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review

Reversibility in Heterogeneous Flow Immunosensing and Related Techniques. A Brief Overview.

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Summary

In the development of many immunochemical techniques the reuse of the immunosorbent is one of the problems to be taken into account. The choice of an adequate desorbent agent which accomplishes an effective desorption without influencing the immunosorbent activity is the key to solving it. This is a review paper of the methods dealing with the problem of reversibility in immunochemical interactions and how the immunosurface life is modified, especially in fields as heterogeneous as flow immunoassays and immunoaffinity extraction and chromatography.

Keywords: immunoassay, reversibility, antigen/antibody interaction, immunoaffinity, immunochromatography

Introduction

The use of immunochemical reagents, antigens and antibodies, as tools in analytical chemistry has been spreading continously, and the number of analytical techniques that use antigen-antibody reactions is growing steadily. This is due to the high sensitivity and selectivity of the immunochemical reactions, and to the higher availability of immunoreagents *(1)*. In the past decades, new techniques of antibody production on a large scale have been developed *(2)*. The antibodies are able to interact not only with macromolecules and with virtually any organic compound (hapten), but also with metal ions such as Hg *(3)*.

Most classic immunoassays are based on the irreversible binding of the analyte to the antibody and the measurement of the extension of this binding by means of the use of auxiliary labeled reagents, thus sending the immunocomplex to waste. This protocol is employed in nearly all batch immunoassays (e.g. enzyme-linked immunosorbent assay, ELISA), and it implies immunoreagent consumption. Also, a high reproducibility in the addition of immunoreagents is required for each assay.

In the last years, continuous-flow immunoassay techniques have been developed. These methods are based on the same principles as batch immunoassays, and use immunoreagents antibodies or antigens – immobilized on a solid support. These flow techniques offer the possibility of reusing the immunosorbent for a large number of assays, thus lowering the cost and raising the reproducibility of the assays. They satisfy the need for breaking the antigen-antibody binding without affecting the immunological activity of the immobilized reagent.

Sensitivity of immunological techniques is directly related to the antigen-antibody affinity constant. Nevertheless, in methods that require the breaking of the antigen-antibody binding, the use of high-affinity antibodies is not recommendable, since in this case the desorption will have to be accomplished by exposing the immunocomplexes to harsh conditions that can damage the immunosorbent *(4)*. A compromise is required between assay sensitivity and immunoreactor reusability. If this compromise is not reached, the immunosorbent must be discarded after each assay, which makes these systems impractical.

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Immunological extraction and clean-up procedures based on the adsorption of the analyte (usually the antigenic species) in a column containing the antibody immobilized on a support are being used. After the adsorption and a further washing step to eliminate the matrix components, a change in the elution conditions allows the desorption of the analyte, that is being collected in the eluate. In these systems, it is desirable that the antigen-antibody reaction be reversible, in order to reuse the immunocolumn.

The current regeneration techniques are based on the exposure of the so-called immunocomplexes to a reagent, which weakens the antigen-antibody interaction and allows the physical separation of both species. Only if the immunoreactor keeps its activity after the desorption process (i.e. there has been no irreversible denaturation) it can be reused.

Hence, in the development of techniques with antigens or antibodies immobilized on a support placed into a column, a critical problem to solve is the reversibility of the antigen-antibody complex. In this sense it is interesting to have information about the methods of reverting immunocomplexes that were employed in previously developed techniques.

In this paper, heterogeneous flow immunoassays and immunoaffinity techniques are reviewed paying attention to the features of the immunosorbent employed in terms of support, immobilized antigen or antibody and immobilization technique, and the method employed for reverting the antigen-antibody interaction, as well as the stability of the immunosurface after repeated regeneration. The aim of this review is to present the most representative works using immobilized immunoreagents, especially those which emphasize the problem of immunosurface reusability and offer practical solutions. Examples of single-use immunosorbents are also given.

Heterogenous Flow Immunoassay Techniques. Flow-Through Immunosensors

Flow immunoassays are immunochemical analysis techniques that combine the sensitivity and selectivity of immunological reactions with the speed, versatility, accuracy and applicability to process control of flow-injection analysis methodology. Bibliographic reviews of immunoassays coupled to flow-injection techniques can be found in the literature *(5)*. The applicability of these methods to the process control has also been reviewed *(4)*.

There are currently instrumental methods able to directly detect immunological interactions, *e.g.* microbalance or surface plasmon resonance, but such methods either are expensive or need the use of defined supports. More common is the employment of auxiliary labelled reagents (tracers) that generate a signal that can be related to the amount of analyte in the sample. The most used labels are radioisotopes, fluorescent dyes and enzymes. Such auxiliary reagents have mainly given rise to two assay formats: competitive (direct or indirect) and sandwich.

Flow immunoassay without tracers

In techniques that measure directly the antigen-antibody interaction, the analyte is put in contact with an excess of immobilized anti-analyte antibody (when the analyte is the antibody, an excess of immobilized antigen is used), and the measurement is directly related to the amount of analyte. The use of these systems is the key to the development of sensors operating without additional reagents and giving responses in real time. The amount of immobilized reagent must be excessive, so that the binding of the analyte is quantitative. The binding reaction must be reproducible after repeated assays.

Gebbert *et al. (6)* have developed a flow-through capacitance cell that can directly monitor immunochemical reactions. In the cell, the antigen (mouse IgG) is covalently immobilized on a tantalum oxide layer by means of film silanization and antigen coupling with cyclohexyl-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate. When the antibody (goat anti-mouse IgG or sheep anti-mouse IgG conjugated to β-galactosidase) binds to the antigen, a change in the capacitance of the cell is produced. The system can be also used with the antibody immobilized and the antigen in solution. The elution of bound species is performed by rinsing the cell with 0.1 M glycine/HCl buffer, pH = 2.1, for 5 min at 0.3 mL min^{-1} flow rate. The activity of the sensor strips decreases to 50% after 40 measurements.

An integrated immunobased elution assay for the on-line determination of tissue-type plasminogen activator and recombinant antithrombin III has been developed by Beyer *et al. (7)*. The antibodies (polyclonal and monoclonal) are immobilized on CNBr-Sepharose 4B, and analytes are fluorimetrically detected during the elution, which is carried out by means of 0.1 M K_3PO_4 buffer pH = 12.3 (fluorescence maximum). The capacity of the cartridges decreases by more than 65% during 7 weeks, although the analytical detection is not influenced.

A special application was proposed by Brecht *et al. (8)*. In their work, the analyte (atrazine) binds to a monoclonal antibody in solution, which inhibits the binding of the antibody to immobilized conjugate atrazine-caproic acid, i.e. an immunoassay with competitive indirect format (see below). The conjugate is immobilized on 10 nm Ta_2O_5–500 nm SiO_2 interference layers by means of the amino groups created on the chip surface with 4-aminobutyldimethyl-ethoxysilane, conversion of amino groups to carboxyl groups by reaction with succinic anhydride, conjugation of carboxyl groups to aminodextrane with ethyldimethylaminopropylcarbodiimide, and coupling of the atrazine-caproic acid with the resulting surfaces with diisopropylcarbodiimide. The derivatized chip is placed into a flow cell designed for reflectometric interference spectroscopy measurements, that detect the binding of the anti-atrazine antibody, and the measurement is related to the amount of free atrazine present in the sample. The chip is regenerated by incubation with 2 mg mL^{-1} pepsin solution in phosphate buffered saline solution, pH = 2, for 9 min. The number of assay cycles that can be carried out with the same chip is not mentioned.

