assays' performance characteristics may be quite different in artificial solvents like buffer and highly purified water compared to real matrices. Therefore, performance must strictly be controlled for each matrix type, which is under determination. In those cases where an analyte must be detected close to the LOD, a pre-concentration step can be necessary. Whenever possible, it should be avoided as it interferes with the field-test nature of an immunoassay. Sometimes sensitivity can be tuned up to a point by means of different target analyte derivatives for tracer and coating antigen synthesis, assay formats, pre-incubation of antibodies and analyte, use of fluorogenic enzyme substrates and signal amplification systems (biotin–avidin and biotin–streptavidin auxiliary labels or enzyme cascades) (Avrameas, 1992; Bauer et al., 1995).

IV.4.1.3.3. Matrix effects

The key reagents in immunoassays are proteins (the antibodies) that are sensitive to non-physiological conditions to a different extent. Concluding from this all physical and chemical factors that can interfere with the protein structure can also adversely affect the immunoassay; these are temperature, sample pH, ionic strength and the presence of organic solvents and surfactants (Manclus and Montoya, 1996; Abad and Montoya, 1997). Moreover, dissolved organic matter like humic acids can also interfere with the assay because of specific (recognition of partial structures of the humic acid as antigenic determinants) and non-specific (adsorption of humic acid covering the openings of antibody binding sites or the active center of the enzyme) interactions with the antibodies or the assay detection system (enzyme tracer) (Keuchel et al., 1992; Matuszczyk et al., 1996; Beyer et al., 1997). In addition, the use of antibodies for the identification of non-extractable pesticide residues (bound residues) in humic substances is a new field of immunological analysis (Dosch et al., 1995; Ulrich et al., 1996; Dankwardt et al., 1997). With indirect ELISAs the sample is removed from the microtiter plate before the enzyme-labeled secondary antibody will be added thus excluding interference with the detection system. Such interferences are rare in clinical applications, i.e. for the determination of target analytes in physiological fluids and tissue homogenates making immunological determinations in clinical chemistry much better interpretable. In contrast, detailed investigation of possible matrix effects is an indispensable element of the immunoassay development.

Both positive (overestimation of target analyte) and negative (underestimation of target analyte) interferences are known. This should be demonstrated with results obtained in our laboratory. The effect of different concentrations of water-miscible organic solvents on the antibody–analyte interaction was studied using indirect pyrene- and 1-nitropyrene-ELISAs (Knopp et al., 1997). In that study the effect of solvents on the optical density of a blank (zero analyte) at different concentrations was determined. As outlined in Figures IV.4.1.2 and IV.4.1.3 it was totally different considering the individual solvents and the two antisera. For example, the addition of acetonitrile caused up to sevenfold increase in optical density at 13.3% organic solvent in the 1-nitropyrene-ELISA. In contrast to this, same solvent amount reduced the signal to about 70% in the pyrene-ELISA. Concluding from this, acetonitrile if present at this concentration, could lead to false negative (1-nitropyrene-ELISA) or false positive (pyrene-ELISA) results.

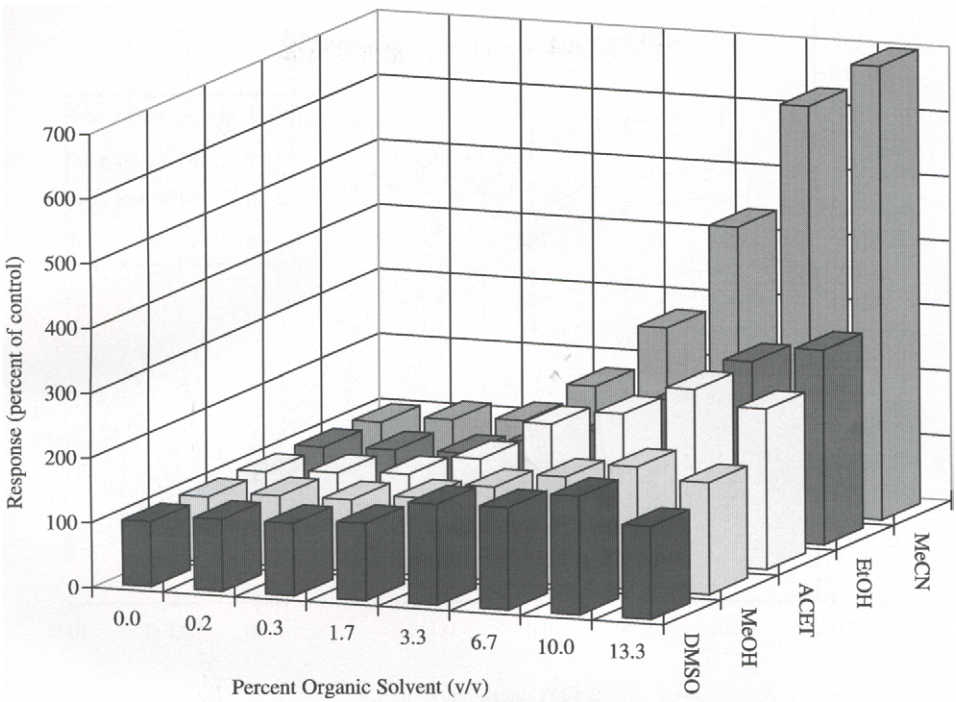

Figure IV.4.1.2. Effect of organic solvents at different concentrations on the optical density of the blank value (1-nitropyrene-ELISA).

In another experiment the effect of humic acid on a direct competitive 2,4-D-ELISA and the indirect pyrene-ELISA was compared (Matuszczyk et al., 1996; Knopp et al., 2000). As shown in Figure IV.4.1.4 with increasing concentration of humic acid, a significant decrease in the maximum optical density was found in the 2,4-D-ELISA. However, the LOD as well as the IC_{50} value changed only slightly to higher concentrations. The same humic acid concentrations had nearly no influence on the signal in the pyrene-ELISA (Fig. IV.4.1.5). Only at the highest concentration (10 mg/l) a small shift of the calibration curve to higher concentrations was observed making this assay much better suited for environmental samples, which contain higher amounts of dissolved organic matter.

IV.4.1.3.4. Sample preparation

As always stated, no or only minimal sample preparation is one of the outstanding properties of immunological methods. However, this depends mainly on the claimed sensitivity and selectivity, the robustness of the assay, the target analyte and on sample matrix type. Generally, a sample preparation can be eliminated with water samples when analyzing groundwater or drinking water, since immunoassays are run predominantly in

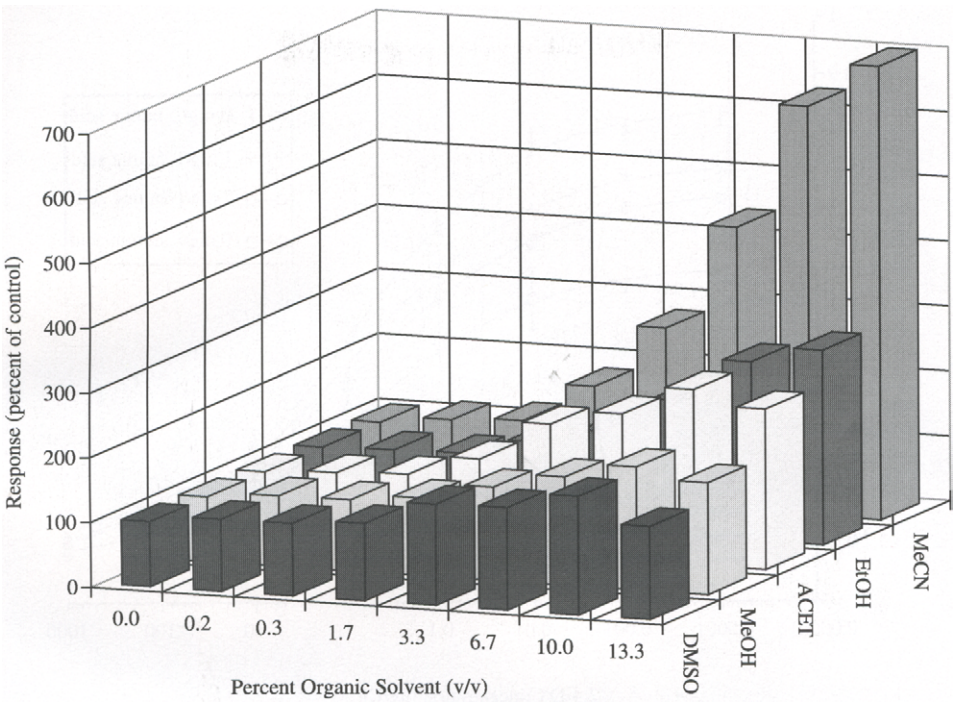

Figure IV.4.1.3. Effect of organic solvents at different concentrations on the optical density of the blank value (1-nitropyrene-ELISA).

