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Review

# Bioseparation and bioanalytical techniques in environmental monitoring

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**Abstract**

The growing use of antibody-based separation methods has paralleled the expansion of immunochemical detection methods in moving beyond the clinical diagnostic field to applications in environmental monitoring. In recent years high-performance immunoaffinity chromatography, which began as a separation technique in biochemical and clinical research, has been adapted for separating and quantifying environmental pollutants. Bioaffinity offers a selective biological basis for separation that can be incorporated into a modular analytical process for more efficient environmental analysis. The use of immunoaffinity chromatography for separation complements the use of immunoassay for detection. A widely used immunochemical detection method for environmental analyses is enzyme immunoassay. The objective of this paper is to review the status of bioaffinity-based analytical procedures for environmental applications and human exposure assessment studies. Environmental methods based on bioaffinity range from mature immunoassays to emerging techniques such as immunosensors and immunoaffinity chromatography procedures for small molecules. 

*Keywords:* Reviews; Immunoaffinity chromatography; Immunoassays; Immunosensors; Affinity adsorbents; Flow injection analysis

## Contents



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## 1. Introduction

In a sense, all immunochemical methods are bioaffinity methods because their ability to separate and identify compounds is based on the interaction between an antibody and an antigen (or analyte). Biologically-based analytical detection methods have gained favor in environmental analytical processes, beginning in the 1980s, when immunochemical methods [1] were recognized as useful screening techniques for the detection of compounds of environmental regulatory concern. The U.S. Environmental Protection Agency (EPA) was joined by the U.S. Food and Drug Administration (FDA), the U.S. Department of Agriculture (USDA), and the U.S. Geological Survey (USGS) in researching immunochemical detection methods for a variety of regulatory and monitoring applications. Environmental matrices can be as seemingly simple as drinking water or as obviously complex as sludge from a hazardous waste site or composite food samples from an exposure assessment study. Compounds of environmental and human health concern can be well-documented like benzene, or newly scrutinized like recent pesticide candidates based on recombinant DNA technology. What all environmental compounds and matrices share is an analytical challenge: to separate the compound of interest from the surrounding matrix and quantify it in terms that support the data quality objectives of the particular study. An even more challenging goal is streamlining this analytical process using more-expedient, less-expensive methods than those currently employed for environmental analysis.

Because successful separation of the analyte of interest from a complex matrix is frequently the first goal of an environmental analysis, the strength and specificity of biological separations is particularly suited to these analytical procedures. Examples of separation methods that rely on bioaffinity discussed in this article are affinity chromatography, immunoaffinity chromatography, affinity capillary electrophoresis and immunoaffinity capillary electrophoresis. The role of biological separations is constantly being refined and is gradually expanding into the environmental area.

Used as the determinative step, bioanalytical methods can contribute by achieving lower detection levels for many compounds of environmental and human health concern. As monitoring or screening methods, they can increase speed of analysis and efficiency while lowering overall cost. The immunodetection methods we discuss are immunoassay, flow injection methods, and immunosensors. Immunoassay has proven to be a valuable analytical tool for environmental and human exposure monitoring. Flow injection methods based on antibodies can expedite high sample loads when formatted in a modular analytical system. Immunosensor research and development activities are focusing on remote sensing and on-line monitoring. The positive attributes of instrumental and bioaffinity methods are being leveraged in hybrid techniques and several examples are presented here. The subject of molecularly imprinted polymers is briefly presented as they may aid in understanding the underlying principles of bioaffinity methods. Molecularly imprinted polymers may eventually be used as the recognition elements in affinity-based techniques for small molecules.

Many of the analytical methods discussed here use selective antibodies as the biorecognition element. An antibody is a protein that selectively recognizes and binds to a target analyte (or antigen) or group of related analytes. Most compounds of environmental concern are small molecules. The small molecule (or

an analog) is coupled to a large carrier molecule as a step in antibody production. Some of the resulting antibodies are able to react with the target analyte. The development of immunoreagents for analytical purposes has previously been described [2,3]. Once the specific antibodies are obtained, they can be used as reagents in many analytical formats.

Sample preparations are frequently time-consuming in environmental analytical procedures. In addition, due to the numerous and sometimes complicated processes involved, it is the step of the analytical scheme where error and sample loss are most likely to occur. Extraction of samples for chromatographic analysis usually requires the use of organic solvents which frequently poses problems in handling, storage and disposal. In contrast, immunoaffinity chromatography, as well as immunoassay detection methods, uses little or no nonaqueous solvents.

## 2. Bioseparation techniques

### *2.1. Affinity chromatography*

Affinity chromatography is a separation technique in which a substance with a selective binding affinity (e.g. antibody or enzyme, etc.) is covalently coupled to a support such as Dextran, cellulose, agarose, or glass beads. The immobilized substance binds its complementary target from a mixture in solution or suspension. Affinity chromatography uses specific and reversible biological interactions between a ligand and a complementary macromolecule. Laboratory guides to affinity chromatography are available, for example, see C.R. Lowe [4].

The specificity of the binding properties of the solid-phase is achieved by covalently coupling a selective binding substance to the support matrix. The solid-phase selectively adsorbs the analyte to be isolated from the sample preparation. After unbound materials have been washed through the column, the extracted (or purified) analyte can be recovered by simply changing the pH or ionic strength of the mobile phase. A key factor in multiple column use is the ability of the covalently bound material to remain bound to the column and biologically active after release of the target. Affinity chromatography can be used to purify large molecules such as enzymes and antibodies, or to extract smaller compounds prior to detection. Cell separations can also be achieved by affinity chromatography techniques.

In designing affinity-based separation schemes, several steps are necessary: selection of a suitable support material, choice of an appropriate activation method, choice of ligand, selection of immobilization method and specification of adsorption or desorption conditions [4]. Usually solid supports are used but some soluble macromolecular materials are occasionally selected for two-phase aqueous affinity partition processes [5]. Affinity chromatography support matrices need to be chemically and biologically inert, easily activated, mechanically stable, and uniform in particle size. Many support materials have been developed for particular applications. A list of commonly used supports and attributes is given in Table 1 [4–6]. This is not an exhaustive list due to the numerous commercially available support matrices.

