Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1000 (2003) 29–52
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Review

# Immuno-based sample preparation for trace analysis

Marie-Claire Hennion*, Valérie Pichon

*Laboratoire Environnement et Chimie Analytique (UMR CNRS 7121), Ecole Supérieure de Physique et de Chimie Industrielles, 10 rue Vauquelin, 75231 Paris Cedex 05, France*

**Abstract**

Immuno-based sample preparation techniques are based upon molecular recognition. Thanks to the high affinity and high selectivity of the antigen–antibody interaction, they have been shown to be a unique tool in the sampling area. Immuno-based sample preparation methods include the widely encountered immunoaffinity extraction sorbents, so-called immunosorbents, as well as membrane-based or ultrafiltration techniques. This review describes the new developments and applications that have occurred in recent years with emphasis on (i) the antigen–antibody interactions, (ii) and their importance for the properties and use of immunosorbents, (iii) multiresidue extractions, (iv) the on-line coupling to chromatographic or electrophoretic separations, and (v) the high potential for improving MS detection. The recent use of artificial antibodies for sample pretreatment, so-called molecularly imprinted polymers, is also described.


*Keywords:* Reviews; Immunoaffinity extraction; Solid-phase extraction; Sample preparation; Molecular imprinting; Immunoextraction; Immunofiltration

## Contents

1. Introduction ..... 30
2. Characteristics of the immuno-based extraction sorbents ..... 30
   2.1. Antibodies ..... 31
   2.2. Effect of the solid support and antibody immobilization procedures ..... 32
   2.3. Capacity and bonding density ..... 33
   2.4. Specificity and cross-reactivity ..... 34
   2.5. Recovery and breakthrough volume ..... 35
   2.6. Polyclonal versus monoclonal antibodies ..... 36
   2.7. Sample percolation and non-specific interactions ..... 36
   2.8. Elution conditions ..... 37
   2.9. Storage and reusability ..... 37
   2.10. On-line coupling to separation techniques ..... 38
      2.10.1. Liquid chromatography ..... 38
      2.10.2. Gas chromatography ..... 39
      2.10.3. Capillary electrophoresis ..... 39
3. Immunologic trapping and immunofiltration ..... 40

*Corresponding author. Tel.: +33-1-4079-4651; fax: +33-1-4079-4776.
*E-mail address:* marie-claire.hennion@espci.fr (M.-C. Hennion).

0021-9673/03/$ – see front matter 
doi:10.1016/S0021-9673(03)00529-6

4. Application to real samples: off-line and on-line coupling to various separation and/or detection techniques ..... 40
4.1. Liquid chromatography ..... 40
4.1.1. LC–UV or fluorescence ..... 40
4.1.2. LC–MS ..... 41
4.1.3. Selected applications for trace analysis of pesticides, organic pollutants, toxins and drugs ..... 42
4.2. Gas chromatography ..... 42
4.3. Mass spectrometry ..... 43
4.4. Bioanalytical assays (immunoassays, bioassays) ..... 45
5. Molecularly imprinted polymers (MIPs) ..... 47
5.1. Synthesis and consequences for the extraction medium ..... 47
5.2. Examples of selective extraction procedures in aqueous samples ..... 49
6. Conclusion and future developments ..... 50
References ..... 50

## 1. Introduction

For the last two decades, intensive research in the area of solid-phase extraction (SPE) has led to the development of novel materials aimed at the selective extraction of analytes from various matrices [1]. Lack of selectivity of the most commonly used SPE sorbents has been widely reported. Co-extraction of analytes and matrix interferences generally occur, and this can become a major problem when analytes of interest are at trace levels and interferences at higher concentrations. Additional clean-up procedures are required, but, then the sample pretreatment involves several steps and consequently the risk of loss or contamination increases and the reliability of the results is reduced. Moreover, today, trends are for more rapid sample preparation methods, which can be coupled on-line to the separation technique. There is a real need for one-step sample pretreatment procedures with capability of automation, which cannot be obtained using non-selective sorbents when sample matrices are complex.

Immunoaffinity extraction (IAE) sorbents, also called immunosorbents (ISs), use biological tools, such as antibodies, and are based upon molecular recognition. Because of the high affinity and high selectivity of the antigen–antibody interaction, they allow a high degree of molecular selectivity. IAE provides unique and powerful techniques, which enables selective extraction and the concentration of individual compounds or classes of compounds from liquid matrices in one step or the sample purification of extracts from solid matrices.

First ISs were described in the biological field because of the availability of antibodies, which can be very selective for large molecules and easily obtained. Many examples have been described for the immunoextraction of antibodies, enzymes, proteins, viruses, hormones, peptides and other subcellular components. Obtaining selective antibodies for small molecules was more difficult and the development of immunochemical methods targeting low-molecular-mass analytes is more recent. However, immuno-based sample preparation methods are becoming increasingly popular. Several reviews have been published in recent years and reporting the basic features of immunoaffinity extraction as sample preparation [2–10]. Especially, it was seen that too much selectivity, i.e. an individual IS for each compound, was not the best procedure and that for many applications, it was better to select antibodies capable of recognizing a whole class of related analytes and/or metabolites. The present review reports on the new developments and applications that have occurred in recent years with emphasis on (i) the antigen–antibody interactions, (ii) and their importance for the properties and use of ISs, (iii) multiresidue extractions, (iv) the on-line coupling to chromatographic or electrophoretic separations, and (v) the high potential for improving MS detection. The last part of this review reports the recent attempts of using new polymers with molecular imprints because their affinity can be comparable to those of natural antibodies.

## 2. Characteristics of the immuno-based extraction sorbents

The immunosorbents are obtained by linking the

antibodies to a solid support, which is then packed in SPE cartridge or precolumn. A typical SPE sequence using an immunosorbent is very similar to that using a conventional $C_{18}$ cartridge. One difference is that the IS should be stored in a wet media, usually PBS (phosphate-buffered saline) solution. Moreover, when synthesizing an IS and developing an immunoaffinity extraction procedure, several parameters have to be carefully controlled and optimized to obtaining a robust SPE method. The affinity between antibodies (Abs) and analytes are strongly dependent on the nature of the antibodies. Close relations exist between extraction recoveries and the affinity and the amounts of the antibodies. In addition, analyte–antibody interaction can be affected by matrix samples and by additives in the samples, and this can also lower extraction recoveries.

### 2.1. Antibodies

The design of the antibodies is the key parameter that defines the potential of the immunosorbent. Both polyclonal (PAbs) and monoclonal (MAbs) antibodies have been selected for immobilization with an increase in the use of MAbs in recent years. Although MAb production is more costly, it guarantees a long-term availability of reproducible antibodies that does not require animals for further large-scale production. In addition, an increasing variety of antibodies are now commercially available. The advantage of bonding antibody fragments (FAb) for increasing the density of the recognition sites has been studied but is not widely encountered in laboratory-made ISs [11]. FAb fragments are easily obtained from PAbs or MAbs using commercial kits. Such fragments have been used for preparing immunoaffinity sorbents for on-line coupling to capillary electrophoresis [12].

Because compounds of low molecular mass are unable to evoke an immune response, before immunization they have to be modified by bonding to a larger carrier molecule, usually a protein such as bovine serum albumin (BSA). Very often it is necessary to introduce a functional group into the selected molecule in order to make possible this coupling. The design of the so-called hapten is still an important variable when trying to obtain antibodies with the required specificity, especially when antibodies are targeted to recognizing a whole class of structurally related compounds. The functional group that is introduced in the antigen molecule should keep as much as possible the identity of the analyte, but the selection is often based on trial-and-error assays, once the antibodies have been obtained. For example, it was experimentally shown that antibodies produced with a hapten based on isoproturon are less class-specific for the phenylurea group than antibodies obtained with a hapten based on chlortoluron [13]. It should be less costly and more attractive if it was possible to be able to determine a priori the preponderant parameters influencing the antigen–antibody recognition. Molecular modeling enables the molar volume and the charge distribution within a molecule to be determined. These two features are important and a few studies have pointed out some relationship between the structural properties of the target compounds and that of the hapten [14,15]. This approach has been used to characterize four haptens and, then, one has been selected to develop anti-trichlorophenols antibodies [14]. With groups like the trichlorophenols which contains only a few related simple molecules, only the charges of the carbon atoms within the aromatic ring were taken into consideration. However, when molecules are more complex, such as triazines or phenylureas, the problem is more difficult because the number of electronic parameters quickly increases and the steric parameters to be considered which can represent the molecule in the hapten molecule can vary significantly. An approach has been proposed and evaluated to obtain an indication of the specificity of antibodies produced with a given compound for a group of structurally related compounds [16]. Recoveries of extraction have been measured using three ISs that were prepared using MAbs obtained from different haptens after chemical modification of dichloroatrazine, atrazine and ametryn molecules. In parallel, data from molecular modeling were examined by principal component analysis. This resulted in distribution maps where the relative position of each hapten based on either dichloroatrazine or atrazine or ametryn, to several various of the triazine group gave information about the future specificity of the antibodies produced using this hapten. In the three cases, the conclusions resulting

from the analysis of the maps fitted well with the experimental results.

Another important feature when making antibodies designed to recognize organic pollutants or drugs is the toxicity of the analytes. Generating antibodies against the very toxic amanitins for the detection an early diagnosis of an intoxication with amanita mushrooms was not easy because the conjugation of amanatoxins to proteins made them more poisonous that the native toxins, enhancing their uptake into protein-consuming cells [17].

### *2.2. Effect of the solid support and antibody immobilization procedures*

The solid support selected for the immobilization of the antibodies is also a critical parameter for the design of the ISs. In addition to basic properties such as chemical and biochemical inertness, good mechanical stability and uniformity in particle size, the solid support should be easily activated to allow antibody attachment, have large pore size because antibodies are large molecules, and should be hydrophilic in order to avoid any non-specific interactions. Finally, the immobilization procedure should retain the biospecific activity of antibodies.

The most common approach consists of a covalent bonding of the antibodies, which is often achieved by reacting free amino groups of the antibody with a support that contains reactive epoxide or aldehyde groups surface or groups that can be activated using carbonyldiimidazole, cyanogen bromide or *N*-hydrosuccinimide. Traditional supports used in immunoaffinity chromatography, include silica, agarose, cellulose and synthetic polymers. Agarose- and silica-based supports are the most common sorbents selected for both commercial and laboratory applications. Agarose-based ISs are mainly designed for off-line applications. However, because they are not pressure-resistant and cannot be operated at high flow-rates, there are an increasing number of silica-based ISs, especially for on-line direct coupling to chromatographic separation techniques.

Several studies have attempted to optimize the antibody immobilization through oriented bonding procedures. The coupling of antibodies to activated agarose-based and silica-based supports is considered random immobilization because it involves covalent coupling via lysine $\varepsilon$-amino groups, which are encountered throughout antibody molecules, allowing thus several different antibody orientations. In contrast, some hydrazide activated supports involves covalent bonding via carbohydrate moieties and are thought to provide greater column binding capacity because immobilization occurs away from the antigen bonding sites and results in a more oriented reaction. Dihydrazide-activated silica can be synthesized and has been used for oriented bonding, but is not commercially available [11]. Prince et al. [18] compared aminopropyl glass beads and ultralinked immobilized hydrazide (Aziactone beads). Despite the theoretical advantage of the oriented immobilization on the latter beads, anti-diazinon ISs prepared using silica beads were found to be more suitable due to greater binding capacity and flow-rate. A recent study also compared the properties of antibody-coated polystyrene particles, -silica particles and agarose beads for the immunopurification of blood serum extracts for the determination of glycoalkaloids [19]. Although paramagnetic polystyrene particles were easier to use, ISs prepared from both polystyrene-based supports appeared to exhibit non-specific affinity for glycoalkaloids and this was shown by bonding non-specific IgG instead of glycoalkaloid antibodies. Another way to contribute to a better orientation and to increase the binding surface area of immobilized antibodies is to employ antibody fragments, because these fragments will increase the number of integral binding sites without causing steric hindrance [12].