aqueous solution. This often comes true also for surface water although with higher cloudiness of the water a filtration step should go ahead. With increasing complexity sample matrix interferences often can be diluted out using purified water or buffer solutions (Knopp et al., 1999). This can only be done with samples that contain higher amounts of target analyte not to get out of the working range of the assay. Solid-phase extraction (SPE) is increasingly used to enrich analytes from aqueous samples (Pollema et al., 1992; Fiehn and Jekel, 1996).

At least for solid samples like solid waste, soils, sediments, plant material and aerosols the sample preparation such as extraction is indispensable. As a rule of thumb with increased enrichment higher reliability and assay sensitivity will be obtained. Therefore, the extent of sample preparation must be in accordance with the later use of the assay. In one case, as for dioxins, a highly sensitive and accurate assay can be aimed at, requiring more extensive sample preparation but still reducing the costs for traditional highly expensive high-resolution GC/MS. In contrast to this, when a great number of samples should be monitored often less sensitive and semiquantitative assays are favored that need only reduced enrichment. Whenever possible, as for polar analytes, sample extraction should be done with aqueous solutions. Anhydrous organic solvents will be necessary to isolate non-polar and lipophilic agents from hydrophobic fractions of environmental samples. After extraction highly volatile solvents can be removed totally or only reduced

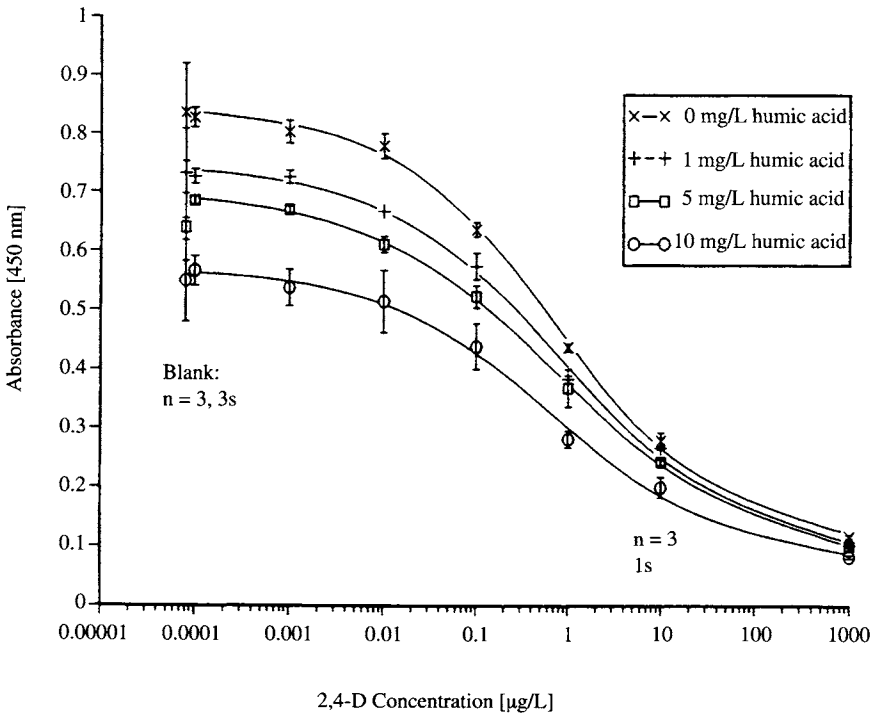

Figure IV.4.1.4. Effect of humic acid on the 2,4-D-ELISA.

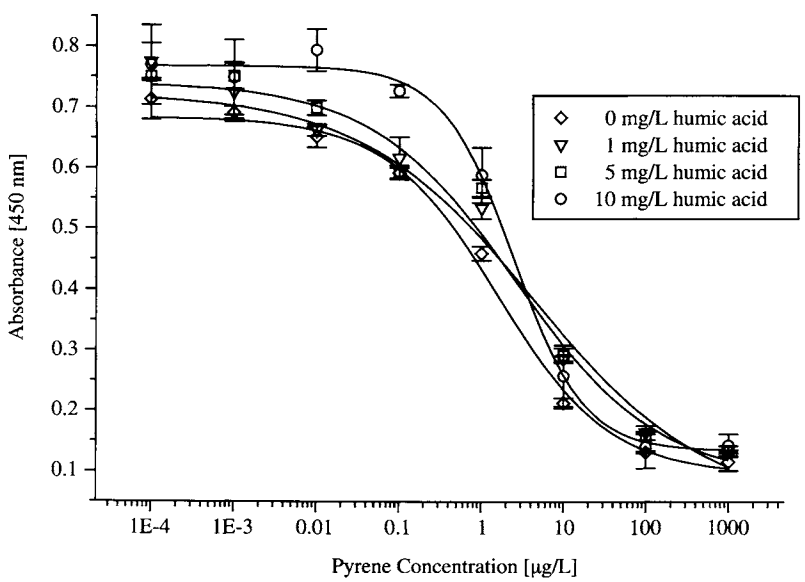

Figure IV.4.1.5. Effect of humic acid on the pyrene-ELISA.

in volume under a stream of nitrogen or under vacuum. In the case of volatile analytes care must be taken to prevent losses. Depending on the solvent compatibility of the assay the dried residue has to be taken up with buffer or another suitable solvent or an aliquot of the extract, mostly a dilution with water or buffer, can be directly transferred to the microtiter plate. Moreover, an organic co-solvent can enhance solubility of the target analyte and therefore prevents wall-adsorption to reaction vessels.

IV.4.1.3.5. Assay validation

Assay validation can be described as a process, which should demonstrate that the analytical method would yield acceptable precise, reproducible and accurate results for a given analyte in a specific matrix. As any other analytical method, immunoassays are prone to several errors. The most important interferences are matrix constituents, which were discussed before. Additional factors are the individual components of the assay system such as hardware (automatic microplate washer and reader), solid-phase material (microtiter plates, tubes, plastic microspheres), pipettes and tips, and the applied reagents (Harrison, 1997). High quality equipments, interfaced with computers to collect and analyze data, are nowadays available from several manufacturers. Microplates, plastic tubes and tips are offered by a vast number of suppliers. Some time is needed to assess the quality and suitability of these materials including monitoring of product lots for lot-wise variability. The same holds true for commercial reagents and chemicals such as enzymes, enzyme-labeled secondary antibodies, blocking agents and substrates; not to forget the quality of in-house reagents like antibodies, coating antigens and buffer solutions. For these substances quality also may vary "lot-wise", i.e. from different preparations and after storage. Whenever possible (sufficient sensitivity, suitable for the matrix of interest...) a critical comparison of an immunoassay with an independent established (non-immunochemical) method should be an inherent part of the immunoassay development. Besides known standards, fortified and unknown samples, different matrices and a sufficient number of field samples of each matrix type from a variety of different locations should be included. Once the results are obtained, the correlation between the methods can be calculated. In addition, any false positives or false negatives generated during the analysis should be noted. False positive results, while not desirable, are not as big a problem, since these samples should be confirmed by means of a reference method. In contrast, falsely identified negative samples, once eliminated from the sample set, are lost to the study. Therefore, a main effort must be directed to exclude as far as possible any tendency to produce false negatives.

