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Review Article

IMMUNOAFFINITY CHROMATOGRAPHY

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ABSTRACT
Methods employing the use of immunoaffinity chromatography for sample preparation or detection as well as purification are gaining popularity as tools in the analysis of biological and nonbiological compounds. Immunoaffinity chromatography is the process in which the binding affinity of an antigen to a parent antibody is utilized as a basis of separation. Owing to their avidity and specificity, monoclonal antibodies have become indispensible for both protein characterization and purification. The article describes the basic procedure of immunoaffinity chromatography. The support matrix upon which the antibody is immobilized and the activation chemistry used to couple the antibody to the matrix affect the immunosorbent performance. Support matrices available for Immunoaffinity chromatography and their activation chemistries including the recent advances have been reviewed. The paper also discusses the developments in the applications of this technique in analysis as well as extraction.

Keywords: Reviews, immunoaffinity chromatography, antibodies, matrices, application, protein purification.

INTRODUCTION
Current advances in recombinant DNA technology have enabled the synthesis of valuable proteins as well as hormones in bacterial cells and in the novel eukaryotic expression systems. These proteins include the proteins for therapeutic use (viz. anticoagulants or fibrinolytics to treat heart attack patients; erythropoietin to treat anemia in patients with kidney failure; interferons to treat cancer, etc.) and proteins for analytical application (e.g alkaline phosphatase used in ELISA). The purification of proteins of interest from the conventional sources (i.e. cell culture as well as novel routes) in a highly purified form necessitates the development of separation techniques capable of recovering proteins from these feed streams in a highly purified form[1,2]. Presence of the endogenous proteins often complicates the purification of therapeutic proteins from the biological sources[2]. Purification methodologies based on ion exchange or adsorption fail to resolve complex protein mixtures to yield a homogeneous protein product from the feed streams.[1]

Purification techniques based on affinity interactions between molecules have evolved using a variety of biological and synthetic ligands.[2] Immunoaffinity chromatography (IAC) is a process in which the binding affinity of an antigen (Ag) to a parent antibody (Ab) forms the basis of separation. The antibody specific to the protein of interest is immobilized onto a rigid solid support to yield an active immunosorbent. A complex mixture of proteins is then passed over the immunosorbent whereby the antibody captures the protein of interest and the other nonproduct proteins are washed away in the column fall through.[1, 3]

IAC has also been extended for analytical purposes which include analysis of hormones and drugs in body fluids, analysis of toxins in food as well as analysis of herbicides and plant hormones in plant and food extracts.[4]

IAC is operated as a column chromatographic process.[5] Key consideration in any chromatographic process is the resolution in less time for analytical purpose and resolution

along with concentration of the analyte for preparatory purpose. Since only protein molecules containing the epitopes recognized by the immobilized antibody will be retained on the column, IAC has a very high resolving power. IAC can produce pure protein in a single step and also has the power to concentrate proteins from very dilute solution. The development of monoclonal antibodies (MAbs) by Kohler and Milstein[6] has led to the explosion in the use of IAC as a tool for protein purification which facilitates selection of antibodies with definite affinity for the antigen. The first protein to be purified by IAC on an immobilized MAb was α2-interferon.[7]

CHROMATOGRAPHIC CONDITIONS

The chromatographic process involves a stationary phase (also called matrix or support) and a mobile phase. The stationary phase retains the analyte depending on its affinity for the analyte thus concentrating the analyte which is later eluted to give pure product. The mobile phase serves as a carrier for the analyte. In IAC the mobile phase is an aqueous buffer with different compositions so as to facilitate binding of analyte and its elution.

Stationary Phase

The stationary phase in IAC consists of antibody or antibody fragments immobilized on matrix or support. For the immobilization of the antibodies or its fragments the supports are activated so that they couple with the antibodies. Choice of the support, the chemistry of their activation as well as the type of the antibodies or antibody fragments used is important for the selectivity and efficiency of IAC.

A) Supports and Support Activation

In IAC the antibodies immobilized on supports are used for separation process. The support material used is an important consideration in the development of successful IAC method.

The ideal support should be[8] -

1) Rigid- IAC can be operated using HPLC (High Performance Liquid Chromatography). As this technique is executed under high pressures, the matrix should be rigid and resist compression at high flow-rates in columns.

2) Uniform size- Narrow size distribution is essential to prevent the blockage of column.

3) Nontoxic- The matrix should be safe to handle as well as nontoxic.

