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review

Basic Principles of the Use of Immunoaffinity Chromatography for Environmental Analysis

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Summary

Usual pollutant extraction procedures applied to environmental samples are not selective. Therefore, the analysis of trace-level of contaminants or their degradation products in complex matrices is often difficult because of the different polarities and low concentrations of the analytes. Additionally, very polar analytes are often not quantitatively retained by the most common solid phase extraction procedures. Immunoaffinity chromatography (IAC) is a separation method based on the specific and reversible interactions between the antigen and the antibody. Because the retention is directed by a different physico-chemical principle, IAC offers an interesting alternative to implement the existing extraction procedures. In this paper we will present the basic principles of the IAC separations. Based on the available literature, the most important criteria for immunosorbent preparation and the operational features of this kind of solid phase extraction method will be discussed. State of the art of the application of IAC for environmental analysis will be presented.

Keywords. pollutant, antigen/antibody interaction, immunosorbent, enviromental analysis

Introduction

Immunoaffinity chromatography (IAC) combines the advantages of the solid-phase extraction techniques with those of immunochemical methods. Immunochemical methods are based on the antibody-antigen molecular recognition, event that ensures high specificity and sensitivity. Antibodies are glycoproteins produced by the immunosystem as a response to the entrance of a foreign substance into the organism. In the immunochemical techniques these antibodies are used as analytical tools in *in vitro* systems.

In IAC an immunosorbent (IS) is prepared by covalently immobilizing an antibody (Ab) on the surface of a rigid or semirigid support. The IS is then used for the selective extraction of a single analyte or a group of analytes (see Fig. 1). Once the IS has been prepared, main steps are loading of the sample, washing and elution of the analytes. Finally the IS is regenerated for further use or storage of the IAC column. The Ab-analyte interaction determines the efficiency of the IAC, as other chromatographic systems are based on physico-chemical variables such as electrostatic or hydrophobic interactions.

In principle, any molecule to which polyclonal antibodies (PAb) or monoclonal antibodies (MAb) can be produced may selectively be extracted by IAC. As reported in the available literature, a broad list of molecules has been purified by IAC including vaccines *(1)*, proteins of pharmaceutical interests *(2,3)*, small biomolecules such as steroids *(4)*, pesticides *(5,6)*, and so on. Most of the applications are intended for clean-up and preconcentration processes although there have also been some studies of the immunoaffinity chromatography as a separation technique. In this paper we will present the basic principles of the IAC and their operational features focussing specially on the application of this technique to environmental analysis.

Preparation of the IS

Solid supports

A suitable support and an appropriate coupling chemistry to attach the ligand are essential for any affinity separation. Besides the valuable information of the

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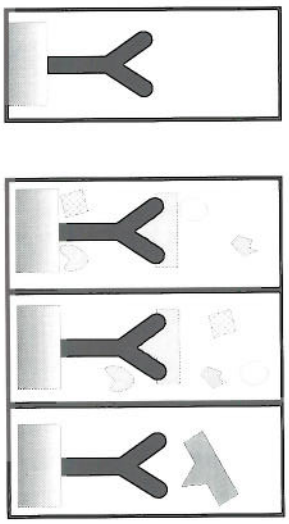


1. Preparation of the IS:
Covalent coupling of the antibodies to an appropriate support

2. Loading of the sample:
Analyte capacity?
Breakthrough volume?

3. Washing step
Wash-out contaminating molecules and nonspecifically bound compounds

4. Elution of the analyte
Reduction of K_a:
pH
Organic solvents
Ionic strength
Chaotropic agents

Fig. 1. Scheme showing the steps involved in immunoaffinity chromatography purification

commercial suppliers, there exist several reviews dealing with these topics *(7–9)*. Important parameters of a solid support for IAC are its *macroporosity, mechanical stability, activation possibility, hydrophilicity* and *absence of nonspecific interactions (10)*.

To achieve an adequate capacity, a high surface area/volume ratio is desirable, which is accomplished by an appropriate *porosity* of the support *(11)*. The pore sizes should be large enough to allow access to the Ab and the antigen. In this context, a study was published on the influence of the porous size of silica supports on the efficiency and capacity of glucosamine columns to analyze concanavalin A and bovine serum albumin (BSA) *(12)*. Despite this there should exist a compromise between the porosity and the *mechanical stability*. A material that is too porous may lack stability.

The support should additionally contain functional groups to allow *activation* through an appropriate linker and immobilization of the Ab by a covalent bond. Additionally the character of the interactions between the IS and the sample matrix should be dictated exclusively by the Ab specificity. In this context, *hydrophilic* materials are usually associated with *low nonspecific interactions*.

We are going to describe briefly the features of the supports that have been most frequently evaluated for environmental analysis (see Fig. 2 for chemical structures):

Polysaccharides. Beaded agarose has been one of the most popular supports for affinity chromatography since it possesses most of the required features mentioned above. The main limitation is its lack of resistance to high presure gradients. To avoid this problem, the commercial companies have produced high cross-linked agaroses. For example, Pharmacia introduced Superose™ whose structural composition has not been reported but maintains the properties of the beaded agarose, increasing resistance to pressure flow gradients. Regenerated cellulose membranes (0.45 μm pore size, 100 μm thickness) have been used as affinity supports, although they have some limitations due to their fixed charge that may confer ionic exchange properties to the clean-up step.