The sol–gel method has been recently used successfully for entrapping antibodies against PAHs [21], 1-nitropyrene [22], *s*-triazines [23] and TNT [24]. It consists in immobilizing antibodies in the pores of a hydrophilic glass matrix. Activation and bonding are no longer necessary and the bonding is carried out in milder conditions, so that one can expect the antibodies to retain their affinity and specificity. The entrapment is a simple and rapid two-step procedure in which hydrolysis is followed by polymerization of tetramethoxysilane after addition of the antibody solution. A good sol–gel format for anti-TNT antibody entrapment was a wet gel prepared by a two step procedure at a silane–water ratio of 1:8 containing 10% PEG [24]. Stakilas et al. [23] optimized the entrapment of anti-atrazine MAbs

with gels containing 6% glycerol. Theoretically, leaching of antibodies can be a problem in sol–gel techniques because of the high porosity of the matrix and the fact that Abs are not covalently bound to it. In the aforementioned studies, no or negligible leaching was observed.

Non-covalent binding can also be used. Streptavidin supports and protein A- or G-based sorbents can interact with these proteins. Especially, the protein G- and the related protein A-supports bind antibody at the Fc region, which orients the antibody with the antigen binding sites away from the surface and towards the solution. These types of IS were successfully employed to characterized combinatorial chemical libraries of small drug compounds, such as benzodiazepines [25]. The binding is quite strong under physiological conditions, but can be easily disrupted by decreasing the pH of the surrounding solution. However, anti-sarafloxacin MAbs were adsorbed to the protein G surface and then covalently cross-linked in place [26]. Since the IS was incorporated in an automatic analytical system, its reusability and durability were important aspects and the home-made ISs have retained consistent analyte binding capabilities even after being used for hundreds of samples over a period of up to 1 year, without any special treatment other that storage at 4 °C in PBS containing 0.02% sodium azide.

An immunoaffinity solid-phase microextraction (SPME) fiber was developed [27]. A theophylline antiserum was covalently immobilized on the surface of a fused-silica fiber, modified with 3-aminopropyltriethoxylane and glutaraldehyde, and used as a selective and sensitive extraction medium for an immunoaffinity SPME determination.

### 2.3. Capacity and bonding density

The bonding density is defined by the number of antibodies linked to the surface of the sorbent and usually expressed in mg/ml of sorbent bed or mg/g of sorbent. It is an important parameter because it determines the future antigen-binding capacity and can be measured experimentally. The determination of the amount of antibody immobilized onto a matrix is usually performed by measuring the concentration of the antibodies in the binding solution by spectrophotometry UV. The bonding density depends on the specific surface area of the solid support accessible for the immobilization of antibodies. Support with small pore sizes have a high surface area, but low accessibility for the large antibody molecules, but on the other hand supports with large pore sizes have good accessibility but small surface area. So a compromise is necessary and previous studies have shown that 30–50 nm pores (the diameter of antibodies is 8–10 nm) were best suited for immunoextraction. The amount of immobilized IgG (bonding density) was extensively studied as a function of the total amount of added Abs expressed in term of the effective number of monolayers that the Abs concentration would have produced if all had become attached to the support [11]. Using an IgG excess of less that one effective monolayer produced a sharp increase in the binding density. Above and up to three layers, the bonding density increased slightly and reached a plateau.

A second important parameter is the degree of purification of the antibody solution. Several studies have used non-purified antisera for bonding, but one can expect a reduced bonding density due to the co-immobilization of other proteins. Recent trends are to use purified PAbs or MAbs. PAb purification is usually performed using a protein A or G affinity column. Further purification of the IgG fraction to isolate the specific antibodies is very difficult because once the Abs linked to a specific analyte, desorption is very almost impossible without organic solvents which destroy free PAbs. Therefore, in most cases, the term ‘‘purified PAbs’’ means purified IgG fractions. In our last review, we listed the available data of bonding density [2]. When given, average values were about 5 mg/ml for agarose gel and 20 mg/g of silica and were not really dependent whether MAbs or PAbs were used

The capacity of an immunosorbent corresponds to the total number of accessible specific immobilized antibodies. This capacity cannot be calculated directly because with polyclonal or monoclonal antibodies, random orientation and steric hindrance might prevent the access of the analyte to the specific complementarity-determining regions of the antibodies. In addition with PAbs the concentration of active antibodies is not known. In a recent study, two ISs were bonded with either anti-isoproturon purified

PAbs or MAbs at a same experimental bonding density of 3.5 mg antibodies per 100 mg silica, pore size 30 nm [28]. The capacity value was determined on the plateau of the capacity curve and results suggested that approximately 50% of the purified PAbs were specific to the antigen.

Because both monoclonal antibodies and the activated solid support used for immobilization are expensive, it is worthwhile to optimize the bonding density. Increasing the density of MAbs from 0.5 to 1 mg per 100 mg silica resulted in a linear increase in capacity, but when the density was 3.5 mg per 100 mg silica, the capacity was lower than expected probably because of steric hindrance between antibodies [28]. Silica and Sepharose were also compared when bonded at densities of 1 mg of anti-isoproturon MAbs per 100 mg silica or per 0.5 ml gel. The experimental capacities were, respectively, $1.35 \pm 0.1$ and $1.34 \pm 0.1$ μg, demonstrating similar MAb accessibility although these values represented around 50% of the theoretical capacity. The reproducibility of the bonding procedure was tested and obtained by making several ISs in two different laboratories.

The capacity value certainly depends on the antibody characteristics. However, the capacities of three ISs obtained using three different MAbs were equal using a well-controlled procedure [16,28]. Watanabe et al. [29] described a new IS with a similar capacity of around 2 μg with a bonding density of 1.8 mg anti-imazalil MAbs per ml of agarose-based gel. However, when using PAbs, the capacity can be lower, depending on the concentration of active antibodies [30]. Therefore, the comparison of capacity from one IS to anther one is difficult, especially when using PAbs, even if the binding is achieved in the same laboratory using similar bonding procedures and the same support [31]. However, the most important feature is that capacities are always in the range of the hundreds of ng to some μg for 1 g of sorbent. Consequently, much care should be given to this value and one has to keep in mind that ISs are only devoted to trace analysis. Moreover, it is very important to not overload the capacity in quantitative analysis because the linear range of the capacity curve corresponds to the linear part of the quantification range.

### *2.4. Specificity and cross-reactivity*

Selective extraction is the primary objectives of using antibodies. The binding of analytes to antibodies is the result of a good spatial complementarity which is a function of the sum of the intermolecular interactions. Therefore, an antibody can also bind one or more analytes with a structure similar to the analyte that has induced the immune response, and this is the so-called cross reactivity of antibodies. It is usually considered as negative feature for an immunoassay, but it is exploited in extraction, because immunoaffinity extraction is followed by a separation allowing individual quantification of each trapped analyte. In the biological field, it is often interesting to determine a group of related drugs and their metabolites. In the environmental field priority lists contain chemical classes of pollutants and not only individual analytes. Commercial ISs have been introduced during the last decade for the clean-up of samples for the analysis of natural food contaminants, such as aflatoxins, ochratoxins and fumonisins, veterinary drugs, such as clenbuterol and analogs and drug of abuse such as LSD and its metabolites. In the environmental field, recent applications have optimized several class-selective ISs for trapping groups of pesticides and priority industrial organic pollutants [2,4,20,31–36].

Enantioselective immunoextraction has also been developed, which employ an immobilized antibody to specifically isolate peptide fragments that have been modified with optically active ibuprofen with the objective of the structural analysis of drug–protein adducts [37].

Antibodies often do not have the same affinity towards different members of the group. An easy experimental estimation of the affinity order may consist in recovery measurements. The stronger is the affinity of the antibodies for an analyte, the higher is the extraction recovery for a given sample volume. So, the measure of the extraction recoveries gives information about the specificity of the immobilized antibodies. However, when 100% recoveries are obtained for several analytes, it not possible to distinguish them. Discrimination can be determined by measuring recoveries, while increasing the sample volume and decreasing the analyte

concentration in order to avoid capacity overloading [2]. The study of the elution conditions also constitutes a good experimental method to evaluate the affinity: the stronger is the affinity, the strongest are the conditions for elution of analytes from the IS. Another experimental test consists of first percolating analytes dissolved in a small volume of water and then, eluting them with small successive fractions containing an increasing percentage of organic solvent [16]. In this case, the stronger the affinity of the antibodies for an analyte, the higher the percentage of organic solvent required to elute was (Fig. 1). Each analyte was studied separately in order to avoid competition between the analytes for the binding site. The analytes are listed in their affinity order. The affinity is good for the nine triazines since they are not eluted with less than 30% methanol whereas dealkylated and hydroxylated metabolites are recovered in the effluent and in the first 10% methanol fraction.

### 2.5. Recovery and breakthrough volume

In addition to the selectivity of the immunoextraction, an important parameter is the extraction recovery, which is linked to the breakthrough volume and the capacity. In order to achieve low detection limits the IS must allow collection from large sample volumes without breakthrough of the analyte. This parameter is not important for serum or plasma samples, but become more relevant with the handling of urine samples, usually in the range 5–25 ml and of environmental samples which can be up to several hundreds of ml when determination is required at the ng/l level using conventional LC with UV detection. Breakthrough of analytes can occur due to the overloading of the capacity or due to an insufficient retention. In the second case, the recovery decreases when the sample volume increases.

The breakthrough volume depends primarily on the affinity between the analytes and the antibodies. An anti-diazinon IS was prepared with a binding density around 45 mg PAbs per g of silica-based IS (capacity not known but >1 μg). Using 1 ml of IS in a cartridge, no loss in recovery was observed up to a sample volume of 500 ml [18]. Using a precolumn containing 250 mg of an anti-propanil IS (bonding density: 4.5 mg PAbs; capacity: 0.6 μg, no breakthrough was observed up to a sample volume of 200 ml [30].

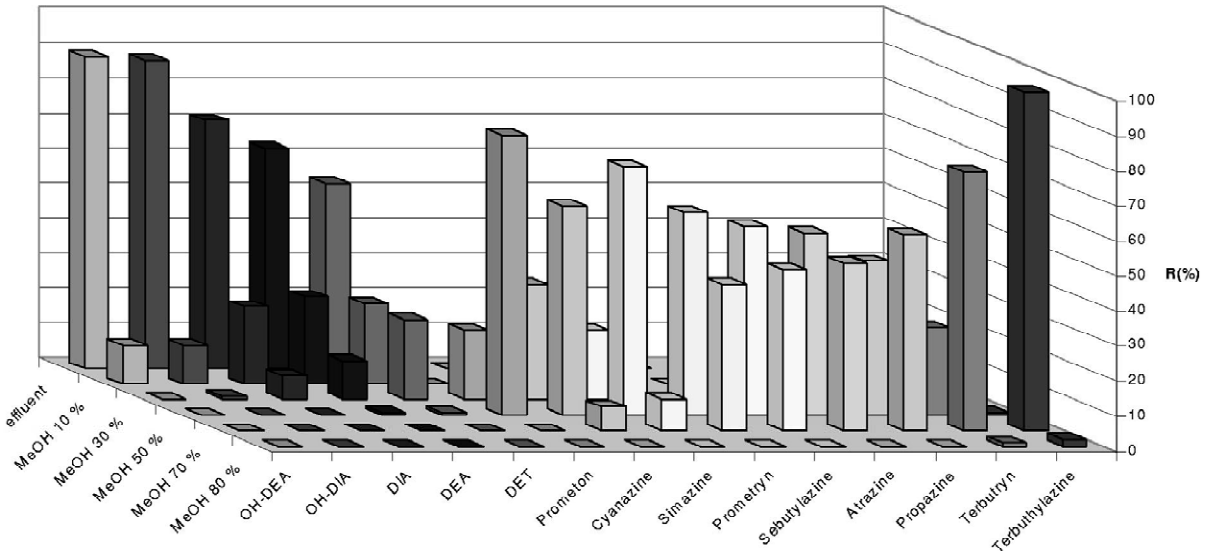


Fig. 1. Step elution of triazines from an IS (100 mg) obtained by bonding anti-ametryn MAbs, (capacity: 1.8 μg) after percolation of 800 ng of each component in 3 ml of ultrapure water and elution with successive fractions of 0.5 ml containing an increasing percentage of methanol [16].