#### *2.1.1. Support materials*

Macroporous supports commonly used in affinity chromatography are usually subject to column compaction, leading to significant pressure drops. Inorganic support materials with covalently bonded affinity ligands have been in use for decades and the utilization of these materials is described in detail in [7]. The advantages of silica, controlled-pore glass (CPG) and other inorganic support materials include a wide range of particle shapes and sizes, insusceptibility to microbial degradation, and robustness with changing solvent conditions and pH. A major advantage of these types of support materials is their ability to withstand high pressure drops without compression. Researchers can take advantage of certain properties of support materials that make them more suitable for specific applications. For example, CPG has physical properties that make it an ideal solid support for flow injection analysis.

Small diameter inorganic support materials with a high mechanical strength enable high flow-rates through the column. This high liquid flow-rate accommodates a high sample volume per unit time and can improve column performance. However, as the diameter of the support material decreases, the problem of pressure drop through the column increases. Perfusive supports reduce this problem due

Table 1
Common support materials used in affinity chromatography

| Type of Support | Attributes |
|---|---|
| Cellulose | An absorbent that is widely used for antibody/enzyme purification which exhibits good flow-rates. Can be limited by fibrous, non-uniform character which impedes macromolecules. |
| Dextran | α-1,6-linked glucose polymer. |
| Cross-linked dextrans | Chemically stable, low porosity, useful for whole cell separations by affinity chromatography. Commercially available as Sephadex. |
| Agar Gels | Fractionation ranges at high molecular weight, good mechanical stability. |
| Agarose gels | Resilient to eluants with high concentrations of salt, urea, guanidine hydrochloride, detergents, or water-miscible organic solvents. Less stable at pH beyond a range of 4–9. |
| Cross-linked agarose | Improved thermal and chemical stability over agarose gels, robust over a pH range of 3–14 and at high temperatures; useable in chromatography up to 70°C, and with many common solvents. Commercially available as Sepharose. |
| Polyacrylamide gels | Stable to most eluants including dilute solutions of salts, detergents, urea and guanidine hydrochloride. Has many modifiable groups allowing for versatility in derivatization technology. Limited use due to low degree of bead porosity. |
| Polyacrylamide-agarose gels | Narrow size distribution of beads allows for better resolution than other chromatography gels; less compressible than conventional gel media permitting higher flow-rates. |
| Porous glass and ceramics | Controlled pore glass is insoluble and unaffected by changes in eluant pressure, flow-rates, pH, and ionic strength; resistant to microbial attack, readily sterilized. Can chemically modify surface. |
| Hydroxylalkylmethacrylate gels | Commercially available in different size exclusion limits as Spheron, similar to agarose and other polysaccharides; hydrophilic. |
| Silica | Stable under pressure and can easily be derivatized to introduce functional ligands. Pore sizes range from 5–400 nm in diameter. Commercially available as Nucleosil. |

to the presence of pores which penetrate the particles and permit convective flow through the particles along the column [8]. This method of perfusion chromatography has varied applications including antibody and enzyme purification, and for affinity-based screening of combinatorial libraries at high flow-rates [9]. In the last example, an anti-β-endorphin antibody, with a known peptide specificity was immobilized to a silica column. The affinity column was coupled to a $C_{18}$ column and automated via a workstation. This system proved effective in screening a soluble peptide combinatorial library, based on the relative affinities of the soluble peptide ligands for the solid-phase antibody.

Concern with environmental compounds that interact with the endocrine system has stirred an interest in analytical methods to measure compounds that may impact hormonal activity. A perfusion chromatography method was evaluated on serum testosterone with excellent precision [10]. The perfusion column with immobilized testosterone antisera was coupled to a flow injection heterogeneous fluorescence immunoassay procedure. The column had a high binding capacity over a range of flow-rates, and a lifetime in excess of 300 runs. The testosterone was incubated with a fluorescence-labeled testosterone analogue in a competitive assay format. The on-line incubation format using the perfusion matrix shortens assay time to about 3 min.

Perfusion chromatography matrices are now available that are very stable, robust, and adaptable, and allow higher sample throughput than CPG. The high stability of the porous structures used in perfusion chromatography allows the use of high temperatures, low pH, and high flow-rates, which can be used to separate molecules from complex samples prior to instrumental analysis. The technique has been coupled to reversed-phase high-performance liquid chromatography (HPLC) for the rapid and accurate detection of whey proteins [11]. The combined

method was used to separate and identify protein variants which differ by a single amino acid. This capability could aid in the accurate identification of protein products resulting from genetic engineering. For example, new plant protection strategies based on bioengineering use sensitive and specific protein analysis for environmental monitoring.

New applications of affinity chromatography include the separation of small molecules of environmental and human health concern in environmental and biological matrices. The development of these methods is not always straightforward and can present challenging difficulties. This is partly due to the fact that affinity chromatography support matrices had their beginnings in the separation and purification of macromolecules. The loose porous network of the insoluble support is designed to permit easy and uniform elution of macromolecules through the column with the smaller immobilized ligands providing selectivity. For analytes of environmental interest, this scenario is reversed. Macromolecular antibodies are commonly the immobilized components providing selectivity for the extraction of small molecules as they elute through the column. However, varied applications of immunoaffinity chromatography have been reported.

### *2.2. Immunoaffinity chromatography*

#### *2.2.1. Theory*

The reaction of a specific antibody with its target analyte (antigen) has many chromatographic applications. B lymphocytes with characteristic immunoglobulin surface markers can be isolated on an immunoaffinity column coupled with the appropriate anti-immunoglobulin [12]. Antibody purification can be accomplished when the solid support is coupled with the corresponding antigen. Alternatively, coupling a specific antibody to the solid support enables the extraction of analyte from a sample. Bacterial proteins A and G specifically react with many antibodies (e.g. IgG molecules of subclasses 1, 2, and 4) distal to the specific binding sites [12]. Proteins A and G are often used as a bridge between the solid support and the antibody to provide the correct orientation of the antibody binding site (Fab region), allowing the site enough freedom to react with the analyte as it elutes through the column. The biotin–avidin recognition system can also be employed as an immobilization technique [12]. When antibodies are used as the stationary phase, the process is often called immunoaffinity chromatography (IAC).