Silica based supports. Silica supports are especially attractive because of their high resistance to the pressure

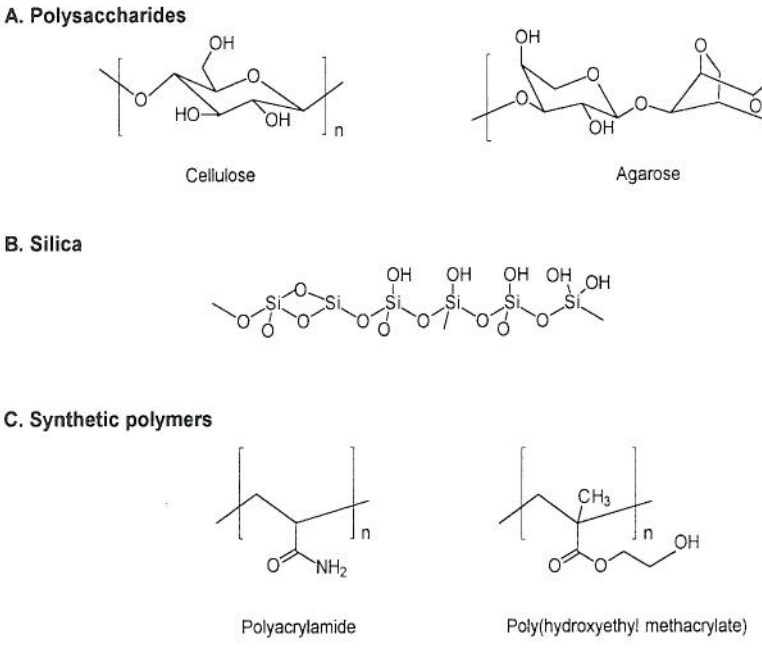


Fig. 2. Chemical structures of the most commonly used supports employed to immobilize antibodies

which makes them suitable for *on line* HPLC procedures or high performance immunoaffinity chromatography (HPIAC) methods. The use of pure silica supports has been limited because of the irreversible adsorption and denaturation of many biomolecules *(8)*. However, modification of the silica beads with hydrophilic substituents has made available more compatible supports. This is the case of the diol-bonded silica or aldehyde derivatized silica beads (see below). Nowadays, these supports are commercially available but can be prepared in the laboratory by chemical modification of the silica primary hydroxyl groups using appropriate silanizing reagents *(13–15)* (see below).

Synthetic polymers. Different polymers have been introduced by commercial companies as excellent supports for affinity chromatography. The monomers and the crosslinking agents used determine the macroporosity, hydrophilicity and mechanical stability of the support. As an example polyacrylamide porous beads are found that certain polymeric supports adsorbed nonspecifically until 83% of the total atrazine was loaded. Uncoupled vinyl polymeric supports bound hydrophobic phenylureas as much as the corresponding anti-isoproturon bonded vinyl polymer *(19)*. Similarly, a significant fraction of carbendazim and other aromatic pesticides was retained unspecifically by a polystyrene-polydivinyl benzene matrix *(20)*.

Recognizing element: Antibody

Antibodies are polypeptides belonging to the immunoglobulin (Ig) family. The antibodies used in most of the immunochemical techniques are IgGs formed in the humoral response of the immune system of the mammals when in contact with a foreign substance (antigen). Both polyclonal or monoclonal antibodies have been successfully applyed to prepare ISs for the analysis of environmental pollutants (Table 1).

Table 1. Features of some immunosorbents used in environmental analysis

Analyte	MAb/PAb	Support	Activation	Capacity *(a)*	Volume *(b)*	Ref.
Atrazine	MAb	Nucleosil 1000 Å, 7 μm	Aldehyde	30 nmol/g IS 0.5 nmol/mol MAb	45 mL	*(46,48)*
	PAb	Silica beads 50 nm	Aldehyde	1.2 μg/g IS	25 mL	*(11)*
	MAb	Lichroprep 2000 Tentacle gel	Hydrazide	2.7 μg/mL IS 1.25 μg/mg MAb	10 mL	*(18)*
		Lichroprep 2000 EMD SH Azlactone gel	Azlactone	50 ng/mg IS*(a)* 2.9 μg/mg MAb	10 mL	*(18)*
		Beaded Cellulose ONB carbonate	Carbonate	16.3 μg/mL IS*(a)* 2.96 μg/mg MAb	10 mL	*(18)*
Carbendazin	MAb	Nucleosil 1000 Å, 7 μm	Aldehyde	15.7 nmol/g IS 35.4 ng/mg MAb	20 x 20 μL (1 mg/L)	*(20)*
		POROS-A1	Aldehyde	11.2 nmol/g IS 11.2 ng/mg MAb	20 x 20 μL (1 mg/L)	
Carbofuran	PAb	Silica beads 500–1000 Å, 30 μm	Aldehyde	38 ng/mg PAb	30 mL	*(6)*
Isoproturon	PAb	Silica beads 30 nm, 100–200 μm	Aldehyde	3.6 μg/g IS	50 mL	*(19)*

(a) theoretical binding capacity; *(b)* not the breakthrough volume, but volume of sample applied to determine column capacity.

hydrophilic gels free of fixed charges. However, Ab immobilization has been reported to be low because of the microporosity of the commercially available polyacrylamide beads *(8,9)*. In contrast, Poly(HEMA), a copolymer of hydroxyethyl methacrylate and ethylene glycol dimethacrylate, shows an appropriate range of pore sizes with an excellent mechanical stability *(16)*. Similarly, azlactone functional copolymer beads, made by copolymerization of azlactone vinyl monomers, acrylamides or acrylates and crosslinking agents such as N,N'-methylene-bis-acrylamide, show excellent properties *(17)*.

Table 1 shows some supports that have been evaluated for a limited number of pesticides. Silica is the most frequently used support for environmental analysis, because of the properties mentioned above and its reasonable cost. Polymeric supports have been compared to silica supports for pesticide analysis; however, results have not encouraged their further use because of the nonspecific interactions. For example, an anticarbofuran poly(methyl methacrylate) column unspecifically retained fluometuron and propoxur *(6)*. Marx *et al. (18)*

The development of successful immobilization procedures has been supported by a good knowledge of the chemical structure of the antibodies. The IgGs (M_r = 150 000) are monomers of Igs formed by two pairs of polypeptide chains linked predominantly by disulfide bridges (see Fig. 3). Two chains contain approximately 440 aminoacids (M_r = 55 000, each) and are known as the heavy chains (H_c) as opposed to the light chains (L_c) with only 220 aminoacids (M_r = 25 000, each). Light and heavy chains contain, each of them, one variable region (V_H or V_L) at their N-terminal and, respectively, one (C_L) or three (C_H1, C_H2, C_H3) constant regions at their C-terminal. The variable regions of both heavy and light chains possess three hypervariable segments of complementarity-determining regions (CDR) that fold together to form the Ab binding site (F_{ab}) of the IgGs. The amino acid sequence of this region is unique on each Ig produced by a single B-cell clone. An important feature of the portion containing the constant regions (F_c fraction) is the presence of sugar residues that have been often used to develop oriented immobilization procedures (see below).

