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Review

Immunoaffinity solid-phase extraction for the trace-analysis of low-molecular-mass analytes in complex sample matrices

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Abstract

Immunoaffinity solid-phase extraction (SPE) sorbents, so-called immunosorbents (ISs), are based upon molecular recognition using antibodies. Thanks to the high affinity and high selectivity of the antigen–antibody interaction, they allow a high degree of molecular selectivity and have shown to be a unique tool in the sample preparation area these last few years. Extraction and clean-up of complex biological and environmental aqueous samples are achieved in the same step and from large volumes when required. Their application to extracts from solid matrixes is solvent-free and more simple than any other clean-up procedure. Single analytes can be targeted, but since an antibody can also bind one or more analytes having structure similar to the one used for its preparation, ISs have been developed for targeting a single analyte and its metabolites. The cross-reactivity was also exploited for developing ISs that could selectively extract a whole class of structurally related compounds. This review describes the current technology used for the synthesis of the ISs, their properties and their field of application. The different parameters governing the antigen–antibody interactions and the solid-phase extraction process are discussed. Emphasis is given to the optimisation of the SPE sequence, especially to the desorption and regeneration steps. The importance of the capacity and its relationship with the analytes recovery and breakthrough volumes is highlighted for class-specific ISs. Multi-class-selective ISs are also presented. Validation studies are reviewed using various certified reference materials. Relevant examples, involving combination with chromatography in both off-line and on-line mode, illustrate the high selectivity provided in various complex matrixes. Miniaturisation is also described, since it allows high throughput of samples.

Keywords: Reviews; Immunoaffinity extraction; Trace-analysis; Organic compounds

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1. Introduction

Despite the advances in the development of highly sensitive analytical instrumentation for the end point determination of analytes in environmental or biological samples, a pre-treatment is usually necessary in order to extract and isolate the analytes of interest in complex matrixes. Conventional liquid–liquid extraction (LLE) has dropped dramatically following the obligatory reduction of chlorinated solvent usage in analytical laboratories. Solid-phase extraction is nowadays widely accepted as a powerful alternative method for sample preparation. These recent years have witnessed important developments in SPE formats and sorbents [1]. Improved n-alkylsilicas, new highly cross-linked copolymers and graphitized carbons are available which have now the capabilities for extracting organic compounds over a wide range of polarity for screening purpose. However, analyte retention is based on hydrophobic interactions and their selectivity is often too low for trace-analysis in many complex environmental, food or biological samples. Co-extraction of analytes and matrix interferences generally occurs, and this can even become a major problem when analytes are at trace levels and interferences at higher concentration. Additional clean-up procedures are required, but, then the sample pre-treatment involves several steps and consequently the risk of loss or contamination increases and the reliability of the results is reduced.

Obtaining extracts free from matrix interferences in a few steps has been recognized as an important goal since the more selective the SPE step is, the more sensitivity is obtained. Therefore, there is a considerable interest in having highly selective sorbents allowing extraction, concentration and clean-up in a single step. Highly selective SPE sorbents have been introduced these recent years using materials involving antigen–antibody interactions, thus providing selective extraction methods based on molecular recognition. Antibodies are covalently bonded onto an appropriate sorbent to form a so-called immunosorbent. Thanks to the high affinity and high selectivity of the antigen–antibody interactions, they allow a high degree of molecular selectivity and have shown to be a unique tool in the sample preparation area these last few years. Extraction, concentration and clean-up from complex liquid samples are achieved in the same step and from large volumes when required. Their application to extracts from solid matrixes is solvent-free and more simple than any other clean-up procedure.

First ISs have been 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, hormones, peptides, enzymes, recombinant proteins, viruses and subcellular components [2,3]. Obtaining selective antibodies for small size molecules was more difficult and the development of immunochemical methods in the SPE field targeting low molecular weight analytes is rather recent [3–6]. The binding of analyte to antibody is the result of a good spatial complementary and is a function of the sum of intermolecular interactions. Therefore, an antibody can also bind one or more analytes with a structure similar to the analyte which has induced the immune response, and this is the so-called cross-reactivity of antibodies. It is usually considered as a negative

feature for an immunoassay, but it was exploited in extraction, so that ISs have been made for single analytes, single analytes and its metabolites or a class of structurally related analytes [4]. 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 or veterinary drugs such as clenbuterol.

In addition to the selectivity of the immunoextraction, an important parameter is the extraction recovery which is linked to the breakthrough volume and to the capacity. In order to achieve low detection limits the extraction sorbent must allow the preconcentration of analytes from large sample volumes without breakthrough of the analyte. First IS applications dealt with serum or plasma samples, with a limited volume in the range 10–500 μl, and the sample volume was not a relevant parameter [7–10]. This parameter became 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 liquid chromatography (LC) with UV detection [11–17]. Since the breakthrough volume depends on the total number of accessible binding sites and on the strength of the interaction between the analytes and the antibodies, the selection of the materials used for the covalent bonding has become of prime importance since it should allow a high capacity and avoid any kind of non-specific interactions with the analytes.

ISs can be used in off-line procedures in disposable cartridges. Desorption should be then made using a minimum volume in order to obtain a high enrichment factor, and this cannot be achieved using the conventional elution aqueous solution of affinity chromatography. Organic solvents are required for the elution of small molecules. This step is often neglected by some scientists coming from the immunoaffinity field. There is also one interest in coupling extraction on-line with LC [1,18,19]. The first extensive studies demonstrating the advantages of coupling immunoextraction to LC have been made by A. Farjam et al. for the determination of estrogens and nortestoserone (19-α/β) in plasma and urine and aflatoxins in milk and urine [12,13,20–23]. They have developed Sepharose-based ISs which were shown to provide very few non-specific interactions but with the drawback of not being pressure-resistant. Therefore desorption occurred at low pressure followed by a re-concentration onto a second precolumn, often a C_{18} silica precolumn, which was then coupled on-line to the LC system. Another extensive study was made by Pichon et al. who developed several silica-based ISs and demonstrated that a single immunoprecolumn could directly be on-line desorbed using a water–organic solvent mixture and easily regenerated for re-use [4,6,24,25].

The objective of this review is to describe the potential of immunoaffinity extraction in its modern form. The characteristics and properties of immunosorbents are presented with emphasis given to the selection of the sorbents used for the synthesis and to the optimization of the SPE sequence, especially to the desorption and regeneration steps. The different parameters governing the antigen–antibody interactions and the solid-phase extraction process are discussed. The importance of the capacity, the best way to evaluate it and its relationship with the analytes recovery and breakthrough volumes are highlighted. Other aspects such as the influence of the flow-rate and the presence of non-specific interactions are discussed.

Relevant examples, involving combination with chromatography in both off-line and on-line mode, illustrate the high selectivity provided to the sample handling for the trace-analysis of several analytes or groups of analytes in various biological and environmental matrixes. Validation studies are reviewed using various certified reference materials. The specific requirements for on-line coupling with LC are discussed. Miniaturization of precolumns and on-line coupling with micro-LC are also described, since it allows high throughput of samples. The high degree of purification permits an efficient coupling with gas chromatography–mass spectrometry (GC–MS), LC–MS or electrophoresis, as shown by several samples.

2. Physico-chemical characteristic of the immunosorbents

2.1. Antibodies

The first step in making an IS is to design antibodies with the capability of molecular recogni-

tion either for one or a group of analytes. Antibodies belong to the mammalian immune system and are produced in response to the presence of a foreign substance (called antigen). Among the five major types of immunoglobulins (A, D, E, G and M), G-types (IgG) are the simplest ones. Their molecular weight is about 150 kDa with a Stoke's radius of 4 nm and an isoelectric point in the range 4.0–8.6. They contain two identical heavy chains and light chains (containing 450–650 and 214 amino acids respectively) that are covalently linked by disulfide bonds in an Y-shaped structure. Two identical antigen binding sites are located at upper ends of the Y. Both light and heavy chains contain regions with constant and variable sequences from one type of antibody to the next. The variable sequences of both chains are characterized by three hypervariable regions that form the specific complementary-determining regions (CDR). The loops and strands of the light and heavy chains in this region interact with the antigenic determinants via hydrogen bridges, ionic, hydrophobic and Van der Waals forces. Although the various binding forces are relatively weak, the large number of interactions and a suitable geometrical fit between antigenic determinant and binding site of the antibody result in a high binding energy. The antigen–antibody complex formed presents an association equilibrium constant of 10^{8}–10^{12} M^{-1} so that the complex can occur even if one of the two compounds is present at very low concentration.

Because compounds of low molecular weight (<1000) are unable to evoke an immune response, before immunization they have to be modified by binding to a larger carrier molecule, usually a protein such as bovine serum albumin. Very often, it is necessary to introduced a functional group into the selected molecule in order to make possible this coupling. The design of this so-called hapten is very important since the functional group should keep as much as possible of the analyte identity [26]. The immunogen or hapten carrier conjugate contains up to 10–20 haptens linked with a single carrier protein. Upon infection of a mammal, the immune system starts to produce a great number of different types of IgG molecules directed against all foreign parts of hapten carrier conjugate molecules. After an immunization period of a few weeks or months, the serum is collected and the IgG fraction is isolated and purified. The purification can be accomplished using various methods such as precipitation with ammonium sulfate, ion-exchange-chromatography, gel filtration or affinity chromatography. These methods generally result in polyclonal antibodies made of an heterogeneous mixture of antibodies that can bind with the antigen with a variety of strengths, because they are directed against the various antigenic determinants (epitopes) on the antigen. When a small molecule is targeted, one can expect the number of specific epitotes to be low. It is difficult to measure the concentration of specific antibodies in the whole IgG fraction. It is commonly found in the literature that the IgG fraction contains about 15% active antibodies.