IV.4.1.4. Immunoassay standardization

The acceptance of the technology will only be obtained if the same test procedure can be exhibited elsewhere under the same conditions and with identical performance as often as needed. Experience with standardization and quality assurance (QA) from the clinical chemistry can be advantageously transferred to environmental immunochemistry but there are numerous problems, which are unique like the immense variability in composition of environmental matrices and the small size of the environmental market.

Inter-laboratory comparisons (round robin studies) can be performed to assess the applicability of an immunoassay for the analysis of selected matrices. For many years, several federal government and regulatory agencies in the US (FDA, EPA, FSIS, ARS), Canada (Laboratory Service Branch of the Ontario Ministry of the Environment and Energy; Quebec Ministry of the Environment) as well as the Association of Official Analytical Chemists (AOAC), the Immunoassay Group of the Central Committee III of the Division of Water Chemistry in the Association of German Chemists (GDCh) and the IUPAC have been involved in the development of guidelines for the evaluation of immunoassay kits. The Analytical Environmental Immunochemical Consortium (AEIC) was established in 1992 in the USA and is comprised of agrichemical and immunochemical companies, academic institutions and other interested parties that develop, provide or use immunochemical methods and associated equipment for environmental chemical analysis. It was formed to promote the use of immunoassays and to provide a credible but impartial source of information about immunoassays. One of its main tasks is to establish performance standards and quality assurance guidelines.

The primary parameters for evaluating analytical method performance are common to a variety of testing systems. They include precision (variability), specificity, sensitivity (LOD and working range), accuracy and systematic errors (bias). Additional important characteristics are reliability (robustness), defined performance limits, defined quality control and quality assurance, cost-effectiveness, versatility, safety and availability of the test system. An immunoassay test kit as it is commercially available from a manufacturer is a packaged system, which contains the principal components (coated solid phase, enzyme conjugate, antibodies, standards and other reagents). Some kits additionally contain everything needed to perform (in the field) the analysis from beginning to end (disposable sample vessels, spatula, extraction and filtration devices). The *User Directory* added should be unmistakably and comprehensively, adequate to the target group and use of the test kit. Besides a description of the principle of the method, the intended use, sample matrix and claimed performance should be outlined. The analytical procedure should be described very detailed referring also to reagent stability and storage conditions, critical steps and a summary of results from earlier validation experiments.

IV.4.1.5. Environmental applications

Since the development of immunological techniques for pesticide residue analysis was first reviewed by Ercegovich (1971) several very helpful reviews of this application have been published (Hammock and Mumma, 1980; Hemingway, 1984; Newsome, 1986; Hammock et al., 1987; Mumma and Brady, 1987; Vanderlaan et al., 1987, 1988; Hammock, 1988; Harrison et al., 1988; Jung et al., 1989; Gee et al., 1990; Kaufman and Clower, 1991; Sherry, 1992, 1997; Van Emon and Lopez-Avila, 1992; Hock, 1993; Meulenberg et al., 1995; Niessner and Knopp, 2001). Until now, immunoassays for chemical contaminants were mainly developed for screening of aquatic and soil contamination but also for food and crop analysis and the biological monitoring of exposed individuals. The vast majority of these methods was applied for pesticides including herbicides, insecticides and fungicides that are generally hydrophilic, non-volatile and stable in water. However, the spectrum was extended also to other trace

contaminants like polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and BTX (benzene, toluene, xylenes) that are lipophilic and in some cases highly volatile. In Table IV.4.1.2, commercial immunochemical test kits are summarized according to the suppliers' information. If taking into consideration also relevant publications from the scientific literature, then the number of environmental chemicals for which an immunoassay has been developed would be even higher.

As always introduced the advantages of immunoassay techniques lie in their sensitivity, specificity, wide applicability, adaptability to laboratory or field situations, speed and low cost. New insights into small-volume scaled processes are possible, too. Single rain droplets can be analyzed without any further manipulation. In its present configuration required sample volume is on the order of microliters, making immunoassays a true microanalytical tool. Often analyses can be carried out directly in the crude sample or with only minimal sample preparation such as simple dilution thus avoiding extraction and cleanup steps. This is of special importance if a huge number of samples has to be measured as it can be necessary for the monitoring of remediation processes, site mapping and identification of "hot spots". Therefore, the application of immunoassays is highly indicated for screening purposes to separate "negative" samples (no target analyte present or only detectable at levels below a set threshold limit). In order to demonstrate some of the advantages and limitations of environmental immunoassays, some preliminary results of a study are summarized below that was conducted at our laboratory to compare immunochemical and traditional analytical determination (HPLC) of PAHs in real-world environmental samples such as groundwater, soil and leaching water from waste deposits (Knopp et al., 1995, 2000; Seifert, 1996).

IV.4.1.5.1. ELISA for polycyclic aromatic hydrocarbons

PAHs are ubiquitous environmental pollutants of natural or anthropogenic origin. They are formed due to incomplete combustion of various materials particularly fossil fuels. Sources of PAHs are power stations, domestic and industrial heating systems, combustion engines (diesel and petrol) and refuse burning. PAHs present in the atmosphere are distributed between gas and particle phases. They are transported over long distances and can be found in wet and dry deposition. Contamination of soils can differ widely depending on local immission situation. Manufactured gas plant sites and cokery sites are known for significant PAH-release into surrounding soil. The International Agency for Research on Cancer (IARC) stated that there is sufficient evidence that some of these compounds are carcinogenic to experimental animals. The US Environmental Protection Agency (US EPA) has identified 16 unsubstituted PAHs as priority pollutants: naphthalene, acenaphthene, acenaphthylene, phenanthrene, anthracene, fluorene, benz[a]anthracene, chrysene, fluoranthene, pyrene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenz[a,h]anthracene, indeno[1,2,3-cd]pyrene, benzo[ghi]perylene. For the analysis of environmental samples, purification and enriching followed by a powerful chromatographic separation and identification are required (Lintelmann et al., 1993; Rose et al., 1993). Most utilized is HPLC with UV (254 nm) or fluorescent detection. Rapid, simpler and more cost-effective methods would facilitate analytical measurements.

Table IV.4.1.2. Commercial immunochemical test kits for priority pollutants (P, plate kit; T, tube kit).