A similar example is the system proposed by Bier *et al. (9)* for *s*-triazine determination. In that device, the

hapten (2-aminohexyl-amino-4-ethylamino-6-isopropyl-amino-1,3,5-triazine) is immobilized on a planar SiO_2-TiO_2 monomode waveguide. The immobilization is performed by silanization of the surface with aminopropyltriethoxysilane, treatment with glutaraldehyde, hapten coupling to the support via Schiff's base formation, and reduction of the Schiff's base with $NaBH_4$. The derivatized waveguide is placed into a suitable flow-through cell that detects the binding of the antibody by monitoring the refractive index. For regeneration purposes, the following sequence is used: washing with 0.1 M glycine/HCl buffer, pH = 1.5 (5 min), washing with phosphate buffered saline, pH = 8.0 (5 min), incubation with 10 μg mL^{-1} proteinase K (10 min), washing with phosphate buffered saline, pH = 8.0 (5 min), and washing with 0.1 M glycine/HCl buffer, pH = 1.5 (5 min). Under these conditions, the same derivatized waveguide is used for more than 600 times over a 2-month period.

Another flow immunosensor for atrazine using an immobilized hapten was proposed by Tom-Moy *et al. (10)*. In that case, the support (quartz) is derivatized with (3-glycidoxypropyl)trimethoxysilane, followed by treatment with periodate in order to oxidize the diol groups to aldehyde groups. Avidin is then coupled to the aldehyde groups, and finally a biotinylated derivative of atrazine is allowed to bind to avidin-derivatized devices. The binding of a polyclonal antibody to the quartz is monitored by means of surface transverse wave measurements. The regeneration of the immobilized hapten is accomplished by treatment with 10 mM HCl for 1 min. Sensors can be used for 100 repeated cycles, although after 48 cycles there is a 30% decrease in the signal response.

Flow immunoassay with competitive format

In heterogeneous immunoassays using labelled reagents, direct or indirect competitive formats are the most common approach found, both in batch immunoassays and flow immunotechniques. The basic operations performed by continuous immunoanalyzers are the same as in batch immunoassays for the two formats. In the competitive direct format, a limited amount of antibody is immobilized on the support. In the indirect competitive format, the antigen or hapten is the immobilized immunoreagent *(11)*. In both cases, the amount of labelled reagent bound to the support is measured, the value being inversely proportional to the amount of analyte present in the sample.

Single-use immunosorbents, as well as systems of highly reusable immunoreactors, can be found in the literature. Between these extremes, there are many intermediate instances.

An example of flow immunoassay employing single-use reactors was proposed by Krämer and Schmid *(12)*. In that work, a polyclonal anti-atrazine antibody is immobilized on a Fluorotrans membrane via protein A interaction, and a conjugate atrazine-peroxidase is used as competitor. The membrane is automatically changed after each assay, so that the same immunosorbent is not reused.

Pollema *et al. (13)* have described the determination of mouse IgG using a commercial immunosorbent containing polyclonal sheep anti-mouse IgG immobilized on magnetic particles, and mouse IgG conjugated to fluorescein isothiocyanate as tracer. A different batch of immunosorbent, which is retained in the reactor or sent to waste by means of a magnetic field created by an electromagnet, is used in each assay.

Pollema and Ruzicka *(14)* have developed a system of exchanging the immunosorbent automatically, based on the jet ring cell, which is especially designed to retain the immunosorbent and send it to waste after the measurement by means of an inversion in the flow direction. The system uses goat anti-mouse IgG bound to agarose beads, and a conjugate mouse IgG-R-phycoerythrin as tracer. Nevertheless, although these methods are simple and fast, they suffer from some disadvantages, such as lack of reproducibility, reagent consumption, and the need of employing special detectors (microscopy) in the case of the jet ring cell. So, the option of reusing the same reactor after a desorption treatment is generally preferred.

One of the first examples of flow immunoassay using a high-reusable system was developed by De Alwis and Wilson *(15)*. This system is able to determine human serum albumin using $F_{ab'}$ fragments of a polyclonal anti-human serum albumin antibody immobilized on Trisacryl GF-2000, and a conjugate human serum albumin-glucose oxidase as enzyme tracer. After the measurement, the regeneration is carried out by flushing 0.1 M phosphoric acid, pH = 2.0, through the reactor for 2 min. Using this treatment, the same reactor can be reused for 600 assay cycles, with a decrease in the immunological activity of less than 5%.

The group headed by Durst has developed two flow immunoassay systems based on the same principle. In both cases, the model analyte used is theophylline, and the tracer is theophylline bound to liposomes that are previously filled with a carboxyfluorescein solution. In the immunoreactor, a moderate affinity anti-theopylline monoclonal antibody is immobilized either on a planar waveguide fabricated on Corning 0211 glass *(16)* or fused silica particles *(17)* by means of a treatment with (3-glycidoxypropyl)trimethoxysilane followed by oxidization of diol groups in order to create aldehyde groups and subsequent coupling of the antibody through its primary amine. In both cases, after the competitive reaction, the adsorbed liposomes are lysed by flushing 1-*O*-octil-β-D-glucopyranoside through the reactor and the carboxyfluorescein released is detected fluorimetrically. The regeneration step is accomplished by rinsing with assay buffer. The Corning glass reactors can be reused 15 times without serious degradation (> 10%) in activity, while the fused silica reactors are reused for more than 300 analysis. Here, the difference in behaviour is due to the support used in each case.

A liposome-based flow immunoanalysis for the determination of alachlor was described by Edwards and Durst *(18)*. The system uses a polyclonal antibody immobilized on glass beads by means of (3-glycidoxypropyl)trimethoxysilane, alachlor conjugated to liposomes filled with potassium ferrocyanide as tracer, and electrochemical detection of the released ferrocyanide. The regeneration of the immunoreactor is accomplished

by treatment with 3 M KSCN for 30 s followed by 0.2% Triton X-100 in phosphate buffered saline for 5–10 min. The system is completely reversible, but the number of assays performed by a single reactor is not mentioned.

The pesticide imazethapyr has been detected using a capillary column flow injection liposome immunoanalysis system *(19)*. The capillary column contains a monoclonal antibody orientatedly immobilized on the inner wall by means of capillary derivatization with (3-glycidoxypropyl)trimethoxysilane, aldehyde formation with periodate, protein A binding to aldehydes, antibody binding to protein A, and covalent linkage formation between the antibody and protein A with dimethyl pimelimidate. A sequential competition is established between the analyte and analyte-tagged liposomes filled with carboxyfluorescein, followed by liposome lysis with n-octyl-β-D-glucopyranoside. The desorption is accomplished by flushing a 30% methanol solution through the column. The capillary life is up to 150 assays when standards are injected and half of that when analysing water samples.

A highly-reusable flow-through immunoanalysis has been proposed by Wittmann and Schmid *(20)*. In that work, both monoclonal and polyclonal anti-atrazine antibodies are immobilized on polystyrene and glass beads by means of the avidin-biotin system. A conjugate atrazine-peroxidase is used as enzyme tracer. After testing several desorption reagents, the best regeneration method was a rinse with 0.01 M glycine/HCl buffer, pH = 2.0, for 1.5 min (flow rate 0.72 mL min^{-1}) followed by a subsequent rinsing step with phosphate buffered saline for 1.5 min. In all cases, the reactor can be used for a minimum of 500 assay cycles without any significant loss of antibody activity or protein amount bound to the support.