IAC has been adapted to many applications, such as antibody purification, separation of large molecules in complex matrices, and separation of small molecules of environmental concern. These noncovalent, reversible purification processes exploit the powerful and highly specific binding interactions between an analyte and a specific antibody. IAC can utilize columns, dialysis membranes, capillaries, or beads. Some characteristics of various IAC methods and their applications are given in Table 2. Although not all of the examples given are of primary environmental applications, Table 2 provides examples that show the breadth of IAC applications for large and small molecules.

Purification of the specific antibodies is the first step in preparing an IAC column. This can be accomplished by precipitation with ammonium sulfate, by ion-exchange chromatography, by gel filtration, or by affinity chromatography. The purified antibody can then be immobilized on activated beads that are typically coated with bacterial proteins A or G. Next, the immunoaffinity support is packed into a stainless steel column and the stability of the column is determined [28]. Elution of analyte from the immunoaffinity column can be performed by changing the ionic conditions of the mobile phase or by using chaotropic buffers. After elution of the target analyte from the column, the extract is usually clean enough for detection and quantitation. High resolution and selectivity are achieved by coupling IAC to HPLC or other chromatographic techniques. A schematic diagram of IAC used prior to HPLC separation and detection by atmospheric pressure chemical ionization (APCI)-mass spectrometry (MS) detection is given in Fig. 1.

Although IAC methods can be difficult to develop for small molecules, once established, they can be easily integrated into instrumental methods, such as HPLC and MS. IAC can provide efficient sample preparations with reduced use of organic solvents. These advantages must be weighed against the large volumes of purified antibody required, varied life

Table 2
Several applications of immunoaffinity chromatography coupled with various detection systems for the analysis of biological and environmental samples

| Target analyte | Matrix | Method details | Detection technique | Reference |
|---|---|---|---|---|
| Aflatoxin $M_1$ | Milk, Corn, Nuts | Aflatest-P affinity column; aflatoxin $M_1$ elutes with methanol. | HPLC–fluorescence | [13] |
| Aflatoxins | Maize | Biocode immunoaffinity column; aflatoxin $M_1$ elutes with methanol. | Fluorescence | [14] |
| Antifluorescein Antibodies | Buffer | Silica Sol is gelatinized in cuvettes; antiflourescein antibodies are combined with fluorescein in a cuvette. | Fluorescence | [15] |
| Atrazine | Water | Diol-bonded silica column; atrazine is desorbed with 0.05 *M* phosphate buffer (pH 2.5). | HPLC–UV | [16] |
| Atrazine | Soil | Silica based immunosorbent column containing anti-atrazine antibodies; atrazine elutes with acetonitrile and water containing ammonium acetate (0.01 *M*). | LC–MS | [17] |
| Carbendazim | Water | Diol-bonded silica column; the carbendazim is desorbed with 0.05 *M* phosphate buffer (pH 2.5). | HPLC–MS | [18] |
| Carbofuran | Surface Water, Potato Extract | Aldehyde-activated silica column; the IgG is desorbed with 0.2% formic acid. | HPLC–MS | [19] |
| Cortisol | Urine, Serum | Aldehyde-activated porous silica column; cortisol is desorbed with methanol–water (60:40). | HPLC–MS | [20] |
| Cytokines | Plant | CNBr-activated Sepharose 4B column; cytokinins elute with methanol. | HPLC–UV | [21] |
| Chloramphenicol (CAP) | Milk, Eggs | Coupling of monoclonal antibodies against CAP to a carbonyl-diimidazile (CDI)-activated support column; CAP is eluted with 0.2 *M* glycine, 0.5 *M* NaCl (pH 8.2). | HPLC UV–vis | [22] |
| Estrogen Steroids | Urine, Plasma | CNBr-activated Sepharose 4B column; the analytes are desorbed with methanol–water (95:5, v/v). | HPLC–UV | [23] |
| Methionyl Granulocyte Colony Stimulating Factor (GCSF) | Serum | CNBr-activated Sepharose 4B column with immobilized polyclonal anti-GCSF antibody; GCSF elutes using 100 m*M* glycine hydrochloride, pH 2.5 at 0.1 ml/min for 10 min. | HPLC–fluorescence | [24] |

expectancy of individual columns, and the unavailability of commercial immunoreagents for some applications.

### *2.2.2. Applications*

IAC has proven to be advantageous for natural food contaminants, such as aflatoxins [13,29],

Table 2. Continued

| Target analyte | Matrix | Method details | Detection technique | Reference |
|---|---|---|---|---|
| Transferrin | Serum | Protein G affinity column; transferin is desorbed with 0.10 *M* glycine, 2% acetic acid at 2 ml/min, a switch valve connects to the C-4 reversed-phase column. | UV | [25] |
| Trenbolone, 17α-Trenbolone (growth promoter) | Urine (Bovine) | IgG antibodies coupled to tresyl chloride-activated Sepharose column; trenbolone elutes with ethanol–water (40:60, v/v). The two epimers are confirmed and separated by TLC. | HPLC–UV | [26] |
| Zearalenone | Milk | CNBr-activated Sepharose 4B column; the analyte is desorbed with methanol. | ELISA | [27] |

fumonisins [30], and ochratoxins [31]. A solid support consisting of agarose beads coated with an antibody selective for fumonisins provided efficient extraction and cleanup. The fumonisins are bound to the immobilized antibodies, eluted with methanol, and derivatized for HPLC analysis. The IAC purification method compared favorably with a strong anion-exchange procedure. Other analytical methods