4) Stable- The analyte sample for the purification or analysis may have very high or very low pH. The matrix should withstand these extremes of pH. Also the matrix should be stable to allow the use of denaturing reagents in the elution stage.

5) Hydrophilic- The support should be hydrophilic because hydrophobicity induces nonspecific binding hence unwanted molecules are retained in the column. These may elute in the elution stage along with the analyte and affect the detection of analyte or purity of extracted product.

6) Macro porous- The matrix used should have pores large enough to allow diffusion of large protein molecules (or antigen) and provide high capacity support. Also the high surface area to volume ratio ensures better separation.

7) Readily derivatized- In IAC the antibodies are immobilized on the matrices. Hence matrices should possess functional groups that can be easily activated so as to react with and immobilize antibodies.

8) Show low nonspecific absorption- The aim of IAC is selective elution of the analyte of interest. Hence the matrix should have the least nonspecific absorbance as possible so that only the analyte is retained and concentrated in the IAC column.[9]

Based on their efficiency, the supports or matrices are classified in three types -

1. Low performance supports:- They are generally beads of carbohydrate-related materials or synthetic organic supports. The low back pressure of these supports facilitates the operation under gravity flow, a slight applied vacuum or peristaltic flow. The use of these gels for IAC is relatively simple and inexpensive. The main disadvantage of these materials is their slow mass transfer properties and their limited stability at high flow rates and pressures.[4]

a. Carbohydrate related materials:- Earlier IAC was performed using fibrous cellulose as support[9], but it is now no longer used owing to the poor flow characteristics of the medium. Beaded agarose can also be used but as such it does not have the required mechanical and chemical stability. So they are crosslinked by the reaction of 2,3-dibromopropanol and subsequent desulfation with borohydride[10] to give a gel with greatly increased mechanical and chemical stability for use as support for IAC.

b. Synthetic organic supports:- Owing to their resistance to biological degradation and the wide range of activation chemistries that can be employed the synthetic supports are finding more use in IAC. These include acrylamide polymers, copolymers or derivatives, polymethacrylate derivatives and polyethersulfone matrices.

Trisacryl, a polymer of *N*-acryloyl-2-amino-2-hydroxymethyl-1, 3-propane diol and *N,N'*-diallyltartradiamide, shows good mechanical strength but is stable only upto pH 11.

2. High efficiency supports:- These supports facilitate use of IAC as HPLC method. The mechanical stability and efficiency of these materials facilitates use with standard HPLC equipment which improves speed and precision for analytical applications of IAC. These supports include derivatized silica, glass and organic matrices such as azalactone beads or polystyrene based perfusion media. The use of these supports along with an antibody or related ligand is referred to as high performance immunoaffinity chromatography (HPIAC).[11]

Controlled pore glass can be used as an IAC support[12] after surface derivatization by silica but is limited only to non-alkaline pH. Azalactone beads are rigid copolymer of *N,N'*-methylene-bis(acrylamide) and vinyldimethyl azalactone.[13] The gel contains reactive azalactone groups for coupling and provides high flow rates and wide pH stability. POROS[14] is a polystyrene/ divinylbenzene polymer with both large (permit through) and small (permit diffusion) pores. Various grades of this polymer are available for use in medium pressure chromatography with activated groups for covalent attachment.

3. Affinity Membranes:- The beaded matrices discussed above undergo compaction when used under high pressure thereby affecting the performance of IAC. Affinity membranes, owing to their high mechanical strength, do not suffer the problems of compaction as encountered with beaded matrices. These devices are used in the form of stacks of membrane filters or hollow-fiber ultrafiltration cartridges[15, 16] in IAC.

Antibodies are immobilized on the support. Hence supports require activation to possess the functional groups that can react with the surface groups on the antibodies. All the supports except the azalactone beads require chemical activation before they will react with and immobilize antibodies.

The most commonly used activation method for polysaccharide matrices employs cyanogen bromide.[17, 18] (Figure 1)

The introduction of new synthetic supports and problem of leakage associated with gel activated by conventional methods induced search for new derivatization techniques that will yield stable matrix-MAb bonds. One of these methods uses FMP (2-fluoro-1-methyl-pyridinium toluene-4-sulfonate) to activate any hydroxyl group and couple it to either an amino or a sulfhydryl group.[19] This reaction may be easily followed by measuring the color in solution of the leaving group.