The breakthrough volume depends on the amount of antibodies immobilized on the IS, as demonstrated using well-controlled ISs based on anti-isoproturon MAbs [28].

### 2.6. Polyclonal versus monoclonal antibodies

Reproducible ISs can only be obtained using MAbs. A general consensus exists in favor of a larger cross reactivity for PAbs due to their heterogeneity. In the literature, many class-selective ISs have been prepared using PAbs, although several ISs prepared using MAbs were also shown to be class-selective. Two ISs made using the same bonding procedure, the same silica and anti-isoproturon MAbs or PAbs and having the same capacity (≈4.2 μg) were compared for their cross reactivity [28]. Their capability to recognize several phenylureas was almost similar. That the cross-reactivity of MAbs is almost as wide as that for PAbs can be explained by the small size of the hapten molecule. For larger molecules characterized by several specific recognition sites, PAbs can be heterogeneous mixture of several antibodies, each of which recognizes a specific part of the molecule. In the development of MAbs, one antibody of the polyclonal mixture is selected. When the targeted molecule is small, the polyclonal mixture cannot contain large number of different specific antibodies for the different parts of the small molecules and the probability is high that PAbs and MAbs have similar properties.

### 2.7. Sample percolation and non-specific interactions

The sample can be modified before application by simple dilution using buffer or water, by adding buffer to adjust the pH, or by adding organic solvent in order to remove some non-specific interactions for non-polar analytes. The flow-rate may affect the binding and it is often said that high flow-rate may prevent analytes from binding to the immobilized antibodies. This problem is of prime importance in on-line methods, since trends are for high throughput, including the sample pretreatment. It depends on the diameter of the precolumns and not so much on the particle size of silica-based IS because they are large compared to the 5–10-μm range used in HPLC. Using 4-mm diameter precolumns, the flow-rate can be up to 2–5 ml/min without any loss in analytes [2]. Using a precolumn of 2 cm×1 mm I.D. packed with an anti-toluene IS, and injecting 1 ml of spiked water sample, an increase in recovery from 25 to 95% was observed as the flow-rate decreased from 2.0 to 0.2 ml/min [36]. No loss was observed for larger samples and with a flow-rate in the range 0.2–0.5 ml/min on precolumn 1 cm×1 mm I.D. prepacked with an anti-phenylurea IS [38].

Sample containing very hydrophobic analytes are often a problem because these analytes have tendency to adsorb everywhere, including the tubes, cartridges and solid-sorbent, via non-selective interactions. The non-selective interactions can be reduced by adding a small amount of organic solvent, usually methanol, acetonitrile or isopropanol, or a detergent such as Tween 20 or Triton X-100 or Brij-35 into the sample before percolation. In order to assess whether non-specificity interactions occurs during the immuno-extraction process, the same sample can be percolated on another IS containing other immobilized antibodies. Water samples (20 ml) spiked at 0.5 μg/l with six volatile PAHs (two or three rings) and containing 10% acetonitrile or Brij-35 were on-line analyzed using precolumns packed either with an anti-fluorene IS or an anti-atrazine IS; both IS having a similar bonding density [33]. The recovery of fluorene was higher with the anti-fluorene IS (53%) than with the anti-atrazine IS (24%) but the recoveries of the more hydrophobic fluoranthene were, respectively, 57 and 43%. Increasing the amount of acetonitrile in the sample could decrease non-specific interactions, but these additions affected the antigen–antibody interaction and lowered the breakthrough volumes, thus lowering recoveries.

Samples containing proteins are also complex and their direct percolation through ISs is not always possible. was used with direct percolation of serum samples. Recoveries were low for glycoproteins on an IS made of antibody-coated agarose beads, whereas the IS was very effective with serum free spiked samples [19]. Diffusion of the analytes towards the antibodies may have been inhibited by the viscosity of the serum or protein–protein interactions may have made the antibody sites less available.

Real life solid samples are usually extracted using

organic solvents, but application of the an extract containing a high percentage of organic solvent to an IS may affect binding of the analyte. This is strongly dependent of the antibodies. Using anti-isoproturon MAbs the percolation of 10 ml of a sample containing above 5% of methanol decreased the recoveries [28]. The sol–gel entrapped anti-TNT antibodies exhibited a high tolerance towards pure acetone, ethanol and acetonitrile, although the tolerance varied with the amount of the entrapped MAbs [24]. Examination of the TNT binding to sol–gel entrapped anti-TNT Abs in the presence of the three organic solvent revealed that interactions between the IS and TNT were not affected up to a concentration of 20%.

### 2.8. Elution conditions

Effective elution solutions should ideally disrupt the analyte–antibody interactions without adversely affecting the immobilized Abs. The most common strategies are reducing the pH to 2 or 3, using water-miscible organic solvents, or chaotropic agents, or a combination of these conditions. The choice first depends on the affinity between the antibodies and analytes. It also depends on the nature of the analyte, because of the ratio between electronic and hydrophobic interactions involved in the antigen–antibody interactions. In most of the reported off-line procedures, including those recommended using the commercial ISs, desorption was achieved with a high percentage of an organic solvent mixed with water, sometimes at low pH [2]. Elution of propanil from an anti-propanil IS required 70% methanol [30]. Several elution solvents mixed with water have been evaluated using an anti-diazinon IS: 50% methanol provided the best recovery at 93% for diazinon while the recoveries for 50 and 60% ethylene glycol, 50% ethanol, and 0.1 *M* glycine pH 3 were much lower, in the range 40–25% [18]. Urea was also evaluated because it is a strong chaotropic agent capable of disrupting ionic interactions hydrogen bonding and weak hydrophobic interactions. The extremely low recoveries for urea (0.3% of bound diazinon for 6 *M* urea), combined with the moderate to high recoveries of 40, 50 and 60% methanol seemed to indicate that the interactions between anti-diazinon antibodies and diazinon were primarily hydrophobic in nature. The disruption of the amanatin–PAbs complex was not completely achieved with the eluent acetone–water (95:5, v/v), but was only possible with acetone–methanol (50:50, v/v) [17]. Combination of organic solvent and acetic acid is sometimes required. An anti-pentachlorophenol IS had a very strong affinity for pentachlorophenol and a lower affinity for tri- and di-chlorophenols [39]. Trichlorophenol could be completely recovered using either water–acetonitrile (20:80, v/v) or water–acetonitrile (30:70, v/v) acidified at pH 3 using TFA. When acidification was achieved by 1% (v/v) acetic acid, a mixture containing only 30% acetonitrile was effective for elution. The elution of pentachlorophenol which has a higher affinity for the PAbs than trichlorophenol was impossible with pure acetonitrile or acetonitrile acidified by TFA. The desorption could only be achieved with mixture water–acetonitrile 20:80 (v/v) containing 1% acetic acid (v/v). Due to this very strong elution conditions, it was possible to apply an additional clean-up to the cartridge between sample percolation and elution. Fig. 2 compares the LC–UV chromatograms obtained for the analysis of an effluent from textile industry. The chromatogram (A) was obtained after extraction using a non-selective styrene divinylbenzene copolymer whereas (B) was obtained after immunoextraction and the application of an intermediary washing step. The effect of this clean-up was to remove the numerous non-polar matrix interferences which were non-specifically co-extracted by the IS. The added selectivity using the immunoextraction procedure to remove matrix interferences is very high.

### 2.9. Storage and reusability

Ideally, as with other SPE sorbents, ISs should not be re-used. However, when they are not commercially available or when they are used in on-line set-up, trends are for reusability, due to the efforts and price of antibodies. They are usually regenerated by percolation of PBS and stored at 4 °C when not in use. An antimicrobial agent, such as sodium azide is often added. However, with time and a number of runs, a decrease in capacity is often observed. After 20 uses, about 70% of the initial capacity of the anti-imazalil IS remained, after 30 uses 60% and

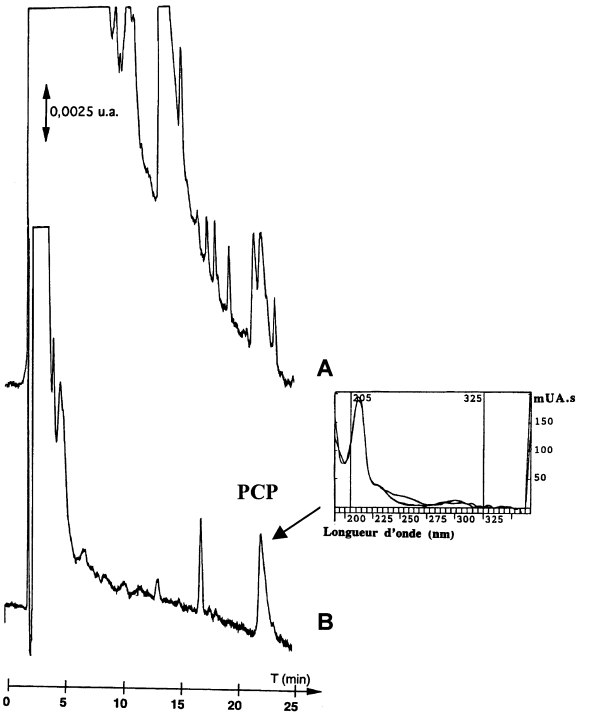


Fig. 2. Off-line analysis of pentachlorophenol in an industrial effluent from textile industry. Percolation of the sample (2 ml) diluted to 20 ml with phosphate buffer at pH 7. Chromatograms obtained after SPE using styrene divinylbenzene copolymer (A) or an anti-pentachlorophenol IS with a a washing step with 2 ml of water–acetonitrile 95:5 (v/v) before elution. UV detection at 215 nm [39].

after 40 uses only 40% [29]. An anti-diazinon IS was re-used 44 times with only a 10% loss in binding capacity [18]. The anti-fluoroquinolone IS was reused hundreds of time in an on-line format [26]. Although eluted with two 15 ml of methanol–acetone (50:50, v/v) at each run, the anti-amanitin IS were easily regenerated and re-used 50 times without any loss in performance [17]. They were stable without use for at last 6 months when stored at 4 °C. The anti-LSD IS prepared from protein A-coated agarose beads was shown to be very robust despite the strong elution conditions by triethylamine at pH 12 [40].

The reusability depends on the robustness of the support used for immobilization. Many studies reported the reusability of commercial and laboratory-made agarose or sepharose-based supports [41]. However, when they are used for large sample volumes, their reusability can be reduced because of their low mechanical resistance.

### *2.10. On-line coupling to separation techniques*

#### *2.10.1. Liquid chromatography*

Automation and on-line coupling of immunoextraction with LC separation are now commonly used methods. The on-line coupling of SPE to LC is commercially available. The main interest in integrating IS into on-line technology is the selectivity of the extraction, because extraction and clean-up are achieved in the same step.

There are two major procedures. The first one is used for IS which are non-pressure resistant ISs. The set-up consists of a two-precolumn procedure: the sample is percolated through the first precolumn containing the IS at low pressure; after washing, the analytes are desorbed by an appropriate aqueous solution and refocused on a second precolumn containing $C_{18}$ silica or an apolar copolymer, which is then coupled on-line to the LC analytical column and desorbed by the LC mobile phase. If the desorption from the IS precolumn can only be achieved with an organic solution, it is necessary to add water to the eluent to allow refocusing on the second precolumn, which requires an additional pump.

The second procedure is very simple and does not differ from that using a simple $C_{18}$ precolumn. In most cases, after sample percolation and washing, the IS pressure-resistant precolumn is directly connected to the analytical column and a water–acetonitrile or –methanol gradient to perform the analytical separation is percolated through the system of precolumn and analytical column. In order that the IS should not be in contact with high concentration of organic solvents for a long time, it is recommended to switch the connecting valve when all the analytes have been transferred.