In the last 20 years, techniques have also been developed that allow the isolation of a specific lymphocyte cell clone, generally with mice, which produces only one type of IgG. The combination of this clone with carcinoma cells results in a cell line that is easy to culture and allows the production of identical antibodies for long-term. These combination antibody-producing long-lived cell lines are known as hybridomas and give a single type of well-defined antibody known as a monoclonal antibody.

In a first approach polyclonal antibodies are cheaper to obtain, but the method suffers of a lack of reproducibility in terms of time of response of an animal, of quantity and even of specificity. In contrast the production of monoclonal antibodies is costly but guarantees a long-term production of reproducible antibodies that does not require animals for further large-scale production.

2.2. Solid supports

The solid support selected for the immobilization of the antibodies is another key parameter for the design of an IS. Most of the procedures consist of a covalent binding. The selected sorbent should be chemically and biologically inert, easily activated, mechanically stable, uniform in particle size, and have large size pores because antibodies are large molecules. It should be hydrophilic in order to avoid any non-specific interactions, and be pressure resistant for direct use in on-line techniques.

Two types of affinity supports have been shown to

be appropriate for making ISs. The first one includes traditional supports used in immunoaffinity chromatography such as agarose and cellulose [7,12–14,20–23,27–35] or polymers such as polymethacrylate derivatives [15,36]. Many of these sorbents are commercially available. They are characterized by a low back pressure resistance thus requiring application of samples under gravity flow or very low flow-rate. They cannot be operated at high flow-rates because of their limited stability: high pressure generates compacting and fouling. Therefore they are appropriate for use in off-line immunoextraction. They generate poor non-specific interactions, present a good chemical stability and are easily derivatized to introduce functional ligands. For example, CNBr-activated Sepharose, an agarose gel, has been widely used. However, this procedure may present some disadvantages such as the introduction of positively charged groups at neutral pH that induce non-specific interactions (ion-exchange effects), unstable leakage of the antibodies and toxicity of CNBr. The majority of the commercial immunosorbents belongs to this category of low-performance supports under various trade marks. Their use have been described in the literature which includes Easi-extract [11,37,38], Aflascan [39], Aflaprep [40,41], Fumonitest [42], Rida [43,44], and ZearalaTest [45].

The second category of appropriate sorbents is pressure-resistant, so-called high-performance supports, and includes derivatized silica, glass beads and hydrophilic organic polymers (vinyl or polystyrene-based). Silica is the most appropriate because of its stability under high pressure and flow-rates. It can easily be activated, has pore sizes of 5–400 nm in diameter and is hydrophilic to avoid non-specific interactions. In the literature, silica activated with various functional ligands has allowed the covalent antibody bonding. Examples of functional groups are aldehyde and glutardialdehyde [8,15–17,25,26,46–59], ester [9], diol [60–63], dihydrazide [64], carbonyldiimidazole [65,66], aminopropyl-aldehyde [67] and epoxy [68]. The disadvantage of silica-based ISs is the limited pH range. Organic matrixes have been also selected such as AvidGel [16], HiPac [69], Poros S [46,70] and Ultraaffinity-EP [71]. Although they are highly hydrophilic, these supports have lead to non-selective interaction between the sorbents and the analytes, mainly due to π-bonds generated by the polymeric sorbent matrix [15,16,46].

2.3. Antibody immobilization

Ideally, the immobilization conditions should keep the biospecific activity of antibodies. Suitable bonding conditions should be in aqueous media of pH 4–9 (at a pH of 0.5–2 units from the isoelectric point of the antibodies), with a ionic strength of 0.01–0.5 $mol.dm^{-3}$, at a temperature of 4–25°C and with short reaction time (less than 16 h).

The most common approach consists in a covalent bonding of the antibodies. This can be achieved by reacting free amine groups of the antibodies with supports that contain reactive epoxyde or aldehyde groups on their surface or that have been activated using carbonyldiimidazole, cyanogen bromide or N-hydrosuccinimide. It results in a random orientation of antibodies bonded to the support. This multiple antibody orientation on the surface may affect the accessibility of the binding site. Using bonding of antibodies via their carbohydrate moieties located on the Fc region (heavy chain), oriented immobilization of antibodies can be achieved with good steric accessibility of antigen binding sites [72]. This is obtained by mild oxidation of these moieties with periodate or enzymatic systems to produce aldehyde residues which can react with a hydrazide or amine-containing sorbent. In a study, oriented immobilization of antibodies against cytokinins did not exhibit crucial effects comparing to a random immobilization via a spacer on AffiGel [33]. In the case of cytokinins, which are rather small molecules, a several carbon atom spacer arm allows enough conformational freedom for the antibody and accessibility for the small molecule to form the antigen–antibody complex. Another way to orient the immobilization is to use sulfhydryl groups that are generated during the production of antibody Fab fragments [73,74].

Non-covalent binding can also be used to couple antibodies to the sorbent. Streptavidin supports and Protein A- or G-based sorbent can interact with these proteins [69,70,75,76]. The protein G and the related protein A support bind antibody at the Fc region which orients antibody with the antigen binding sites away from the surface and towards solution. The

binding is quite strong under physiological conditions but can be easily disrupted by decreasing the pH of the surrounding solution. In general, frequent replacement of the antibodies is necessary and this method requires much larger amounts of antibodies than covalent immobilization.

Another method, the so-called sol–gel method, consists in immobilizing antibodies in the pores of a hydrophilic glass matrix. Compared with other immobilization techniques, it offers a number of advantages. Antibodies, in this case, retain their affinity and specificity to the antigen because the bonding is carried out under mild conditions, the pores of the matrix are filled with an aqueous solution, so that the orientation of the trapped antibodies is not fixed and access to the CDRs is not sterically hindered. This technique avoids activation and bonding reactions as required for covalently binding and thus all affinity losses associated with the chemical reactions. The hydrophilic matrix reduces non-specific adsorption of apolar analytes and narrow pores prevent the diffusion of size large molecules and of bacteria or proteolytic enzymes. So, no bacteriostatic agent must be added for storage. Immunosorbents against polycyclic aromatic hydrocarbons (PAHs) have been prepared with this method [77,78].

3. Solid-phase extraction properties of the immunosorbents

3.1. Bonding density

The bonding density is defined by the number of antibodies linked to the surface of the sorbent. It is usually expressed in milligrammes per millilitre of bed sorbent or milligrammes per gramme of sorbent. It is an important feature because it determines the future antigen-binding capacity and it can be measured experimentally. It depends first of the specific surface area of the sorbent accessible for the immobilization of the antibodies. Supports with small pore sizes have high surface areas, but they are not accessible for the large antibody molecules. On the other hand, supports with large pore sizes have good accessibility but small surface areas. So, a compromise is necessary. A study realized with pore sizes of 10–400 nm has suggested that 50 nm pores were best suited for immunoextraction of low molecular weight peptides [65]. Fifty nanometres corresponds to about 10 times the antibody diameter. The reasonable minimal pore size is in the order of 2–5 times antibody diameter for immunoextraction of small molecules.

A second factor is the degree of purification of the antibody solution. Several studies have used non purified antisera for the bonding [7,17,47,48,75]. One can expect a reduced bonding density due to co-immobilization of other proteins. In contrast, better antibody density will occur using purified IgG fraction. In the literature the purification is mainly performed using a Protein A or G affinity column or ammonium sulfate precipitation. Although it is theoretically possible to isolate specific antibodies in the IgG fraction, it is not often described because this can only be performed using a structurally related analyte within the targeted group which shows enough affinity for retaining antibodies but not too much for allowing their desorption without organic solvent. Using monoclonal antibodies, these drawbacks are circumvented. Another strategy consists in bonding only the moiety of the antibodies which contains the antigen binding site (e. g. the Fab or Fv fragments) [65,76]. As these fragments have molecular weight of about 25 kDa, the bonding density may be notably improved.

In the literature, different bonding densities are encountered. Values can be compared for agarose and silica-based ISs. Table 1 presents the bonding densities found for agarose and silica depending on monoclonal or polyclonal antibodies. Average values are about 5 mg/ml for agarose gel and 20 mg/g of silica and are not really dependent whether monoclonal or polyclonal antibodies are used. As the efficiency rate of the immobilization step is generally between 80 and 100%, it is relatively easy to know approximately the amount of antibodies necessary to design an immunosorbent with a pre-determined bonding density. The determination of the amount of antibodies immobilized onto a matrix consists in measuring the concentration of the antibodies in the bonding solution by spectrophotometry UV at 280 nm. Therefore, the antibody surface coverage of the support can be calculated. For example, an anti-atrazine immunosorbent that has been bonded with 9

Table 1
Target analytes used for the preparation of the antibodies and bonding densities encountered in literature for agarose and silica supports coupled to polyclonal or monoclonal antibodies

Target analyte	Sorbent	Antibody type	Bonding density	Reference
Thiabenzadole	Agarose	Monoclonal	0.8 mg/ml	[79]
Chloramphenicol			5.4 mg/ml	[27]
Forskolin			6.5 mg/ml	[30]
Ampicillin, Cloxacillin			7.2 mg/ml	[80]
β-19-Nortestosteron		Polyclonal	10 mg/ml	[13]
Diethylstilbestrol			5 mg/ml	[14]
Chloramphenicol			0.9 mg/ml	[29]
Aflatoxin B1			3.8 mg/ml	[31]
1α,25-Dihydroxyvitamin D_3			2 mg/ml	[32]
Cytokinins			13.3 mg/ml	[71]
Anabolic steroid			3 mg/ml	[81]
Avernectin B1			10 mg/ml	[82]
β-19-Nortestosteron			0.6 and 4.6 mg/ml	[12]
Oestrogen			10 mg/ml	[20]
Carbendazim	Silica	Monoclonal	4.5 mg/g	[46]
Atrazine			9 mg/g	[60]
Atrazine			10 mg/g	[61]
Δ^9-Tetrahydrocannabinol		Polyclonal	0.5 mg/g	[68]
Atrazine, Chlortoluron			50 mg/g	[25]
Atrazine, Isoproturon			5–25 mg/g	[16]
Toluene			16.5 mg/g	[64]
Isoproturon			20 mg/g	[50]
Fluorene			20 and 25 mg/g	[52]

mg of antibodies per gramme of silica (25 m^2/g) had an antibody coverage of 0.14 monolayers [60].