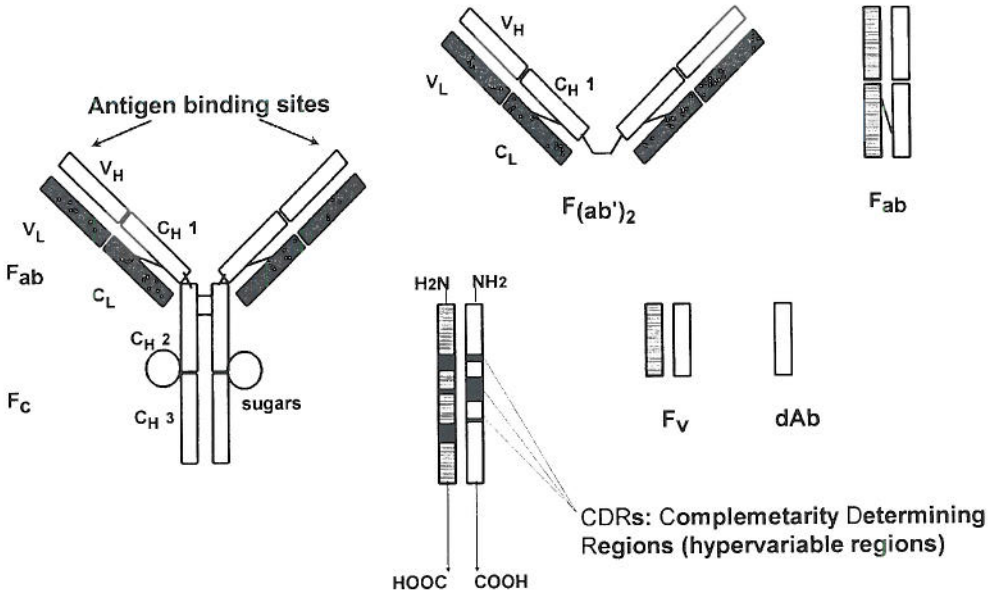


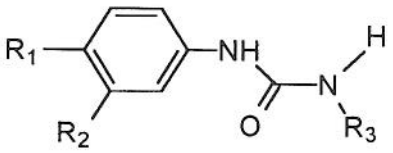
Fig. 3. Structure of an immunoglobulin G and its most important fragments used in IAC. Some immobilization procedures are designed to keep the antigen binding sites free

Ab fragments keeping the F_{ab} region have been obtained by using specific enzymes (pepsin: $F_{(ab')_2}$ and F_c fragments; papain: 2 F_{ab} and 1 F_c fragment) or recombinant DNA techniques *(21,22)*. Finally »paralog« peptides (M_r = 1000–2000) of the CDRs can be produced chemically by solid-phase synthesis *(23)*. Other kinds of Ab fragments, used in some immobilization procedures (see below), are obtained by using reducing agents that dissociate the disulfide bonds to generate free sulphydryl groups. As an example mercapto ethylamine reduces the disulfide bond of the hinge region without dissociating the heavy and light chains *(24)*. The use of Ab fragments for immunoaffinity purification is still rare although some authors have pointed out the potential of these reagents to reduce the cost of the technique *(25)*.

The immunoresponse is a complex process where molecular recognition and communication between specialized cells take place through certain mediators to produce a cascade of events leading to the multiplication of specific clones of B-cells in the serum. These cells are responsible for secretion of IgGs with highly defined avidity towards the antigen. For more extensive information on the mechanisms involved in the immunoresponse the reader is addressed to other sources *(26–29)*. Antigens below certain molecular size do not trigger the immunoresponse.

Many environmental pollutants, nowadays reason for public concern, have relative molecular masses below 1000 and consequently do not elicit an immunoresponse. To raise antibodies against pollutants it is necessary to chemically synthesize an appropriate hapten. The chemical structure of the immunizing hapten greatly affects the *avidity* and *specificity* of the resulting antibodies *(30–32)* and will consequently determine the properties of the studied IS. It has been proved that immunoaffinity columns prepared with high *avidity* antibodies provide greater breakthrough volumes to the analyte (see below) *(5)*. To obtain high avidity antibodies it is essential that the hapten preserves as much as possible of the chemical structure, electronic distribution and conformation of the target analyte *(31)*. Regarding *specificity*, on clean-up and screening applications it is generally preferred that the antibodies recognize a wide range of pesticides belonging to the same family. As an example, ISs for phenylurea herbicides were prepared using antibodies raised against both isoproturon and chlortuluron (see Table 2, for chemical structures) *(5)*. After percolation of 50 mL of LC grade water spiked at 0.5 μg/L with a mixture of 15 phenylureas, it was observed that the antiisoproturon IS only showed a high recovery for isoproturon (98%) while other phenylurea herbicides presented values below 50% (i.e., methoxuron, 5%; chlortoluron, 46%; linuron, 19%; chlorbromuron, 31%; etc.). On the contrary, the antichlortoluron IS was found to be more appropriate for screening purposes since recoveries were higher than 75% for 10 of the 15 phenylureas studied. This difference in behavior is mainly related to the chemical structure of the immunizing hapten. Isoproturon has a bulky group placed at the *para* position of the aromatic ring, and this is probably an important antigenic determinant, not present in most of the phenylureas analyzed (see Table 2). In contrast, chlortoluron with a methyl group at the *para* position and a chlorine atom in *meta*, exhibits more similarities with other members of the phenylurea group (Table 2), broadening, thus, the number of herbicides retained by the IS. Thus, methoxuron, linuron and chlorbromuron possessing a chlorine atom in the same position are reocvered by the antichlortoluron IS in the amount of 80, 85 and 102% respectively, under the conditions mentioned above. In certain applications it may be an interesting approach, to combine antibodies with different selectivities on the same IS. Hence, antibodies to chlortoluron and isoproturon immobilized simultaneously on a silica support have been used for the enrichment of water samples containing various phenylurea herbicides *(33)*.