The on-line coupling of immunoextraction with micro-LC has the advantage of providing a high sensitivity while decreasing the amount of antibodies required. One main advantage is to reduce the sample volume. Using a precolumn 1 cm×1 mm I.D. containing an anti-phenylurea IS and an analytical column of the same diameter, the on-line extraction of a 5-ml sample was sufficient for determination of

several phenylureas at the 50 ng/l level in contaminated surface water [38]. Another advantage is the possibility of performing very rapid extraction when the amount of antibodies is greatly reduced. An on-line chromatographic set-up was employed to measure the non-bound (or free) fraction of drugs using millisecond-scale extraction on small IAE column [42].

*2.10.2. Gas chromatography*

Immunoextraction has also been coupled to GC although interfacing between immunoextraction and the non-aqueous GC part is not as easy as it is with LC. One relevant example was described for triazine analysis using a two-precolumn procedure [43]. The first IS was a cellulose-base IS with immobilized anti-atrazine MAbs and the second, packed with a reversed-phase polymer, was used for refocusing the analytes after elution with an acidic buffer. After clean-up and drying with nitrogen, desorption and transfer was done with ethyl acetate via an on-column interface. The selectivity of the system was such that a non-selective flame ionization detection could be used to detect several triazines in river, wastewater and orange juice with detection limits in 10-ml samples in the range 15–25 ng/l.

*2.10.3. Capillary electrophoresis*

Capillary electrophoresis (CE) and capillary electrochromatography (CEC) have the advantages of instrumental simplicity, low consumption of sample and reagent and short run times. However, this advantage also leads to a severe limitation of poor concentration sensitivity. Often high analyte concentrations are required to have sufficient material for detection in the capillary. In this regards, a series of microextraction devices that are broadly termed ‘‘analytes concentrators’’ have been developed for selective and non-selective preconcentration on-line with the CE capillary [44–46]. Several strategies have been reported for positioning a small section of packing material in the electrophoresis capillary to capture sample components. In a subsequent step, a small volume of eluting solution is passed through the packing to remove the adsorbed material from the so-called ‘‘enrichment chamber’’. The standard set-up of an immunoaffinity preconcentration contained a solid-support immobilizing the antibodies. The enrichment was achieved by pressurizing the sample through the solid-support onto which the analytes will bind to antibodies. In the subsequent step, the eluting solution, whose goal was primarily to achieve dissociation of the analyte–antibody complex, is composed of a small plug of an organic solvent, a solution with increase ionic strength, or a buffer containing special additives, depending on the compound to be analyzed. In this approach a larger injection volume, up to 50 μl could be introduced in the CE system, thus effectively greatly improving the concentration sensitivity. In such a system, the capacity of the preconcentration chamber had to be high, but at the same time the chance of clogging and band broadening had to be low. The simplest enrichment chamber was made of a portion of the CE capillary containing antibodies on its wall, but the limited surface area resulted in very low enrichment factors. A microscale-format affinity device was also described and used for the on-line IAE of the immunoreactive gonadotropin-releasing hormone (GnRH) in serum and urine [12]. The concentration limit of detection for GnRH in the serum, utilizing IAE–CE with UV detection was 1 ng/ml. The system could be re-used for at least 10 times.

Thomas et al. [47] described a system where enrichment was achieved by immunoaffinity capillary electrochromatography (IACEC), which eliminates the need for pressure-driven flow. The IS consisted of anti-biotin antibodies immobilized onto aldehyde-activated silica. Fluoresceinated biotin served as a fluorescent analog suitable for detection. Samples were electroosmotically injected into a fused-silica capillary, the first 3–10 mm of which was packed with the immunoaffinity support. The authors paid much attention to the desorption strategy and buffer used, one condition being that they should be suitable for electroosmotic flow (EOF). Desorption was achieved with a 2-propanol–4 m*M* sodium tetraborate buffer (20:80, v/v) solution at pH 9.2. The column was regenerated and re-used many times.

The last currently available preconcentration chambers, also manufactured without the use of frits, beads or membranes, consisted of a bundle of small-diameters capillaries. Other developments are new phases, such as monoliths that can be polymerized in situ, thus avoiding the use of frits. Many develop-

ments are currently under study into with the microchip technologies where separation mode is electroosmatically driven.

## 3. Immunologic trapping and immunofiltration

Supported liquid membrane (SLM) extraction was combined with an immunologic recognition in order to obtain a high degree of selectivity in sample preparation [48]. The SLM employs a hydrophobic polymer to support the immobilization of an organic solvent, thus forming a non-porous membrane, which was able to separate the aqueous simple on one side (donor) from a receiving aqueous phase on the other side (receptor). The extraction involved the partition of neutrals compounds between the sample solution, continuously pumped alongside the membrane, and the membrane. From the membrane re-extraction took place in a second aqueous phase containing antibodies specific for the target analyte(s). When the antigen–antibody complex forms, the antigen could no longer redissolve in the organic membrane and remained trapped in the acceptor. Consequently, the concentration gradient of free antigen over the membrane was ideally not affected, this being the driving force for the process. When Abs were in excess, the concentration of analyte in the receiving phase will exceed the initial sample concentration. The set-up was applied to the extraction of 4-nitrophenol. The complex 4-nitrophenol–Abs was quantified on-line using a fluorescein flow immunoassay in a sequential injection analysis set-up. The coupling of the immunologic trapping with the immunoassay allowed the determination of 4-nitrophenol in spiked wastewater sample, thus demonstrating that IAE can be suitable when dealing with complex matrices.

Immunofiltration has been described for the sample clean-up for the immunochemical detection of β-agonists in urine samples [49]. In the applied format, free (non-immobilized) anti-salbutamol PAbs were mixed with the urine sample (0.25 ml, 50-fold diluted in PBS) in an ultra-filtration device having a cut-off of 30 000 Dalton and the sample was removed. The antibody bound β-agonists were freed from the antibodies by addition of a mixture of methanol and 0.1 *M* acetic acid (1:1, v/v) and centrifugation. The filtrate containing the free β-agonists was evaporated to dryness. In the presented study, the residue was analyzed by the β-agonist ELISA and by GC–MS for confirmation. The anti-salbutamol antibodies recognized several β-agonists and the combination of the immunofiltration with the β-agonists ELISA resulted in a 30-times lower detection limits compared to results obtained using non selective sample preparation methods.

The use of receptor instead of antibodies is so similar that it is considered as part of immunotechniques by some authors. Onorato and Henion [50] have evaluated the estrogenic activity of triterpene glycosides contained in a popular herbal formulation, black cohosh, used for the treatment of symptoms associated with menopause. Affinity ultrafiltration was used for sample extraction. Binding experiments were performed on sample reservoirs of centrifugal filter with a total incubation volumes of 100 μl. Three triterpene glycosides contained in black cohosh were combined with the ligand binding domain of estrogen receptor β (ER-β) and allowed to incubate for 1 h at room temperature. The samples were then centrifuged at 10 000 *g* for 5 min and spin-rinsed with sorbent to remove all unbound components. The decomplexation was then accomplished by adding 1% TFA in acetonitrile and then again centrifuged and washed to elute the previously bound components. The analytes were further analysed by LC–MS.

## 4. Application to real samples: off-line and on-line coupling to various separation and/or detection techniques

### *4.1. Liquid chromatography*

#### *4.1.1. LC–UV or fluorescence*

The main advantage of IAE before LC separation is that it allows the use of simple detectors such as UV or fluorescence, and give with low detection limits because of the clear base line due to the removal of matrix interferences. Although fluorescence detection provides its own selectivity, some matrices are so complex that IAE provide chromatograms that are more exploitable. This was illustrated by the analysis of the 16 priority PAHs after ex-

traction using supercritical fluids from a certified reference sludge samples [33]. Since a certified reference matrix was used, the whole procedure including the immunoclean-up could be validated.

*4.1.2. LC–MS*

The advantage of IAE as a good sample preparation method for LC–MS was already emphasized 4 years ago [51]. Owing to the tremendous increase in the use of LC–MS coupling and to the trend to high-throughput and miniaturization, IAE for MS detection, with or without intermediary separation has become more and more common. Cai and Henion [52] were the first to demonstrate the potential of IAE when it was coupled to LC followed by ion-spray tandem MS detection. They used a two-precolumn system for the automated determination of LSD analogs and metabolites in urine samples. Analytes were first trapped on a small IS precolumn and then on-line refocused onto $C_{18}$ precolumn which was further on-line coupled to a capillary LC for optimum sensitivity and coupling with ion-spray tandem MS detection. From 50 ml of diluted urine samples, it was possible to detect analytes at the 2.5 ppt level which was 20-fold below the limit of detection obtained when the $C_{18}$ precolumn was used alone.

In addition to a better sensitivity using tandem MS, immunoextraction allows the use of simple quadrupole spectrophotometer. Rolcik et al. [53] compared the LC–electrospray MS chromatogram obtained after $C_{18}$ extraction and after IAE for the determination of melatonin in human serum samples. The IAE was performed off-line and was very simple: percolation of serum diluted with PBS through an agarose-based anti-melatonin IS, elution with methanol, evaporation and reconstitution. The selectivity provided by the IS extraction is illustrates in Fig. 3. Detection limits as low as 10 fmol were obtained.

It is well known that MS–MS can easily remove matrix effects, provided that no ionization suppression effect occurs in the ionization chamber due to the matrix components. LC–MS was used after IAE for the rapid and specific detection of amanatins in body fluids for an early diagnostic of an intoxication with amanita mushrooms [17]. The LC–MS methods was previously developed after conventional SPE of

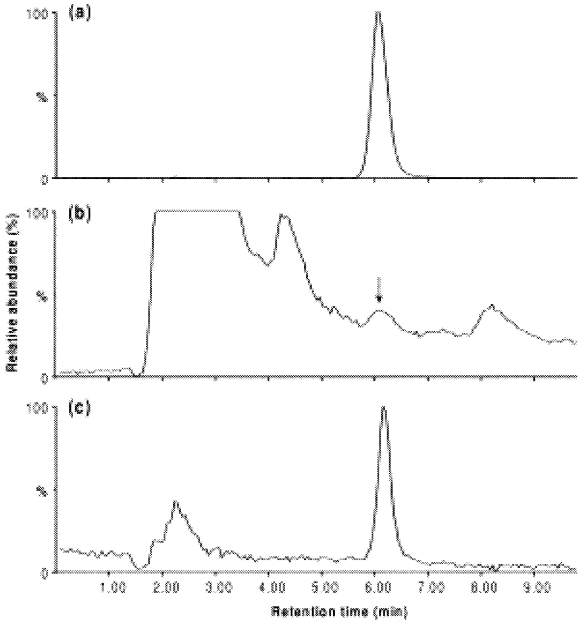


Fig. 3. Comparison of LC–MS analyses of human serum samples processed by $C_{18}$ solid-phase extraction (b) and by immmunoaffinity extraction (c) and LC–MS analysis of melatonin standard (a). Respective amounts of injected melatonin were 500 (a) and 68 fmol (c). The ion chromatograms were obtained by measurement at $m/z = 174$ [53].

urine samples, but could not be validated due to many problems arising from urine matrix compounds co-chromatographing with the analytes. They disturbed the detection of α- and β-amanitin by hampering their ionization in the electrospray chamber. The interfering matrix could not be removed by modification of the SPE nor by modification of the chromatographic system. The authors overcame the problem by developing a sepharose-based IS using PAbs against β-amanitin. applied. The IAE allowed reducing the influence of the matrix by a factor of 100. Using IAE, the abundances of the analytes with and without matrix were the same. Thus, LOD for both α- and β-amanitin in urine could be lowered to 2.5 ng/ml.