3.2. Immunosorbent capacity

The capacity is defined as the total number of immobilized active antibodies (specific for the target analyte) which cannot be measured directly because the concentration of active antibodies in the IgG fraction is not known. Moreover, the random orientation and the steric hindrances may prevent the antigen from access to the CDRs. But, for a standard immobilization procedure, the theoretical binding capacity can be estimated. If, for example in the case of polyclonal antibodies, with the hypothesis that (i) 10% of a purified IgG fraction is considered as specific, (ii) the antibody activity is fully retained and (iii) active site accessibility is complete, then taking into account for the bonding density, the molecular weight of an antibody (150 kDa) and two binding sites per antibody, it is possible to estimate the theoretical capacity of an immunosorbent.

In many studies, the binding capacity is measured using the antigen. One reported method was using frontal analysis [83]. A more common method consists in measuring the amount of analyte adsorbed as a function of the analyte concentration in the percolated sample. Fig. 1 represents a capacity curve obtained on an anti-atrazine IS using silica with a

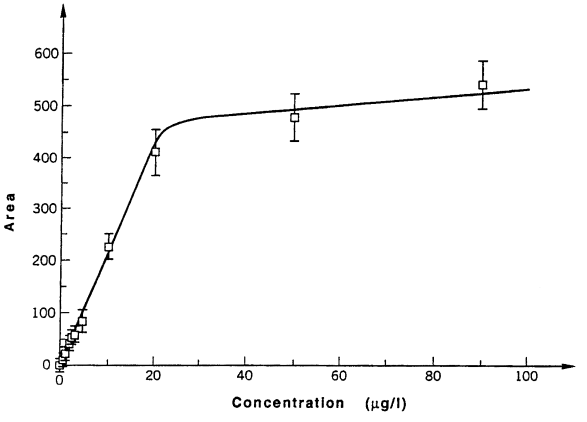

Fig. 1. Binding-capacity curve of an anti-atrazine IS as measured by the amount of atrazine adsorbed onto the IS as a function of the analyte concentration in the percolated samples [6].

pore size of 50 nm [6]. This curve has a Langmuir shape with a linear part and a plateau where the capacity can be easily estimated experimentally. Table 2 reports the capacities which were measured with the analyte-antigen. Whereas the bonding densities are similar, the capacities were strongly dependent on the antibody and were respectively 0.8, 2.5 and 19.3 μg/g of IS prepared from anti-simazine, anti-atrazine and anti-isoproturon antibodies. These differences also showed that concentrations of active antibodies may be strongly different.

In general, the experimental capacity is lower than the theoretical one. Although Hydrazide gel allows an oriented immobilization, the experimental capacity of an anti-atrazine IS was 3.8 μg/ml instead of a theoretical capacity of 6.2 μg/ml, whereas the same capacity values (12.9 μg/ml) were found for the same antibody bonded onto cellulose [35]. Another study reported a difference between experimental and theoretical capacities (21 versus 34 μg/ml of sorbent) for an anti-atrazine prepared using cellulose beads [34].

When an IS targets several structurally related analytes, the definition of the capacity is more complex. The competition process between the compounds in a group can be illustrated by the measurements of capacities using mixture of these structural analogs. This was achieved with an anti-isoproturon IS [50]. Table 3 presents the capacities measured by the amount of each phenylurea adsorbed on the IS after percolation of 50 ml of water spiked at 100 μg/l of each analyte. The isoproturon capacity was still much higher than the capacities measured for the other phenylureas, which depend on the affinity of the polyclonal antibodies for these compounds, but it was lower than that obtained when isoproturon is percolated alone on this IS (11.8 instead of 19.3 μg/g). A remarkable result was that the sum of the capacities for the ten phenylureas in a mixture was close to the capacity measured with the antigen alone.

Table 2
Capacity of three immunosorbents measured using the analyte-antigen [50][a]

Antibody	Immunosorbent		
	IgG[b]	Capacity[c]	Capacity[d]
Isoproturon	35±2	19.3±0.6	0.55±0.04
Atrazine	47±2	2.5±0.2	0.053±0.005
Simazine	54±2	0.80±0.05	0.015±0.002

[a] Silica with 50 nm pore sizes.
[b] Amount of IgG in mg/g of silica-based sorbent.
[c] Capacity in μg/g of IS.
[d] Capacity in μg/mg of IgG.
Capacity measurements with increasing concentrations (5–150 μg/l) of analytes in 50 ml of LC-grade water for isoproturon, and in 25 ml for atrazine and simazine.

Table 3
Capacities measured for each phenylurea after the percolation of 50 ml of water spiked at 100 μg/l of each of the 10 phenylureas in mixture [50]

Analyte	Experimental capacity (μg/g)
Buturon	0.52±0.05
Chlorbromuron	1.21±0.08
Chlortoluron	0.58±0.05
Difenoxuron	0.95±0.06
Diflubenzuron	0.51±0.04
Fenuron	0.29±0.02
Isoproturon	11.8±0.2
Linuron	0.72±0.07
Metoxuron	0.40±0.05
Monuron	0.49±0.05

3.3. Immunoextraction procedure

ISs are generally packed into disposable cartridge or LC precolumn. Very few applications using capillary column or filter membrane have been related [84,85]. Fig. 2 represents the three steps of an immunoextraction sequence.

The most important parameters in this sequence are the sample volume which can be percolated without loss in recovery and the desorption conditions.

3.3.1. Recoveries

According to the many examples described in the literature for the trace extraction from aqueous matrixes, the sample volume can vary from 25 μl (determination of cortisol in serum [8]) to 1 l (determination of chloramphenicol in milk [66]). During an extraction process, recoveries are determined and linear curves of calibration are plotted in order to allow quantification of unknown samples.

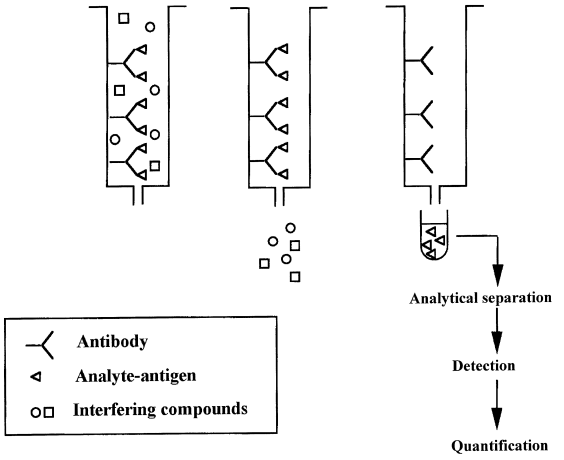

Fig. 2. Off-line procedure used for the immunosample pretreatment on immunosorbents. (a) percolation of the sample; (b) washing to eliminate the nonretained compounds; (c) elution of compounds retained by the immobilized antibodies.

The high affinity for the analyte-antigen is seen by the high sample volume that can be handled without breakthrough. The group of Stevenson reported that 1 l of water containing 100 ng/l of isoproturon could be handled with a 100% recovery using 1 g of silica modified by 200 μl of crude anti-isoproturon serum [49]. High breakthrough volumes were also reported using anti-triazine or anti-phenylureas ISs when water is spiked with the analyte-antigen alone [24,50].

Two parameters can affect the recovery which are the IS capacity and/or affinity of antibodies towards compounds. Insufficient retention induces a low breakthrough volume and an incomplete recovery. As seen above, capacities are rather low compared to that of other reversed-phase sorbents for which overloading the capacity is unlikely to occur. So, it is essential to verify if experimental conditions do not induce an overloading of the IS capacity or of the breakthrough volume, because then calibration curves are no longer linear [4]. In general, immunoaffinity extraction is used to extract compounds at trace levels. So, the greater the breakthrough volume and the capacity are, the better the limit of detection is. This important point has to be kept in mind. A compromise between cost and sufficient amount of bonded-material has to be found.

3.3.2. Elution conditions

During the sample percolation, the antibody–antigen complex is formed primarily by electrostatic forces, which attract and orient them. Then, this elicits the formation of secondary hydrogen bonds, pulling the molecules closer together, and excluding water. Finally, Van der Waals forces are initiated to form a stable non-covalent bond, the target residue therefore being adsorbed [86]. To desorb the analyte, this complex has to be disrupted. As the biochemical interaction energies are high, the desorption will occur only by notably modifying the experimental conditions. Different approaches are possible to induce elution of the analyte(s): displacer agents, chaotropic agents, temperature increase, pH variations or water–organic modifier mixtures. Table 4 presents several elution conditions encountered in the literature.