Table 2. Recoveries of the IAC extraction of phenylurea herbicides using antiisoproturon and antichlortoluron ISs*(a)*



Analyte	R_1	R_2	R_3	Antiisoproturon IS*(b)*	Antichlortoluron IS
Isoproturon	$(CH_3)_2CH$	H	CH_3	98	90
Chlortoluron	CH_3	Cl	CH_3	46	95
Methoxuron	CH_3O	Cl	CH_3	5	80
Linuron	Cl	Cl	CH_3	19	85
Chlorbromuron	Br	Cl	OCH_3	31	102
Monuron	Cl	H	CH_3	39	78
Neburon	Cl	Cl	C_4H_9	51	92

(a) The immunosorbents were prepared by immobilizing antibodies directed against isoproturon and chlortoluron on a silica support. *(b)* 50 mL of LC-grade water were spiked at 0.5 μg/L with a mixture of 15 phenylureas and percolated through the column. Data extracted from Pichon *et al. (5)*.

Coupling chemistry

The activation and coupling chemistry procedures determine the efficiency of the immobilization procedure. A variety of supports are commercially available from different suppliers (Pharmacia, Biorad, Pierce, etc.) with different types of activations. Usually, activation of the support occurs through their functional groups (*i.e.* hydroxyl groups: agarose, silica; carboxy group: alkaline hydrolysis of polyacrylamide etc), which are appropriately derivatized to introduce a spacer arm with the desired chemistry. The coupling methods described in the literature can be mainly grouped in two categories:

A) The antibodies are randomly oriented. Usually the ε-NH2 group of the lysine is the functional group of the antibodies most frequently used for coupling. Lysine residues are distributed throughout the Ab molecule leading to a randomly oriented immobilization of the antibodies. If immobilization occurs through the F_{ab} sites, the Ab ability to interact with the analyte may be reduced, and consequently also the column capacity.

Cyanylation of the hydroxyl groups of the agarose is one of the most popular activation procedures. Cyanogen bromide (CNBr) has been frequently used, although, due to its toxicity, other cyanylating agents have been introduced such as p-nitrophenylcyanate (pNPC) or 1-cyano-4-(dimethylamino)-pyridinium tetrafluoroborate (CDAP) *(34,35)*. The method is simple and yields high coupling rates with the amino groups of the antibodies in a wide range of pHs (4 to 10). However, the isourea bond formed (Fig. 4, A) is unstable resulting in a slow leakage of the Ab, especially if nucleophiles are present in the samples. Another disadvantage is that the isourea can be positively charged at neutral and acidic pH giving an ion exchange character to the IS.

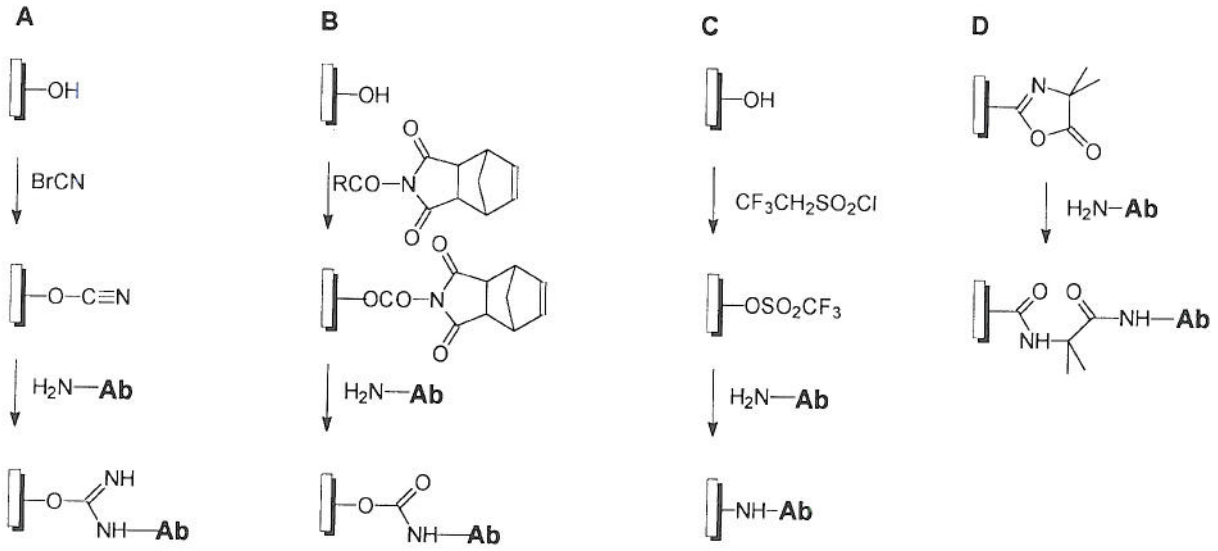


Fig. 4. Examples of some support activation procedures and coupling chemistry. A: Cyanylation; B: Carbonates; C: Sulphonyl esters; D: Azlactone

Carbonyl imidazole activated supports react with nucleophiles such as amino groups of the proteins to produce uncharged carbamates overcoming the ion exchange properties of the above mentioned coupling procedure. Reaction takes place at basic pH (8.5–11) leading to high coupling yields. Similarly activated **carbonate** supports have been used to obtain carbamate bonds after the coupling reaction (Fig. 4, B). Monoclonal antibodies for atrazine were immobilized on a beaded cellulose carbonate gel with a 100% yield *(18)*. In that study, from a group of five different activated supports, the cellulose carbonate support provided the best properties for immunoaffinity purification of triazines.