The same system was further developed by Dietrich and Krämer *(21)* using a monoclonal anti-terbutryne antibody. In that work, the enzyme tracer is a conjugate terbutryne-peroxidase. The regeneration of the reactor is performed by flushing 1 mL of 0.1 M glycine/HCl, pH = 2.1, for 2.38 min. Under these conditions, each reactor is reused between 60 and 100 times, with more than 60% activity loss.

A flow immunoassay system for continuous on-line process monitoring has been developed by Nilsson *et al. (22)*. In that work, α-amylase from a fermentation reactor is determined using a polyclonal anti-α-amylase antibody immobilized on a Sepharose column (the immobilization method is not described) and a conjugate α-amylase-peroxidase as enzyme tracer. Column desorption is accomplished by a 30 s pulse of 0.1 M glycine/HCl buffer, pH = 2.2. The column slowly loses its binding capacity, so it has to be discarded at the end of a working day. This phenomenon is interpreted as being due to the presence of proteases in the sample that attack the immobilized antibodies, since when monitoring α-amylase in standard solutions one column can be used for 270 assays.

The reusability performance of a competitive fiber-optic based flow immunosensor has been studied by Oroszlan *et al. (23)*. In that work, a monoclonal anti-atrazine antibody is immobilized on silanized quartz fibers by physical adsorption, and the competition is established between the analyte (atrazine) and fluorescein-labelled atrazine, which is fluorimetrically detected through the fiber-optic. The regeneration of fibers is accomplished by applying a combination of pH = 3 buffer and 12% 1-propanol for 5 min. In those conditions, a linear signal decay can be observed, producing a reduction of the absolute signal from ≈0.2 to ≈0.1 fluorescence intensity units in 325 consecutive assay cycles.

Two flow-through immunosensors have been developed by González-Martínez *et al. (24)* for the analysis of the insecticide carbaryl. The direct format system uses a monoclonal anti-carbaryl antibody immobilized on a hydrazide-derivatized agarose gel (Affi-Gel Hz), a conjugate 6-[((1-naphthyloxy)carbonyl)-amino]hexanoic acid-peroxidase as enzyme tracer and fluorometric detection. The immunosensor regeneration is accomplished by injecting 0.35 mL of 0.1 M glycine/HCl buffer, pH = 2.0, at 0.5 mL min^{-1} flow-rate. The activity of the immunosensor remains constant for 60–70 assay cycles. On the other hand, the indirect format system uses a conjugate 6-[((1-naphthyloxy)carbonyl)-amino]hexanoic acid-bovine serum albumin immobilized on a *N*-hydroxysuccinimide-derivatized agarose gel (Affi-Gel 15) as immunosorbent, a different monoclonal anti-carbaryl antibody in solution and a conjugate polyclonal rabbit anti-mouse IgG-peroxidase as secondary antibody. The immunosorbent regeneration is carried out by injecting 3 mL of 0.1 M glycine/HCl buffer, pH = 2.0, at 2 mL min^{-1} flow rate. The same immunoreactor can be used for 160–200 assay cycles.

Flow immunoassay with sandwich format

The sandwich format is applicable to antigen analytes with multiple antigenic determinants, and its name is due to the fact that the analyte is sandwiched between a solid phase immobilized antibody and a labelled antibody. An analog format is used in order to detect the presence of specific antibodies in the sample, in which the antibody is sandwiched between the immobilized target antigen and a labelled secondary antibody *(11)*. This format has some advantages over the competitive format, such as higher sensitivity *(25)* and direct relationship between the signal measured and the amount of analyte present in the sample *(26)*. The main drawback of this format is the lack of applicability to the determination of haptens.

Single use reactors can be found in flow immunoassays using sandwich format. One example is given by Pollema and Ruzicka *(14)*. In that work the immunosorbent used, as well as the technique for exchanging it, are the same as those described earlier for the competitive assay format.

Another example is given by Huet and Bourdillon *(27)*. In that case the analyte is rabbit IgG, and the antibodies used to form the sandwich are goat anti-rabbit IgGs, the secondary one being conjugated to glucose oxidase. The immunosorbent is made by physical adsorption of the primary antibody on a glassy carbon electrode placed in an electrochemical flow cell. After a measurement, the electrode is re-anodized by applying a potential of 2.2 V *vs.* saturated calomel electrode while flushing a 1.6 M HNO_3 solution, so that all the bound

species, including the primary antibody, are quantitatively desorbed from the electrode.

Anyway, the reuse of immunoreactors in flow-injection sandwich immunoassays is a more popular choice. An earlier sandwich flow immunoassay was designed by De Alwis and Wilson *(25)*. The system determines mouse anti-bovine IgG using a column of bovine IgG immobilized on Reactigel-6X as recommended by the manufacturer, and a secondary goat anti-mouse IgG conjugated to glucose oxidase. The bound antibodies are desorbed from the column by flushing 0.1 M phosphate buffer, pH = 2.0, for 5 min at a 0.5 mL min^{-1} flow rate. The immunosorbent reactor is shown to be stable for up to 3 months of repeated use (at least 500 assays).

Another flow immunoassay with sandwich format has been presented by Shellum and Gübitz *(28)*. In that work mouse IgG is determined by sheep anti-mouse IgG immobilized on carbonyldiimidazole-activated Trisacryl GF-2000 following the manufacturer's instructions, and acridinium ester-labelled goat $F_{(ab')_2}$ fragments of anti-mouse IgG. Reactor regeneration is carried out by means of 0.1 M phosphoric acid, pH = 1.8, for 2 min at 0.3 mL min^{-1}. The number of assays performed by a single reactor is not specified, but after five days the reactor activity, expressed as the slope of the calibration graph, decreases from 42 to 26.

Lee and Meyerhoff *(26)* have developed a sandwich flow immunoassay using human IgG and α-acid-glycoprotein as model analytes. In both cases the immunosorbents used are a polyclonal antibody immobilized on carbonyldiimidazole-activated controlled-pore glass, and adenosine deaminase as label for the secondary labelled antibody. The column is regenerated by washing with 0.1 M glycine/HCl buffer, pH = 2.2, for 2 min at 0.7 mL min^{-1} flow rate. The immunoreactors can be used for several hundred assays (e.g. 300) without a significant decrease in antigen binding.

The parathyroid hormone is determined by Hage and Kao *(29)* using an automated flow-through immunoaffinity assay. In that study, the polyclonal antibody is purified by immunoaffinity chromatography. The antiserum is introduced into a column containing the antigen immobilized on CNBr Sepharose 4B and after a washing step, the bound antibody is eluted from the column by flushing with 0.1 M phosphate buffer, pH = 3.0. Only the fraction of the antibody separated from the antigen under those conditions is collected and further immobilized on Nucleosil Si-1000 by Schiff's base formation in order to prepare the immunosorbent. The secondary antibody is labelled with acridinium ester. The desorption is performed by application of 0.1 M phosphate buffer, pH = 3.0, to the column for 3 min. Over 250 injections of standard solutions, or 200 injections of clinical samples, can be made with a single column with no apparent signs of column degradation.