A displacer is a highly concentrated cross-reacting molecule able to induce a biospecific desorption. The displacer molecules compete with the bound analyte molecules and the large excess of displacer ensures a quantitative desorption of the analyte. For optimum performance, the displacer has to meet several criteria: (i) a high cross reactivity with the immobilized antibodies, (ii) a retention time significantly different from that of the analytes because the large excess causes a prominent peak which can easily interfere with the detection of the analytes, (iii) a good stability and a high purity because impurities present at level as low as 0.01–0.1% can disturb the chromatogram, and (iv) a weak price, a non-toxic characteristic, an absence in real samples and a low detectability comparing to the analytes. Moreover, a large volume of elution solution is necessary which imposes a reconcentration on a classic SPE support before analysis. This type of desorption was used in an on-line set up for the determination of the anabolic hormone β-19-nortestosterone and its metabolites α-19-nortestosterone in calf urine by Farjam et al. [12]. Urine was directly loaded on the immunoprecolumn packed with Sepharose-immobilized polyclonal antibodies against β-19-nortestosterone. Because Sepharose was not pressure resistant it was necessary to use a second C_{18} precolumn for the selective desorption and re-concentration of analytes from the immunocolumn. A displacing solution containing 5% acetonitrile and a high concentration

Table 4
Examples of elution conditions of analytes from IS encountered in the literature

Technique	Eluent	Analyte	Matrix	Ref.
Displacer	E3 and E2Ac steroids	Oestrogen	Urine	[20]
	norgestrel	β-19-Nortestosterone	Urine, bile, muscle, meat, liver, kidney	[13] [12]
Chaotropic solution	6.3 *M* urea in buffer	Proinsulin	Horse serum	[88]
pH	Acetic acid 2%	LSD	Human urine	[69]
	Formic acid 0.1% (pH 2.6)	Carbofuran	Crude potato	[15]
	Citric acid 0.1 M	Cytokines	Human body fluid	[76]
	0.2 *M* glycine–HCl	Atrazine	Water	[35]
pH +organic modifier	Acetonitrile–trifluoroacetic acid 0.1%	S-8921 (M_w=540)	Human urine	[89]
	Ethanol–buffer (1/1) (pH 2)	Isoproturon, Chlortoluron	River water	[47]
	Methanol–propionic acid 1 *M* (1/1)	Dexamethasone	Horse urine	[36]
organic modifier	Acetonitrile	Aflatoxin M1	Cheese	[37]
	Acetonitrile–water (70/30)	PAHs	Water, sludges	[55] [58]
	Acetonitrile–water (40/60)	Pyrene	Water	[78]
	Acetonitrile–water (30/70)	Okadaic acid	Mussels, algae	[59]
	Acetone–water (95/5)	Oestradiol–17–β	Bovine plasma, milk	[7]
	Ethanol–water (80/20)	Clenbuterol	Beef liver, meat	[43]
	Ethanol–buffer (50/50)	Chlortoluron	Water, urine, plasma	[17]
	Ethanol–buffer (40/60)	5,5-Diphenylhydantoin	Human plasma	[9]
	Ethanol–buffer (35/65)	Isoproturon	River water	[67]
	Methanol	Ochratoxin A	Blood, serum, milk, Foodstuffs	[38]
	Methanol–buffer (90/10)	Aflatoxin M1	Milk	[28]
	Methanol–water (80/20)	Fumonisin B1	Canned and frozen sweet corn	[42]
	Methanol–water (80/20)	Microcystins	Water, cells	[57]
	Methanol–water (70/30)	Triazines, phenylureas	Water	[25]
	Methanol–water (50/50)	Dexamethasone	Bovine urine	[90]
	Methanol–water (30/70)	Imidazolinone	Urine, kidney, corn, wheat plant	[91]

of the cross-reacting steroid hormone norgestrel was used for the desorption and the transfer to a second C_{18} precolumn. Then the classical on-line C_{18} precolumn/C_{18} analytical column coupling was used. The chromatogram represented in Fig. 3 corresponds to the on-line analysis of 26.5 ml of urine, filtered and diluted in the same volume of water containing 10% of acetonitrile and spiked with 300 ng/l of each α- and β-19-nortestosterone. The selectivity of the immunoextraction is clearly demonstrated by this chromatogram.

Solutions of chaotropic ions disrupt the water structure around an antibody, which induces a rupture of the hydrophobic interactions in the antibody structure and between the analyte and the antibody. The most common chaotropic ions are chloride, iodide, perchlorate and thiocyanate ions at concentration of 1.5–8 *M*. In fact, the various aqueous solutions, which have been successfully applied for many years for the desorption of proteins from IS were shown to be unable to desorb small molecules [12,16,87]. Probably, the desorption of the protein is based mainly on changes in the structure of the bound protein (partial denaturation), and not on the changes in the structure of the immobilized antibodies. Therefore, the elution of the small molecules, which are not sensitive to denaturation, needs much more rigorous conditions.

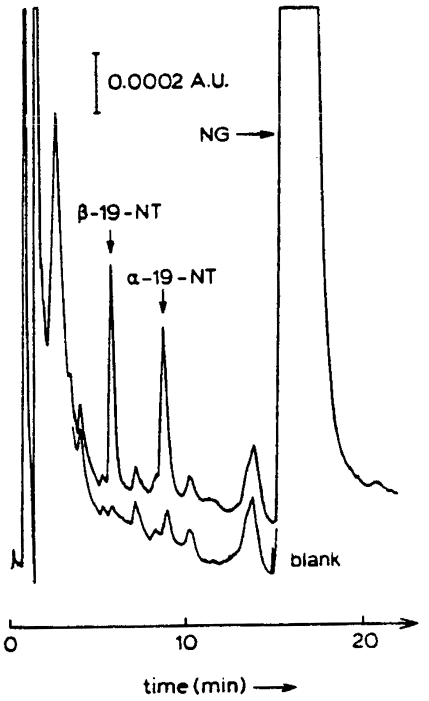

Fig. 3. Chromatogram of a calf urine sample spiked with 300 ng/l of each β-19-nortestosterone and α-19-nortestosterone, and the corresponding blank urine. The samples were filtered and diluted with an equal amount of acetonitrile–water (10/90). An aliquot of 53 ml (containing 26.5 ml urine) was loaded on the precolumn (10×10 mm I.D.) packed with an immunosorbent made of sepharose and immobilized antibodies against β-19-nortestosterone. On-line elution with a displacer according to the text with permission from Ref. [12].

With a temperature increase from 4 to 43°C, the dissociation constant of antibody–analyte interactions may be increased by two orders of magnitude. This method was not applied to the immunoextraction of small molecules.

Elution with low pH solutions is often carried out to desorb small molecules from IS. But, three units of pH from the antibody isoelectric point are required. This kind of elution (shift of pH without altering the ionic strength) avoids damaging labile antibodies. One inconvenient is that large volumes are required for complete desorption so that these solutions have some drawbacks when used in off-line procedures. Further evaporation of large volume of aqueous buffers is then required. They have been described in on-line procedure with reconcentration on a second precolumn [29,45]. Common elution buffers consist in a 0.05 *M* phosphate or in formic acid solution at pH 2.5.

A water–organic modifier mixture is often required to elute small molecules from IS. The presence of non-polar solvents reduces the hydrophobic binding component of the antibody–analyte interaction. However, it also affects the stability of hydrophobic bonds, maintaining the antibody tertiary structure and results in the release of the antigen. This harsh eluting condition can irreversibly denature antibodies but, as small volume are required, contact times can be minimized. In most of the off-line procedures, including those recommended using the commercial ISs, desorption is achieved with a high percentage of an organic solvent (see Table 4). The combined effects of a low pH with an organic solvent allowed to desorb phenylureas in only 2 ml of a PBS solution containing 50% of ethanol at pH 2 [47].

3.3.3. Immunosorbent regeneration and reusability

Immunosorbent reusability involves a possible regeneration. It depends on the concentration and conformation of the bonded antibodies and the chemical stability of the support and bonding because deterioration can occur under external pressure or by swelling with the different solvents. The last two points can be circumvented by an appropriate support choice. The most critical step is the dissociation of the antibody–analyte complex, which is induced by a solvent change. The key parameter to follow the evolution of an immunosorbent is its capacity because it is defined as the total number of immobilized active antibodies. Several studies have been performed to characterize the effects of temperature, pH and elution solution. It has been observed that ISs maintained at 4°C could be recycled approximately 200 times before a detectable loss in capacity whereas only 50 cycles could be applied when ISs were operated at room temperature [76]. Using a too low pH for desorption (2.3 instead of 2.5) improved the elution but also induced a more rapid loss of antibody activity [60]. Narhi et al. have compared the effect of three elution solutions on the conformation of monoclonal antibodies [93]. The antibodies remained almost completely folded in the 0.1 *M* glycine at pH 2.9, with an intact β-sheet

secondary structure. In a 7 *M* urea solution with 50 m*M* sodium acetate at pH 4.0, the antibody was partially unfolded, with some of the β-sheet structure converted to disordered structure. In 6 *M* guanidine HCl with 50 m*M* sodium acetate at pH 4.0, the antibody was completely unfolded, with no secondary and tertiary structure present. The antibodies exposed to glycine or urea were refolded into phosphate-buffered saline (PBS) solution which is widely used in immunoaffinity for regeneration and storage because it is closed to physiological conditions. Other studies have confirmed the low denaturation effect of glycine buffer [29,66]. Glycine buffer and methanol or ethanol have been compared. Fig. 4 shows the evolution of the total column capacity with the two eluents. In contrast to glycine buffer, using methanol induces a significant drop of immunosorbent capacity. The denaturation was not irreversible, but the kinetic of regeneration was quite long (a few days). The observed differences between glycine and organic solvent was explained by the fact that glycine buffer reduces the interactions between analyte and antibody by variation of the pH while in addition the organic solvent denatures the antibodies. Other studies have pointed out some decreases in effectiveness induced by organic solvent [8,13,16,38,82].