Beaded cellulose, Sepharose, Spheron, Trisacryl, and TSK Toyopearl are activated using norbornene carbonochloridate[20] and their coupling to protein can be determined spectrophotometrically at 270nm. Methacrylate polymers activated using 4,6-diphenylthieno[3,4-d]-1,3-dioxol-2-one-5,5-dioxide (TDO) couple rapidly at neutral pH.[21]

B) Antibodies

IAC utilizes antibodies immobilized to stationary support. The high specificity and avidity of antibodies is used for better separation. These antibodies act as ligands where specific analytes from complex mixture bind to it with high avidity to resolve the mixture. Polyclonal antibodies were once used for IAC but due to high non-specific absorption and with the production of MAbs their use declined. In IAC purification, the choice of MAb used is of utmost importance for determining the success or failure of the system. For a MAb to be used as an IAC ligand, MAb must retain its biological activity during the coupling reaction and then under the conditions necessary to achieve elution of the antigen.[22, 23] Over many years, an empirical approach was used where MAbs were immobilized to stationary phase, followed by investigation of whether it still binds antigen and if so what conditions were necessary to achieve elution. This conventional empirical approach being expensive of time and material, a rational approach was developed. One of the first steps was taken by Homsey *et al.*[24] who attempted to define a set of potential eluants and determine if the antigen would withstand the treatment. Recently a rapid ELISA plate technique has been developed to determine whether a MAb will retain its biological activity following its exposure to a range of eluants.[25] Some methods of immobilization have been reported to completely inactivate the MAb for no apparent reason.[26]

The synthetic peptides with sequences based on the hypervariable regions of MAbs serve as an alternative to MAbs.[27] With the advent of genetic engineering the synthesis of antigen binding fragment (Fab) as well as the fragment variable section (Fv) became possible. Their immobilization through their sulfhydryl groups yielded more efficient stationary phase. The Fv fragments are produced in *Escherichia coli* where the molecule can be tailored with, for example, a signal peptide so it is secreted into the growth medium[28] or with a histidine-rich C-terminus such that it may be purified readily by metal ion affinity chromatography.[29] Fv

fragments specific for hen-egg lysozyme have been used to purify the antigen from spiked serum in a single step[30] and were more efficient than whole antibody.

C) Antibody Immobilization

There are many different methods for antibody immobilization to both low- and high-performance supports. Immobilization of antibodies is most usually achieved by coupling through the free amino groups of lysine residues, hydroxyl, carboxyl or sulfhydryl groups on the surface of the protein.[16]

Approaches to immobilization of antibodies:-

1. Covalent immobilization: In this technique the antibodies are immobilized by formation of covalent bonds with the activated supports. The two types of covalent immobilization are

a. Non-site-selective:-It is done by reacting the free amine groups on antibodies with activated supports (supports are activated using agents such as N, N'-carbonyl diimidazole, cyanogens bromide, N-hydroxysuccinimide and tresyl chloride/tosyl chloride or with supports that have been treated to produce reactive epoxide or aldehyde groups on their surface). Though the use of antibody amine groups serves the easiest route for immobilization it can give rise to less than optimum activity because of the random orientation or denaturation of the immobilized antibody.

The sorbents so derivatized are inefficient in binding antigen[31] because of

i. Incorrect orientation of the antibody due to which the antigen binding site is involved in coupling the MAb to the matrix.
ii. Multipoint attachment of the MAb to the matrix which deforms the antigen binding site and renders it inactive.
iii. At high levels of matrix substitution the close proximity of MAb molecules sterically inhibits the approach of antigen to a binding site. These problems have led to the development of the concept of site-directed immobilization of MAbs.[32]

b. Site-selective:- Antibodies, or antibody fragments can also be covalently immobilized through site-selective methods. For example –

i. Free sulfhydryl (-SH) groups generated during the production of antibody Fab fragments can be used to couple these fragments to supports using appropriate activation chemistry. For example: divinylsulfone, epoxy, maleimide, iodoacetyl/bromoacetyl, TNB-thiol, or tresyl chloride/tosyl chloride can be used to activate the sulfhydryl group.[4]
ii. Carbohydrate residues of antibodies can be used to couple them after mild oxidation of these residues using periodate or enzymatic systems to produce the aldehyde residues. These aldehyde groups can be reacted with a hydrazide or amine-containing support for antibody immobilization.[25]

Advantage of the sulfhydryl and carbohydrate based approaches is that the immobilized antibodies or antibody fragments produced have fairly well defined points of attachment and greater accessibility to the antigens in solution. This results in IAC columns that have higher relative binding activities than comparable columns made by amine-coupling methods.