A different sandwich flow immunoassay has been proposed by Liu *et al. (30)*. In that case the determination of monoclonal mouse anti-bovine IgG is carried out by means of a membrane reactor with the antigen (bovine IgG) immobilized on an Immobilon AV membrane and a conjugate goat anti-mouse IgG-peroxidase as secondary antibody. The elution is performed with 0.1 M phosphate buffer, pH = 2.2, for 10 min at 0.1 mL min^{-1} flow rate. The membrane reactor loses 20% of capacity after 3 regeneration cycles and must be changed.

The protein hirudin has been determined with a sandwich fiber-optic based flow immunosensor *(23)*. This device uses a monoclonal anti-hirudin antibody covalently immobilized on the fiber optic by silanization of the fiber with (mercaptomethyl)-dimethylethoxysilane followed by antibody cross-linking with *N*-succinimidyl-4-maleimidobutyrat, and a fluorescein-labelled polyclonal anti-hirudin antibody. Regeneration of the immunosurface is achieved by using a glycine/HCl buffer, pH = 2.5 for 5 min. A total of 91 assay cycles can be performed with the sensor with an exponential signal decay of about 50%.

The reusability features of flow immunoassay techniques are summarized in Table 1.

Extraction and Chromatography Techniques Based on Immunoaffinity

Immobilized antibodies and antigens have found extensive use in solid-phase extraction procedures and in chromatographic techniques whose analyte-stationary phase interactions are based on antigen-antibody binding. Extraction, clean-up and preconcentration of an analyte can be performed by applying a large volume of the sample to a column containing an antibody raised against the analyte, washing thoroughly the column with buffer and eluting the analyte with a small volume of an adequate desorbent. If the analyte is the specific antibody, the correspondent antigen would be immobilized into the column. The eluted analyte can be further determined, off-line or on-line, by a suitable method of analysis. If the analyte can be detected directly, a suitable detector can be connected at the end of the immunoaffinity column, thus having an immunoaffinity chromatography technique. Immunoextraction as sample pretreatment for the determination of pesticides was recently reviewed *(31)*.

In all cases, it is desirable that immunosorbent columns are reusable, in order to perform a high number of runs with the same column, maintaining the reproducibility of the immunochemical process. Furthermore, the elution method (volume ratios, nature of the desorbent, dynamic parameters such as flow rate or pressure) must be compatible with the process that follows the immunoextraction, or with the detector employed in immunoaffinity chromatography.

In this review, immunoextraction and immunoaffinity chromatography techniques are classified according to the process that follows the immunochemical separation.

Preparative immunoaffinity chromatography

The number of works employing immunoaffinity chromatography with a preparative target is very large *(26,29)*, since in many instances the purification of specific antibodies for further use in immunological techniques is carried out by preparative immunoaffinity chromatography. In that case, the obtained product (antiserum, cell culture supernatant, or ascites fluid) is ap-

plied to a column containing the immobilized antigen on a support (*e.g.* agarose), so that the specific fraction of antibody is retained in the column and the other proteins pass through. The specific antibody is further eluted by applying a suitable desorbent and collected. The antibody must be then inmediately separated from the desorbent, or the collected solution mixed with a solution that neutralizes the denaturing effect of the desorbent.

Preparative affinity chromatography applied to antigens is also widely employed. A variety of commercial supports derivatized for antibody immobilization can be found, and the commercial firms offer detailed instructions for immobilizing the antibody and eluting the antigen from the immunosorbent *(32)*.

A peculiar example of preparative affinity chromatography was proposed by Knox and Galfre *(33)*. The technique is applied to the separation of haptens; nowadays the preparative affinity chromatography is generally used to isolate proteins or other macromolecules from biological fluids. Furthermore, the separated haptens are the enantiomers of abscisic acid, which indicates the high separation possibilities of immunointeractions. In that work, a monoclonal antibody raised against (+)-abscisic acid is immobilized on CNBr-Sepharose 4B, and the elution of the enantiomer retained in the column is carried out by washing with 1 M NH_4SCN solution. Columns used in this study are reused over 10 times without any substantial loss of capacity.

An habitual use of preparative affinity chromatography has been the purification of recombinant proteins produced in microorganisms by genetic engineering, since the obtained product (a cell lysate) is a complex mixture. As an example, Agraz *et al. (34)* use an immunoaffinity chromatography technique to separate recombinant hepatitis B surface antigen from a yeast extract. The immunoaffinity column contains a monoclonal antibody immobilized on Sepharose CL-4B previously activated by the CNBr method. The optimal elution solution is 3 M KSCN in 20 mM Tris-HCl buffer, pH = 7.8, known as a hepatitis B surface antigen stabilizer. The column is stable for up to 30 runs. Further runs affect rapidly the recovery, mainly due to the presence of proteases in the sample.

Another example of recombinant protein purification has been described by Yao *et al. (35)*. In that work, a column with a monoclonal antibody immobilized on CNBr-activated Sepharose 4B is used to separate recombinant human lymphotoxin from an *Escherichia coli* cell lysate. The elution of the bound fraction is carried out

Table 1. Immunosorbent regeneration in flow immunoassay techniques

Analyte	Immunosorbent[a]	Regeneration procedure	No. of cycles (work time)	Remaining activity	Ref.
IgG	Ag on tantalum oxide	0.1 M Gly/HCl, pH = 2.1, 1.5 mL	40	50%	*(6)*
Recombinant proteins	MAb and PAb on CNBr Sepharose 4B	0.1 M K_3PO_4, pH = 12.3	N. m. (7 weeks)	35%	*(7)*
Atrazine	Atrazine-caproic acid on Ta_2O_5	2 mg mL^{-1} pepsin at pH = 2, 9 min	N. m.	N. m.	*(8)*
s-triazine	s-triazine on SiO_2-TiO_2	0.1 M Gly/HCl, pH = 1.5, 2×5 min + 10 μg mL^{-1} proteinase K, 10 min	600 (2 months)	≈100%	*(9)*
Atrazine	Atrazine-biotin on quartz	0.01 M HCl, 1 min	48	70%	*(10)*
Atrazine	PAb on membrane via prot. A	Membrane changement	1	–	*(12)*
Mouse IgG	PAb on magnetic particles	Magnetic field	1	–	*(13)*
Mouse IgG	PAb on agarose beads	Jet ring cell	1	–	*(14)*
Albumin	PAb ($F_{ab'}$) on Trisacryl GF-2000	0.1 M H_3PO_4, pH = 2.0, 2 min	600	>95%	*(15)*
Theophylline	MAb on Corning 0211 glass	Rinsing with buffer (mass action)	15	>90%	*(16)*
Theophylline	MAb on fused silica particles	Rinsing with buffer (mass action)	300	≈100%	*(17)*
Alachlor	PAb on glass beads	3 M KSCN + 0.2% Triton X-100	N. m.	N. m.	*(18)*
Imazethapyr	MAb on capillary column	30% Methanol	150 standards	≈100%	*(19)*
Atrazine	MAb and Pab on polystyrene and glass beads	0.01 M Gly/HCl, pH = 2.0, 1.1 mL	500	≈100%	*(20)*
Terbutryne	MAb on polystyrene and glass	0.1 M Gly/HCl, pH = 2.1, 1 mL	60–100	>40%	*(21)*
α-amylase	PAb on Sepharose	0.1 M Gly/HCl, pH = 2.2, 30 s	210 standards (24 h samples)	0%	*(22)*
Atrazine	MAb on quartz by adsorption	12% 1-Propanol at pH = 3.0, 5 min	325	≈50%	*(23)*
Hirudin	Mab on fiber optic	Gly/HCl, pH = 2.5, 5 min	91	≈50%	*(23)*
Carbaryl	Mab on Affi-Gel Hz	0.1 M Gly/HCl, pH = 2.0, 0.35 mL	60–70	≈100%	*(24)*
Carbaryl	HPT conjugate on Affi-Gel 15	0.1 M Gly/HCl, pH = 2.0, 3 mL	160–200	≈100%	*(24)*
Mouse IgG	Ag on Reactigel-6X	0.1 M Phosphate, pH = 2.0, 5 min	500	N. m.	*(25)*
Human IgG, glycoprotein	PAb on controlled-pore glass	0.1 M Gly/HCl, pH = 2.2, 1.4 mL	300	≈100%	*(26)*
Rabbit IgG	PAb adsorbed on glassy carbon	Anodization + 1.6 M HNO_3	1	–	*(27)*
Mouse IgG	PAb on Trisacryl GF-2000	0.1 M H_3PO_4, pH = 1.8, 0.6 mL	(5 days)	≈62%	*(28)*
Parathyroid hormone	PAb on Nucleosil Si-1000	0.1 M Phosphate, pH = 3.0, 3 min	250	≈100%	*(29)*
Mouse IgG	Ag on Immobilon membrane	0.1 M Phosphate, pH = 2.2, 1 mL	3	80%	*(30)*