The IS can be re-used several times without apparent loss in recoveries if the initial capacity was high enough. Therefore several studies using commercial IS have reported the regeneration conditions for reusability of the commercial ISs. A anti-clenbuterol IS could be regenerated for at least 18 times for the clean-up of liver extracts with an elution carried out using 2 ml of 80% ethanol in water [44]. Regeneration was obtained by 5 ml of water followed by 5 ml of PBS at pH 7.4 and storage at 4°C. Before each sample analysis, 3 ml of an aqueous

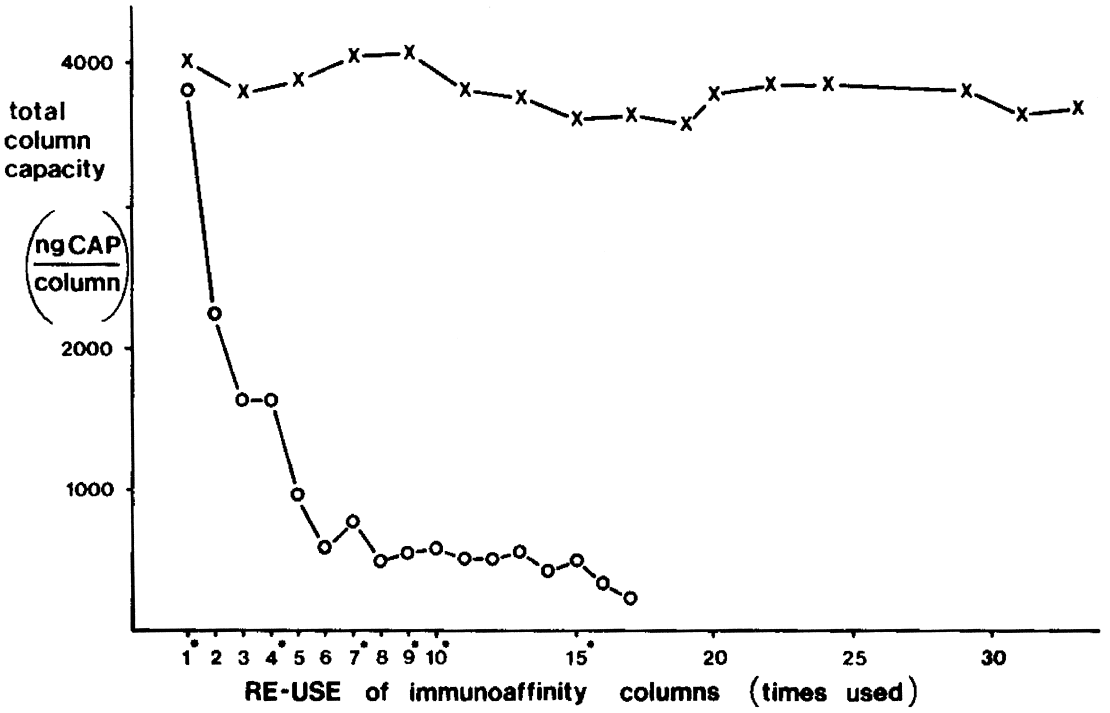

Fig. 4. The effect of storage of the immunosorbent in PBS at 4°C and of the type of eluent used on the total column capacity by reuse of the immunoaffinity columns. The successive antibody-mediated extraction cycles (saturation, washing, elution and regeneration) were performed with two identical immunoaffinity columns. One column was used for the methanol elution (○–○), the other column for the glycine–NaCl elution (×–×). The numbers provide with an asterisk are the first capacity determinations performed on a new day. The capacity determinations were made over a period of 1 month with permission from Ref. [66].

solution of clenbuterol standard were passed through the IS and analyzed by LC to ensure that the cartridge functioned properly. Another commercial IS could be regenerated for 20 successive analysis of ochratoxin A in blood serum, but only provided small amounts of matrix was used at each run [38]. However, the re-usability of disposable off-line cartridges with real samples is never recommended when real complex samples are analyzed. Regeneration is more important using on-line procedures with direct elution using a water–acetonitrile gradient. Then by switching the valve before the end of the analysis in order to avoid percolation of too high proportions of solvent (>50%), the same anti-phenylurea IS could be re-used more than 50 times for the analysis of dirty surface water samples. After each run, the IS was washed with 5 ml of 70% methanol in water and 6 ml of PBS [24]. When not in used, the IS should be stored in PBS at 4°C. A decrease of 10% in capacity was measured after 30 runs with dirty samples. All these studies show that the chemical bond greatly increased the stability of antibodies in solution containing organic solvents.

3.3.4. Flow-rate effect in the sample application step

The flow-rate is another parameter that was shown to affect recoveries of analytes, especially with low-performance sorbent because of their slow mass transfer properties. During the sample percolation step, high flow-rates may prevent analytes from binding to the immobilized antibodies. The binding flow-rate effect has been studied [27,46,60–62,64,82]. The effect can occur using on-line techniques, where sample application is performed via a pump. An increase in recovery from 25 to 95% was observed as the flow-rate decreased from 2.0 to 0.2 ml/min when 1 ml of 50 ppb toluene in water was percolated through a 2 cm×1 mm I.D. precolumn packed with a silica-based anti-toluene IS [64]. Using a 1.5 cm×4.6 mm I.D. precolumn packed with a silica-based IS containing anti-carbendazim antibodies, a similar effect of the flow-rate was observed between 0.2 and 10 ml/min but the recovery decrease was from 75 to 60% [46]. Other studies did not notice any loss when increasing the sample flow up to 2–5 ml/min using 4.6 mm I.D. precolumns [24]. The effect of the flow-rate is certainly more important with 1 mm I.D. precolumns.

3.3.5. Non-specific interactions

Non-specific interactions can occur between the IS matrix, the antibody proteins, the analyte and the sample matrix. Most commonly, hydrophobic and ionic forces are responsible of undesirable adsorption, which results in a decrease of the selectivity and limit of detection. Silica- and agarose-based ISs have been shown to minimize the interactions generated by the solid support. When the bonding procedure involves activated silica, the bonding reaction is usually followed by the neutralization of the remaining free activated groups on the carrier. The effect of adsorption sites of the IS surface can also be reduced by applying a blocking agent [78]. Non-specific interactions occur with the handling of highly hydrophobic analytes which have tendency to adsorb everywhere. The non-selective interactions were reduced by adding a small percentage of organic solvent (methanol or acetonitrile) or a detergent such as Tween 20 or Triton X-100 into the sample before percolation [10,51,55,57–59,78,94]. When analytes of interest are polar or moderately hydrophobic, the co-extraction of interfering hydrophobic analytes can be reduced or removed by applying a washing solution made of water with a small percentage of organic solvent.

In order to investigate the retention mechanism of the analyte on the IS it is interesting to compare its behaviour on three different columns, one constituted by the non-bonded sorbent, another one by the sorbent bonded with non-specific antibodies and the last one by the sorbent bonded with the specific antibodies of interest. This kind of study gives an indication about the occurrence of the non-selective interactions in the retention mechanism, and may indicate whether they are mainly due to the support or to the constant region of the antibody, the spacer or the coupling groups. For example, it has been shown that an anti-fluorene IS develops both non-specific and specific interactions with the antigen and five other PAHs [51]. PAHs are so hydrophobic that they are partially retained by an anti-atrazine IS. These non-specific interactions are also involved in the retention of PAHs on anti-fluorene IS but it was shown that the retention was better due to the

addition of the specific antigen–antibody interactions. Another way to address the occurrence of non-specific interactions is to look at the differences in binding behaviour between native and intentionally denatured immobilized antibodies. This was achieved with an anti-biotin IS and the denaturation was induced by passing acetonitrile–4 m*M* tris buffer (80/20) through the column for 1 h [93].

4. Applications to real samples

A large numbers of papers have been published that illustrate the various features of immunoextraction with relevant applications. Some selected examples have been reported in Table 5 showing that many applications deal with natural food contaminants, drugs and various organic pollutants such as pesticides and toxins. It is surprising that a major part of them are followed by LC analysis and that a great part of them have been on-line coupled to LC systems. Finally, many examples take advantage of the cross-reactivity of the antibodies to develop multiresidue analysis targeting a parent compound and its metabolites or a class of structurally related analytes.

4.1. Off-line immunoextraction

Table 5 shows that many off-line techniques are in fact used as clean-up of extract from various solid matrixes. After a washing step to remove a part of undesirable sample components, elution is achieved using an organic solvent in water or buffer as shown earlier. As common in SPE, the elution solution is often evaporated to dryness under mild conditions and reconstituted in a solvent that is compatible with the method to be used for quantitation. The advan-

Table 5
Examples showing the multiplicity of matrixes, compounds and separation detection methods associated with the indication about an off- or on-line way

Target analyte	Matrices	Analysis	Off- or on-line	Single or Class selective	Ref.
Aflatoxin B_1, B_2, G_1, G_2, M_1, Q_1	Urine	LC–Fluo	Off	MR	[40]
Aflatoxin M_1	Milk	LC–Fluo	Off	S	[11]
Aflatoxins	Airborne dust	LC–Fluo	Off	MR	[39]
Aflatoxicol	Urine	LC–Fluo	Off	MR	[41]
Atrazine	River, orange juice	GC–FID	On	MR	[34]
Atrazine	Apple, carrot, celery, corn, potato, peas	LC–DAD, GC–MS	Off	MR	[97]
Carbendazim	Creek, fountain waters	LC–UV, LC–MS	On	S	[46]
Clenbuterol	Liver, muscle	LC–UV	Off	S	[44]
Clenbuterol	Urine	LC–MS	On	MR	[75]
Clenbuterol	Liver, urine	GC–MS	Off	MR	[92]
Chloramphenicol	Muscle, liver, kidney, urine	GC–ECD	Off	MR	[29]
Cortisol	Serum, urine	LC–UV, RIA	On, off	S	[8]
Diethylstilbestrol	Urine, plasma	GC–MS	Off	MR	[14]
Fluorene	Water samples	LC–DAD	On	MR	[51]
Forskolin	Roots, culture	IA	Off	MR	[30]
Isopentenyladenosine	Maize seeds	LC–DAD	Off	MR	[33]
LSD	Urine	LC–MS	On	MR	[69]
Oestradiol-17-β	Plasma, milk	RIA	Off	MR	[7]
PAHs	Soil, soot	LC–DAD	Off, on	MR	[95]
Simazine	Water samples	LC–DAD	Off, on	MR	[25]
Tetracycline	Milk	Fluo	Off	S	[99]
Thiabenzadole	Water	Scintillation	Off	MR	[79]
Toluene	Water samples	LC–MS	On	MR	[64]
Zearalenone	Corn	LC–Fluo	Off	S	[45]