Target analyte	Manufacturer
Acetochlor	Coring-System (P)
Alachlor	Mallinckrodt Baker (T), Coring-System (P,T)
Aldicarb	Mallinckrodt Baker (T), Coring-System (P,T)
Atrazine (triazines)	Mallinckrodt Baker (T), Coring-System (P[a],T), Riedel de Haen (P)
Bioresmethrin	Coring-System (P)
Captan	Mallinckrodt Baker (T)
Carbaryl	Mallinckrodt Baker (T)
Carbendazim/benomyl	Mallinckrodt Baker (T)
Carbendazim/MBC	Coring-System (P,T)
Carbofuran	Mallinckrodt Baker (T), Coring-System (T)
Chlordane	Coring-System (T)
Chlorpyriphos	Mallinckrodt Baker (T), Coring-System (P)
Chlorpyriphos-methyl	Coring-System (T)
Chlorothalonil	Mallinckrodt Baker (T)
Chlorsulfuron	Coring-System (P)
Cyanazin	Mallinckrodt Baker (T), Coring-System (P)
Cyclodiene	Coring-System (T)
2,4-D	Mallinckrodt Baker (T), Coring-System (P,T)
DDT	Coring-System (T)
Endosulfan	Coring-System (P)
Diazinon	Coring-System (P)
Fenitrothion	Coring-System (P,T)
Harnstoffherbizide	Coring-System (P)
Hexazinon	Coring-System (P)
Isoproturon	Coring-System (P)
Lindan	Coring-System (T)
Metalaxyl	Coring-System (P)
Methoprene	Coring-System (P)
Methomyl	Mallinckrodt Baker (T)
Metolachlor	Mallinckrodt Baker (T), Coring-System (P)
Metsulfuron-methyl	Coring-System (P)
Microcystin	Coring-System (P,T)
Molinat	Coring-System (P)
Paraquat	Mallinckrodt Baker (T), Coring-System (P)
Parathion/parathion-methyl	Coring-System (P)
Pirimiphos-methyl	Coring-System (P)
Procymidone	Mallinckrodt Baker (T), Coring-System (P,T)
Silvex	Mallinckrodt Baker (T), Coring-System (T)
Thiabendazol	Coring-System (P)
Toxaphene	Coring-System (T)
Triasulfuron	Coring-System (P)
Triclopyr	Mallinckrodt Baker (T)
Trichlorpyridonol	Mallinckrodt Baker (T)
BTEX	Mallinckrodt Baker (T), Coring-System (T)

(*continued*)

Table IV.4.1.2. (Continued)

Target analyte	Manufacturer
Pentachlorophenol	Mallinckrodt Baker (T), Coring-System (T[a])
Petroleum hydrocarbons	Coring-System (T)
PCB	Mallinckrodt Baker (T), Coring-System (T[a])
PAH	Mallinckrodt Baker (T), Coring-System (T[a]), Quantix (P)
TNT	Mallinckrodt Baker (T)
RDX	Coring-System (T)
Mercury (II) ions	BioNebraska (P[a],T)

Summarized according to the available literature and the suppliers' information. If not signed otherwise the test kits are based on polyclonal antibodies.
[a]Monoclonal antibody-based test kit available.

Commercial immunochemical test kits for the determination of total PAH-content in soil were offered in the past by *Mallinckrodt Baker* (LOD 70 μg/kg, tube kit), *Coring-System* (LOD 100 μg/kg, tube kit), *Quantix* (LOD μg/kg, microtiter plate kit) and *Merck* (600 μg/kg, immunofiltration device). Application of these kits for water analysis is also possible: *Mallinckrodt Baker* (LOD 0.7 μg/l), *Coring-System* (LOD 1 μg/l), *Merck* (8 μg/l) and *Quantix* (LOD 50 μg/l). Moreover, *Mallinckrodt Baker* offered a carcinogenic PAH-ELISA (LOD 0.2 μg/l in water and 20 μg/kg in soil). The PAH-ELISA used in the described study was developed at our laboratory and was based on polyclonal antibodies raised in rabbits against a pyrene–KLH conjugate (Meisenecker et al., 1993). Test format was an indirect competitive ELISA performed in 96-well micotiter plates. The linear dynamic range of the method calibrated with pyrene was 0.05–5 μg/l (from 20 to 80% inhibition) for aqueous standards and 0.25–20 μg/l (from 20 to 80% inhibition) for aqueous standards containing 10% acetonitrile as they were used for the analysis of extracts from soil.

IV.4.1.5.1.1. Groundwater monitoring

Because of building activities in the area of a former gas plant site, a mobilization and wash-out of PAHs from the contaminated soil with the lowered groundwater level were noticed. For purification the water was removed from the quarternary groundwater layer downstream of the contaminated area and cleaned with multilayer filters and activated charcoal. Purification process was controlled routinely by HPLC-analysis of the PAH-content. Including sample preparation throughput was about 15 analyses every 3 days. Supposing the immunochemical results (pyrene-equivalents as calculated from pyrene calibration curve) to be approximate values of the total PAH concentration then these data compare very well ($r = 0.82$) with the sum of 16-EPA PAHs as measured by HPLC (without acenaphthylene which cannot be detected by fluorescence) (Fig. IV.4.1.6). Based on 114 determinations the immunoassay generated an 18% false positive (defined as a positive response for a sample that contains the 16-EPA PAHs below the claimed action level of 0.2 μg/l) and no false negatives (defined as a negative response for a sample that contains the 16-EPA PAHs at the action level of 0.2 μg/l). The majority of the false

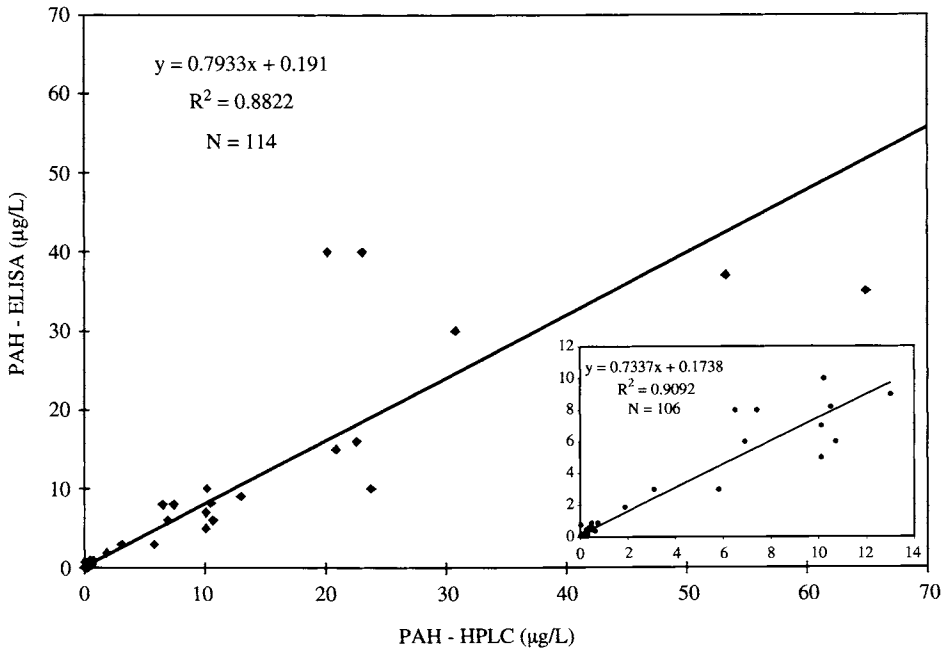

Figure IV.4.1.6. Comparison of HPLC- and ELISA-PAH determination in contaminated groundwater of a former gas plant site. Individual values represent the sum of 16-EPA PAHs as measured by HPLC (without acenaphthylene) and were correlated to the amount of pyrene-equivalents as calculated from the pyrene calibration curve.

positive results were at concentrations near the detection limit. However, as can been seen from the slope of the regression line, ELISA results were on average significantly lower than corresponding summarized PAH concentrations from HPLC. This was not surprising because the antibodies will never exhibit identical binding affinity to each of the single PAH compounds. In this case, only those chemicals, which are structurally most related to pyrene (the immunizing hapten) and which exhibit some higher water solubility could bind to the antibodies to a certain extent. These are first of all fluoranthene and phenanthrene as was found in cross-reactivity studies with calibration standards that were prepared to cover water-soluble concentration ranges. At present, nearly nothing is known whether PAH metabolites can also contribute significantly to the ELISA signal. This is mainly caused by the lack of available metabolite standards to measure cross-reactivity. It is known from the literature that a wide variety of bacteria, fungi and algae have the ability to metabolize PAHs starting with the incorporation of molecular oxygen (Shuttleworth and Cerniglia, 1995). As an example, the cross-reactivity of 1-hydroxypyrene was found to be 180% in this ELISA pointing to the importance of biodegradation products as potential interfering compounds. In accordance with these findings, Li et al. (2000) reported an overestimation of PAHs in water and sediment samples by ELISA over GC–MS which was attributed to, at least in part, PAH metabolites. The presence of 1-hydroxypyrene was confirmed by HPLC-fluorescence in that study. This is of special

interest for those immunoassays that aim at a whole group of target analytes as in the case of PAHs.