[a] Covalent immobilization unless mentioned.
Ag: antigen; HPT: hapten; MAb: monoclonal antibody; PAb: polyclonal antibody; N. m.: not mentioned

with 0.1 M triethylamine, pH = 11.5, and more than 100 runs are performed in the same column without any decrease in efficiency.

Forskolin has been purified from plant extracts by immunoaffinity chromatography *(36)*. The immunosorbent used in that work consists of a monoclonal anti-forskolin antibody immobilized on CNBr-activated Sepharose 4B. After testing several elution buffers, the desorbent used is 45% methanol in phosphate buffered saline. The immunocolumn can be used over 10 times without any substantial loss of capacity.

A novel immunosorbent for use in low-pressure immunoaffinity chromatography has recently been developed by Cichna *et al. (37)*. That immunosorbent consists of an antibody entrapped in the pores of a silicate glass prepared by the sol-gel technique. Employing pyrene as model analyte and a polyclonal anti-pyrene antibody immobilized in that way into an immunoaffinity column, the isolation and preconcentration of pyrene and other related polyaromatic hydrocarbons can be carried out with good recovery and preconcentration factor. The elution of the analyte is carried out by washing the column with an acetonitrile:water (40:60, volume ratio) solution. The columns show no change in specific retention properties after 10 absorption-desorption cycles.

Analytical immunoaffinity chromatography

Although the preparative immunoaffinity chromatography is frequently carried out in low-pressure systems, in the development of an immunoaffinity chromatography technique with analytical purposes a possibility to be taken into account is the use of high-pressure systems (high-performance immunoaffinity chromatography, HPIAC). Since the support to be used in HPIAC techniques must be able to resist the high pressure required, the supports employed are the same as those used in conventional high-performance liquid chromatography (HPLC) derivatized for the immobilization of antibodies by covalent linking. Many works describe the assay of a new support for its use in HPIAC techniques. As in HPLC, an eluent gradient instead of a sudden change in the eluent composition can be used for elution purposes *(38)*.

An example of analytical immunoaffinity chromatography with a low-pressure system is the work presented by Hara *et al. (39)*. That work describes the development of a two-column system connected in series and switched on and off by means of valves. The columns contain controlled-pore glass 3000 with polyclonal anti-human serum albumin and anti-ovalbumin immobilized by a periodate method *(40)*. The eluted proteins are post-column detected by a chemiluminescent method. The elution of the proteins is performed by washing with a solution 0.0042 M HCl and 0.025 M KCl, pH = 2.5. The affinity columns can be used for about 20 replicate analyses.

An immunosupport for HPIAC has been developed by Babashak and Phillips *(38)*. The support is made of glass beads with streptavidin immobilized on its surface by silanization with 3-aminopropyltriethoxysilane, treatment with succinyl chloride and triethylamine, active ester formation with N-hydroxysuccinimide and N,N'-dicyclohexylcarbodiimide, and coupling of the streptavidin. A biotinylated monoclonal anti-human B27 antigen antibody is then attached to the streptavidin-derivatized support. The bound antigen is eluted and detected spectrophotometrically at 280 nm. The elution conditions assayed are acidic (0.1 M HCl, pH = 1.0) and chaotropic (2.5 M NaSCN), with an eluent gradient in a 10-min period followed by another 10-min application of the desorbent. The effective column life is 10–20 regenerations with the acidic elution and 20–40 regenerations with the chaotropic elution.

Tresyl-5PW, which consists of tresyl-derivatized TSK gel G5000PW, was assayed as HPIAC support by Nakamura *et al. (41)*. Polyclonal antibodies raised against some human serum proteins are directly immobilized on the activated support. Proteins are spectrophotometrically detected at 280 nm after elution, which is carried out by means of a stepwise change from assay buffer (0.1 M phosphate, pH = 7.4) to 0.1 M citric acid/HCl, pH = 1.6. The column containing anti-human serum albumin antibody is reused 100 times with a human serum albumin binding capacity decrease of 10%, whereas the column containing anti-human IgG antibody is reused 300 times without loss of capacity.

De Ruiter *et al. (42)* have detected fungal carbohydrate antigens by HPIAC using a HiTrap protein A column with an immobilized polyclonal antibody raised against extracellular polysaccharides of *Mucor racemosus*. The immobilization is carried out by protein A interaction followed by covalent linking with dimethyl ester of heptane diimidic acid in triethanolamine. The polysaccharides are pulsed-amperometrically detected after post-column alkalinization with NaOH. The desorbent used for elution is 0.05 M sodium citrate buffer, pH = 2.0 (4 mL at 0.2 mL min^{-1}). Under those conditions, the column exhibited a negligible loss of performance after the analysis of more than 200 samples for one year.

Another application of HPIAC is the purification and determination of glutamine synthetase performed by Alhama *et al. (43)*. A polyclonal anti-glutamine synthetase antibody is immobilized in an Ultraffinity-TM prepacked epoxy-activated silica analytical column by direct coupling to the oxirane groups followed by blocking the remaining active groups with glycerol. The eluted enzyme is detected spectrophotometrically at 280 nm and its activity is further measured. The elution is performed by a pulse (2 mL, 0.5 mL min^{-1} flow rate) of assay buffer (0.05 M 3-(N-morpholino)propanesulphonic acid, 0.25 M KCl, 0.001 M EDTA, pH = 7) supplemented with 10% acetonitrile and 2.5 M $MgCl_2$. Furthermore, the column is washed every 2 or 3 assay cycles with 30% acetonitrile in order to desorb the unspecific proteins adsorbed. The column capacity appears to remain constant after 50 runs.

Albumin and creatinine in human urine have been simultaneously determined using a HPIAC system by Ruhn *et al. (44)*. Human serum albumin from the urine sample is retained in a column containing the immunosorbent, which consists of a polyclonal anti-human serum albumin antibody immobilized on diol-bonded Nucleosil Si-1000 by antibody oxidation with periodate and coupling the oxidized antibody to the hydrazyde-deriva-

tized support, while creatinine passes through the immunocolumn and is determined downstream in a flow-injection system with alkaline picric acid. Human serum albumin is then eluted from the column with 0.1 M phosphate buffer, pH = 2.5, for 2 min at 1 mL min^{-1}, and spectrophotometrically detected at 280 nm. The HPIAC column is used for over 650 injections before any apparent degradation is noted.