MR: multi-residue, S: single analyte, IA: immunoassay, RIA: radioimmunnoassay.

tage of off-line procedures are that the extract can be analyzed using GC without taking care of the residual water or using two complementary techniques for confirmation or can readily be derivatized. The selectivity is the most important feature of these SPE materials and has been demonstrated in complex solid matrixes such as soil, sediments, sludges, biological and plant tissues or food. For solid samples, supercritical fluid extraction coupled to immunoextraction clean-up has been investigated for the trace analysis of organic pollutants including PAHs and pesticides from soil and soots [95]. The analysis of phenylureas and triazines in several food samples (carrots, celery, corn, grapes, onions, potatoes, and strawberries) was also shown to be highly simplified [96,97]. Methanolic extracts of the plant tissues were simply concentrated and then diluted with water before percolation through the IS. Thanks to the high degree of clean-up, this approach was very rapid compared to actual methods and avoid the requirements of solvents such as hexane, dichloromethane, acetone and others commonly used for adsorption chromatographic clean-up of sample extracts. PAHs could be determined in waste sludges and sediments using LC–UV diode array detector (DAD). The method was validated using certified reference sludges and sediments and the clean-up provided by an anti-fluorene IS was shown to be better than that obtained using conventional silica clean-up [54].

Liquid samples include serum, plasma, urine, milk and water. Urine is often diluted in an equal volume of PBS. The main advantage is that extraction and clean-up are performed using a single cartridge and even if the price is higher, that should be compared to the use of two cartridges and the laborious and sometimes difficult use of Florisil sorbent for clean-up. In addition, the fact that the analytes are selectively extracted on a molecular recognition basis reinforced the identity of the analytes.

4.2. On-line immunoextraction

Automated devices which allow the on-line coupling of SPE to LC are commercially available. One main interest for integrating IS in on-line technology is the selectivity of the extraction, because extraction and clean-up are achieved in the same step. So, chromatograms present a clear base-line, allowing quantification at low level and better identification of the analytes using classical UV diode array detectors. Therefore, the sample volume can be reduced.

First examples dealing with on-line techniques used antibodies immobilized on Sepharose [12,13,20–23]. Because Sepharose was not pressure resistant it was necessary to use a second C_{18} precolumn for the desorption and re-concentration of analytes from the immunocolumn. But as far as desorption could not be made without a high proportion of organic solvent, direct desorption and re-concentration became impossible without modification of the basic on-line set-up [12]. This problem could be solved by using a displacing solution containing 5% acetonitrile and a high concentration of the cross-reacting steroid hormone norgestrel for the desorption and transfer to the second precolumn. Then the classical C_{18} precolumn/C_{18} analytical column system was used (see Fig. 3). Another option when using a Sepharose precolumn in an on-line set up was given for the determination of the aflatoxins [22]. After percolation through the immunoprecolumn, the analytes were desorbed with methanol–water (70:30, v/v), diluted on-line with water, subsequently re-concentrated on a second C_{18} precolumn and finally transferred to the LC separation column. The classical on-line set was modified by the addition of a pump for adding water to the desorption solution from the immunoprecolumn. The selectivity of the system was demonstrated with spiked urine and the immunoprecolumn could be re-used. Unfortunately its re-usability was strongly shortened with milk samples and that was explained by the occurrence of proteolytic enzymes in milk which may be at the origin of the degradation of the immobilized antibodies. A solution was to add a dialysis unit to the system [23].

This two-precolumn set-up is also necessary using supports based on antibodies that are adsorbed to protein A or G. Rule et al. reported on-line immunoaffinity chromatography coupled with LC for the extraction and detection of two basic drugs in diluted urine using a protein G immunoaffinity sorbent [98]. This two-precolumn set-up was also selected even when a first silica-based precolumn containing the immobilized antibodies was used. Although the direct desorption can be performed, some authors

prefer using large volumes of aqueous buffer at low pH for eluting the analytes and therefore they need a second precolumn for reconcentration. One advantage is that the size of the first precolumn can be large since it does not belong to the on-line system, allowing thus higher sample flow-rate. Another advantage is the use of totally aqueous solutions through the IS, which is often considered a better choice for the life-time of the IS. Using a 3 cm×4.6 mm I.D. precolumn packed with aldehyde activated-silica with immobilized anti-carbofuran antibodies, the excellent specificity toward the single analyte carbofuran was demonstrated with direct extraction and detection at low levels (40 ng/l) in spiked water [15]. Water samples were pumped through the IS at 4 ml/min followed by a washing with 15 ml of PBS and elution with 15 ml of pH 2.6 formic acid at 3 ml/min. The selectivity was shown with the analysis of a crude potato extract.

The on-line set-up using a single silica-based immunoprecolumn is very simple and does not differ from that using a single C_{18}. Precolumns packed with a silica-based IS containing either anti-triazine or anti-phenylurea antibodies have been used for loading aqueous samples or extracts [24,25]. After a washing with water, the immunoprecolumn is directly connected to the analytical column and a water–acetonitrile gradient allowing the analytical separation was percolated through the system precolumn-analytical column. In order that the IS should not be in contact with too high amounts of acetonitrile for a long time, the connecting valve was switched when the percentage of acetonitrile was 50%. At each cycle, the IS was washed with 5 ml of a solution containing 70% methanol and then was regenerated with 6 ml of PBS. The same precolumn could be reused more than 50 cycles including a half of dirty samples. The ISs can be submitted to a high proportion of organic solvent, which destroy the antigen–antibody interaction, but in a reversible way, because antibodies have been stabilized by the covalent bonding to the silica. Several ISs have been operated in the same way containing various antibodies against benzidine, fluorene, or pyrene [51,55,56]. Anti-triazine and anti-phenylurea ISs have been validated in both off-line and on-line procedures [52,53]. An automated method was performed using a single on-line immunoprecolumn combined with LC coupled directly to atmospheric pressure chemical ionization (APCI)-MS in positive operation mode [52]. After the percolation of 20 ml of samples high recoveries were obtained and due to the selectivity and the sensitivity of the extraction, detection limits ranging 1–50 ng/l were obtained in ground water. The method was validated through an interlaboratory study with Aquacheck certified samples.

Immunoextraction was also coupled to GC although the interfacing between the immunoextraction and the GC part is not as straightforward as it is with LC. After trace-enrichment of analytes on a 10×3 mm immunoprecolumn packed with monoclonal anti-atrazine antibodies immobilized onto cellulose, they were desorbed and re-concentrated on a reversed-phase precolumn packed with a copolymer by means of an acidic buffer [34]. After clean-up and drying with nitrogen, desorption and transfer to the GC was done with ethylacetate via an on-column interface. The selectivity of the system was such that non-selective flame ionization detection (FID) could be used to detect several triazines in river, waste water and orange juice. The detection limits for 10 ml samples were 15–25 ng/l for FID and about 1.5 ng/l for nitrogen-phosphorus detection.

4.3. Multiresidue immunoextraction

The affinity is usually the highest between antibodies and the analyte selected for inducing the immune response (so-called analyte–antigen). However, for analytes such as aflatoxins which are very closely related, the cross-reactivity is unavoidable and was exploited as soon as the first ISs have appeared. Therefore, few ISs target single analyte whereas many examples have been published for immunoextraction of a single analyte and its metabolites or a whole group of related analytes. Atrazine and its major metabolites could be analyzed at low nanogramme per litre range using an IS obtained by bonding a monoclonal anti-triazine antibody [61]. A two-precolumn on-line system as described above was used. A typical chromatogram of atrazine degradation products is given in Fig. 5. From a 45-ml sample, it is possible to detect as low concentration as 60 ng/l of de-isopropylatrazine using LC–UV.

Relevant examples have been described also in the biological field such as trace determination of LSD

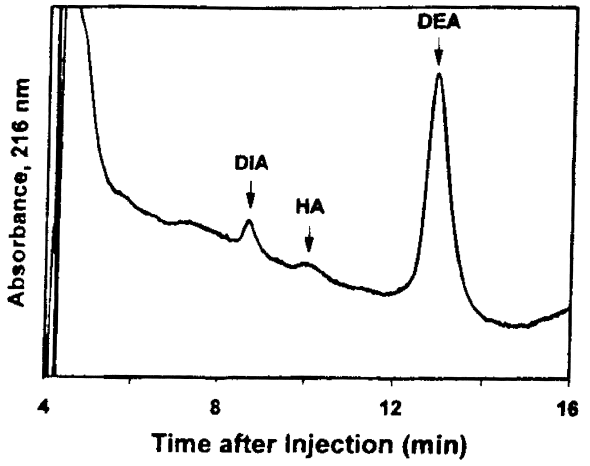

Fig. 5. Typical chromatogram for atrazine degradation products in a 45 ml groundwater sample. The concentrations measured for de-ethylatrazine (DEA), hydroxyatrazine (HA) and de-isopropylatrazine (DIA) were 210, 10 and 60 ng/l, respectively. The sample was on-line preconcentrated on a precolumn containing monoclonal anti-triazine antibodies bonded to a silica-based sorbent, a reversed-phase LC (RPLC) column and then an RPLC analytical column with permission from Ref. [61].

drug and its metabolites in urine [69]. Raw human urine (25 ml) diluted 1:1 with PBS was pumped through a 2.1 mm I.D. protein G immunoaffinity precolumn with non covalently immobilized antibodies against LSD and was analyzed by electrospray-MS after reconcentration onto a packed capillary trapping column. Since the elimination half-lives of LSD metabolites are longer than those of the parent LSD, their presence may be detectable much longer after the level of LSD has dropped the limit of detection. Five metabolites could be detected at concentration as low as 2.5 ng/l.