Organic sulphonyl chlorides have been used to activate hydroxyl-containing supports *(36)*. The sulphonyl ester is easily displaced by nucleophiles such as the thiol or amino groups of the proteins to form stable bonds. The most popular activators are the tresyl and tosyl chlorides (Fig. 4, C). The tresyl-activated supports may react with nucleophiles even at pH = 4 combining certain selectivity toward thiol groups in the presence of amines. Silica can be reacted with 3-sulfanylpropyl trimethoxysilane (SPTMS) to introduce the desired activation.

Azlactone polymeric supports may be directly used to couple the Ab with a very easy chemistry (Fig. 4, D). Azlactone polymeric supports have been used to immobilize atrazine monoclonal antibodies with a 71% yield *(18)*.

Aldehyde groups can be introduced on supports containing diol vicinal groups by $NaIO_4$ oxidation. Similarly, silica supports are activated with silanizing agents (i.e. 3-glycidoxypropyl trimethoxysilane) to introduce a spacer arm that contains a diol group (diol-bonded silica) which can subsequently be oxidized to obtain the aldehyde (Fig. 5, A). New aldehyde functionalized silanes are now commercially available, allowing direct introduction of the aldehyde group on silica supports *(15)* (Fig. 5, B). Aldehyde-activated silica support has been the preferred support of most of the immunoaffinity applications in environmental analysis (Table 1). Reaction of aldehyde activated supports with amino groups under mild conditions (pH = 7–8) leads to the formation of unstable imine bonds (Schiff base) that should then be reduced to a secondary amine using $NaCNBH_3$ (Fig. 5, A).

Amino groups can also be introduced on silica supports by using appropriate silanizing agents (Fig. 5, C). These amino groups can later react with the antibody through amino acids containing carboxylic groups or through the C-terminal end of the polypeptide chains thus forming amide bonds. Similarly, secondary amines can be formed after reaction with aldehyde groups generated on the sugar residues of the antibody molecule (see below).

Supports containing carboxylic acids can be activated through the formation of the corresponding **N-hydroxysuccinimide** esters (Fig. 5, D). Carboxylic acids can be introduced into hydroxyl containing supports by reaction with diacids (one carboxylic group forms an ester group with the support and the other is free for activation), or by appropriate silanizing agents on the silica supports. Similarly, polymeric supports such as poly(HEMA) containing an amide group can be hydrolyzed to yield free carboxylic group (Fig. 2). Coupling of amino groups to these activated supports takes place under mild aqueous conditions to produce amide bonds. A polymeric matrix activated as NHS ester (Affi-Prep®, BioRad) has been used to immobilize polyclonal antibodies to carbo-

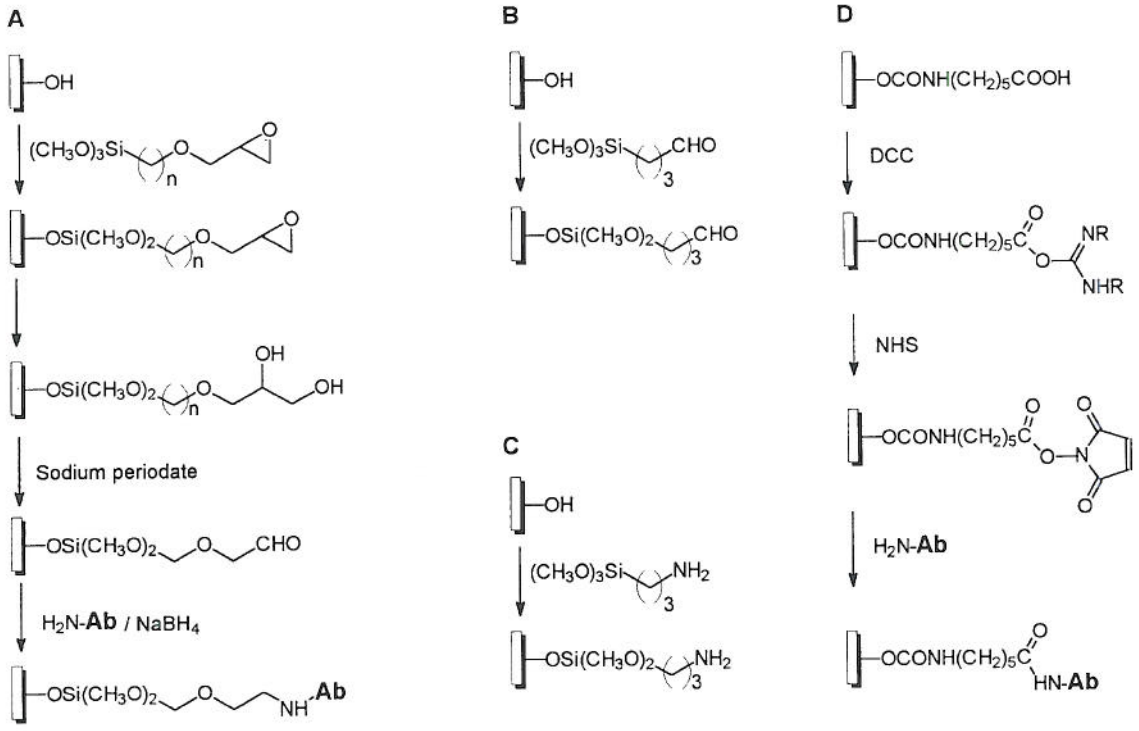


Fig. 5. Methods of activation of silica supports: A: diol; B: aldehyde; C: amine; D: N-hydroxysuccinimide ester

furan *(6)*; however no further applications of this support have been reported because of the nonspecific adsorption shown by the polymeric support. In contrast we have obtained high specific recoveries of atrazine by coupling polyclonal antibodies to an NHS activated agarose (NHS activated Superose^{TM}, Pharmacia) *(37)*.