2. Noncovalent immobilization:- Noncovalent immobilization involves the adsorption of antibody to the support. Site selective approaches in non-covalent immobilization are

a. Oxidizing the carbohydrate residues of the antibodies to produce the aldehyde group which after reaction with the biotin-hydrazide can bind the antibodies noncovalently to an immobilized streptavidin support.[4]

b. Adsorbing the antibody to secondary ligands such as protein A or protein G which bind strongly as well as selectively to the stem region of antibodies leaving the antigen binding site free to interact, under physiological conditions.[32] This binding can be disrupted by decreasing the pH of the surrounding solution.

3. Other approaches:- An alternative coupling method involves first generating free sulfhydryl groups in either intact or fragmented MAb. The MAb or $F(ab')_2$ fragments may be reduced with β-mercaptoethylamine to produce free –SH groups at the hinge region of the MAb. The fragments may then be immobilized to a matrix containing iodoacetyl groups to give a stable thioether link[32] or to maleimide groups.[33]

The effect of site directed immobilization on improving the performance of IAC support does not provide any evidence to arrive at any conclusion. Hoffman and O'Shannessy[34] showed that binding could be very efficient but that the coupling step was markedly less efficient than could be achieved with CNBr activation. They also showed that the method was more successful with polyclonal rather than with monoclonal antibodies. Other workers have shown the overall strategy to be not very satisfactory[35, 36] primarily owing to the variable and poor coupling of the MAb. It has also been shown that high efficiencies of

antigen binding are only achieved at low MAb coupling levels[37] rendering the purification process less productive than it might be.

CHROMATOGRAPHIC SEPARATION

The separation of the analyte using IAC involves three steps, viz. binding, washing and elution as depicted in general scheme for IAC in figure 2.

Binding

Binding is an essential step that has a profound influence on the purity of the final product of an IAC process.[38] Binding is performed at or slightly above the neutral pH in a phosphate or borate buffer containing NaCl and a nonionic detergent.[39] This facilitates only the binding of analyte of interest and the non-specific absorption on the stationary phase is minimized. Presence of salt in buffer is essential to reduce the ionic interactions of the charged surface groups of immobilized MAb. Detergents reduce hydrophobic interactions. Use of detergents for potential therapeutic products is irrational owing to the inherent difficulties in removing detergents from proteins. Inclusion of polyethylene glycol (PEG) in the binding buffer helps reduce nonspecific binding.[40]

Washing

Washing is essential in the chromatography to remove any material bound nonspecifically to the matrix. Hydrophobic forces are more likely the cause of the presence of unwanted molecules. The low molecular weight compounds which may be retained in the gel beads will elute in 1 to 2 column volumes. The compounds retained through the hydrophobic forces may be removed by washing with low-ionic-strength buffers or with water.

Elution

Antigens and antibodies are bound to each other by a web of forces, which include ionic bonding, hydrophobic interactions, hydrogen bonding, and van der Waals attractions. The strength of Ag-Ab complexes depends on the relative affinities and avidities of the antibodies. In addition, steric orientation, coupling density, and nonspecific interactions can also influence the binding. The objective of the elution step is to recover the specifically bound protein at a high yield, purity, and stability. Elution conditions, which might denature the protein product, have to be avoided. Examination of the literature suggests a wide variety of elution conditions and the choice of an eluant seems empirical. However, a logical sequence of available elution strategies can be considered when selecting an appropriate elution protocol.