Another example of matrix ionization suppression effect was found in the determination of steroid estrogens in biologically treated effluent from a sewage plant by Ferguson et al. [54]. They developed an IS selective to relevant steroid estrogens by bonding Mabs anti-17β-estradiol (E2) and estrone

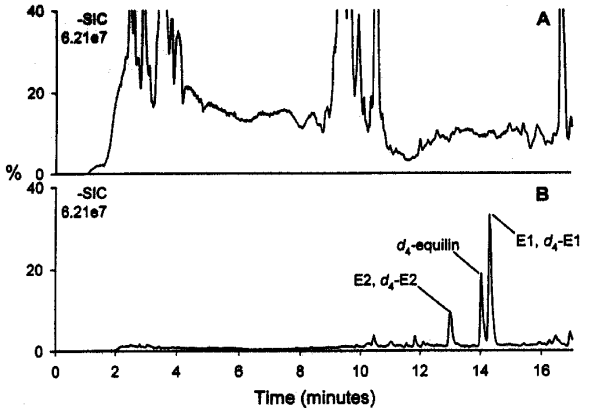


Fig. 4. Summed ion chromatograms of raw (A) and immuno-treated (B) extracts from sewage effluent, analyzed by negative polarity HPLC–ESI–MS. Peaks corresponding to analytes surrogates and internal standards are labeled [54].

(E1) to controlled-pore glass particles, surface-modified with hydrazide groups. The mixed IS extracted E1 and E2 with good recoveries from effluent. Fig. 4 shows the total ion chromatograms of raw and immuno-treated water extracts of sewage effluents. The estrogens, their surrogate and the internal standard are clearly distinguishable after immunoclean-up. The effluent that was not immuno-treated contained a large amount of background signal for the ions monitored and there are numerous isobaric interference peaks (Fig. 4A). It is interesting to note that that there is no detectable signal even for equilin-$d_4$ internal standard which was added to the sample just prior to analysis. This essentially indicates that ionization of the analytes and the standards was suppressed at a level approaching 100% in this particular sample. Although MS–MS would virtually eliminate the isobaric noise shown in Fig. 4A, it would not remove the suppression effect since the process occurs in the MS source during ion formation.

#### *4.1.3. Selected applications for trace analysis of pesticides, organic pollutants, toxins and drugs*

In recent years, there had been many developments of ISs for the trace analysis of pesticides and organic micropollutants mainly for environmental analysis and food control. Table 1 reports some selected recent applications (see Ref. [2] for previous applications).

Toxins have been targeted these recent years. Mycotoxins are secondary metabolites produced by various fungi growing in a wide range of food and animal feedstuffs (nuts, peanuts, corn, cereals, grain, oilseeds, dry figs and raisins, milk, apple juice). Because about 20% of food products, mainly of plant origin, are contaminated, monitoring is very important. Many applications also deal with the determination of mycotoxins in foodstuffs that include aflatoxins, ochratoxins, fuminosins, patulin and zearalenone. Immunoclean-up was introduced in the early 1990s and has been accepted in many validated and official methods. As a consequence, several ISs are commercially available. Gilbert and Anklam [59] published an extensive review on the validation of analytical methods for determining mycotoxins in foodstuffs, where the methods validated with the use of IAE for sample clean-up are listed. Other toxins are produced by aquatic microalgae that are responsible of shellfish and fish contamination. Paralytic shellfish poisoning toxins (PSP) should be controlled in shellfish. The regulatory method is still the mouse bioassay, which is greatly criticized. Alternative methods are urgently required. Diarrhetic shellfish poisoning (DSP) is also encountered worldwide.

Microcystins are cyanobacterial toxins, which represent an increasing environmental hazard. Some relevant examples dealing with the determination of various toxins have been reported in Table 2.

Another class of widely monitored analytes consists of veterinary drugs such as antibiotics. Although the use of synthetic corticosteroids as growth promoters is prohibited in Europe, it continues for commercial reasons because the meat that is produced is more appealing to consumers. Clenbuterol is a β-agonist drug frequently used for the treatment of obstruction in the bronchial tubes of animal and also as a growth promoter in animals. Selected applications have been reported in Table 3.

### *4.2. Gas chromatography*

Several relevant examples of off-line immunoaffinity extraction methods followed by GC analysis have been reported in Table 4.

Table 1
Selected recent applications using immunoaffinity extraction followed by LC separation techniques for the determination of pesticides and organic pollutants in environmental and biological matrices

| Analytes | Matrix | Extraction technique | Method of analysis and LOD or LOQ | Ref. |
|---|---|---|---|---|
| Imazalil | Citrus fruits (lemon, orange, grapefruit) | Anti-imazalil MAbs immobilized on Agarose gel Methanol extraction of sliced and homogenized fruits, dilution with PBS before IAE, washing with PBS–methanol (9:1, v/v), elution with PBS–methanol (2:8, v/v). Reusability at 30 times | LC–UV LOD<0.5 ppm | [29] |
| Diazinon | River water, spring water, apple juice | Anti-diazinon PABs immobilized on various supports. Evaluation of several elution strategies. Re-usability more than 40 times | LC–UV | [18] |
| Avermectins | Swine liver | Anti-avermectin PAbs immobilized on Sepharose. Extraction with methanol, dilution with PBS before IAE. Elution with methanol | LC–APCI–MS LOD: 5 μg/kg (abamectin and ivermectin) | [55] |
| Propanil and phenylureas | River water and lemon juice | Anti-propanil PAbs immobilized onto silica. Direct off-line and on-line use. Elution with methanol–water (7:3, v/v) | LC–UV LOD<0.1 μg/l in river water | [30] |
| PAHs | River water, sludges, biological tissues | Anti-pyrene PAbs immobilized onto silica. On-line and off-line use for aqueous samples. Extraction of sludges using microwave assisted extraction with toluene–acetone (50:50, v/v) | LC–UV–Fluorescence, LOQ<20 ng/l in river water | [35,56] |
| 1-Nitropyrene | Herbs | Sol–gel generated IS with anti-nitropyrene PAbs. Acetonitrile extraction followed by size exclusion clean-up before IAE clean-up | LC–Fluorescence LOD: 0.3 μg/l | [22] |
| Benzidine, congeners and related dyes | Surface water and industrial effluents | Anti-benzidine PAbs immobilized onto silica. On-line preconcentration of diluted samples. | LC–UV–Visible LODs: 0.1–1 μg/l | [57] |
| Bisphenol A | Serum samples | PAbs immobilized onto Sepharose. Centrifugation and dilution before IAE. Elution with methanol–water (80:20, v/v). No retention of phenol | LC UV/Fluorescence | [58] |

LOD for limits of detection and LOQ for limit of quantification.

### 4.3. Mass spectrometry

Immunoextraction can be directly coupled to MS. The ability of matrix-assisted laser desorption time-of-flight MS (MALDI-TOF MS) to analyze complex mixtures makes it more suitable than other techniques for biological extracts. Although MALDI-TOF MS is tolerant of impurities compared to other MS techniques, maximum sensitivity is achieved from pure analytes in solution. The coupling of immunoextraction has been made by attaching Abs to the surface of a MALDI probe tip via a thin nitrocellulose film [83]. This allowed the corresponding antigen to be selectively captured and concentrated on the probe tip from complex plasma solutions for MALDI MS analysis. The method was successfully applied to determine a therapeutic peptide at relevant doses

An immunosorbent was developed for the trace-level determination of potato glycoalkaloids in blood serum [19]. Glycoalkaloids were first extracted using $C_{18}$ SPE and then selectively purified on antibody-coated agarose beads. The agarose beads were washed with waters and the glycoalkaloids were eluted with methanol. MALDI-TOF MS was used to detect the glycoalkaloids in the methanol eluent. It

Table 2
Selected recent applications using immunoaffinity extraction followed by LC separation techniques for the determination of various toxins in environmental and biological matrices

| Analytes | Matrix | Extraction technique | Method of analysis and LOD or LOQ | Ref. |
|---|---|---|---|---|
| Aflatoxins M1 | Dairy products (milk, dry milk, for infant formula, yogurt | Commercial IS. Survey of 400 samples. Aflatoxin M1 was detected in 78% milk samples with only four samples of dry milk over the legal limits established by the EU | HPLC–Fluorescence LOD<1 ng/kg | [60] |
| Aflatoxins M1 | Sesame seed and tahini | Commercial IS. Extraction and IAE purification according to the AOAC method 968.22. Elution with methanol. Derivatization with trifluoric acid | LC–Fluorescence | [61] |
| Fumonisins B1 and B2 | White rice flour, cornstarch, cornmeal and glucose | Commercial IS. Extraction with methanol–acetonitrile–water mixtures (25:25:50, v/v) and dilution with PBS before IEA purification. Elution with methanol. Derivatization with *o*-phthaldialdehyde/mercaptol | LC–Fluorescence and LC–ESI–MS | [62] |
| Fumonisins B1 and B2 | Corn products | Evaluation of both silica-based and Sepharose-based IS using the same anti-fumonisin B1 PAbs. Direct IAE clean-up for detection at the 20 ng/g level. Preliminary clean-up with strong anion-exchanger cartridge before IAE purification for lower detection. The silica-based IS was re-used more than 10 times | LC–fluorescence after prechromatographic derivatization LODs = 2–5 ng/g in corn flour and nacho chips | [63] |
| Ochratoxins A | Wines | Comparison of two commercial ISs. Direct percolation of wine samples adjusted at pH 7.8, elution with pure methanol | LC–Fluorescence LOQ: 0.02–0.045 μg/l | [64] |
| Ochratoxins A | Wines and vinegar | Commercial IS. First acidic chloroform extraction and then IAE purification | LC–Fluorescence LOD: 0.002 μg/l | [65] |
| Ochratoxins A | Roasted coffee | Commercial IS. Extraction of grind coffee with methanol, then first clean-up using aminopropyl silica and then IAE purification. Derivatization to its methyl ester derivative | LC–Fluorescence LOD: 1 ng/g | [66] |
| Ochratoxins A | Baby food | Commercial IS. First extraction with TBME, dilution with PBS before IAE purification. Elution with methanol. Post column derivatization with ammonia. Validation through interlaboratory study | LC–Fluorescence LOD<0.05 ng/g | [67] |
| Zearalenone and ochratoxin A | Wheat, rye, barley and oat samples | Commercial IS cartridges. First extraction with acetonitrile–water (60:40, v/v) and use of the automated SPE system (ASPEC) for purification of diluted extracts in PBS. Elution with methanol–acetic acid (98:2, v/v) | LC–Fluorescence LODs: 0.1 μg/kg (ochratoxin A) and 1.5 μg/kg (zearanalenone) | [68] |
| Saxitoxin and neosaxitoxin | Water and algae samples | Evaluation of PAbs and MAbs against saxitoxin and neosaxitoxin for use in immunoassays and IAE. | LC–UV | [69] |
| Okadaci acid and related dinophysis | Shellfish | Anti-okadaic acid PAbs immobilized onto silica. First extraction with methanol. Many interferences coming from the matrix and from the derivatization reaction. IAE clean-up of derivatized extracts. Simpler method when LC–MS is used. | LC–fluorescence after derivatization or LC–MS. LOD: 1 μg/g hepatopancreas. | [70] |
| Okadaci acid | Strains | Anti-okadaic acid PAbs immobilized onto silica. Simple IAE of methanol extracts | LC–fluorescence after derivatization or LC–MS. | [71] |
| Microcystins | Water and algae samples | Anti-microcystin-LR PAbs immobilized onto silica. Direct percolation of water samples through the IS | LC–UV and LC–MS. LOD <0.2 μg/l in water | [72] |
| Microcystins | Blue-green algae, fish, water | Anti-microcystin-LR PAbs immobilized onto Sepharose or silica. Extraction of fish and algae with methanol–water (75:25, v/v). Elution with methanol–acetic acid (80:20, v/v) | LC–UV LOD: 0.03 μm/g (algae and fish); 0.02 ng/ml in water | [73] |
| Microcystins | Lake water | Anti-microcystin-LR PABs immobilized onto cellulose-based beads. First extraction of 1-l samples using a styrene divinyl-benzene cartridge before IAE clean-up of extracts | LC–UV and LC–ESI–MS LOD: 0.005 μg/l in lake water | [41] |

Table 3
Selected recent applications using immunoaffinity extraction followed by LC separation techniques for the determination of antibiotics and drugs in biological matrices

| Analytes | Matrix | Extraction technique | Method of analysis and LOD | Ref. |
|---|---|---|---|---|
| Fluoroquinolones | Chicken liver, milk | Anti-sarafloxacin MAbs immobilized onto Poros polymer containing bonded protein G. Extraction using 0.1 *M* sodium hydroxide with subsequent neutralization with the addition of phosphoric acid, PBS and 5% methanol before IAE purification. | LC–Fluorescence LOQ <1 ng/ml for all individual fluoroquinolones | [26] |
| Corticosteroids (dexamethasone, flumethasone, etc.) | Feed and biological matrices | Commercial IS. Feed samples: extraction with TBME, dilution with water and percolation through the IS cartridge. Elution with water–methanol (30:70, v/v). Urine samples: hydrolysis before IAE purification | LC–UV: LOD of 50 μg/kg in feed samples LC–MS: LOD: 10 μg/kg in feed and 0.5 μg/l in urine | [74] |
| Clenbuterol | Plasma | Anti-clenbuterol PAbs immobilized onto silica. Dilution of plasma with PBS (1:9, v/v) before IAE. Elution with methanol–PBS (50:50, v/v). Improvement of reproducibility by precipitating the plasma proteins with acetonitrile | LC–UV LOD <100 ng/ml | [75] |
| β-Agonists | Urine, feeds, chicken muscles and liver, bovine bile, plasma | Review of IAE applied for these analytes | | [76] |
| LSD and related | Urine samples | Commercial IS. Confirmation of positive samples after immunoassay screening. | LC–Fluorescence LOD<100 ng/ml | [77,78] |

was shown that immunoaffinity sample purification effectively reduced the signal suppression observed during the analysis of unpurified samples. It was possible to detect α-chaconine and α-solanine in serum spiked with 1 ng/ml of each analyte (Fig. 5).