B) The antibodies are uniformly oriented keeping free the F_{ab} sites for the recognition of the analytes. Oriented immobilization procedures use the following strategies:

i) The support is derivatized with elements recognizing the F_c region of the antibodies (anti-IgG antibodies, protein A/G, etc.) A 100% immobilization yield with a full Ab activity was reported using a Protein A-agarose support *(38)* (Fig. 6, A). Protein A is covalently attached to the F_c region of the IgGs by using a homofunctional cross-linker such as dimethyl pimelidate (DMP). The use of cobalt iminodiacetate resins as immunoaffinity media has also been described. The F_c fraction possesses a histidine-rich sequence located at the C- terminal region of the heavy chain, which is responsible for the binding of the antibodies to metals *(39)*. No examples are reported of the use of these strategies in environmental analysis.

ii) Covalent coupling through the sugar residues of the F_c side of the antibodies to **amine** or **hydrazide** activated supports (Fig. 6, B). Reactive aldehydes can be formed on the F_c region after periodate oxidation of the IgGs under mild conditions *(40)*. Subsequent reaction, under reductive conditions, with an amine or hydrazide support will lead to the formation of a stable secondary amino group. Besides the theoretical advantages of this coupling method, the randomly oriented immobilization procedures have often rendered superior results because of their simplicity, good coupling yields and mild reaction conditions. Thus, the coupling rate of atrazine monoclonal antibodies to a hydrazide derivatized support was only 61%, and additionally, only 46% of the theoretical binding capacity was used to retain atrazine *(18)*. Similarly, it has been reported that oxidation of the carbohydrate residues has more success with polyclonal than with monoclonal antibodies *(41)*. Some authors have explained this behaviour by the presence of carbohydrate moities located on the F_{ab} part of the monoclonal antibodies *(42,43)*.

iii) Covalent coupling through thiol groups of antibodies fragments. Thiol groups generated from whole Ab molecules or from F_{ab} fragments by reducing agents, such as mercaptoethanol, DTT or mercaptoethylamine, can specifically react with **iodoacetyl-** or **maleidimide-**activated supports (i.e. Sulfolink® coupling gel, Pierce), keeping free the F_{ab} sites. Although it is an interesting approach, low antigen binding has been reported for these supports *(44)*.

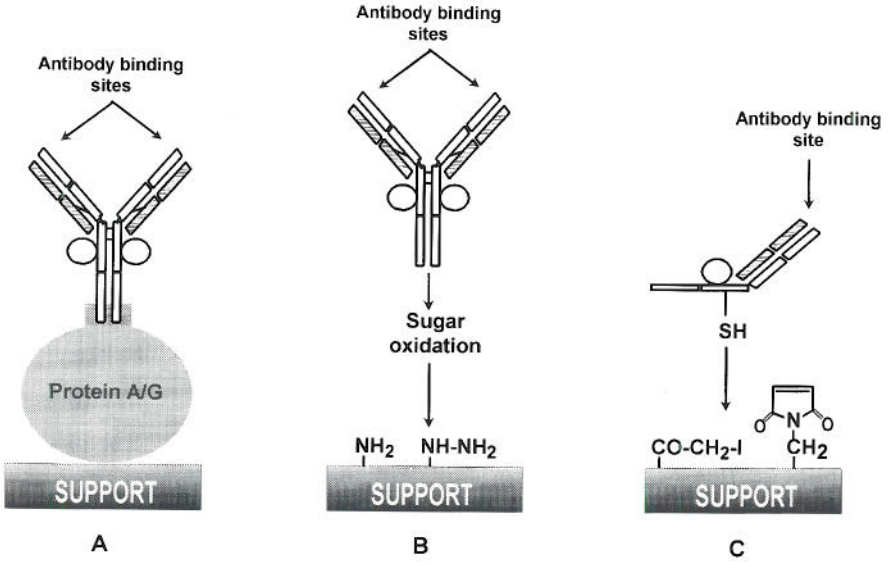


Fig. 6. Antibody-oriented immobilization methods: A: Protein A; B: Oxidation of the sugar residues; C: Thiol groups generated from antibody fragments

Operation in immunoaffinity chromatography

Loading the column

Capacity of the column and the *breakthrough* volume are important parameters to consider when loading the immunoaffinity column. The *capacity* of the column indicates how much analyte can be retained by the IS. This fact is strongly dependent on the support, its coupling capacity and the quality of the antibodies used. The theoretical coupling capacity of the support is usually provided by the commercial supplier, but the amount of immobilized antisera should nevertheless be measured. On the other hand, as mentioned before, Ab quality is

strongly dependent on the hapten design and procedures employed to raise antibodies *(31)*. In this context, Pichon *et al. (19)* showed the differences encountered in the analyte extraction recoveries when the IS had been prepared with antisera from distinct bleedings or raised against different immunizing haptens *(5)*. Although it has been sugested that the use of the hapten specific affinity chromatography isolated fraction of the polyclonal antibodies increases IS capacity, there may exist the risk of an incomplete recovery of the highest affinity fraction of the antisera.

Capacities values of immunoaffinity columns for a single analyte have been reported (Table 1). However, pesticides are usually applied in combination. Often two or more members of the same family can be detected together in an environmental sample. Because of the cross-reactivity exhibited by the antibodies, structurally related analytes will compete for the same Ab binding sites, modifying the behavior of the IS. Hence, 90 ng of atrazine were retained by an antiatrazine IS when the analyte was applied together with a mixture of nine other triazines (2.5 μg each), but 630 ng when the same amount was percolated alone using the same sample volume *(5,45)*. Nevertheless this behavior seems to be dependent on the analyte concentration and Ab nature as well. In fact, it has been demonstrated that the column capacity is not directly related to the avidity of the antibodies used. Thus, analyzing mixtures of phenylureas with an antiisoproturon IS, a greater retention of isoproturon at low concentration values was observed. However, the amount bound reaches a plateau while for other phenylureas the amount trapped is constantly increasing.