Immunoextraction coupled to batch immunoanalysis

Immobilized antibody columns have also been extensively used for solid-phase extraction, clean-up and preconcentration of the analyte as a previous step before applying an analytical technique. Immunoaffinity becomes a sample conditioning tool coupled (on-line or off-line) to another analytical method. Batch immunoanalysis following immunoextraction is a combination that has been exploited in many instances. One example of coupling immunoextraction with batch immunoanalysis has been presented by Glencross *et al. (45)* for the determination of oestradiol-17β in bovine plasma and milk. The immunosorbent used in that work is a polyclonal anti-oestradiol-17β antibody immobilized on CNBr-activated Sepharose 6B. After the application of the sample, the bound steroid is eluted with 3 mL of a mixture water:acetone (5:95, volume ratio). The eluate is then evaporated to dryness and determined by radioimmunoassay using the same antibody as the immobilized one. The columns are stable at room temperature for twelve months and after at least 50 times of repeated use.

Azcona *et al. (46)* have developed a tandem immunoaffinity-ELISA for the determination of zearalenone in dehydrated skimmed milk after reconstitution. The immunoextraction is performed in columns containing a monoclonal anti-zearalenone antibody immobilized on CNBr activated Sepharose 4B. The bound zearalenone is eluted from the column with 2 mL of methanol, which is then evaporated and resuspended in phosphate buffered saline before the ELISA determination using the same antibody. Typically, 10 samples can be passed through the same column with no observable loss of activity.

The determination of urinary 3-methyladenine by immunoaffinity clean-up coupled to ELISA has been reported by Prevost *et al. (47)*. The clean-up is performed in columns containing a polyclonal anti-3-methyladenine immobilized on protein A-Sepharose CL4B gel. The analyte is recovered from the column by washing with 1 M acetic acid (2 mL). The eluate is then reduced to dryness and reconstituted with phosphate buffered saline for monoclonal antibody-based ELISA. Columns may be recycled up to 30 times with no apparent loss of efficiency or detectable carry-over between samples.

Pickett and Sauer *(48)* have determined clenbuterol in bovine urine by enzyme immunoassay following concentration and clean-up in an immunoaffinity column filled with a polyclonal anti-clenbuterol antibody immobilized on CNBr-activated Sepharose 4B. The analyte bound to the column is eluted for 20 min with 10 mL of a solution prepared by diluting 0.1 M glycine buffer 20-fold in methanol and adjusting the pH to 2.0 with HCl. The eluate is subsequently collected, dried and redissolved in phosphate buffer prior to enzyme immunoassay. Immunoaffinity columns are reused at least 10 times.

Immunoextraction coupled to gas chromatography

The tandem immunoaffinity extraction-gas chromatography (GC) combines the selectivity of immunochemistry with the high potential of GC, especially when using mass spectrometry detection (GC-MS). However, little attention has been paid to this combined technique, since gas chromatography can only be applied to volatile analytes. Several discussions about the applicability of the combination of immunoextraction-GC-MS for multiresidue analysis are available *(49,50)*.

An off-line immunoextraction-GC-MS tandem has been proposed by Chiabrando *et al. (51)* for the determination of thromboxane B_2 and 2,3-di-nor-thromboxane B_2 in urine. In that work, a polyclonal antibody raised against thromboxane B_2, which shows 35% cross-reactivity with 2,3-di-nor-thromboxane B_2 is immobilized on CNBr-activated Sepharose 4B and placed into a column. After applying the sample containing a deuterated thromboxane B_2 as internal standard, the bound species (thromboxane B_2, its 2,3-di-nor metabolite, and the internal standard) are eluted by incubating the column with 0.5 mL of acetone : water (95:5, volume ratio) for 15 min, followed by an additional washing step with 1 mL of the same mixture. The collected eluate is then derivatized for GC-MS analysis. Columns can be used for 15 times with no observable loss of extraction efficiency.

Van Ginkel *et al. (52)* have developed an off-line multi-immunoextraction-GC-MS for the determination of nortestosterone and methyltestosterone in muscle tissue. Immunoaffinity columns contain two polyclonal antibodies raised against both analytes immobilized on Tresyl-activated Sepharose following the supplier's instructions. After the application of the sample extract which contains 10% (volume fraction) methanol in water, the analytes are eluted with 2 mL of 50% (volume fraction) ethanol-water solution, followed by an exhaustive washing with 10 mL of 90% (volume fraction) aqueous ethanol, 5 mL of water, 5 mL of 0.1 M sodium acetate, and 5 mL of 0.5 M phosphate buffer, pH = 7.5. After removing the ethanol, the eluate is derivatized with heptafluoro butyric anhydride-acetone (1:4, volume ratio) and sent to GC-MS analysis. Columns are used more than 25 times (over a period of more than one year) without a significant decrease in capacity.

The automation of an on-line tandem immunoaffinity-GC has been described by Farjam *et al. (53)*. The system developed can monitor β-19-nortestosterone, or simultaneously the related steroids norethindrone and norgestrel, in urine samples. The on-line connection between the immunoaffinity column and the GC is performed with an interface that consists of a reversed-phase precolumn and a GC retention gap. The immunocolumn contains a polyclonal antibody raised against β-19-nortestosterone, which shows cross-reactivity with norethindrone and norgestrel, immobilized on Tresyl-activated Sepharose. Analytes retained in the immunoaffinity column are eluted with 2 mL of methanol-water (95:5, volume ratio) followed by a washing step with ad-

ditional 2.5 mL of methanol-water mixture. When not in use, the columns are kept in methanol-water mixture. The immunoaffinity columns can be used for at least 70 analyses.

Chloramphenicol has been determined in animal tissues and urine by off-line tandem immunoaffinity-GC with electron-capture detection *(54)*. The samples are extracted, allowed to evaporate to dryness and redissolved in 10% (volume fraction) ethanol in water prior to the application of immunoextraction, which is carried out in columns containing a polyclonal anti-chloramphenicol antibody immobilized on agarose gel (no details of immobilization are given). Two elution agents are tested: 15 mL of 70% (volume fraction) ethanol in water and 40 mL of 0.2 M glycine-0.5 M NaCl, pH = 3. Ethanolic elution leads to no more than 20 reuses, while with glycine-NaCl buffer the column can be reused for more than 100 times.

Immunoextraction coupled to high-performance liquid chromatography

The use of immunocolumns for sample conditioning prior to HPLC analysis is very popular, mainly due to the high analytical potential of HPLC. Also, the on-line coupling of immunoaffinity with HPLC does not require additional devices, as it is the case of tandems immunoaffinity-GC *(53)*. The development of derivatized supports for antibody immobilization, conditioned for its use in high-pressure systems, makes easier the development of these analytical systems.

In on-line systems the desorbent employed in the immunoextraction must be compatible with the chromatographic process. This compatibility does not exist in many instances, since the immunoaffinity column works in aqueous media and the HPLC system usually requires the use of non-polar mobile phases. The solution to this problem consists of entrapping the analytes that have been desorbed from the immunoaffinity column in a reverse-phase (RP) precolumn, thus preconcentrating them, and their re-eluting from the precolumn by using an eluate compatible with the HPLC system. Another suitable option is the use of an RP-HPLC system, but this option depends on the analyte.