### *4.4. Bioanalytical assays (immunoassays, bioassays)*

Immunoassays are very sensitive to matrix effects, which can provide a high number of false positives that need to be confirmed. Usually the same antibodies are used for extraction and for detection in an ELISA format. Commercial immunoassays, which are designed for use in urine samples, are frequently used for non-urine matrices in forensic settings and there has been a considerable interest in the detection of drugs of abuse from whole blood by immunoassays. One relevant example is the immunochemical extraction and detection of LSD in whole blood [40]. The authors set-up indirect ELISA but there was an overall decrease in precision when whole blood was used in place of urine due to the increased complexity of the matrix. IAE was used to isolate LSD from blood and urine samples before ELISA measurement. The affinity support was prepared by covalently attaching anti-LSD antibodies to protein-A coated agarose beads. No pretreatment of the samples was required other than addition of PBS. Sub-ng/ml concentrations of LSD were routinely extracted with greater than 80% recovery of drugs.

IAE was also applied to soil extract for the detection of chlorimuron-ethyl, a new sulfonylurea herbicide that is applied at very low dose [84]. Although sensitive the competitive indirect ELISA that was developed was affected by the presence of co-contaminants of the matrix and was unable to provide accurate determination. The Sepharose-based IS was able to remove most of the matrix interferences, but the clean-up made more efficient by passing the sample through a column containing epoxy-coupled 1,6-diaminohexane Sepharose 4B to remove organic acids prior to IAE. Assay sensitivity was increased 100-fold.

Water blooms of toxic cyanobacteria should be monitored in the field because 70% of blooms have

Table 4
Selected recent applications using immunoaffinity extraction followed by GC separation techniques for the determination various compounds in environmental and biological matrices

| Analytes | Matrix | Extraction technique | Method of analysis and LOD | Ref. |
|---|---|---|---|---|
| Anti-fouling trazinic biocides | Marina seawater | Anti Irgarol 1051 PAbs immobilized to agarose-based beads. Percolation of 250 ml water samples without breakthrough. Desorption with ethanol–water (70:30, v/v) | GC–NPD and GC–EI–MS. Detection at the ng/l level | [79] |
| PAHs | Sludges | Anti-pyrene PAbs immobilized onto silica. First microwave assisted extraction with toluene–acetone. Dilution in water and elution with water–acetonitrile (30:70, v/v). Validation of the whole protocol using a certified reference sludge sample (CRM 088). | GC–MS LODs: 0.1–0.8 mg/kg | [56] |
| Fipronil | Soil and vegetation | Anti-fipronil PAbs immobilized onto Sepharose. First extraction with acetonitrile–acetone (70:30, v/v). Two-step clean-up using first a silica cartridge before the IAE cartridge. Degradation studies under tropical conditions | GC–ECD and GC–MS | [80] |
| Exogenous 19-nor-androsterone | Urine | Sepharose-based IS. Preliminary purification if urine by SPE after hydrolysis by β-glucuronidase and further isolation of unconjugated 19-NA was by IAE. Washing step with methanol–water (20:80, v/v) to remove androsterone, etiocholanolone and cholesterol before elution with 80% methanol | GC/combustion/isotope ratio mass spectrometry (GC/C/IRMS) LOD: 400 ng | [81] |
| Delta9-THC (tetrahydro-cannabinol) and its major metabolites | Urine, plasma and meconium | Anti-THC Abs immobilized onto Sepharose First enzymatic hydrolysis before derivatization of the samples as TMS derivatives | GC–EI–MS with SIM Monitoring. LODs from 0.5 to 2.5 ng/ml. | [82] |
| s-Triazines | Water and soil | Anti-atrazine MAbs encapsulated in sol–gel glass matrix. | GC–NPD LODs 0.02–0.1 μg/l in water | [23] |

proved to be toxic. Rapid detection of the presence of toxins is required when blooms occurs in recreational areas or in drinking water reservoirs. Commercial ELISA kits and laboratory-made phosphatase bioassays for the analysis of microcystins have been evaluated [72]. A strong matrix effect was observed with some spring and surface waters. A clean extract free from any matrix interferences and the easy-to-obtain enrichment factor of 10 greatly improve the determination at the 0.1 μg/l level in surface water using these two bioanalytical assays. The best available technique for rapid monitoring of toxic blooms is the combination of a simple immunoextraction with phosphatase inhibition tests because it combines a structure recognition tool with a bioassay based on the toxicity mode. Tsumiso et al. [85] have developed the coupling IAE–ELISA for the rapid monitoring of drinking water. The combined methods could detect pg to μg/l of equivalent microcystin-LR in tap water.

An IS was developed for the determination of peanut protein from food matrixes. After chocolate extraction and immunoclean-up the amount of protein was determined by an ELISA [86]. Overall recovery from chocolate spiked with 0.2–3.2 μg/g of peanut protein averaged 77% and the limit of detection was 0.1 μg/g. Direct extracts of chocolates could not be measured by the ELISA.

As already described in Section 3, immunofiltration was shown to be a good clean-up procedure before ELISA since the combination of the immunofiltration with the β-agonists ELISA resulted in a 30

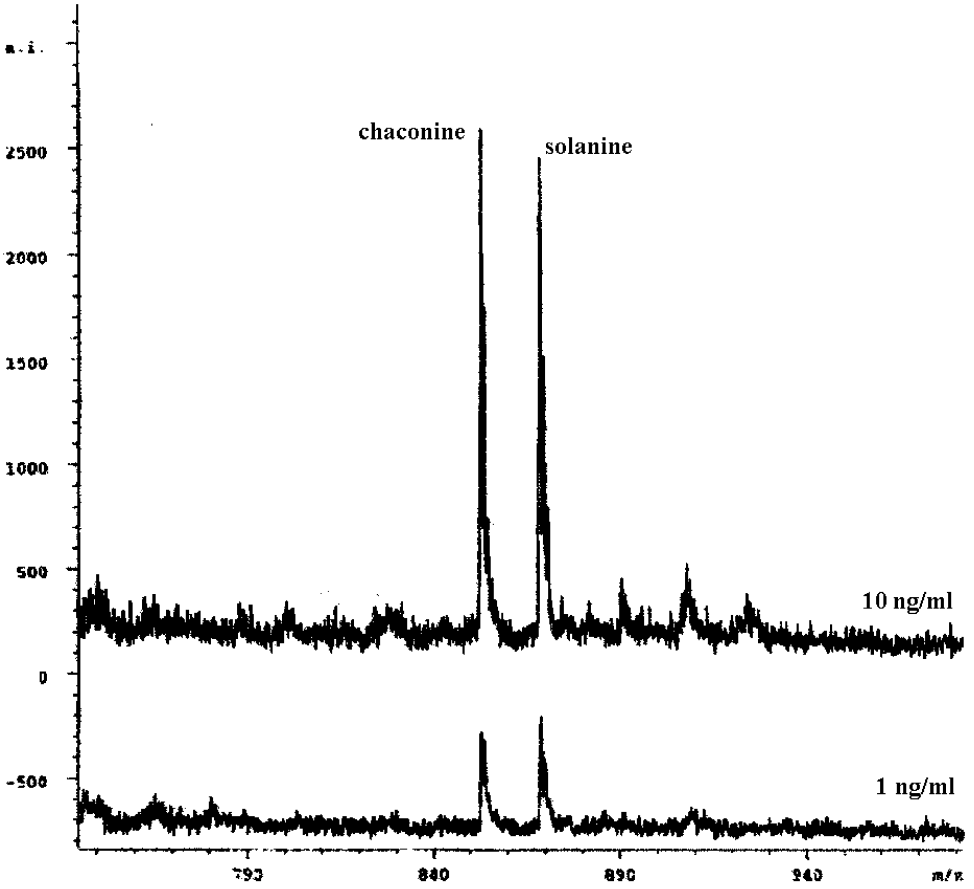


Fig. 5. MALDI-TOF spectrum of serum glycoalkaloids purified by SPE and immunoaffinity on agarose beads [19].

times lower detection limits compared to results obtained using non selective sample preparation methods [49].

## 5. Molecularly imprinted polymers (MIPs)

The development of immunosorbents takes a long time and is very expensive. Theses drawbacks have led to the recent development of synthetic antibody mimics or plastic antibodies, so-called molecularly imprinted polymers (MIPs). Molecular imprinting consists of the synthesis of highly crossed-linked resins in the presence of a given molecules which acts as a template. Once thoroughly washed, the cavities are complementary in size and shape to the print molecule. Therefore, the MIP can be utilised to selectively rebind the template and its closed analogs from complex matrices. Their use for sample preparation has been recently reviewed [87–89]. Some recent applications have been reported in Table 5.

### *5.1. Synthesis and consequences for the extraction medium*

The most versatile approach to the MIP synthesis is based on self-assembly of the template and a complementary functionalised monomer prior to polymerisation. Thus, the template remains associated with the growing polymer during the synthesis

Table 5
Selected applications of molecular imprinted polymers for the extraction of organic compounds in various environmental and biological matrices

| Analytes | Template molecule | Matrices | Monomers | Porogenic solvent | Ref. |
|---|---|---|---|---|---|
| Alkylphosphonates | Various methyl phosphonates | Serum (after transfer of solutes into acetonitrile) | MAA | Acetonitrile | [90] |
| Chlorophenoxyacetic acids | 2,4,5-T | Water | VP | Water–methanol | [91] |
| Bisphenol A | *p*-tert.-Butylphenol | Water | VP | Toluene | [92] |
| Clenbuterol | Clenbuterol | Urine | MAA | Acetonitrile | [93] |
| Nicotine and oxidation products | Nicotine | Chewing gum | MAA | Dichloromethane | [94] |
| 4-Nitrophenol | 4-Nitrophenol | Water | VP | Acetonitrile | [95] |
| Phenylureas | Isoproturon or fenuron | Water | MAA | Acetonitrile or toluene | [96] |
| Sulfonylureas | Met-sulfuron-methyl | Water, soil | TFMAA | Dichloromethane | [97] |
| Triazines | Atrazine | Liver | MAA | Acetonitrile | [98] |
| Triazines | Propazine | Water and diluted sediment extracts | MAA | Dichloromethane or toluene | [99] |
| Triazines | Simazine | Water, urine, apple (after $C_{18}$ SPE) | MAA | Dichloromethane | [100] |
| Triazines | Terbutylazine | Water | MAA | Dichloromethane | [101,102] |
| Triazines | Terbutylazine | Water (after mixed-mode extraction)* | MAA | Toluene | [103] |
| Triazines | Terbutylazine | Water (after $C_{18}$ SPE) | MAA | Various solvents | [104] |
| Triazines | Dibutylmelamine | Water | MAA | Chloroform–water | [105] |

MAA, methacrylic acid; VP, 4-vinylpyridine; TFMAA, trifluoromethylacrylic acid.
* Restricted access MIP (exclusion + hydrophobic interactions).

and the addition of a large portion of cross-linking monomer allows the formation of complementary sites that remain stable after template removal. The monomer is chosen in order to develop strong non-covalent interactions with the template. Widely used monomer and cross-linker are, respectively, methacrylic acid and ethyleneglycol dimethacrylate (see Table 5). The appropriate solvent of such a polymerisation is generally an aprotic and non-polar solvent. As a consequence, the main interactions that are developed between the template and the molecular imprint are hydrogen bonds and dipole–dipole interactions when methacrylic acid (MAA) is selected as monomers. It has been largely demonstrated that MIPs offer the highest selectivity when samples are in the solvent used for the MIPs preparation. Consequently, when a MAA-based molecular imprint is used for solid-phase extraction (MIPSE) a selective procedure deals with the handling of samples dissolved in an appropriate organic solvent. Therefore, examples of MIPSE protocols based on a selective adsorption generally include a step where the sample is modified or extracted with the organic solvent prior to application. In those cases, MIPSEs are rather used as clean-up sorbents. For aqueous matrices (usually environmental waters or biofilms), the adsorption of analytes is non selective when the samples are directly applied to the MIPSE cartridge. Then the MIPSE should be washed with a selective solvent capable of disrupting only the non-specific interactions of the matrix components with the polymeric matrix.