Breakthrough occurs when the analytes are not retained either because they are not sufficiently recognized by the immobilized antibodies or because overloading of the IS has taken place. Unfortunately, this column feature is often omitted in many of the published papers on pesticide immunoaffinity purification. Since the IS exhibits different affinities for the analytes of a same family, the breakthrough volume should be measured for individual analytes. An exhaustive study on the breakthrough volumes of 9 triazine herbicides using antiatrazine and antisimazine IS has been reported *(5)*. The study shows that breakthrough occurs at 50 mL, for most of the compounds except for those analytes highly recognized by the antibodies. Thus, using an antiatrazine IS atrazine, terbutylazine and propazine resulted in high recovery values (85, 84 and 100%, respectively) when 150 mL sample volume was applied. The breakthrough volumes of 16 phenylurea herbicides (100 ng each, were evaluated using an antiisoproturon IS *(19)*. At 200 mL five phenylureas showed recoveries higher than 50%, and isoproturon presented a recovery of 100% even with 500 mL sample volume. In contrast, using an antichlortoluron IS, because of its broader specificity (see Table 2), chlortoluron, isoproturon, diuron, chlorbromuron and neburon showed 100% recovery even with 150 mL sample volumes *(5)*. On a MAb-based antiatrazine beaded cellulose column, 500 mL volume solutions of atrazine and terbutylazine (20 μg/L) yielded recoveries of 95 and 96%, respectively *(18)*. In contrast to the capacity values, the differences encountered in the calculated breakthrough volumes of the different phenylurea herbicides had a better agreement with the avidities expected from these antibodies *(5)*.

Besides this, other important parameters determining IS capacity are the Ab immobilization procedure employed and the availability of the Ab binding sites (see above) and the flow rate applied. Flow rate should allow efficient analyte-Ab interaction. It has been reported that high flow rates may diminish the capacity of the column, while low flow rates (0.5 to 1 mL/min) seem to be adequate for the retention of carbofuran by the IS *(20)*.

Washing step

The function of the washing step is to remove unwanted materials from the matrix and nonspecifically bound compounds. The number of column volumes necessary to remove nonspecifically bound substances depends on the adsorption association constant (K_{ad}). It is desirable that the used supports show low nonspecific adsorption $K_{ad} < K_a$. In that case, the buffer used for the loading step is also adequate for the washing procedure. If $K_{ad} \leq K_a$, harsher conditions would be required to remove the unwanted nonspecifically bound impurities and the joint elution of the specifically retained analyte may occur.

Elution of the analytes

Unfortunately, an universal system that ensures an efficient desorption of the analytes does not exist. Every immunosorbent should be checked for the best elution. The strategy of elution is based on creating a local environment that reduces the value of the association constant K_a. Some of the methods that have been employed to accomplish this condition are:

i) Changing of the buffer pH above 10 or below 2.5 is one of the most often employed strategies. Since higher pH may lead to irreversible denaturation of the antibodies, lowering the pH is the preferred strategy. Triazine metabolites and carbendazim were completely desorbed from a silica-based IS by using 10 mL of phosphate buffer (0.05 M, pH=2.5) *(20,46)*. At lower pH a rapid loss of the Ab activity was observed. On an on-line silica-based IAC/LC/MS system, carbofuran was trapped by a C_{18} column after desorbing it from the IS by flushing at 3 mL/min with a 0.2% formic acid (pH=2.6) solution during 5 min *(6)*. However, this step is strongly dependent on the support employed. By comparing several desorbing systems, Marx *et al. (18)* obtained recoveries close to 100% when eluting atrazine with 0.2 M Glycine-HCl, pH=2.2 from a beaded cellulose ONB carbonate (Eurochrom-Knauer, Berlin) support. In contrast, the same buffer system used on LiChroprep 2000 columns (Merck, Darmstadt) yielded only between 60 and 80% of the total loaded atrazine.

ii) Organic solvents alter the polarity of the system reducing consequently the value of K_a. Each Ab-antigen system may show a different behavior with increasing amount of organic solvents. Thus some solvents can be tolerated up to 60 or 70% without interfering with the association of the immunocomplex, while others readily decrease the value of K_a at very low percentage *(47)*. A volume of 4 mL of a 7:3 methanol:water solution was

necessary to efficiently elute triazine and phenylurea herbicides from a silica-based IS *(19)*. In that work, acidic buffers or chaotropic solutions did not produce desorption of the analyte, while 0.2% formic acid solution allowed recovery only of the weakly bound herbicides (low K_a). In an *on-line* procedure, the same analytes were also efficiently desorbed by applying an acetonitrile gradient in phosphate buffer at 1 mL/min *(5)*. Phenylurea herbicides have also been desorbed from a silica-based IS using mixtures of ethanol in phosphate buffered saline (PBS) solutions at low pH *(33)*.

iii) Alteration of the ionic strength causes changes in the association constant and may mildly desorb analytes. However, no applications of this strategy have been reported in environmental analyses.

iv) Chaotropic agents disrupt water structure, break hydrogen bonds and weak hydrophobic interactions. However, the risk of protein denaturation has reduced the application of these agents and no examples to elute analytes of environmental interest have been found in the literature.

Regeneration and storage of the IA column

Regeneration of the column is necessary to start a new purification cycle or to store the column. Removing the elution buffer rapidly will ensure a longer Ab half-life. Storage conditions are usually at 4 °C with phosphate buffer in the presence of a preserving agent such as sodium azide or thirmersoal.

Environmental applications of IAC

The application of IAC for environmental analysis is still rare. Usual methods of analysis of environmental pollutants require a clean-up and/or preconcentration step, which is mainly accomplished by solid-phase extraction (SPE) techniques. Besides the wide variety of stationary phases offered by the commercial suppliers, efficient retention of high polar compounds from water samples is still difficult. In contrast, polarity is not a limitation for immunoaffinity extraction since it is based on specific interactions between the IS and the analyte, allowing thus a selective extraction of the target compounds.