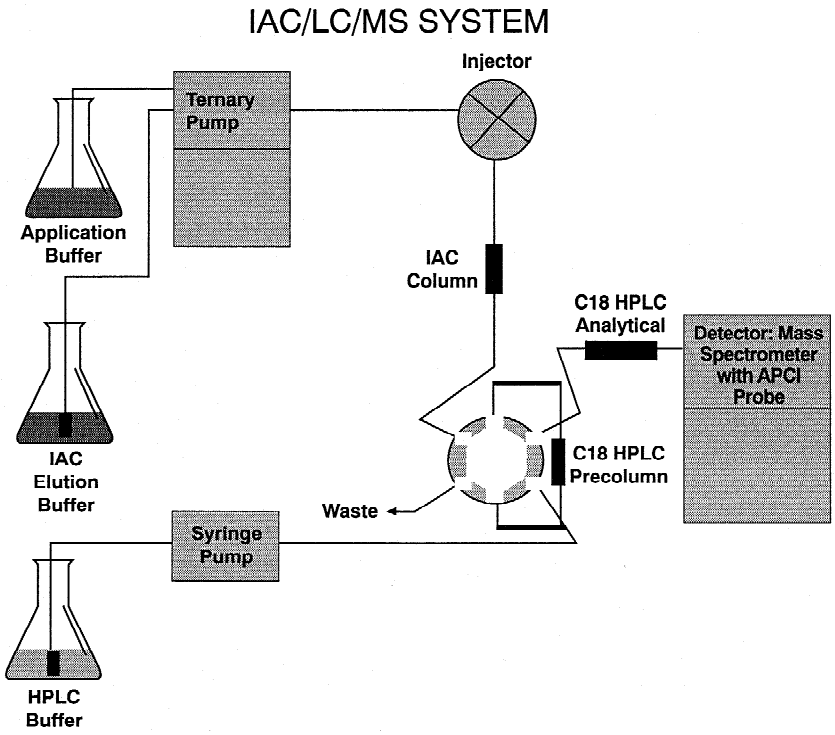


Fig. 1. Experimental setup for IAC–HPLC–MS. In repetitive analysis, samples are introduced through the IAC to the HPLC column, and detected by APCI-MS. (Reprinted with permission from Ref. [28], copyright 1996, American Chemical Society.)

for determination of these compounds (i.e., thin-layer, liquid, and gas chromatography) required extensive sample preparation, making the process slow and costly. Immunochemical methods proved capable of generating results without a complicated and expensive sample preparation. The success of these methods in food analysis has lead pesticide chemists to adapt these promising methods for their applications.

It is possible to use antibodies for extraction of pesticides from a wide range of environmental and food matrices, such as surface runoff water and potato extracts [19]. The analyte of interest in one study was carbofuran, a carbamate pesticide which is used to control insects and nematodes on agricultural crops. Traditionally, sample extraction for carbamates has been accomplished by the use of organic solvents. IAC was developed as a cost reduction and pollution prevention measure. The support used was an anti-carbofuran IgG coupled to an aldehyde-activated silica based column. The specific antibodies were bound to columns that can be used on-line prior to analysis by reversed-phase liquid chromatography–mass spectrometry (LC–MS). These IAC columns were linked to the LC–MS system by an automated column switching technique. This study shows the feasibility of on-line IAC columns for sample cleanup prior to LC–MS analysis.

When low ppb levels are important, high-performance immunoaffinity chromatography (HPIAC), HPLC, and detection by either UV-vis diode-array detector (DAD) or MS provides an on-line method for extraction, preconcentration, and analysis. Some characteristics of high-performance IAC are (1) smaller size of the support material (1–10 μm in diameter), (2) higher pressure, (3) shorter chromatographic cycle, and (4) higher sensitivity. HPIAC–HPLC–DAD and HPIAC–HPLC–MS methods were evaluated against an enzyme-linked immunosorbent assay (ELISA) for quantitation of the pesticide carbendazim [18]. The first column in the procedure is a nucleosil HPIAC column (10 mm×4.6 mm I.D., with 7.0 μm particle size) with immobilized carbendazim antibody. It is followed by a $C_{18}$ trapping column and, finally, the analytical column ($C_{18}$-bonded silica, 250 mm×2.1 mm I.D., particle size 5 μm). The linear range of the calibration curve for 200-μl injections of carbendazim is 0.025 to 100 μg/l for HPIAC–HPLC–MS and 0.075 to 100 μg/l for HPIAC–HPLC–DAD. One major advantage of the HPIAC–HPLC–MS system is that it responds to mass rather than to concentration. This allows the analyst to reanalyze by simply adding additional sample.

An earlier study was conducted to evaluate the use of tandem HPIAC with reversed-phase HPLC for the analysis of atrazine in water samples [16]. Atrazine, a triazine herbicide, is widely used in the U.S. and Europe to protect crops from weeds. The U.S. Safe Drinking Water Act requires that public water supplies be regularly monitored for atrazine, which has a maximum allowable level of 3 μg/l [32]. This study showed that the HPIAC/HPLC system can provide reliable quantitative results for atrazine and its degradation products without a separate sample derivatization step.

Other immunosorbents have been coupled on-line with LC–MS for the detection of pesticides [17]. An extraction procedure was developed and validated for triazine and phenylurea herbicides in sediments, groundwater, and sea water. Anti-atrazine and anti-chloroluron antibodies were immobilized onto a silica support and used as immunoaffinity ligands to extract the target analytes. The immunosorbents used allowed a multianalyte extraction due to the cross reactivity of the antibodies for related compounds.

A reusable, dialysis-based IAC system was designed to work with an LC system and fluorescence detection to analyze raw milk samples for aflatoxin $M_1$ [33]. The dialysis step is used to efficiently remove interfering compounds in milk samples, which normally require extensive cleanup. Though IAC columns are commercially available for aflatoxin $M_1$ in water, the enzymes present in milk samples cause degradation of the immobilized antibodies, rendering the system non-reusable. The desirability of IAC, and its ability to be connected to the LC system via an automated column-switching system, led researchers to consider a novel method of removing the interfering milk components. Two dialysis units were evaluated in this study: a flat membrane unit and a hollow-fiber unit. The flat membrane dialysis unit was a 15 000 Da molecular mass cut-off cellulose acetate membrane. The separated sample is quantified by fluorescence. Better reproducibility was achieved with the hollow-fiber

dialysis unit. Using this unit, more than 70 untreated milk samples can be analyzed with no decrease in performance. Full automation of the system is possible by using a peristaltic pump to flush the dialysis unit. The dialysis unit was inserted into a sample vial equipped with a magnetic stirrer. When not in use, the unit was stored in a sodium azide buffer solution in LC-grade water. A series of pumps and switching valves, controlled by computer, took the sample from the preconcentration step, through the dialysis step, then precolumn, separation column, and detection.