The cross-reactivity of antibodies was also exploited for developing ISs that could selectively extract a whole class of structurally related compounds. ISs have been tailored by several authors for the extraction of groups of organic compounds including aflatoxins [39,40], β-agonists [75], corticosteroids [36], triazines and phenylurea pesticides [4], BTEXx (benzene, toluene, ethylbenzene and xylene isomers) [64], PAHs [51,54,55,77,78], benzidine and related azo dyes [56]. For class-selective ISs the selection of the compounds which will be used for immunization is of prime importance. That was shown for the group of phenylureas, for which ISs containing polyclonal antibodies against isoproturon were not able to recover all the class whereas IS containing anti-chlortoluron antibodies were able to recover almost all the phenuylureas which are now used in agriculture (11 phenylureas of the 13 in the selected mixture) as shown in Fig. 6. The direct on-line analysis of phenylureas allowed quantification at the 0.1 μg/l level from a sample volume as low as 50 ml of surface water (River Seine in Paris) and using a simple UV DAD. No interferences from humic acids and other analytes are observed.

The 16 priority PAHs could be monitored in surface water at the level of 20 ng/l each from a sample as low as 20 ml using an anti-fluorene IS on-line connected to a LC system with fluorescence detection (Fluo) [52,55]. Due to their hydrophobicity, the addition of an organic solvent in the samples before percolation was necessary in order to avoid the adsorption of the PAHs on container walls, connection tubes and to reduce the non-specific interactions with the immunoextraction sorbent. An

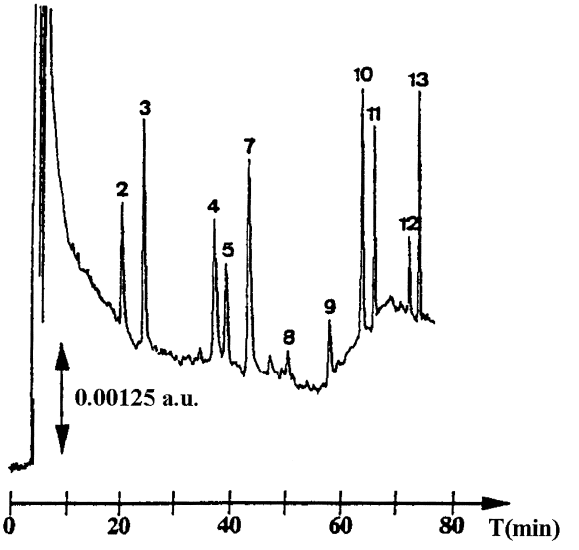

Fig. 6. On-line solid-phase immunoextraction-LC analysis-UV diode array detection of 50 ml of river Seine (Paris city) water sample spiked with a mixture of 13 phenylureas at a concentration level of 0.5 μg/l for each compound, using a precolumn packed with an anti-chlortoluron IS. (1) fenuron, (2) metoxuron, (3) monuron, (4) metabenzthiazuron, (5) chlortoluron, (6) fluometuron, (7) isoproturon, (8) difenoxuron, (9) buturon, (10) linuron, (11) chlorbromuron, (12) difluzbenzuron, (13) neburon. UV detection shown at 244 nm [6].

anti-pyrene IS was used to monitor some priority PAHs in urban sludges before their use for soil amendment.

In order to recover the whole class, sometimes two antibodies have been mixed in the cartridge bed. Mixed-bed sorbents using antibodies against chlortoluron and isoproturon have been obtained for recovering the group of phenylureas [49]. A mixed-bed IS was also tailored for trapping ampicillin and cloxacillin [80].

Since the desorption conditions from cartridges packed with anti-phenylurea or anti-triazine IS were

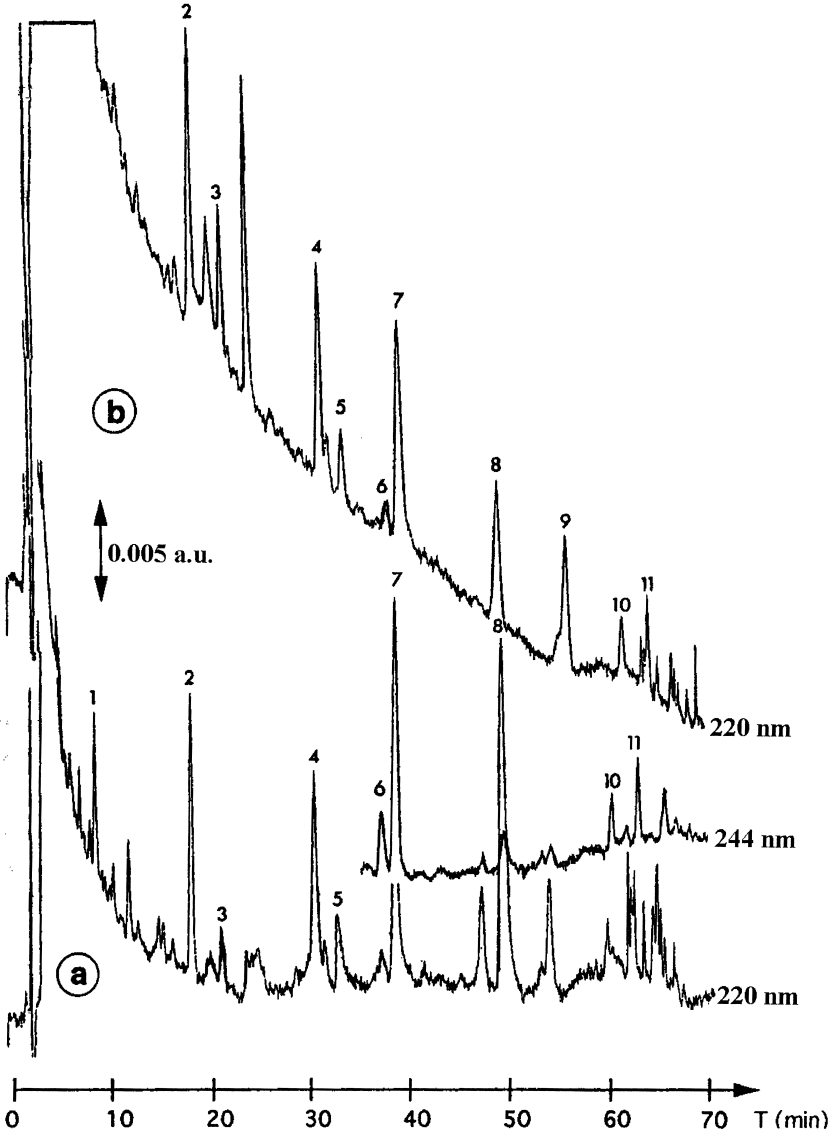

Fig. 7. Multiresidue analysis for two classes of herbicides using (a) a mixed bed IS (anti-phenylurea and anti-triazine) and (b) a non-selective PLRP-S extraction sorbent for the analysis of 50 ml of surface water spiked with 0.5 μg/l of each analyte. RPLC with water–acetonitrile gradient. Chromatograms obtained with UV DAD detection shown for (a) and (b) at 220 nm and for (a) partly at 244 nm. (1) de-ethylatrazine, (2) simazine, (3) monuron, (4) atrazine, (5) chlortoluron, (6) isoproturon, (7) diuron, (8) propazine, (9) terbuthylazine, (10) linuron, (11) chlorbromuron [4].

identical, a mixed-bed sorbent was made for multi-class residue analysis. The interest lies in the fact these two classes of herbicides are often detected together in surface water because of their applications in similar cultures. Fig. 7a shows the potential of a mixed-bed anti-phenylurea/triazine IS for multiresidue determination of several phenylureas and triazines in a spiked surface water sample (50 ml). The difference with the chromatogram corresponding to a non-selective extraction is shown in Fig. 7b, thus illustrating the high selectivity for the polar early eluted compounds which are not detectable because of the humic substances interfering peaks at the beginning of the chromatogram. De-ethylatrazine can be easily detected using the IS whereas it cannot in Fig. 7b.

A procedure involved recycling immunoextraction for the simultaneous measurement of a number of analytes in a single sample [76]. It was based on the passage of a fluorochrome labelled sample through a battery of small immunoaffinity columns, each column extracting a single specific analyte. The set-up is shown in Fig. 8a. Detection was achieved by elution of the bound analytes and laser-induced fluorescence. Elution of each column is achieved by isolating the column via combination of the switching valves. The system was applied to a number of different biological fluids and was found capable of isolating and measuring up to 10 different cytokines in a 25-μl sample of body fluid. A chromatogram produced by passing eluted blood spot samples through the battery of extraction columns is given in Fig. 8b.