Performance of IAC has been demonstrated only with very few pollutants (Table 3). Many of the scientific papers published are focussed on atrazine, its metabolites *(18,19,46,48,49)* and phenylurea herbicides *(5,19,33,45,50,51)*. To a lesser extent immunoaffinity purification of other pesticides has been reported, such as carbofuran *(6)* and carbendazim *(20)*. Purification or preconcentration procedures may be *off-line* using cartridges containing the IS, where the selectively eluted fraction is subsequently analyzed by HPLC, GC or immunoassay *(19,49,50)*. However, automated *on-line* procedures have also been reported *(6,19,20,46,49)*. In this case, it is particularly important that the supports employed are able to resist the pressure conditions required by the chromatographic system (see above). The sensitivity reached is highly dependent on the detector system used. Thus, it is possible to reduce the amount of sample required and simultaneously reach very low limits of detection when mass-spectrometry is used *on-line* with the chromatographic system (Table 3).

Water samples from different origin (tap, drinking, river, runoff, etc.) have been the most commonly analyzed samples *(6,19,20,33)*. However, interesting applications to more complex matrices such as plant extracts have been reported to analyze phenylurea and triazine herbicides *(49,50)*. It is worth noting that on some vegetable materials no further clean-up steps had to be introduced other than IAC, to quantitate these herbicides. Table 3 summarizes some of these applications and the detection limits obtained in each case.

Atempts to use IAC as a separation technique have been reported although elution conditions had to be adjusted to selectively desorb the different analytes belonging to the same family, obtaining, at the same time, narrow peaks for proper resolution. An *on-line* reverse phase cartridge should usually be introduced to avoid

Table 3. Examples of IAC application in environmental analysis

Analyte	MAb/ PAb	Analytical System	Sample	Sample size	LOD	Ref.
Atrazine	MAb	*on-line* HPIAC/RPLC/UV	Buffer	45 mL	6 ng/L	*(48)*
	PAb	SPE-SAX, IAC, LC/UV	Apple, carrot, celery, corn, potato and peas	5 g	2–10 ng/g	*(49)*
Hydroxyatrazine	MAb	*on-line* HPIAC/RPLC/UV	Buffer	45 mL	6 ng/L	*(48)*
Deethylatrazine	MAb	*on-line* HPIAC/RPLC/UV	Buffer	45 mL	10 ng/L	*(48)*
Deisopropylatrazine	MAb	*on-line* HPIAC/RPLC/UV	Buffer	45 mL	10 ng/L	*(48)*
Carbendazim	MAb	*on-line* HPIAC/RPLC/DAD	Reagent, pond, creek and fountain water	200 μL *(a)*	75 ng/L	*(20)*
		on-line HPIAC/RPLC/MS	Reagent, pond, creek and fountain water	200 μL *(a)*	25 ng/L	
Carbofuran	PAb	*on-line* HPIAC/RPLC/MS	Surface runoff water	50 mL	40 pg/mL	*(6)*
Phenylurea herbicides	PAb	IAC, SPE-HPLC/UV	Drinking water	200 mL	0.1 μg/L	*(5,19)*
		on-line HPIAC/RPLC/DAD	Seine river			
	PAb	IAC, HPLC/UV	Potatoes, carrots	5 g	25 ng/g	*(50)*
	PAb	IAC, SPE-SAX, LC/UV	Grape, onion, celery, corn, strawberries	5 g	25 ng/g	*(50)*

(a) Up to 40 mL can pass through the column without changing the column performance

this problem. More frequent is the connexion of an IAC column to an analytical RPLC column to separate the analytes (i.e. *(48)*), although, in this case, it is also necessary to introduce a RP precolumn to preconcentrate the sample even more. Another application of the immunoaffinity is the enzyme immunoassay. The immunoassay is performed inside the column by mixing the samples with an appropriate enzyme tracer. In the last step, the eluted product of the enzymatic reaction is measured to quantitate the analyte *(52,53)*. It has been reported that applying this method the presence of phenylurea herbicides in water samples can be visually detected at levels close to 0.1 μg/L.

Conclusion

In this paper we have presented the basic principles of the IAC and the most commonly used approaches for IS preparation. Each analyte/Ab system may perform better with particular supports and under specific operational features. The optimum condition should be investigated separately to render an efficient and economic immunopurification process. Although a variety of activated supports are available nowadays in the market, a knowledge of their properties is necessary to diminish nonspecific interactions. In most of the environmental applications reported to date, silica supports have been the preferred materials. Polysaccharide supports show excellent properties, limited only by their susceptibility to gradient pressure. In contrast, most of the reported studies using polymeric materials point to the nonspecific adsorption of the analytes to the unbonded matrix.

IAC offers a great potential for the selective purification of a wide variety of environmental contaminants, especially of those polar compounds which are difficult to isolate by other commonly used supports. However, besides its demonstrated versatility and specificity, IAC is still seldom used in routine environmental analyses. Preparation of polyclonal or monoclonal antibodies for small molecules is an intricate work, which determines the unavailability of a wide range of selectivities. This fact has also an influence on the actual high price of the immunoreagents available on the market. Compared to other immunochemical techniques such as ELISA (enzyme-linked immunosorbent assay), the amount of Ab necessary to prepare an IS is much higher, increasing thus the cost of the technique. In this context recombinant DNA techniques will hopefully decrease the price, facilitating the widespread commercial application of immunoaffinity purification methods.

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Osnovne zasade u primjeni imunoafinitetne kromatografije za analizu okoliša

Sažetak

Uobičajeni postupci ekstrakcije nisu selektivni za uzorke iz okoliša. Stoga je analiza malih količina onečišćavača ili njihovih produkata u kompleksnim uzorcima često otežana zbog različite polarnosti i malih koncentracija pojedinih sastojaka. Osim toga, polarni se sastojci često nepotpuno izdvajaju uobičajenim postupcima ekstrakcije u krutoj fazi. Imunoafinitetna je kromatografija (IAC) način odvajanja koji se zasniva na specifičnim i reverzibilnim interakcijama između antigena i antitijela. Budući da retencija ovisi o drukčijim fizičko-kemijskim svojstvima, IAC omogućuje alternativni pristup primjeni postojećih postupaka ekstrakcije. U radu su prikazani osnovni postupci IAC-odvajanja. Prema podacima iz literature razmotrene su najvažnije značajke priprave imunosorbensa i način provođenja postupka ekstrakcije u krutoj fazi. Prikazana je primjena IAC za analizu okoliša.