An on-line immunoaffinity-RP-HPLC tandem for the analysis of phenytoin in plasma samples was described by Johansson *(55)*. The immunoprecolumn used in that work contains a polyclonal anti-phenytoin antibody immobilized on LiChrosorb Si 60. The immobilization was accomplished by support convertion into its γ-glycidoxypropyltrimethoxy derivative, oxidation to the corresponding carboxylic acid derivative, active ester formation with N-hydroxysuccinimide, and antibody attachment to the active ester. The samples are loaded in the immunoaffinity column and after a washing step, the analyte is eluted and sent to the RP-HPLC by flowing a mixture of 0.1 M phosphate buffer (pH = 7.2):ethanol (60:40, volume ratio) for 2 min. That desorbent solution was used as eluent in HPLC. The same column can be used for a three-month period (approximately 200 assays) maintaining the linearity and slope of the calibration standard curves.

A similar system has been developed by Rybacek *et al. (56)* for the determination of recombinant leukocyte interferon α-2. The system uses an immunocolumn filled with a monoclonal antibody immobilized on a polyhydroxylated silica support (GF-200) by periodate oxidation, antibody coupling via Schiff's base formation, blocking of the remaining active sites with ethanolamine, and reduction of the Schiff's base with sodium cyanoborohydride. The sample (a cellular extract) is injected into the immunocolumn and the adsorbed analyte is eluted with 4 mL of 0.2 M Na_2SO_4 solution containing 0.2% trifluoroacetic acid (pH = 2.3). The eluate is directly transferred to an RP-HPLC column. No data of the immunocolumn life are mentioned.

Two systems have been developed for the determination of β-19-nortestosterone (a) and its metabolite α-19-nortestosterone (b) *(57,58)* using a polyclonal antibody immobilized on CNBr-activated Sepharose 4B. In both cases the system used is a tandem immunoaffinity-HPLC with an RP precolumn as interface. The polyclonal antibody shows affinity for β-19-nortestosterone (100%), α-19-nortestosterone (70%), norgestrel (20%) and 17-β-trenbolone (40%). The elution of bound species is carried out with 33 mL of 5% acetonitrile aqueous solution containing 190 μg L^{-1} norgestrel (a), or with 20 mL of 5% acetonitrile aqueous solution containing 250 μg L^{-1} norgestrel (b) followed by a washing step with 20 mL of methanol : water (70:30 volume ratio) solution in order to desorb the norgestrel retained on the immunosorbent. In (a) the column is reused 100 times with a decrease in capacity from 450 to 180 ng; meanwhile in (b) the column can be reused for 600 assays with a reduction of its capacity from 250 to 60 ng.

In a later work, Farjam *et al. (59)* performed a study of the applicability of different desorption techniques to an on-line immunoextraction-HPLC system, with a C_{18} RP precolumn as interface, applied to the determination of estrogen steroids. The immunoextraction is carried out in a column containing a polyclonal anti-estrogen antibody immobilized on CNBr-activated Sepharose 4B. The reagents tested in that work for an effective desorption are: acidic solutions (e.g. 0.1 M glycine/HCl buffer, pH = 2.0 or 0.2% (mass fraction) trifluoroacetic acid), micellar solutions (e.g. 3.54 M sodium dodecyl sulphate or 3.1 M hexadecyltrimethylammonium chloride), chaotropic solutions (e.g. 4 M urea), displacer solutions (an estrogen with cross-reactivity), thermodesorption (heating the column), and aqueous methanol solutions. The best desorbent agent found is 3.7 mL of methanol-water (95:5, volume ratio), and the capacity of columns decreases to 50% after 100 analyses. The reduction is maximal in the first assays, probably due to a leaching of non-effectively immobilized antibodies.

Van der Water *et al. (60)* have determined chloramphenicol in milk and eggs by immunoaffinity clean-up off-line coupled to HPLC. In that case, the immunocolumn contains a monoclonal anti-chloramphenicol antibody immobilized on carbonyldiimidazol-activated trisacryl GF-2000. The elution of the analyte is performed with 20 mL of 0.2 M glycine/0.5 M NaCl (pH = 2.8) solution at 1.2 mL min^{-1} flow rate. The eluate is further extracted, preconcentrated and transferred to the HPLC

system. The immunocolumns can be used for 33 assays.

Thomas *et al. (61)* have developed a high-performance three-column system for the determination of atrazine, some atrazine metabolites, and the related pesticide simazine in river and well water samples. The system consists of an immunocolumn containing a monoclonal anti-atrazine antibody which shows cross-reactivity for hydroxyatrazine, deisopropylatrazine, deethylatrazine and simazine, a C_{18} RP precolumn, and a C_{18} analytical column. The antibody is immobilized on diol-derivatized Nucleosil Si-1000 by the Schiff's base method. The compounds retained in the immunocolumn are desorbed by injection of 10 mL of 0.05 M phosphate buffer, pH = 2.5, at 1.5 mL min^{-1} flow rate. The immunocolumn is stable for about one year, during which it may be used for more than 700 assay cycles. A gradual decrease in the activity of immobilized antibody can be observed, but this is not a problem since the column contains a large excess of binding sites.

A new application of on-line tandem immunoaffinity-HPLC systems for the determination of pesticides

Table 2. Immunosorbent regeneration in immunoaffinity-based extraction and chromatography techniques