Therefore, the choice of both the monomer and the porogenic solvent is very important because it will indicate the medium in which selective extraction can be obtained. Other monomers such as 4-vinylpyridin are able to develop $\pi$–$\pi$ interactions. As for the selection of the hapten for the synthesis of immunosorbent, the interest of using molecular modelling has been shown for the selection of the nature of the monomers and the ratio between monomer and template molecule [106].

After adsorption, the analyte needs to be efficiently desorbed from the MIPSE in the smallest possible volume in order to obtain large enrichment factors. For MAA-based MIPSE, analytes are easily desorbed with protic and polar organic solvents such methanol or water. When analytes are strongly

bound, efficient elution has been obtained with addition of small amounts of acids (acetic or trifluoroacetic acids) or base (triethylamine) [108].

### 5.2. Examples of selective extraction procedures in aqueous samples

The selectivity of a MIP for ametryn and other related triazines in tap water was shown and compared with the use of a $C_{18}$ SPE cartridge [107]. Since the MIP worked well in organic solvents, a first extraction was performed onto a $C_{18}$ SPE cartridge before purification on the MIPSE by mean of a solvent elution. The operation could be automated by an on-line methodology. The selectivity of the extraction was demonstrated by the strong reduction of the humic peak at the beginning of the chromatogram. However, the recoveries of the overall methodology were in the range 10–40%. Low recoveries have also been obtained for the determination of four triazines in water using a similar methodology [100]. An interesting on-line coupling was described for the determination of triazines in natural waters by using as first column a restricted access material (RAM) instead of a $C_{18}$ precolumn in order to limit the amount of humic substances on the MIP [103].

Very few studies have described direct extraction of compounds from aqueous matrix [95,101,102]. In contrast to immunosorbent, the direct use of MIP in aqueous sample is difficult because the selective interactions via hydrogen bonds are weak in water. A recent study described the optimization of a SPE procedure for the class extraction of triazines including polar degradation products with the direct percolation of aqueous samples through the MIP as first step [102]. The MIP used for this study was synthesized using methacrylic acid as monomer and terbutylazine as template. During the percolation of the water sample, retention is mainly due to hydrophobic interactions with the polymeric matrix of the MIP and the retention mechanism is well known. Breakthrough depends on the polarity of the analytes and the interactions are not selective, so that many other organic compounds present in the aqueous samples are co-extracted depending on their polarity. The possibility was studied to transform the non specific interactions into specific hydrophilic interactions (hydrogen bonds) by applying a few ml of porogenic solvent, dichloromethane. It was necessary to closely adjust its volume in order to allow the diffusion of the triazines into the specific cavities and their link to the polymer, with the simultaneous elution of the co-extracted analytes. Since water and dichloromethane are not miscible, a drying step had to be introduced between the first two steps. The third step was the elution from the MIP, which was easily performed by methanol. In order to assess whether the MIP specifically retained triazines during the whole SPE procedure, the recoveries were compared using the triazine MIP and a similar—but non imprinted—polymer. Recoveries in the range 80–100% were obtained for triazines and main metabolites on the imprinted polymer and lower than 10% on the non-imprinted one. When the experiments were realized with mineral and tap water spiked with three triazines and two metabolites, a strong matrix effect was observed with extraction recoveries lower than 20%. This loss of retention was explained by the presence of cations in the water sample. Because the polymer was synthesized from methacrylic acid possessing carboxylic acid function ($pK_a$ value of 4.65), an ion-exchange between the carboxylate functions of the polymer and the divalent cations present in natural waters occurred during the percolation of the water sample. The consequence of this phenomenon was the removal of the necessary donor groups of hydrogen bonds for the selective retention on MIP. The triazines possessing only acceptor group of hydrogen bonds needed hydrogen from the carboxylic functions of polymer to develop hydrogen bonds. The matrix effect was easily solved by regenerating the interaction sites via an acidic wash before application of dichloromethane. Fig. 6 compares the chromatograms obtained after extraction through a conventional polymeric SPE sorbent and through the terbutylazine MIP for the analysis of an industrial effluent from textile industry spiked with a mixture of triazines and phenylureas. The selectivity that was provided by the MIP for triazines was comparable to that obtained using immunosorbents.

MIPs present a number of advantages compared to antibodies with respect their ease, cost and time of preparation, and a high thermal and chemical stability. They are easy to use for clean-up of samples

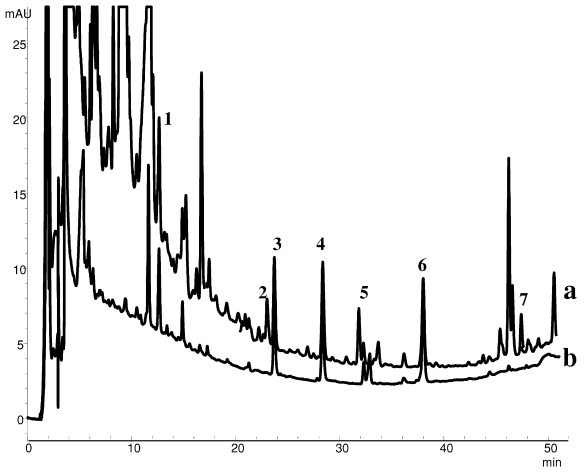


Fig. 6. Comparison between MIP and conventional support. Chromatograms obtained after preconcentration through a styrene divinylbenzene copolymer SPE (a) and after preconcentration through the terbuthylazine MIP cartridge (b) of 50 ml of an industrial effluent spiked at 1 μg/l of a mixture of triazines and phenylureas. 1=DEA, 2=monuron, 3=DET, 4=atrazine, 5=diuron, 6=terbuthylazine, 7=neburon. UV detection at 220 nm. After sample application, the MIP was successively washed with 1 ml of 0.1 *M* HCl before drying, 5 ml of dichloromethane and 3 ml of methanol [102].

dissolved in an organic solvent that should be similar to the porogenic solvent. The direct use in water is actually more delicate. However, the affinity of a MIP for an analyte occurs through interactions with the polymer, the analyte or both. The affinity increases with the number of interacting groups. Each individual interaction is strongly dependent on the properties of the solvent, e.g. protic or aprotic, polarity, dielectric constants, etc. Therefore, molecular imprints have a high potential for application in solid-phase extraction.

## 6. Conclusion and future developments

There is no doubt of the numerous advantages provided by immuno-based sample preparation methods. The large number of recent applications presented in this review have shown that for complex matrices IAE is more rapid, simple, easy-to-use and cost-effective than conventional clean-up methods. Moreover, it can be coupled on-line to separation methods. Commercial ISs are still only available for a restricted range of analytes, mainly mycotoxins, veterinary drugs and drugs of abuse, but today ISs are considered to be a powerful technique which greatly simplifies sample pretreatment, as seen by the increasing number of laboratories who are now developing tailor-made ISs. The weak point is still the antibody production, which is expensive and time-consuming. Making synthetic antibodies is still a challenge that will certainly be overcome in a near future. On another hand, making artificial antibodies via molecular imprinted polymers is a very active area. They have a high potential in the near future even if up to date they have not achieved similar capabilities to natural antibodies. The antigen–antibodies interactions are very complex, and we still have much to learn about this, which will certainly be very useful for the optimization of molecular imprints.

The coupling of highly specific immunoaffinity methods to capillary electrophoresis and microchip technology has proved to be a method of choice for many clinical and forensic applications. The superior selectivity of the immunoextraction combined with the high resolution power of CE coupled to mass spectrometry is a technology that has the power to revolutionize chemical and biological analysis, in many fields such as the environment, forensic analysis, pharmaceuticals, biotechnology, and food.

## References

[1] M.C. Hennion, J. Chromatogr. A 856 (1999) 3.
[2] N. Delaunay, V. Pichon, M.C. Hennion, J. Chromatogr. B 745 (2000) 15.
[3] D.S. Hage, J. Chromatogr. B 715 (1998) 2.
[4] V. Pichon, M. Bouzige, M.-C. Hennion, Anal. Chim. Acta 376 (1998) 21.
[5] J.M. Van Emmon, C.L. Gerlach, K. Kbowman, J. Chromatogr. B 715 (1998) 211.
[6] V. Pichon, M. Bouzige, C. Miege, M.C. Hennion, Trends Anal. Chem. 18 (1999) 219.
[7] D.S. Hage, Clin. Chem. 45 (1999) 593.
[8] Z. Tang, H.T. Karnes, Biomed. Chromatogr. 14 (2000) 442.
[9] D. Tsikas, J. Biochem. Biophys. Methods 49 (2001) 705.
[10] V. Pichon, M.-C. Hennion, in: J. Pawliszyn (Ed.), Sampling and Sample Preparation for Field and Laboratory, Comprehensive Analytical Chemistry, Vol. XXXVII, Elsevier, Amsterdam, 2002, p. 1081.
[11] W. Clarke, J.D. Beckwith, A. Jackson, B. Reynolds, E.M. Karle, D.S. Hage, J. Chromatogr. A 888 (2000) 13.