IV.4.1.5.1.2. Soil monitoring

In a further study the application of this ELISA for soil samples was investigated. Generally, immunological determination of soil matrices is (1) more complex compared to aqueous samples because the target analyte has to be extracted and (2) interferences by soil constituents such as humic acids are often found. Starting with fortification experiments using well-characterized standard soils and several extractive procedures were compared to study PAH-recovery (Fig. IV.4.1.7).

Efficiency was comparable using ultrasonication (acetonitrile) or soxhlet extraction (tetrahydrofurane) and was on average some lower (about 10%) using agitation (acetonitrile). PAH determination again was performed in parallel with HPLC and ELISA. The same extractive procedures were tested with aged field samples from several sites including forest soil, farmland, grassland, city ground, soil of a former gas plant site, and a reference material that was certified with EPA SW-846 methods 3540 and 8270 by 20 laboratories. As measured with HPLC, PAH concentration ranged between 0.15 and 703 μg/g of soil. As with the spiked samples soxhlet extraction and ultrasonication showed similar efficiency whereas agitation resulted in a loss of recovery of about 10%. In comparison to the previous groundwater samples ELISA determination of soil extracts resulted in a much greater underestimation of PAH concentration when comparing the pyrene-equivalents with the sum of the 16-EPA PAHs (without acenaphthylene) from HPLC (Fig. IV.4.1.8). Again this was not surprising looking at the PAH profile in the soils and taking into consideration the cross-reactivity pattern of the antiserum (Fig. IV.4.1.9).

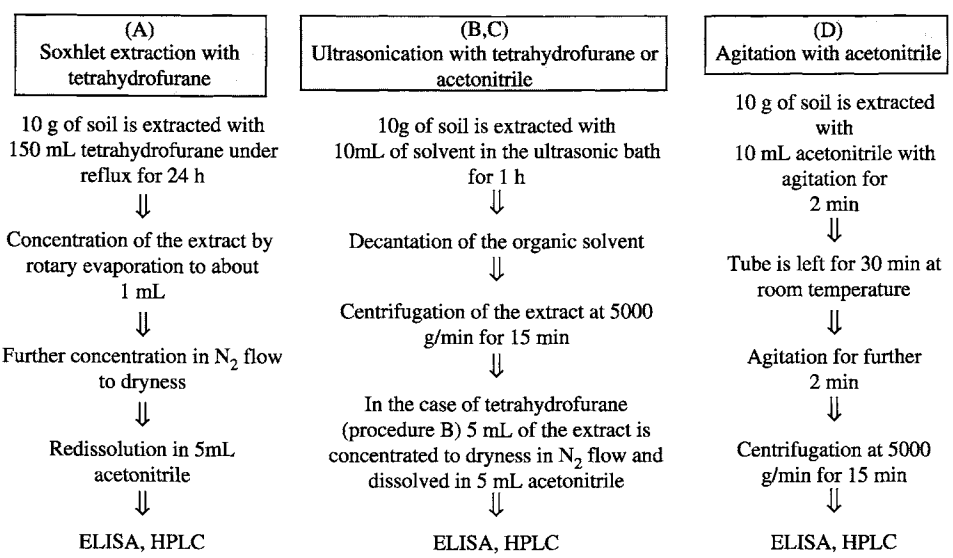

Figure IV.4.1.7. Extraction procedures tested for the recovery of PAHs from soil.

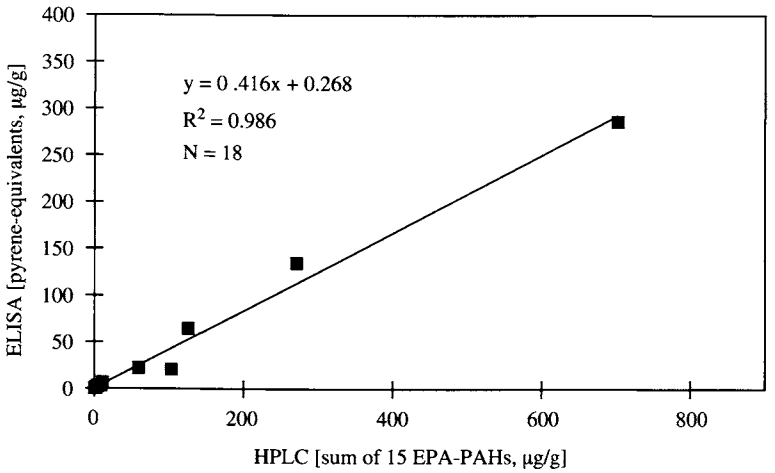

Figure IV.4.1.8. Comparison of HPLC- and ELISA-PAH determination in soil extracts. Individual values represent the sum of 16-EPA PAHs (without acenaphthylene) as measured by HPLC and were correlated to the amount of pyrene-equivalents as calculated from the pyrene calibration curve.

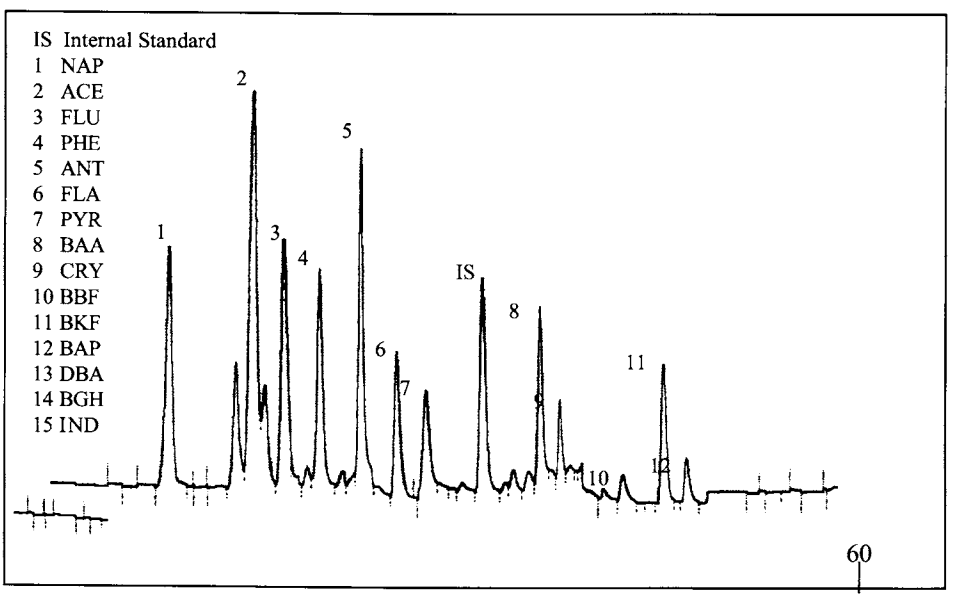

Figure IV.4.1.9. HPLC chromatogram of a highly contaminated soil from a former gas plant site. Extract was diluted 1:500. Used abbreviations of PAH compounds: NAP, naphthalene; ACE, acenaphthene + acenaphthylene; FLU, fluorene; PHE, phenanthrene; ANT, anthracene; FLA, fluoranthene; PYR, pyrene; BAA, benz[a]anthracene; CRY, chrysene; BBF, benzo[b]fluoranthene; BKF, benzo[k]fluoranthene; BAP, benzo[a]pyrene; DBA, dibenz[a,h]anthracene; BGH, benzo[ghi]perylene); IDP, indeno[1,2,3-cd]pyrene.