In response to the European ban on anabolic steroids for bovine growth promotion, an automated multi-immunoaffinity chromatography (MIAC) system was developed for the simultaneous extraction of several anabolic steroids in bile and urine [34]. The system has been used to analyze α-nortestosterone, zeranol, trenbolone, diethylstilbestrol, boldenone and dexamethasone. Samples are passed through a column containing multiple antibodies immobilized onto an inert gel support. The purified extracts can then be characterized by instrumental or immunochemical detection methods. In this study, six different antibodies could be effectively coupled to 1.5 ml of support gel. The column life expectancy was diminished to 20 analyses since the samples did not undergo a pretreatment prior to the immunoaffinity procedure. A robotic system transfers the column extracts to immunoassay plates for quantitation. The false positive rate for bile ranged between 3–5% and from 0.7–1.4% for urine. These results were considered quite satisfactory, especially since bile and urine can be difficult samples to analyze. Immunochemical methods and the incorporation of a robotic system allows a robust and reliable method for multianalyte screening of these regulated steroids.

Another related application of IAC separation is in the analysis of metabolites in body fluids such as urine and serum. Due to the low detection limits required and the structural complexity of these analytes, tandem immunochemical methods may be employed. Though ELISA detection methods are extremely sensitive, they do not provide in-depth information about the structure of the analyte. HPLC provides additional separation power but components carried over from the IAC column can be misidentified if HPLC is used for detection.

As can be seen in some of the preceding examples, an ideal analytical module would have three components: IAC column for removal of the bulk of the interfering matrix components, HPLC to resolve analyte isoforms, and a postcolumn immunochemical detection technique [24,35]. This type of system has application for environmental analyses, and demonstrates the feasibility of building modular analytical systems in which bioaffinity plays more than one key role. For example, a class selective antibody that responds to a wide range of structurally similar pesticides or metabolites could be used in the first step for the extraction of multiple compounds from a sample. The second step would be compound resolution using HPLC and final quantitation by immunoassay or in some cases MS.

By immobilizing antibodies to various chromatographic supports, practitioners have a flexible, highly selective separation technique that can be used for many compounds of environmental concern. The strength and flexibility of immunochemistry can be seen on several levels: multiple antibodies on a column, many columns in series, or the coupling of immunochemical procedures with other analytical methods.

### *2.3. Affinity capillary electrophoresis*

Capillary electrophoresis (CE) is a powerful separation technique based on the tendency of ions to migrate in an induced electric field. Whereas in (reversed-phase) HPLC, species are typically separated on the basis of hydrophobicity, CE exploits the charge-to-mass ratio of molecules [36]. In certain situations CE can be used to estimate affinities of monoclonal antibodies with fast reaction kinetics and to compare kinetics of antibodies with similar binding constants [37]. By measuring migration shifts at different antigen concentrations, it is possible to estimate dissociation constants.

Proteins, such as albumin, can be used as chiral selectors in affinity capillary electrophoresis (ACE). The technique has shown greater enantiomer separation than specialized HPLC columns [38]. ACE has also been used to study the binding of monovalent ligands to bivalent antibodies to determine dissociation constants [39]. The interaction of Ig antibodies and monovalent ligands of low molecular masses can be quantified using ACE to study binding

phenomena. The affinity of several *s*-triazines (hydroxyatrazine, ameline, atraton and ametryn) for dissolved humic substances was performed with ACE [40]. The information from similar studies may elucidate secondary aspects of pollution such as environmental fate, transport, and weathering.

#### *2.3.1. Immunoaffinity capillary electrophoresis*

By exploiting the sensitivity of CE and the strength of the reaction between an antibody and an antigen, researchers are able to develop hybrid capillary immunoaffinity CE (IACE) methods. It is often difficult to detect the small zones of analyte separated by capillary zone electrophoresis (CZE). Sample stacking and other preconcentration manipulations are usually nonselective and can lead to the concentration of interfering materials in the final analysis. Immunoaffinity preconcentration procedures have been developed to improve selectivity. An immunoaffinity stationary phase consisting of anti-insulin antibodies was used in tandem with CZE in both on-line and off-line modes [41]. The on-line mode enabled injection of the desorbed zones directly onto the electrophoresis system. Insulin samples (1 ml) were loaded onto the immunoaffinity column. Desorption volumes as low as 1 μl could be accommodated resulting in a 1000-fold preconcentration. This study indicated that the use of separate columns for preconcentration and electrophoresis was advantageous in protecting the column and maintaining separation efficiency.

An alternative to immunoaffinity chromatography preconcentration is the formation of solution-phase immunocomplexes prior to CE. In one study, fluorescently labeled tracer and antibody were added to serum samples for the determination of cortisol [42]. Cortisol in the sample and the labeled tracer compete for the antibody binding sites. After equilibrium is achieved, a small aliquot of the solution is subjected to CE and quantitated by fluorescence intensity. A high amount of cortisol in the sample yields a large signal for the free labeled tracer.

Though some clinical applications exist [43], IACE has not yet been evaluated for its use in environmental analysis. Another hybrid technique with potential application in this arena is the on-line coupling of CE with immunoassays [44]. The history of bioanalytical methods, beginning in clinical laboratories and being successfully adapted for environmental use, may portent a successful future for these new areas.

## 3. Bioanalytical detection methods

### *3.1. Immunoassays*

#### *3.1.1. Theory*

Immunoassays have revolutionized many field and laboratory analyses and are gaining acceptance in regulatory agencies in the Unites States and other countries [45]. The most common immunoassay format in environmental analysis is the ELISA. Various ELISA formats are available, including the indirect enzyme immunoassay, where the analyte and immobilized coating antigen compete for a limited amount of antibody. Quantitation is based on competition for antibody binding sites and is determined by an enzyme-labeled second antibody [3]. Heterogeneous assays require the separation of bound from unbound label and typically utilize a solid-phase, whereas homogeneous assays require no separation. Although simpler to perform, homogeneous assays are more hampered by matrix interferences. Quantitation by ELISA has become more accepted in the past decade and is now a laboratory standard for environmental analysis, frequently surpassing traditional methods in sensitivity, selectivity and cost [46]. In addition, ELISA can be optimized to provide quick results in field applications. Many field and laboratory studies have been conducted that compare ELISA results with those from traditional chromatographic procedures. Researchers in public and private practice continue to expand the role of immunoassays in environmental analysis and human exposure assessment.