Analyte	Immunosorbent[a]	Regeneration procedure	No. of cycles (work time)	Remaining activity	Ref.
Abscisic acid	MAb on CNBr-Sepharose 4B	1 M NH_4SCN	10	≈100%	(33)
Hepatitis B surface Ag	MAb on CNBr-Sepharose CL4B	3 M KSCN in buffer pH = 7.8	30	≈100%	(34)
Lymphotoxin	MAb on CNBr-Sepharose 4B	0.1 M Triethylamine, pH = 11.5	100	≈100%	(35)
Forskolin	MAb on CNBr-Sepharose 4B	45% Methanol in buffer	10	≈100%	(36)
Pyrene	PAb on sol-gel	40% Acetonitrile	10	≈100%	(37)
Human B27 antigen	MAb on glass beads	0.1 M HCl, pH = 1, 10 min gradient + 10 min pure	10–20	N. m.	(38)
Human B27 antigen	MAb on glass beads	2.5 M NaSCN, 10 min gradient + 10 min pure	20–40	N. m.	(38)
Albumin	PAb on CPG-3000	4.2 mM HCl, 25 mM KCl, pH = 2.5	20	N. m.	(39)
Albumin	PAb on Tresyl-5PW	0.1 M Citric acid/HCl, pH = 1.6	100	90%	(41)
Human IgG	PAb on Tresyl-5PW	0.1 M Citric acid/HCl, pH = 1.6	300	≈100%	(41)
Carbohydrate	PAb on HiTrap protein A	0.05 M Citrate buffer pH = 2.0, 4 mL	200	≈100%	(42)
Glutamine synthetase	PAb on Ultraffinity epoxy-activated silica	10% Acetonitrile, 2.5 M $MgCl_2$ in buffer, 2 mL	50	≈100%	(43)
Albumin	PAb on diol Nucleosil Si-1000	0.1 M Phosphate, pH = 2.5, 2 mL	650	≈100%	(44)
Oestradiol	PAb on CNBr Sepharose 6B	95% Acetone, 3 mL	50	≈100%	(45)
Zearalenone	MAb on CNBr Sepharose 4B	Methanol, 2 mL	10	≈100%	(46)
3-Methyl-adenine	PAb on protein A-Sepharose CL4B	1 M acetic acid, 2 mL	30	100%	(47)
Clenbuterol	PAb on CNBr Sepharose 4B	5 mM Gly/HCl in 95% methanol, pH = 2.0, 10 mL	10	N. m.	(48)
Thromboxane and 2,3-di-nor-thromboxane	PAb on CNBr Sepharose 4B	95% Acetone, 0.5 mL, 15 min	15	≈100%	(51)
Nortestosterone, methyl-testosterone	PAbs on Tresyl Sepharose	50% Ethanol, 2 mL + 90% ethanol, 10 mL + 0.1 M Na acetate, 5 mL	25 (1 year)	≈100%	(52)
Nortestosterone and two analogs	PAb on Tresyl Sepharose	95% Methanol, 4.5 mL	70	N. m.	(53)
Chloramphenicol	PAb on agarose gel	70% Ethanol, 15 mL	20	N. m.	(54)
Chloramphenicol	PAb on agarose gel	0.2 M Gly–0.5 M NaCl, pH = 3.0, 40 mL	100	N. m.	(54)
Phenytoin	PAb on LiChrosorb Si 60	40% Ethanol in buffer, 2 min	200	≈100%	(55)
Leukocyte interferon α	MAb on polyhydroxylated silica GF-200	0.2% Trifluoroacetic acid in 0.2 M Na_2SO_4, 4 mL	N. m.	N. m.	(56)
Nortestosterone	PAb on CNBr Sepharose 4B	190 μg L^{-1} Norgestrel in 5% acetonitrile, 33 mL	100	40%	(57)
Nortestosterone	PAb on CNBr Sepharose 4B	250 μg L^{-1} Norgestrel in 5% acetonitrile, 20 mL	600	24%	(58)
Estrogens	PAb on CNBr Sepharose 4B	95% Methanol, 3.7 mL	100	50%	(59)
Chloramphenicol	MAb on carbonyldiimidazol-activated Trisacryl GF-2000	0.2 M Gly–0.5 M NaCl, pH = 2.8, 20 mL	33	N. m.	(60)
s-triazines	MAb on diol Nucleosil Si-1000	0.05 M Phosphate, pH = 2.5, 10 mL	700	N. r.	(61)
Phenylurea pesticides	PAb on silica	Gradient 50% acetonitrile + 70% methanol, 5 mL	30	90%	(62)
s-triazines	MAb on diol Nucleosil Si-1000	0.05 M Phosphate, pH = 2.5, 7.5 mL	700–1000	N. r.	(63)
Carbendazim	MAb on Nucleosil 1000–7	0.05 M Phosphate, pH = 2.5, 5 mL	200	N. r.	(64)

[a] Covalent immobilization unless mentioned

MAb: monoclonal antibody; PAb: polyclonal antibody; N. m.: not mentioned;
N. r.: not relevant due to a large excess of binding sites

has been described by Pichon *et al. (62)*. The system developed can determine 13 phenylurea derivative pesticides in drinking and surface waters with an immunocolumn switched to a C_{18} RP-HPLC system. The immunocolumn contains a polyclonal anti-isoproturon antibody immobilized on a silica support (the immobilization method is not described). The elution of the analytes from the immunocolumn is performed by means of a gradient of acetonitrile in phosphate buffered saline from 0 to 50% at 1 mL min^{-1}, followed by a washing step with 5 mL of 70% aqueous methanol solution. The same immunocolumn can be reused for more than 50 cycles, including more than 20 analyses of dirty river samples. A loss of 10% in capacity after processing 30 dirty samples can also be observed.

Atrazine and its degradation products have been determined in water by tandem HPIAC-RPLC *(63)*. The online system uses an immunocolumn containing a monoclonal antibody immobilized on diol-bonded Nucleosil Si-1000 *(61)*, an RP precolumn and an RPLC analytical column. The analytes are eluted from the immunocolumn by applying 0.05 M phosphate buffer, pH = 2.5, for 5 min at 1.5 mL min^{-1} flow rate. The HPIAC column is found to be quite stable, a consistent performance being obtained over 700–1000 sample injections and over a 10–12 month period. Only a gradual decrease in column activity is noted, but this is not a problem since the column originally contains a large excess of binding sites relative to the amount of injected triazines.

Carbendazim has been determined in water by online HPIAC-RPLC *(64)*. The immunocolumn used contains a monoclonal antibody immobilized on Nucleosil 1000–7 by (3-glycidoxypropyl)trimethoxysilane derivatization of the support, periodic acid oxidation to generate carbonyl groups and antibody coupling by Schiff's base formation. An immunosorbent consisting of the same antibody immobilized on aldehyde-activated POROS support is also tested, but it is discarded due to the unspecific adsorption of several pesticides – including carbendazim – on the support. The elution of carbendazim bound to the immunocolumn is carried out with 0.05 M phosphate buffer, pH = 2.5, for 5 min at 1 mL min^{-1} flow rate, and the eluate is transferred to a C_{18} reverse phase trapping column and then to a C_{18} reverse phase analytical column. The immunocolumn can be used for over two months (more than 200 cycles) with a decrease in the activity of immobilized antibody that is not a problem since the column originally contained a large excess of binding sites.

A summary of immunosorbent regeneration in immunoaffinity-based techniques is given in Table 2.

Conclusions

The enormous dispersion found in works describing the development of immunological techniques that require immunosorbent regeneration leads to the need for assaying and optimizing the desorption process when developing an analytical technique based on immunochemical reactions that have to be reversed. In this sense, general rules cannot be given. Every antigen-antibody system behaves in a different manner, and a desorption protocol that works well in one immunosystem does not necessarily perform properly in another.

Acidic buffers are the most often employed desorbent agents, although organic media are also frequently used, especially in immunoextraction processes coupled to chromatographic techniques. Mimic molecules can be a solution in order to use non-denaturing conditions. Also, tensioactive reagents may have a potential. Basic research into this should be carried out in the future.

The support plays an important role in the performance of the systems. As the immunoreagent activity is a key factor, oriented immobilization is preferred to random immobilization. Solid support nature is important in relation to dynamic properties, derivatized agarose being the most frequently used support. Protein A and G, hydrazide, diol and aldehyde based supports perform good antibody orientations. On the other hand, covalent immobilization is basically used instead of physical adsorption, since it leads to immunosorbents with a longer column life. Tubular reactors containing the immunosorbent are preferred to other kinds of immunoreactors such as those bound to membranes, plates or magnetic particles, because these systems give better precision and sensitivity and reduce the volume of reagents used.

The new technologies developed to obtain antibodies with desired features, as well as the development of new supports and immobilization chemistries, will lead in the future to immunosystems with enhanced sensitivity and stability properties.

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Reverzibilnost u heterogenom protočnom imunosenzorskom postupku i srodnim tehnikama

Sažetak

U razvoju mnogih imunokemijskih postupaka ponovna uporaba imunosorbensa jedan je od problema o kojem treba voditi računa. Način da se to riješi jest izbor odgovarajućeg agensa za desorpciju koji ne utječe na aktivnost imunosorbensa. Ovaj pregled opisuje primijenjene postupke uzimajući u obzir reverzibilnost u imunokemijskim interakcijama te način na koji se površina antigena imunološki mijenja, osobito u tako heterogenim područjima kao što su protočno imunokemijsko određivanje, imunoafinitetna ekstrakcija i imunokromatografija.