Table IV.4.1.3. Classification of ELISA results.

Class	Contamination level	ELISA-pyrene equivalents (μg/g)	Estimated PAH-level (sum of the 16-EPA PAHs, μg/g)
1	Very low	<0.35	<1
2	Low	0.35–3.5	1–10
3	High	3.5–35	10–100
4	Very high	>35	>100

The ELISA data were classified according to Table IV.4.1.3 to consider if it can be used for a semiquantitative estimation of the PAH contamination in soils. Applying this for all the data obtained so far with the soil extracts about 5% of the estimated PAH concentration was either false positive or false negative (Table IV.4.1.4) pointing again to the effect of target analyte partition and relative response factors (reactivity of the analytes in the test sample relative to the reference compound in the assay) on the correctness of results in class specific ELISAs. The false negative sample from this study

Table IV.4.1.4. Assessment of the classification set-up.

Soil type	ELISA-pyrene equivalents (μg/g)	Class	HPLC-sum of 16-EPA PAHs (without acenaphthylene) (μg/g)	Estimation of PAH-contamination level with the ELISA
Reference soil	0.02	1	0.26	Correct
Reference soil	0.03	1	0.15	Correct
Reference soil	0.05	1	0.33	Correct
Grassland	0.05	1	0.18	Correct
Reference soil	0.13	1	0.25	Correct
Forrest soil	0.17	1	2.56	False negative
Forrest soil	0.35	2	2.11	Correct
Reference soil	0.54	2	0.57	False positive
Farmland	1.0	2	1.78	Correct
Farmland	2.2	2	4.61	Correct
Grassland	2.94	2	4.22	Correct
City ground	3.1	2	8.9	Correct
City ground	6.6	3	11.2	Correct
EPA-reference soil	20.6	3	98.2	Correct
City ground	22.2	3	59.57	Correct
Gas plant soil	64.22	4	126.09	Correct
Gas plant soil	134.6	4	271.59	Correct
Gas plant soil	286	4	702.92	Correct

contained a phenanthrene proportion greater than 70%. Because this compound shows a cross-reactivity of only about 10%, the extrapolation of the ELISA-"pyrene-equivalents" led to an underestimated PAH-level. Perhaps, it will be possible for this PAH-ELISA as for class specific ELISAs in general that the rate of false negative results can further be reduced and accuracy enhanced by a fairly simple approach: Determination of the target analyte partition of a few random samples from the contaminated site with a traditional technique as the base for the calibration of the ELISA with the known cross-reactivities of the individual compounds (site-specific calibration). However, this has to be proved in the future.

IV.4.1.5.1.3. Leaching water monitoring

Depending on the type of the waste deposit leaching water can constitute an extremely complex matrix difficult to be analyzed by immunochemical techniques. In this study, leaching water from two different waste deposits was analyzed in regard to the PAH contamination. While the water from the municipal waste deposit showed only a weak cloudiness, the sample from the special waste deposit was a dark fluid covered by an oily layer disseminating a strong smell of coal tar. As was found with HPLC, PAH concentration was rather low (28 μg/l) in the sample from the municipal waste deposit but was very high in the aqueous (8.15 mg/l) and oily (540 mg/l) layers of the sample from the special waste deposit. The lower condensed higher water-soluble 2-, 3- and 4-ringed PAHs dominated in both samples with a high excess of naphthalene in the sample from the special waste deposit. Water from the municipal waste deposit as well as the aqueous layer of the special waste deposit could be analyzed with the ELISA after at least 1:100 pre-dilution of the samples, as was found with fortification experiments. Underestimation of the PAHs was in the same order as described for the soil samples, i.e. recovery was 25% (special waste deposit) and 40% (municipal waste deposit) compared to HPLC. For the immunochemical analysis of the oily layer from the special waste deposit it was pre-diluted 1:10,000 with acetonitrile and then applied as acetonitrile/water (10:90, v/v) solution directly in the ELISA. Recovery rate found for PAHs in this sample was only about 2% compared to HPLC, obviously owing to matrix interferences. PAH profile in both layers was very similar but the sum of the 16-EPA PAHs by a factor of about 66 was higher in the oily layer (Figs. IV.4.1.10, IV.4.1.11a and IV.4.1.11b).

To summarize, the PAH-ELISA was proven to be applicable to groundwater samples from a gas plant site for screening out samples of PAH concentration (as the sum of the 16-EPA PAHs) below 0.2 μg/l (threshold limit as set by the German Drinking Water Act) and thus, as the main advantage in this case, leading to reduced sample load for the time-consuming and more costly traditional analytical method. Application to different soil samples ($n = 18$) revealed that 5% of the estimated PAH concentration was either false positive or false negative that was mainly caused by the PAH partition in the sample. ELISA results can be classified to be used for an estimation of the PAH concentration at levels < 1, $1-10$, $10-100$ and > 100 ppm. PAHs can be recovered from soil with high yield using 1-h ultrasonication with acetonitrile (for laboratory ELISA). Extraction efficiency is lower with agitation but is acceptable as part of a first-step on-

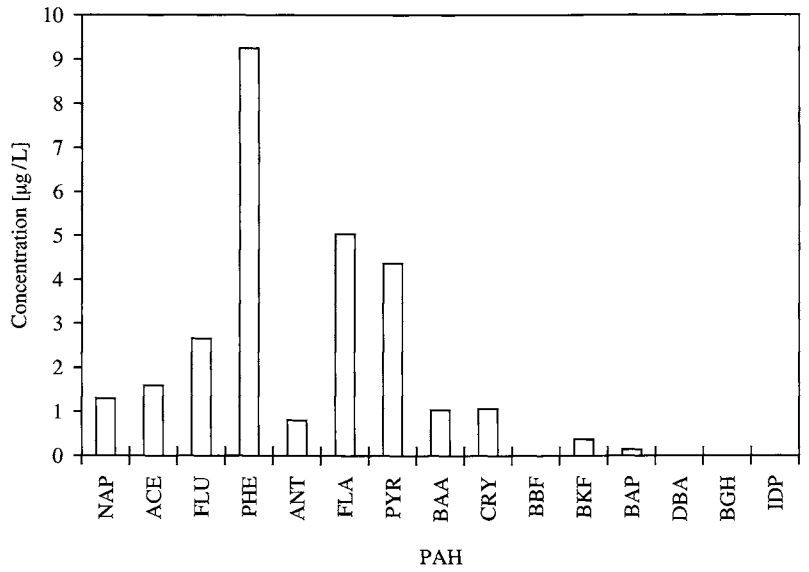

Figure IV.4.1.10. PAH-profile in the leaching water from a municipal waste deposit. PAH order and used abbreviations: as in Figure IV.4.1.9.

site field test to provide rapid, semiquantitative and reliable test results for making environmental decisions. While groundwater samples can be measured directly, soil and leaching water require at least 1:100 dilution prior to immunochemical analysis to remove matrix interferences. The classification set-up proposed for the estimation of the PAH-level in soils was also applicable to the leaching water samples from municipal and special waste deposits as well.