#### *3.1.2. Applications*

One of the most widespread uses of environmental immunoassay has been in the field of pesticide analysis [2]. Immunoassay formats are available for screening and quantitative analysis. Some pesticides for which immunoassays have been developed include alachlor, aldrin, atrazine, benomyl, chlordane, chlorpyrifos, dieldrin, endrin, heptachlor, metolachlor, paraquat, parathion, terbutryn, and 2,4,5-T. An

Table 3
Immunochemical methods of environmental and human exposure significance

| Class | Method | LOD | Matrix | Reference |
|---|---|---|---|---|
| Atrazine | ELISA | 0.22 μg/l | Ground water | [47] |
| Cadmium (II) | ELISA | 7 ng/ml | Ambient water | [48] |
| Chlorpyrifos | ELISA | 0.10 ng/ml | Water | [49] |
| Irgarol 1051 (Antifouling agent) | ELISA | 0.020 μg/l | Seawater | [50] |
| *Listeria* spp. | ELISA | 1 to 10 cfu[a]/25 g | Food | [51] |
| Saxitoxin and Neosaxitoxin | ELISA | 80 μg/100 g | Shellfish | [52] |
| Pyrethroids | ELISA | 2.5 μg/l | Aqueous (buffer) | [53] |
| Pyrethroids | ELISA | 10 μg/kg | Fish | [54] |
| Metribuzin | ELISA | 0.04–3.0 μg/l | Stream water | [55] |
| Triazine | ELISA | 0.1 μg/l | Rainwater | [56] |
| Triazine | FIA[b] | 0.05–0.10 μg/l | Aqueous (buffer) | [57] |
| Triazine | ELISA | <0.1 μg/l | Water (spiked) | [58] |
| Triclopyr | ELISA | 0.1 ng/ml | Water | [59] |

[a] Colony forming units.
[b] Fluoroimmunoassay.

advantage of immunoassay is its adaptability to a wide range of analytes and matrices of environmental concern. In Table 3 some representative examples of compounds and matrices, for which immunoassays are available and suitable, are shown.

Many environmental applications involve the determination of trace concentrations of a compound or group of compounds in complex matrices. Human exposure studies, for example, focus on several aspects of exposure, including long-term exposure to ingested pesticide residues in food, trace pesticide levels in drinking water, or foliar dislodgeable residues (FDRs) on edible and non-edible plants. In one study, an indirect ELISA [60] was used to analyze chlorpyrifos residues on various agricultural products. A volume of 200 μl, each of antibody and test material was used. Standards and samples were placed on a microtiter plate in 100 μl volumes. The microtiter plates had been previously coated with chloropyrifos antigen at 50 ng per well. Antibody and antigen dilutions were optimized for ELISA by checkerboard titration. Standards were prepared from stock solution at several concentration levels. This incorporation of an ELISA into the established procedure of FDR analysis yielded results that agreed well with those from the standard HPLC method. Studies such as this one are defining the role of immunoassay technology in human exposure assessment studies.

Immunoassay tests can be particularly suitable for difficult matrices such as food. In a recent study, ELISA test kits for nine pesticides (alachlor, aldicarb, atrazine, carbaryl, carbendazim, carbofuran, cyanazine, 2,4-D, and metolachlor) were used to analyze food samples from the U.S. FDA Total Diet Study [61]. Baby foods containing potato, chicken, broccoli, carrots, cheese, noodles, and bananas were also tested. The samples were extracted using supercritical fluid extraction (SFE) and analyzed by ELISA. Though recovery rates were low for some of the compounds in various food matrices, it is believed that refinements in the SFE procedure will improve results. Multianalyte immunoassays would increase the efficiency of food analysis surveys.

A quantitative ELISA was used to determine the concentration of Aroclors 1242, 1248, 1254, and 1260 in soil and sediment samples from two U.S. EPA listed Superfund sites [62]. The polychlorinated biphenyls (PCBs) are classified as suspected carcinogens by the U.S. EPA and their manufacture and use was banned by the U.S. Congress in 1976. Their persistence in the environment and their tendency to bioaccumulate make them a continuing concern to scientists studying human and ecological health. The traditional method for PCB analysis with Soxhlet extraction followed by gas chromatography (GC) quantitation, is a costly and labor-intensive procedure. ELISAs were performed on samples following

a simple methanol shake extraction and on samples which, after the methanol shake, were extracted by Soxhlet or SFE. Results support that the extraction procedure is crucial to the total method performance. In this study, the overall results for the ELISA samples tracked, and were consistent with the chromatographic data when comparable sample preparations were used. This is an important consideration when evaluating new detection procedures.

Recognizing the importance of evaluation studies, the U.S. EPA has evaluated immunoassay test kits as part of the Superfund Innovative Technology Evaluation (SITE) program for the past several years. A competitive ELISA for benzene, toluene, xylene (BTX) compounds was evaluated in the field for potential use in the monitoring of underground storage tanks [63]. The immunoassay was shown to perform well for screening purposes (at the 200 ng/g level). By modifying the critical response level of the test, it could be adapted for use at the 25 ng/g decision level.

In a series of SITE demonstrations, field-portable immunoassay test kits were evaluated for screening of pentachlorophenol, a wood preservative, in water and soil matrices [64]. Results were compared with those from GC–MS. Of the four immunoassay procedures evaluated, two could be used for screening, one was useful for semiquantitative analysis, and the last, a 96-well microtiter plate assay, although semiquantitative, yielded no false positive results. The assay formats differed greatly and the inherent variability of the confirmatory method contributed to uncertainty in evaluation of the immunoassay results.

ELISAs are being applied to many monitoring situations. A magnetic-based ELISA test kit was developed for the herbicide metribuzin in stream water samples [55]. The ELISA kit performed well (achieving agreement with predicted values in the 99–100% range) for control spike samples in deionized water. The results against predicted values for stream water were in the 106–130% range, indicating some matrix interference. It is possible that some false positive bias can be attributed to dissolved organic compounds.

Until recently, immunoassays were not considered appropriate for the detection of inorganic compounds. As the technology matures and advances, the list of analytes and applications increases. A portable immunoassay has been developed for ionic cadmium, which is regulated by the U.S. EPA at 4 ppb in drinking water. The immunoassay uses a monoclonal antibody bound to a cadmium–ethylenediaminetetraacetic acid (EDTA) complex [48]. Although Hg (II) may cause false positives at concentrations greater than 1 $\mu M$, the assay compared favorably with atomic adsorption spectroscopy in its ability to quantify cadmium in spiked Louisiana bayou water samples. Another inorganic compound that can be detected by immunoassay is indium.