[12] N.A. Guzman, J. Chromatogr. B 749 (2000) 197.
[13] V. Pichon, L. Chen, N. Durand, F. Le Goffic, M.C. Hennion, J. Chromatrogr 725 (1996) 107.
[14] S. Ballesteros, D. Barcelo, F. Sanchez-Baeza, F. Camps, M.-P. Marco, Anal. Chem. 70 (1998) 4004.
[15] R. Galve, F. Camps, F. Sanchez-Baeza, M.-P. Marco, Anal. Chem. 72 (2000) 2237.
[16] N. Delaunay-Bertoncini, V. Pichon, M.C. Hennion, J. Chromatrogr. A (2003) in press.
[17] H.H. Maurer, C.J. Schmitt, A.A. Weber, T. Kraemer, J. Chromatogr. B 748 (2000) 125.
[18] A.E. Prince, T.S. Fan, B.A. Skoczenski, R.J. Bushway, Anal. Chim. Acta 444 (2001) 37.
[19] D.R. Drieger, P. Sporns, J. Agric. Food Chem. 49 (2001) 543.
[20] V. Pichon, L. Chen, M.C. Hennion, R. Daniel, A. Martel, F. le Goffic, J. Abian, D. Barcelo, Anal. Chem. 67 (1995) 2451.
[21] M. Cichna, P. Markl, P. Knopp, R. Niessner, J. Chromatrogr. A 919 (2001) 51.
[22] B. Spitzer, M. Cichna, P. Markl, G. Sontag, D. Knopp, R. Niessner, J. Chromatrogr. A 880 (2000) 113.
[23] C. Stalikas, D. Knopp, R. Niessner, Environ. Sci. Technol. 36 (2002) 3372.
[24] M. Altstein, A. Bronshtein, B. Glattstein, A. Zeichner, T. Tamiri, J. Almog, Anal. Chem. 73 (2001) 2461.
[25] M.L. Nedved, S. Habibi-Goudarzi, B. Ganen, J. Henion, Anal. Chem. 68 (1996) 42238.
[26] C.K. Holtzapple, S.A. Buckley, L.H. Stanker, J. Agric. Food Chem. 47 (1999) 2963.
[27] H. Yuan, W.M. Mullet, J. Pawliszyn, Analyst 126 (2001) 1456.
[28] N. Delaunay-Bertoncini, V. Pichon, M.C. Hennion, Chromatographia 53 (2001) S224.
[29] E. Watanabe, Y. Yoshimura, Y. Yuasa, H. Nakazawa, Anal. Chim. Acta 433 (2001) 199.
[30] A. Krasnova, V. Pichon, M.C. Hennion, Chromatographia (2003) in press.
[31] A. Houben, E. Meulenberg, T. Noij, C. Gronet, P. Stoks, Anal. Chim. Acta 399 (1999) 69.
[32] S. Perez, I. Ferrer, M.-C. Hennion, D. Barcelo, Anal. Chem. 70 (1998) 4996.
[33] M. Bouzige, V. Pichon, M.-C. Hennion, J. Chromatogr. A 823 (1998) 197.
[34] R.K. Bentsen-Farmen, I.V. Botnen, H. Noto, J. Jacob, S. Ovrebo, Int. Arch. Occup. Environ. Health 72 (1999) 161.
[35] M. Bouzige, V. Pichon, M.C. Hennion, Environ. Sci. Technol. 33 (1999) 1916.
[36] S. Ouyang, Y. Xu, Y.H. Chen, Anal. Chem. 70 (1998) 931.
[37] S. Ikegawa, N.M. Ria Isriyanthi, M. Nagata, K. Yahata, H. Ito, N. Mano, J. Goto, Anal. Biochem. 296 (2001) 63.
[38] E. Schoenzetter, V. Pichon, D. Thiebaut, A. Fernandez-Alba, M.C. Hennion, J. Microcol. Sep. 12 (2000) 323.
[39] M. Bouzige, PhD thesis, University of Paris VI, March 2000.
[40] S. Kerrigan, D.E. Brooks, J. Immunol. Methods 224 (1999) 11.
[41] F. Kondo, Y. Ito, H. Oka, S. Yamada, K. Tsuji, M. Imokawa, Y. Niimi, K. Harada, Y. Ueno, Y. Miyazaki, Toxicon 40 (2002) 893.
[42] W. Clarke, A. Roy Chowdhuri, D.S. Hage, Anal. Chem. 73 (2001) 2157.
[43] J. Dalluge, T. Hankemeier, R.J.J. Vreuls, U.A.Th. Brinkman, J. Chromatogr. A 830 (1999) 377.
[44] N.H.H. Heegart, S. Nilsson, N.A. Guzman, J. Chromatogr. B 715 (1998) 29.
[45] N.A. Guzman, R.J. Stubs, Electrophoresis 22 (2001) 3602.
[46] T. Stroink, E. Paarlberg, J.C.M. Waterval, A. Bult, W.J.M. Underberg, Electrophoresis 22 (2001) 2374.
[47] D.H. Thomas, D.J. Rakestraw, J.S. Schoeniger, V. Lopez-Avila, J. van Emon, Electrophoresis 20 (1999) 57.
[48] E. Thordarson, J.A. Jonsson, J. Emneus, Anal. Chem. 72 (2000) 5280.
[49] W. Haasnot, A. Kemmers-Voncken, D. Samason, Analyst 127 (2002) 82.
[50] J. Onorato, J.D. Henion, Anal. Chem. 73 (2001) 4704.
[51] J. Henion, E. Brewer, G. Rule, Anal. Chem. 70 (1998) 650A.
[52] J. Cai, J. Henion, Anal. Chem. 68 (1996) 72.
[53] J. Rolcik, R. Lenobel, V. Siglerova, M. Strnad, J. Chromatogr. B 775 (2002) 9.
[54] P. Lee Ferguson, C.R. Iden, A.M. McElroy, B.J. Brownawell, Anal. Chem. 73 (2001) 3890.
[55] Z. Wu, J. Li, L. Zhu, H. Luo, X. Xu, J. Chromatogr. B 755 (2001) 361.
[56] C. Miège, M. Bouzige, S. Nicol, J. Dugay, V. Pichon, M.C. Hennion, J. Chromatogr. A 859 (1999) 29.
[57] M. Bouzige, P. Legeay, V. Pichon, M.C. Hennion, J. Chromatogr. A 846 (1999) 317.
[58] M. Zhao, Y. Liu, Y. Li, X. Zhang, W. Chang, J. Chromatogr. B 783 (2003) 401.
[59] J. Gilbert, E. Anklam, Trends Anal. Chem. 21 (2002) 468.
[60] F. Galavano, V. Galofaro, A. Ritieni, M. Bognanno, A. De Angelis, G. Galavano, Food Addit. Contam. 18 (2001) 644.
[61] D. Nilufer, D. Boyacioglu, J. Agric. Food Chem. 50 (2002) 3375.
[62] E.K. Kim, P.M. Scott, B.P. Lau, D.A. Lewis, J. Agric. Food Chem. 50 (2002) 3614.
[63] J.F. Lawrence, C. Menard, J. Yeung, S.B. Rejeb, J. AOAC Int. 83 (2000) 597.
[64] M. Castellari, S. Fabbri, A. Fabiani, A. Amati, S. Galassi, J. Chromatogr. A 888 (2000) 129.
[65] P. Markaki, C. Delpont-Binet, F. Grosso, S. Dragacci, J. Food Prot. 64 (2001) 533.
[66] L. Sibanda, S. De Saeger, C.V. Peteghem, J. Chromatogr. A 959 (2002) 327.
[67] P. Burdaspal, T.M. Lagarda, J. Gilbert, J. AOAC Int. 84 (2001) 1445.
[68] M. Eskola, M. Kokkonen, A. Rizzo, J. Agric. Food Chem. 50 (2002) 41.
[69] E. Dietrich, R. Burk, E. Schneider, E. Martlbauer, J. AOAC Int. 84 (2001) 1649.
[70] N. Delaunay, V. Pichon, J.P. Le Caer, M.C. Hennion, Anal. Chim. Acta 407 (2000) 173.
[71] L. Ten-hage, N. Delaunay, V. Pichon, A. Couté, S. Puiseux-Dao, J. Turquet, Toxicon B 38 (2000) 1043.
[72] C. Rivasseau, M.C. Hennion, Anal. Chim. Acta 399 (1999) 75.

[73] J.F. Lawrence, C. Menard, J. Chromatogr. A 922 (2001) 111.
[74] A.A.M. Stolker, P.L.W.J. Schwillens, L.A. Van Ginkel, U.A.Th. Brinkman, J. Chromatogr. A 893 (2000) 55.
[75] B.A. Rachid, P. Kwasowski, D. Stevenson, J. Pharm. Biomed. Anal. 21 (1999) 635.
[76] F.J. Dos Ramos, J. Chromatogr. A 880 (2000) 69.
[77] S.A. Reuschel, D. Eades, R. Foltz, J. Chromatogr. B 733 (1999) 145.
[78] T. Grobosch, U. Lemm-Ahlers, J. Anal. Toxicol. 26 (2002) 181.
[79] P.B. Carrasco, R. Escola, M.P. Marco, J.M. Bayona, J. Chromatogr. A 909 (2001) 61.
[80] H. Fenet, E. Beltran, B. Gadji, J.F. Cooper, C.M. Coste, J. Agric. Food Chem. 49 (2001) 1293.
[81] M.C. Descroches, J.C. Mathurin, Y. Richard, P. Delahaut, J. de Ceaurriz, Rapid Commun. Mass Spectrom. 16 (2002) 370.
[82] S. Feng, M.A. ElSohly, M.Y. Salem, J. Anal. Toxicol. 24 (2000) 395.
[83] X. Li, D.M. Lubman, D.T. Rossi, G.D. Nordblom, C.M. Barsdale, Anal. Chem. 70 (1998) 498.
[84] C. Sheedy, J.C. Hall, J. Agric. Food Chem. 49 (2001) 1151.
[85] T. Tsutsumi, S. Nagata, A. Hasegawa, Y. Ueno, Food Chem. Toxicol. 38 (2000) 593.
[86] W.H. Newsome, M. Abott, J. AOAC Int. 89 (1999) 666.
[87] B. Sellergren, Molecularly imprinted polymers in solid-phase extractions, in: F. Lanza (Ed.), Molecularly Imprinted Polymers—Man-made Mimics of Antibodies and Their Applications in Analytical Chemistry, Techniques and Instrumentation in Analytical Chemistry, Vol. 23, Elsevier, Amsterdam, 2001, p. 355, Chapter 15.
[88] F. Lanza, B. Sellergren, Chromatographia 53 (2001) 599.
[89] O. Ramström, C. Skudar, J. Haines, P. Patel, O. Brüggemann, J. Agric. Food Chem. 49 (2001) 2105.
[90] M. Zi-Hui, L. Qin, Anal. Chim. Acta 435 (2001) 121.
[91] C. Baggiani, C. Giovanolli, L. Anfossi, C. Tozzi, J. Chromatogr. A 938 (2001) 35.
[92] T. Kubo, K. Hosoya, Y. Watabe, T. Ikegami, N. Tanaka, T. Sano, K. Kaya, J. Chromatogr. A (2003) in press.
[93] C. Berggren, S. Bayoudh, D. Sherrington, D. Ensing, J. Chromatogr. A 889 (2000) 105.
[94] A. Zander, P. Findlay, T. Renner, B. Sellergren, Anal. Chem. 70 (1998) 3304.
[95] N. Masqué, R.M. Marcé, F. Borrull, P.A.G. Cormack, D.C. Sherrington, Anal. Chem. 72 (2000) 4122.
[96] A. Martin-Esteban, E. Turiel, D. Stevenson, Chromatographia 53 (2001) S434.
[97] Q.-Z. Zhu, P. Degelman, R. Niessner, D. Knopp, Environ. Sci. Technol. 36 (2002) 5411.
[98] M.T. Muldoon, L.H. Stanker, Anal. Chem. 69 (1997) 803.
[99] E. Turiel, A. Martin-Esteban, P. Fernandez, C. Perez-Conde, C. Camara, Anal. Chem. 73 (2001) 5133.
[100] B. Bjarnason, L. Chimuka, O. Ramström, Anal. Chem. 71 (1999) 2152.
[101] I. Ferrer, F. Lanza, A. Tolokan, V. Horvath, B. Sellergren, G. Horvai, D. Barcelo, Anal. Chem. 72 (2000) 3934.
[102] F. Chapuis, V. Pichon, F. Lanza, B. Sellergren, M.C. Hennion, J. Chromatogr. (2003) in press.
[103] R. Koeber, C. Fleischer, F. Lanza, K.S. Boos, B. Sellergren, D. Barcelo, Anal. Chem. 73 (2001) 2437.
[104] T. Pap, V. Horvath, A. Tolokan, G. Horvai, B. Sellergren, J. Chromatogr. A 973 (2002) 1.
[105] J. Matsui, K. Fujiwara, S. Ugata, T. Takeuchi, J. Chromatogr. A 889 (2000) 25.
[106] I. Chianella, M. Lotierzo, S.A. Piletsky, E. Tothill, B. Chen, K. Karim, A.P.F. Turner, Anal. Chem. 74 (2002) 1288.
[107] I. Ferrer, D. Barcelo, Trends Anal. Chem. 18 (1999) 180.
[108] P. Martin, I.D. Wilson, G.R. Jones, Chromatographia 52 (2000) S19.