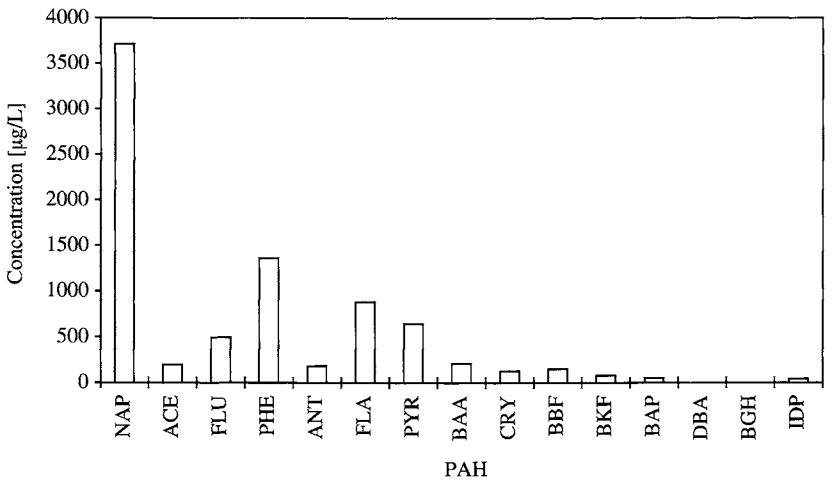

Figure IV.4.1.11a. PAH-profile in the leaching water from a special waste deposit. PAH order and used abbreviations: as in Figures IV.4.1.9 and IV.4.1.10.

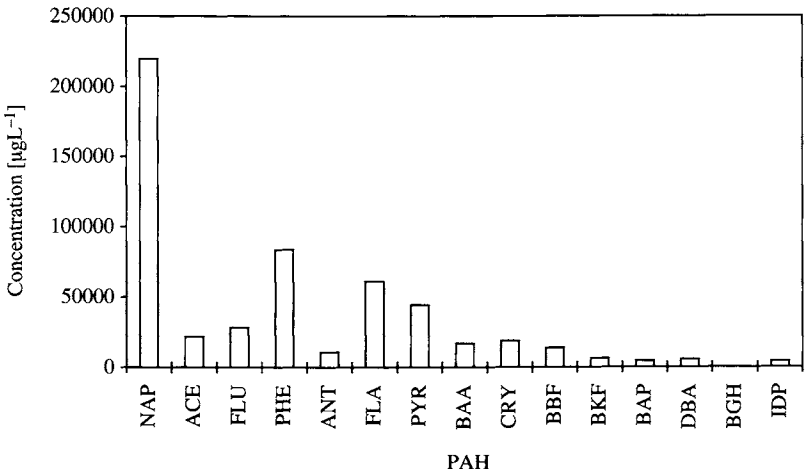

Figure IV.4.1.11b. PAH-profile in the oily layer of the leaching water from a special waste deposit. PAH order and used abbreviations: as in Figures IV.4.1.9, IV.4.1.10 and IV.4.1.11a.

IV.4.1.6. Future immunochemical techniques

There are several emerging concepts of immunoanalysis. Flow injection analysis (FIA) and immunology have been combined to create flow injection immunoanalysis (FIIA; antibody-based flow-through immunosensor) that enables analysis to be carried out in a rapid, on-line and automated way. It has been employed in variety of devices (Liu et al., 1991; Pollema et al., 1992; Gunaratna and Wilson, 1993; Whelan et al., 1993; Ruzicka, 1994; Kumar et al., 1996; Gonzalesz-Martinez et al., 1997; Krämer et al., 1997; Narang et al., 1997; Fránek et al., 2000; Bjarnason et al., 2001; Nistor et al., 2001). Other efforts are directed on the development of test-strips or immunofiltration arrangements based on immunological principles that could be very suitable to perform a semiquantitative, rapid, inexpensive and non-instrumental analysis on-site (Rittenburg et al., 1990; Schneider et al., 1991; Anonymous, 1993; Dankwardt and Hock, 1993; Giersch, 1993; Keuchel and Niessner, 1994; Siebert et al., 1995; Del Carlo et al., 1998; Morais et al., 1999). Evaluation can be done visually or spots can be read out by a pocket reflectometer. Some additional future growth areas are high-performance immunoaffinity chromatography, multianalyte immunoanalysis and molecular imprinting techniques that could be of special interest for the monitoring of solid waste sites and therefore, should be introduced in some more detail below.

IV.4.1.6.1. High-performance immunoaffinity chromatography (HPIAC)

Normally, the initial sample preparation steps where analytes must be purified and enriched from environmental samples are the most time-consuming part of an analytical method. Nowadays, several solid-phase materials (chemical adsorbents) in the format of cartridges or discs are increasingly used for the isolation of target analytes from complex

matrices (El Harrak et al., 1996). However, these adsorbents are not very selective but rather will retain chemical compounds by their non-polar or polar nature. Higher selectivity can be obtained using an immobilized secondary molecule (ligand; that may also be a biomolecule) on the solid-phase material that exhibits higher affinity to the target molecules (affinity chromatography) (Cass and Ligler, 1988). Immunoaffinity chromatography, as the term suggests, exploits the fine specific and reversible interaction between an antibody and its antigen to purify and concentrate the antigen from a crude sample that may include soluble and insoluble impurities. In the opposite manner an immobilized antigen can be used to isolate its antibody from a polyclonal antiserum. Typically, the antibody is immobilized on an insoluble support matrix by simple adsorption or covalent coupling, which mainly depends on matrix type and antibody stability (Schramm et al., 1993). The ideal support material is of high mechanical stability, macroporosity, ease of activation, hydrophilicity and inertness. The most popular support matrices for affinity chromatography are beaded agarose, polyacrylamide gels, azlacton functional copolymer beads and silica-based packings (bonded-phase silicas, glass beads, coated glass beads). There currently exist many methods for activation of the supports that can be divided into those that produce randomly oriented immobilized ligands (attachment of the ligand to the support via primary amino groups on the protein is favored) and those that produce more uniformly oriented species (attachment via the Fc region). A new approach is the physical entrapment of the antibodies in the pores of a silicate glass prepared by the sol–gel technique (Avnir et al., 1994; Dave et al., 1994; Avnir, 1995; Lev et al., 1995; Zühlke et al., 1995; Turniansky et al., 1996; Roux et al., 1997; Cichna et al., 1997a,b; Doody et al., 2000; Lan et al., 2000; Altstein et al., 2001; Pulido-Tofino et al., 2001). This support offers a number of advantages like improved stability and no antibody leaching even under harsh elution conditions. Although there were published first results which were obtained with real samples, the method is still in its infancy (Scharnweber et al., 2000; Spitzer et al., 2000; Cichna et al., 2001).

The principal stages of immunoaffinity separations are loading, washing, elution and regeneration. Following loading, the immunoaffinity support is washed to remove impurities present in the sample as well as those bound non-specifically to the matrix. During the elution step, the captured analyte is removed from the column by using a solvent that reduces the affinity of the analyte to the antibody such like extreme pH, chaotropic agents, organic solvents, low ionic strength and others. A regeneration step should follow to prepare the column for loading again if the affinity support is to be reused. No single schedule seems to work best for all antibodies and is still a trial-and-error procedure (Yarmush et al., 1992a,b).

Complementary features of immunoaffinity chromatography and traditional chromatographic techniques (HPLC, GC, CE) allow them to be combined to produce a highly efficient technology with superior selectivity, speed and sensitivity. This can be realized off-line using cartridges or on-line with a tandem system using an immuno precolumn (high-performance immunoaffinity column) and column switching. Based on the results of numerous applications mainly from residue and pesticide analysis, it can be speculated that this approach will be extended to other classes of environmental pollutants and their degradation products as well in the near future (Farjam et al., 1991; Orthner et al., 1991; Van Ginkel, 1991; De Frutos and Regnier, 1992; Kim et al., 1993; Stanley et al., 1993; Vanderlaan et al., 1993; Kussak et al., 1994; Rule et al., 1994; Thomas et al., 1994; Marx

et al., 1995; Matuszczyk et al., 1995; Pichon et al., 1995, 1996; Wong et al., 1995; Lawrence et al., 1996; Nedved et al., 1996; Rollag et al., 1996; Strong et al., 1996; Van Emon and Lopez-Avila, 1996; Hage et al., 1997; Shelver et al., 1998; Weller, 2000; Delaunay et al., 2000; Bou Carrasco et al., 2001). Whether the principle of immunoaffinity also can be used successfully for routine monitoring of air-borne pollutants such as indoor air studies, workplace monitoring, and studies on air-mediated transport of organic compounds will depend first of all on the development of devices suitable to keep necessary liquid, which is required for the sampling antibodies.