Sometimes ELISA is used as a standard method against which results from a new candidate method are measured. A recent evaluation of a flow injection immunoaffinity analysis (FIIAA) system compared results for triazine mixtures in river water to those obtained using ELISA [65]. Thus, the perception of ELISA has changed in the past decade from being considered an innovative environmental analytical alternative to being accepted as a reliable quantitative method. New refinements of immunoassay technology, such as microchip-based methods and multianalyte immunoassays, while still in research, will certainly take their place in the environmental monitoring scenarios of the future.

### *3.2. Flow injection methods*

Flow injection analysis (FIA) is a continuous format of ELISA which can be used for the rapid analysis of small molecules. Antibody-based flow injection provides a powerful analytical tool for semicontinuous operation or for high sample throughput scenarios. FIA provides an alternative or complementary technique to solid-phase immunoassay by providing real-time monitoring data [66].

Continuous-flow systems are easier to automate than assays using tubes, or microtiter plates and can provide rapid results and sensitive detection. Flow-injection enzyme immunoassays can be used with electrochemical, spectrophotometric or fluorometric detection methods [67]. Chemiluminescence has been used with FIA for the analysis of $\alpha$-fetoprotein [68] and human IgG [69]. Conventional UV-vis absorptiometry, a standard method in chromatographic analysis of ligands, is ideally suited to FIA for bioligand interactions [70].

The usefulness of flow-injection immunoaffinity

analysis has been demonstrated for diuron and atrazine in water [71]. The method was developed as a cost-effective screen for determining compliance with the European drinking water directive. Using a flow-through fluorimeter, detection in the desired concentration range of 0.1 μg/ml was achieved. With the prototype system, one analysis including column regeneration, took about 50 min. The column material was regenerated up to 1600 times over a two and a half month period. The flow-injection results compared favorably with the corresponding ELISA. Additional research is anticipated in developing a multi column device for environmental monitoring. This type of device is well suited for monitoring drinking water where the preponderance of samples are expected to fall well below the regulatory limits, and where trace contaminant levels can be crucial.

### *3.3. Immunosensors*

Biosensors are analytical systems comprising an immobilized biological sensing element and a physical transducer. When the biological component is an antibody, the biosensor is called an immunosensor. Physical transducers can be piezoelectric, electrochemical or optical. Though biosensors are used in clinical applications, their development for environmental analysis has been slow. Theoretically, immunosensors are capable of continuous and reversible detection but, because the antibody–antigen interactions have high affinity constants, reversibility is difficult to achieve in practice. Since cost and time are critical factors in environmental monitoring, this makes biosensors less desirable than alternatives that are already available, namely immunoassay test kits, unless on-line, real-time analysis makes biosensor development cost-effective.

There are several classes and subclasses of immunosensors, each with advantages for environmental analysis (Table 4). Piezoelectric sensors (including bulk acoustic and surface acoustic wave) use an external alternating electric field to directly measure properties of the antibody–antigen interaction. Electrochemical sensors (including potentiometric, amperometric, capacitative, and conductimetric), while still in the development stage for environmental applications, may offer inexpensive analytical alternatives for effluent monitoring. Optical sensors (including fiber optic and evanescent wave biosensors) measure the absorption or emission of a wavelength of light and base detection on fluorescence, absorbance, luminescence, or total internal reflectance fluorescence. Surface plasmon resonance (SPR) is an optical-electronic technique in which an evanescent electromagnetic field generated at the surface of a metal conductor is excited by light of a certain wavelength at a certain angle [81]. Atrazine detection has been achieved using SPR [82]. The rapid advancements in electronics, especially microprocessors used in signal processing, should help the science of sensors.

A grating coupler immunosensor for the measurement of four *s*-triazine herbicides was evaluated [75]. Analysts were able to detect terbutryn in a range of 15 to 60 n*M*. Sensitivity at the 15 n*M* level can be achieved by using a dynamic system response. The grating coupler waveguides are placed in a flow-through cell and connected to a constricted flow-path system. A peristaltic pump is used to split reagents through the flow cell. Refractive index change on the sensor surface due to antibody binding to analyte is measured by the incoupling angle. This label-free competitive immunosensor may be regenerated and recalibrated.

An innovative use of immunosensors is in the detection of whole cells and spores and other large biological particles. An automated system has been developed [83] that is capable of detecting levels low enough to be valuable for environmental applications. The system has three elements: incubation, capture-filtration, and detection by a light-addressable potentiometric sensor. During incubation the reagents (i.e., analyte, biotinylated antibody, fluorescein-labeled antibody and streptavidin) are mixed in solution. The two antibodies form a ‘sandwich’ immunocomplex with the analyte. The analyte must be large enough to bind two antibodies simultaneously. Streptavidin anchors the immunocomplex to the biotin-coated membrane. Next the immunocomplex is filtered through the microporous membrane which sequesters and detects only completely formed complexes. Finally, the membrane is pressed against a silicon chip where change in pH is monitored as a function of time and expressed as a voltaic rate. The signal generated is directly proportional to the