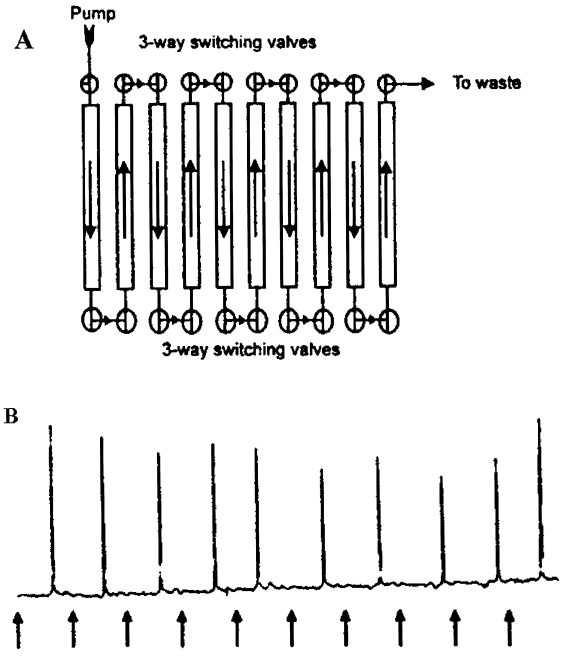

Fig. 8. (a) Diagram of the recycling anti-cytokine immunoaffinity system. Samples are passed through the battery of immunoaffinity columns as indicated by the arrows. The immobilized antibodies in each column retain their specific analyte. (b) Recycling chromatogram obtained by passing a plasma sample, containing a mixture of cytokines through the battery of immunoaffinity columns. Peak eluted in the order of IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNFα and γIFN according to the position of each column in the battery. Arrows indicate initiation of the elution phase for each column. The test mixture contained 100 pg/ml of each cytokine. IL: interleukine, TNFα: tumour necrosis factor alpha and γIFN: gamma interferon with permission from Ref. [76].

4.4. High sample throughput with on-line coupling with microchromatography

On-line coupling of immunoextraction with microLC has the advantage to provide a high sensitivity. The potential of on-line IS-coupled column packed capillary LC-ion spray tandem MS was demonstrated for the multiresidue determination of five β-agonists in bovine urine [75]. Trace-enrichment and preliminary sample clean-up was performed on-line using 25 ml of bovine urine diluted with 25 ml of PBS and percolated through a 10×2 mm I.D. IS precolumn. The column switching process involved elution and reconcentration onto a mini-bore 20×1 mm I.D. precolumn which was further on-line coupled to a microbore (320 μm or 1 mm I.D.) analytical column for optimum sensitivity in LC–MS–MS. Fig. 9 shows the chromatogram obtained in selected reaction monitoring (SRM) mode corresponding to a urine sample spiked with 100 ng/l of each analyte. The advantage of the LC–MS–MS is its high sensitivity and the fact that a complete separation is not required so that it can be as rapid as 8 min. Lower levels for quantification were in the range 10–50 ng/l. The 50-ml sample could be percolated at 4 ml/min and the elution was made using 20 column volume of 2% acetic acid with a flow-rate of 200 μl/min. The on-line pro-

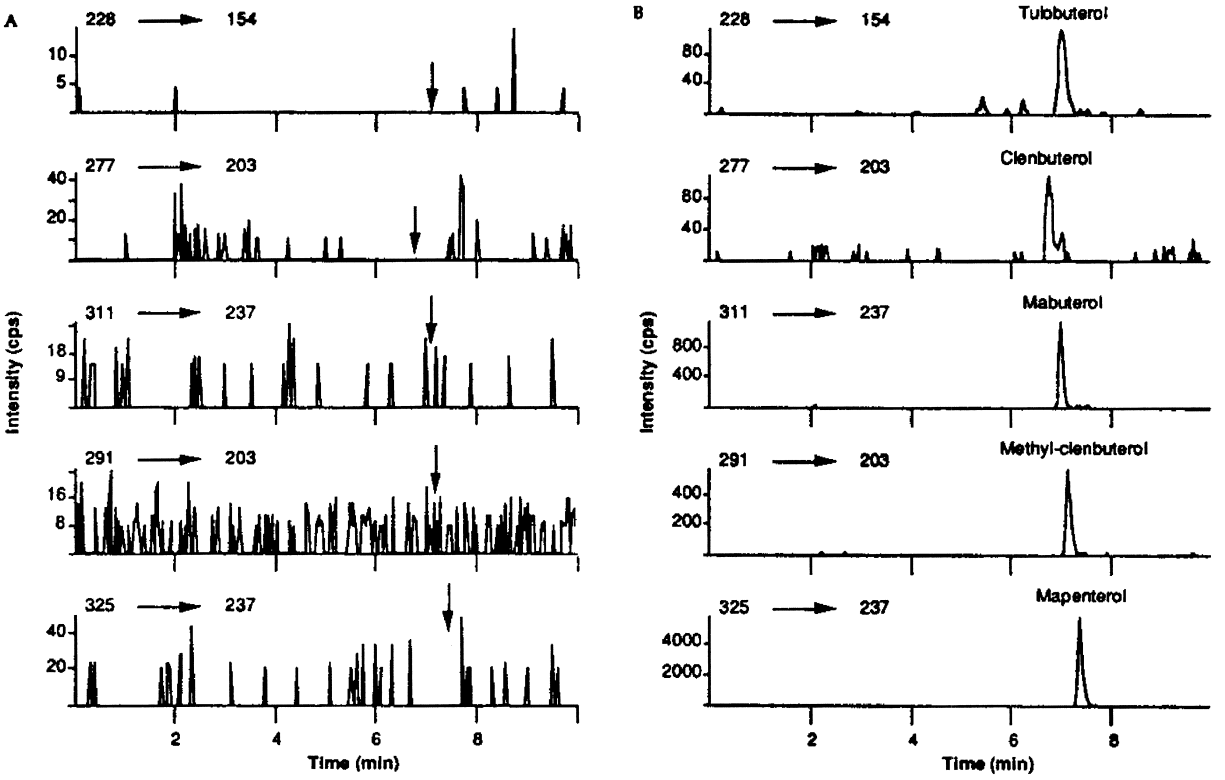

Fig. 9. Selected reaction monitoring mode/immunoextraction/LC/LC/MS of bovine urine using a 1 mm I.D. microbore analytical column. (a) blank urine, (b) urine spiked with 5 β-agonists at 100 ppt levels with permission from Ref. [75].

cedure and the possibility of loading the sample during the analysis of a previous sample increased the throughput. The advantage of immunoextraction as a good sample preparation method for LC/MS/MS was recently emphasized [100].

The potential for high sample throughput was demonstrated with the direct on-line coupling of IS with microLC-UV DAD. The direct on-line set-up was performed using a 10×1 mm I.D. precolumn prepacked with a silica-based anti-phenylureas IS and a 250×1 mm I.D. analytical column. Thanks to the high selectivity of the extraction and the sensitivity of the system although the detection system was UV DAD, 5 ml were enough for obtaining quantification limits of 10 ng/l [101]. Fig. 10 illustrates the high selectivity of the immunoextraction as compared as the use of the same precolumn packed with C_{18} silica. Four phenylureas could be easily identified by their UV spectrum in the surface water sample whereas this was impossible in the chromatogram obtained using a non-selective sorbent. This analysis demonstrates the interest of a class-selective IS. Concentrations were around 50 ng/l for both chlortoluron and linuron, 150 ng/l for isoproturon and 200 ng/l for diuron. Especially since there is no co-extraction of humic materials, the analysis can be more rapid because usually the first eluted peak is delayed in order to be after the humic acid peaks. The analysis of diuron used in antifouling paints could be done in sea water in less than 10 min. This was possible because the 40-μm silica particles used for preparing the IS which allowed a rapid sample flow-rate and the sample volume is low.

5. Conclusion

The large number of examples presented in this review has demonstrated that the coupling of im-

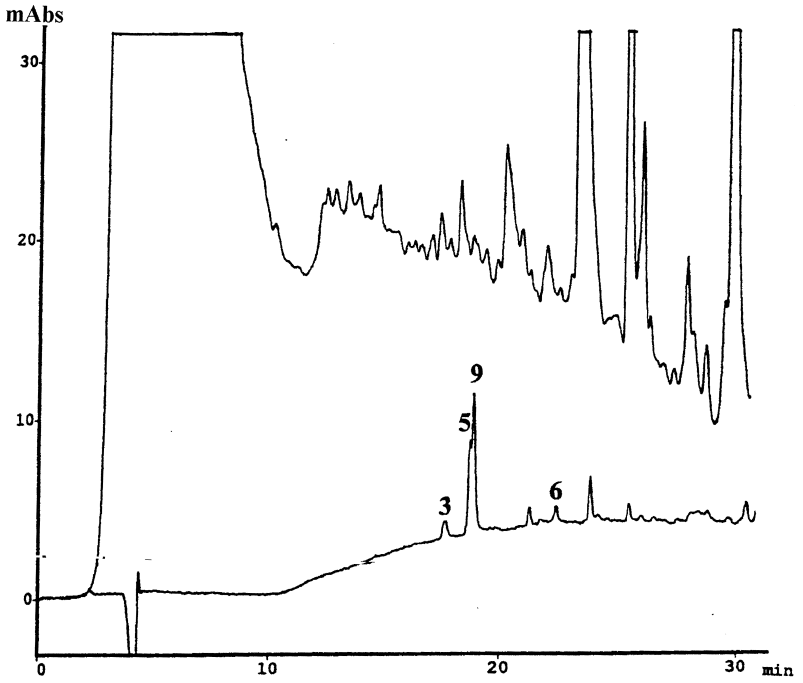

Fig. 10. Comparison of chromatograms obtained after on-line preconcentration of 5 ml of river Seine (Paris city) water using a non-selective C_{18} precolumn (top) and a selective anti-phenylureas immunosorbent (bottom). (3) chlortoluron, (5) isoproturon, (6) linuron, (9) diuron [101].

munoaffinity SPE in off-line or on-line procedures is a powerful technique for the determination and easy quantification at low level of many organic compounds in various complex samples. The sample handling step is greatly simplified. The selectivity in the extraction step also allows easier identification of analytes of interest and their confirmation is reinforced since trapping occurs on the basis of a structure recognition. Moreover, a clear baseline enhances the overall sensitivity of the analysis and allows the handling of a lower sample in comparison to non-selective extraction procedures. Multiresidue IS and class-selective immunoextraction are expected to be developed.

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