IV.4.1.6.2. Multianalyte immunoassays

A limitation of an immunoassay compared to common chromatographic techniques often specified by environmental analysts consists in its single-compound analysis. However, this is only partly true. Because antibodies target epitopes, not the whole antigen, multiple substances may have the same or similar epitopes. When this is the case the cross-reactivity of antibodies can be used for a multiresidue analysis of structurally related compounds that might be a class of pesticides such as the triazines including a number of metabolites. If there is available a library of antibodies (an antibody array) exhibiting a different pattern of affinity for a series of structurally related compounds, an appropriate statistical analysis (multivariate statistical techniques, parametric models) has the power to turn the problem of cross-reactivity into an advantage. Moreover, the number of antibodies required will typically be less than the number of target analytes. Multiple immunoanalysis using an immunoarray, a panel of less selective antibodies with differing affinity patterns, can proceed in different directions. One is mixture analysis in which samples are assumed to contain mixtures of analytes coming from a known small set of cross-reacting compounds (usually no more than four). In another approach, samples are assumed to contain only one unknown analyte from a class of possible compounds (Jones et al., 1997). The immunoarray responses are used first to identify the analyte and then to estimate the concentration. Further, the application of neuronal networks for pattern recognition has also been applied to the analysis, with the inputs being either the untransformed responses or an estimated concentration of a chosen reference analyte (Wittmann et al., 1997).

In contrast to the above approaches, Ekins and co-workers in a series of publications described a miniaturized microspot multianalyte immunoassay system (microarray-based immunoassays) based on labeled antibodies and permitting the simultaneous determination of many analytes in the same small-volume sample (Ekins and Chu, 1995; Chu et al., 1997). The authors estimate that the technique has the potential to detect hundreds of different analytes in a 1-ml sample or less. Minute amounts of different antibodies are to be located in different microspots forming an array on a solid support such as membranes, glass slides, quartz optical fibres or chips. It reflects the realization that the use of vanishingly small concentration of antibody yields assays that are faster and more sensitive than others. For quantification of occupied or unoccupied capture antibodies fluorescent labeled secondary antibodies can be used together with laser scanning confocal microscope or CCD camera (Weller et al., 1999; Bernard et al., 2001). The breakthrough of this technology will mainly depend on the availability of improved solid supports and antibodies (or antibody fragments), and by the development of more simple instruments,

e.g. a compact disc-based microarray technology (Kido et al., 2000). The same concepts are applicable to assays that rely on the use of oligonucleotide probes for genetic testing (McGown et al., 1995).

IV.4.1.6.3. Artificial antibodies

Biological recognition elements like antibodies and enzymes in some cases lack storage and operational stability. This limits their use in industrial, pharmaceutical and environmental analytical chemistry. For several years, considerable efforts are being made to develop synthetic recognition systems specific for a given molecule (Andersson et al., 1993; Wulff, 1993, 1995; Bartsch and Maeda, 1998; Sellergren, 2001). This technique, referred to as molecular imprinting or as template polymerization, involves arranging polymerizable functional monomers and cross-linkers around an analyte of interest (print molecule, template) by non-covalent or covalent interactions prior to initiation of polymerization. Subsequently, the print molecules are extracted or chemically cleaved leaving recognition sites in the rigid polymer network with specific shape (three-dimensional geometry) and functional group complementarity to the original template. The polymer may then be used as an artificial receptor to selectively rebind the template from a mixture of chemical species. The principal means for exerting specificity are ionic interactions and hydrogen bonding between the analyte and the polymer functional groups. The choice of the functional monomer, or combination of monomers, depends on the chemical properties of the analyte that the molecular imprinted polymer (MIP) is being made for. Other variables in polymer synthesis include type and relative amount of cross-linking reagent used, type of solvent, and the time and temperature at which the polymerization is carried out (Hosoya et al., 1996; Mayes and Mosbach, 1996).

Intended applications of MIPs, which are already partly realized, are as substitutes for biological recognition structures in biosensors, as tailor-made solid-phase material for chromatographic separations and as catalytically active polymers or enzyme mimics (preparation of active centers) in organic synthesis (Kriz et al., 1995, 1997). Further, the use as artificial antibodies in ligand binding assays (molecularly imprinted sorbent assay) was reported (Vlatkis et al., 1993; Muldoon and Stanker, 1995; Andersson, 1996; Haupt et al., 1998).

Most of the work so far has involved the development of MIPs for sugars, amino acids and their derivatives, but also therapeutic drugs and other chemical compounds (Kriz et al., 1994, 1995; Andersson et al., 1995; Kempe, 1996; Matsui et al., 1996; Levi et al., 1997). However, MIP preparation was also reported for pesticides, mainly atrazine, one of the most widely used herbicide in the world. These MIPs were used as solid-phase adsorbents in binding assays, HPLC, sensors and SPE columns (Matsui et al., 1995; Piletsky et al., 1995; Siemann et al., 1996; Muldoon and Stanker, 1995, 1997a,b; Ferrer et al., 2000; Köber et al., 2001). From the present point of view it seems questionable, whether binding affinities with MIPs comparable to natural antibodies can be obtained in the near future. At present, for many analytes there is a gap, which comprises several orders of magnitude. This is, however, an advantage for affinity chromatography as it facilitates regeneration and repeated use of the chemical polymers. The high chemical, solvent and thermal stability, and the low cost of preparation make them a valuable complement to antibodies for use in environmental analysis, especially for poorly water-soluble contaminants.

In this context, there is a desire to perform immunoassays in anhydrous organic solvents (Russell et al., 1989; Wetall, 1991; Francis and Craston, 1994; Abad and Montoya, 1997; Dankwardt et al., 1997; Stöcklein et al., 1997; Horácek and Skládal, 2000). Analysis of crude organic solvent extracts may be possible and this would greatly simplify many current procedures. Still, the applicability for difficult environmental matrices has to be proven.

IV.4.1.7. Conclusions

Immunological techniques are increasingly being recognized as rapid, sensitive and inexpensive methods in environmental analytical chemistry. By screening out negative samples (no target analyte present or only detectable at levels below a set threshold limit) they can constitute a valuable tool to decrease the demand for chemical analyses by more time-consuming and highly sophisticated instrumentation. At present, corresponding tests are available mainly in the microtiter plate and tube format. Still the limited provision of highly affine and well-characterized antibodies for environmental contaminants must be considered as a constraint for further dissemination of these methods. This will change, as growing progress made in molecular biology will be reflected in this area. Some of the main efforts are directed to the development of ready-to-use and easy to perform field tests, which can be used on-site for monitoring remediation processes, site mapping and identification of "hot-spots". Increasing activities in validation and standardization of immunochemical tests as were initiated by federal government and regulatory agencies of several countries and well-recognized national and international organizations will lead to more transparency and uniformity in method development and evaluation and therefore, will contribute that the assays get rid of their smack as "dubious biological tests", sometimes found in discussions with analytical chemists. Immunological methods cannot be assessed, dependent from the view, simply as "good" or "bad" but rather as suitable for an application or not.

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For further information

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