Table 4
Types of immunosensors for environmental monitoring

| Type of sensor | Analyte | Procedure | Reference |
|---|---|---|---|
| Potentiometric using field-effect transistor (FET) devices | 2,4-D | 2,4-D antibodies are immobilized on membranes; competitive enzyme immunoassay is used with 2,4-D conjugated to horseradish peroxidase (HRP); pH-sensitive FET measures a decrease in the signal when 2,4-D is present in samples. | [72] |
| Piezoelectric quartz crystals with resonant frequency of 10 MHz. | 2,4-D | 2,4-D is bound directly to silanized surface of crystals; crystals are incubated with anti-2,4-D antibodies and sample; competition for IgG binding sites results in frequency decrease which is indirectly proportional to concentration of free 2,4-D. | [73] |
| Fiber optic using evanescent florescence | Polychlorinated Biphenyls (PCBs) | Anti-PCB antibodies are immobilized onto quartz fibers; fluorescein bound to 2,4,5-trichlorophenoxybutyrate (TCPB) compete with PCBs in the sample for antibody binding sites. Bound PCBs lead to a decrease in florescence detection. | [74] |
| Fiber optic using a grating coupler of $SiO_2/TiO_2$ | Triazine | Triazine is immobilized with glutaraldehyde; *s*-triazines are detected by label-free competitive immunosensing; regeneration of transducer is achieved by incubation with proteinase. | [75] |
| Fiber optic using reflectometric interference spectroscopy (RIFS) | Atrazine | Atrazine-caproic acid is coupled to the glass chips as part of the interfacing layer of the RIFS. Antibody and analyte are introduced in the flow-cell transducer by FIA. Detection is by diode array simultaneous spectrometer. Regeneration of the transducer is achieved by using pepsin. | [76] |
| Fiber optic using evanescent fluorescence | Cyclodiene Insecticides | Anti-chlorendic caproic acid (CCA) is immobilized to quartz fibers. Florescent labeled CCA and analyte compete for antibody sites on the fibers. As little as 0.1 n*M* chlordane is detectable within 1 min. Studies use single-use fibers. | [77] |
| Fiber optic using evanescent florescence | Imazethapyr Herbicide | Anti-imidazolinone antibody is immobilized on the fibers. Fluorescein-labeled imazethapyr analog and free-imazethapyr compete for the antibody sites on the fiber. Regeneration of quartz fibers is achieved by washing with phosphate buffered saline (PBS). | [78] |
| Electrochemical using conducting electroactive polymers (CEP) | PCBs | Anti-PCB antibodies are immobilized onto CEP sensing electrode. The sensing electrode is coupled with periodic pulsed waveforms in the presence of PCBs. Induced changes in the CEP allows detectable interactions with PCBs in a reversible manner. | [79] |
| Automated flow-through system using controlled-pore glass (CPG) | Carbaryl Pesticides | The anti-carbaryl antibody is immobilized on the silanized CPG. A synthetic hapten of carbaryl is coupled to HRP. This enzyme tracer and sample carbaryl compete for antibody sites on the CPG. $H_2O_2$ mixed with a fluorogenic substrate is used for detection of a fluorescent signal. Desorption with a glycine/HCl mixture and washing with PBS regenerates immunosensor. | [80] |

quantity of immunocomplex (and analyte) that was formed and captured.

### *3.4. Molecularly imprinted polymers*

Molecular imprinting is a nonbiological method for creating binding sites for target molecules. Since, in some ways, the binding mechanism of molecularly imprinted polymers (MIPs) resembles the biological response between an antibody and an antigen, MIPs can be thought of as synthetic antibodies. Molecularly imprinting involves the arrangement of polymerizable functional monomers around a template. A template is covalently bound to the monomer during molecular imprinting and, after cleavage of the template, rebinding takes place by noncovalent interactions [84]. Efficient molecular recognition can be achieved by introducing functional groups associated with the imprinted binding sites. Specific adsorbents can be prepared that are able to selectively rebind the compound used as a template [85]. MIPs are quite versatile and considerable recent research has been directed toward their expanded use.

In a simple preparation, the functional monomer and the template are allowed to self assemble by covalent interactions and the resulting assemblies are then copolymerized with the crosslinking monomer. The binding sites are then generated by a simple wash procedure. The rebinding to these sites resembles the recognition events in biological systems. These highly stable synthetic polymers may offer an improvement over antibodies, which may be too unstable for environmental applications [86]. MIPs are especially suitable for immunosensor applications. The technology has already been applied in an indirect electrochemical sensor format for morphine [87].

Molecular imprinting is currently being assessed for separations that require strong and selective binding of small molecules [88]. The best studied use of MIPs is as stationary phases for affinity HPLC. The use of MIPs as selective matrices for affinity CE was also recently demonstrated [89]. Recent breakthroughs in MIPs have resulted in the development of enantioselective imprinted CE columns used in chiral separations of drugs [90,91]. This capability would also be useful to resolve pesticide enantiomers.

As MIP technology matures, it may be possible to improve polymer binding capacity and selectivity, making MIPs a possible alternative to the current biological recognition elements in biosensors or the antibodies used in ELISA.

## 4. Conclusion

The synergistic effect achieved when one facet of bioanalytical technology increases the sensitivity or applicability of another is a reflection of a broader partnership: that of the biological and chemical sciences. As scientists continue to explore the complex interactions at the molecular and cellular level, the potential for wider applications of biological reagents comes closer to realization. The immunoselectivity of biorecognition principles will undoubtedly be further explored and result in new methods for identifying and quantifying trace analytes in complex matrices.

## 5. Abbreviations

| | |
|---|---|
| 2,4,5-T | 2,4,5-trichlorophenoxyacetic acid |
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| ACE | Affinity capillary electrophoresis |
| APCI | Atmospheric pressure chemical ionization |
| BTX | Benzene, toluene, xylene |
| CE | Capillary electrophoresis |
| CPG | Control pore glass |
| CZE | Capillary zone electrophoresis |
| DAD | Diode array detector |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| EPA | U.S. Environmental Protection Agency |
| FDA | U.S. Food and Drug Administration |
| FDRs | Foliar dislodgeable residues |
| FIA | Flow injection analysis |
| FIIAA | Flow injection immunoaffinity analysis |
| GC | Gas chromatography |
| HPIAC | High-performance immunoaffinity chromatography |
| HPLC | High-performance liquid chromatography |
| IAC | Immunoaffinity chromatography |
| IACE | Immunoaffinity capillary electrophoresis |
| LC–MS | Liquid chromatography–mass spec- |

| | |
|---|---|
| | trometry |
| LIFD | Laser-induced fluorescence detection |
| LOD | Level of detection |
| MECC | Micellar electrokinetic capillary chromatography |
| MIAC | Multi-immunoaffinity chromatography |
| MIPs | Molecular imprinted polymers |
| MS | Mass spectrometry |
| ORD | Office of Research and Development |
| PCBs | Polychlorinated biphenyls |
| SFE | Supercritical fluid extraction |
| SITE | Superfund Innovative Technology Evaluation |
| SPR | Surface plasmon resonance |
| TLC | Thin layer chromatography |
| USDA | U.S. Department of Agriculture |
| USGS | U.S. Geological Survey |
| UV-vis | Ultraviolet-visible |

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