The various elution strategies include –

1. Specific elution: Certain antibodies bind to their respective antigens under high pH or in the presence of metals like calcium or magnesium or in the presence of chelating agent like EDTA. Antigens bound to such antibodies can be eluted under gentle conditions where lowering the pH or adding EDTA to the elution buffer or adding divalent metals to the elution buffer causes the Ag-Ab complex to dissociate.
2. Use of extreme pH:
a) Acid elution: This is the most widely used method of desorption and is normally very effective. The commonly used acid eluants are glycine-HCl, pH 2.5; 0.02 M HCl and sodium citrate, pH 2.5. Upon elution, the pH of the eluant sample is quickly neutralized to 7.0 with 2 M Tris base, pH 8.5, to avoid acid-induced denaturation. In some cases increased hydrophobic interactions between antigen and antibody gives low recovery with acid elution. Incorporation of 1 M propionic acid, or adding 10% dioxane or addition of ethylene glycol to the acid eluant, is effective in dissociating such complexes.
b) Base elution: It is less frequently employed than acid elution. Typically, 1 M NH_4OH, or 0.05 M diethylamine, pH 11.5 have been employed to elute membrane proteins (i.e., hydrophobic character) and other antigens that precipitate in acid but are stable in basic conditions.
3. Use of Chaotropic agents: These agents disrupt the tertiary structure of proteins and, therefore, can be used to disrupt the Ag:Ab complexes. Chaotropic salts are particularly useful as they disrupt ionic interactions, hydrogen bonding, and sometimes hydrophobic interactions. The relative order of the effectiveness of chaotropic anions is $SCN^- > ClO^{4-} > I^- > Br^- > Cl^-$. Chaotropic cations are effective in the order of Mg>K>Na. Eluants such as 8 M urea, 6 M guanidine-HCl, and 4 M NaSCN are effective in disrupting most Ag-Ab interactions. To avoid and minimize chaotropic salt-induced protein denaturation, rapid desalting or dialysis of the eluant is essential.
4. Use of denaturants: The denaturants most frequently used in IAC are 6-8M urea and 3-4M guanidine hydrochloride. These reagents were more commonly used in conjunction with polyclonal antibodies with a very high affinity for the antigen and in cases where the antigen is stable to the treatment.[41]
5. Use of organic solvents: Organic solvents have found favor as eluants in some circumstances but with the possible exception

of ethylene glycol are infrequently employed in IAC.[42]

6. Changes in ionic strength: Raising the ionic strength of the solvent can be a mild method of elution but is rarely effective when using NaC1. Of more use are the salts of divalent cations such as Mg^{2+} and Ca^{2+}. The ionic strength of a 4.5M $MgC1_2$ solution is 13.5M and the chloride concentration is 9M. This may in some way explain the effectiveness of such solutions as eluants in terms of very high concentrations of a weakly chaotropic ion in combination with an ability to disrupt ionic interactions. In addition Ca^{2+} and Mg^{2+} are more chaotropic than either Na^+ or K^+.

APPLICATIONS

IAC, due to its high specificity is widely used for protein purification. It's application has also been extended for analytical purposes. These applications of IAC are discussed below –

Preparative Applications

IAC has been used to purify a wide range of products. The use of IAC for production of enzymes[43] as well as a variety of proteins[42] has been reviewed. The products of therapeutic importance include Monoclate and Mononine, IAC-derived human Factor VIII and Factor IX. The products prepared on large scale include tissue plasminogen activator[44] and calf intestine alkaline phosphatase.[45] Other proteins purified by IAC have been enlisted in Table 1.

Analytical Applications

Owing to its high selectivity, IAC has also been used for analysis. The analysed substances include various proteins, hormones, toxins, drugs and even the antibodies employing the various IAC techniques from a range of matrices. The various IAC techniques are

a) IAC with direct detection
b) Immunoextraction
c) Chromatographic immunoassays

a) IAC with direct detection:

This is the simplest format for IAC in analytical applications. It involves the adsorption of the test solutes in an immobilized antibody column, followed by elution and detection of the analytes.

The sample of interest is first injected onto the IAC column under the mobile phase conditions in which the analyte will have strong binding to the immobilized antibodies in the column. Owing to the specificity of the antibody-analyte interaction, other solutes present in the sample are not retained and washed through the column by the application buffer. After the removal of the nonretained solutes, a second buffer is applied that dissociates the retained analyte which is detected as it elutes from the column in the elution buffer. The immobilized antibodies are regenerated by application of the initial buffer after complete elution of the test analyte. The advantages of of this approach include its relative simplicity, good precision, and potential for fast sample analysis when performed using HPIAC. Applicaitons of IAC with direct detection are listed in Table 2.

b) Immunoextraction

Immunoextraction involves the use of IAC for the removal of a specific solute or group of solutes from a sample prior to its determination by a second analytical method. Operating scheme is same as that of IAC, but involves combining the immunoaffinity column either off-line or on-line with some other method for quantitation of analytes.

i) Off-line immunoextraction:

Off-line immunoextraction serve as the easiest way for combining immunoaffinity columns with other analytical techniques. This approach utilizes antibodies immobilized onto low performance support which is packed into a small disposable syringe or solid- phase extraction cartridge.[58-60] After conditioning the column with application buffer or conditioning solvents, the sample is applied and undesired sample components are washed away. The test analyte is collected as it is removed from the column upon application of the elution buffer. The eluted fraction can be analyzed directly by the second technique, but mostly the collected fraction is first dried down and reconstituted in a solvent that is compatible with the method to be used later for quantitation. The collected solute may be derivatized, when required, to improve detectability or alter physical property before analysis by other techniques. The applications of off-line immunoextraction are given in Table 3. One advantage of off-line immunoextraction is that the samples collected from the IAC columns can be readily derivatized or placed into a different solvent in between sample purification step and quantitation step especially when combining IAC with GC. Also it is easy to set up once an appropriate antibody preparation has been obtained.

ii) On-line immunoextraction

Off-line immunoextraction involves manual sample preparation which limits the potential speed and precision of this approach. The on-line immunoextraction methods overcome this problem. The direct coupling of immunoextraction with HPLC by incorporation of IAC into HPLC system makes this appealing as a means for automating immunoextraction

methods and for reducing the time required for sample pretreatment. Majority of the application of IAC with HPLC involve reversed phase liquid chromatography but the techniques involving IAC coupled to size exclusion[70] and ion exchange chromatography[71,72] have also been reported. Mostly high performance supports are used in this approach. There are relatively less reports of on-line immunoextraction by coupling IAC with GC except the work reported by Farjam *et al.*[73] Successful attempts have also been made by coupling on-line immunoextraction with capillary electrophoresis.[74-76] Some of the applicaitons of on-line immunoextraction are enlisted in Table 4.

c) Chromatographic immunoassays

The use of immobilized antibody (or immobilized antigen) columns to perform various types of immunoassays is known as chromatographic immunoassay. The use of IAC to perform immunoassays is particularly valuable in determining trace analytes that, by themselves, may not produce a readily detectable signal. In chromatographic immunoassays the use of labeled antibody or labeled analyte analog for indirect analyte detection overcomes this problem. Various formats of chromatogtaphic immunoassays are

i) Competitive binding immunoassays

The basic principle in competitive binding immunoassay involves the incubation of analyte in the sample with a fixed amount of a labeled analyte analog in the presence of a limited amount of antibodies that bind to both the native analyte and labeled species. Of the several approaches for these assays, the simplest approach is to mix the sample and labeled analyte analog and simultaneously apply these to the IAC column. This approach is known as a simultaneous injection competitive binding immunoassay and is the format used in most of the competitive binding techniques listed in Table 5.

ii) Sandwich immunoassays

Sandwich immunoassay involves the use of two different types of antibodies that each bind to the analyte of interest. The first of these two antibodies is attached to a solid-phase support and is used for extraction of the analyte from samples. The second antibody contains an easily measured tag (i.e. an enzyme or fluorescent label) and is added in solution to the analyte either before or after this extraction. The second antibody serves to place a label onto the analyte, thus allowing the amount of analyte on the immunoaffinity support to be quantitated. Some of the applications of sandwich immunoassays have been given in Table 6.

iii) One-site immunometric assays

In one-site immunometric assay, the sample is first incubated with a known excess of labeled antibodies or Fab fragments that are specific for the analyte of interest for binding. After binding, this mixture is then applied to a column that contains an immobilized analog of the analyte. This column extracts any antibodies or Fab fragments that are not bound to sample analyte where as the antibodies or Fab fragments bound to the sample get eluted to give a nonretained peak, quantitated using an appropriate technique. The example of this approach is the assay of α-(difluoromethyl)ornithine from plasma detected by fluorescence by Gunaratna P.C. *et al.*[88]

CONCLUSION

The efficiency of the IAC process is dependent on the efficiency of the coupling of antibodies to support and the activation chemistries used in coupling as they affect the avidity of antibodies. IAC, due to its high selectivity has been a method of choice for the purification of biomolecules. Also it has been applied for drug, pesticide and food contaminant analysis. IAC serves a promising approach for selective analysis of very dilute analyte samples and also bioanalysis of drugs.

Table 1: List of proteins purified by IAC

Purified constituent	Sorbent	Eluant
Human parainfluenza virus type 3 glycoproteins[46]	Affi-Gel HZ Mab	0.1M glycine pH 2.5
Human insulin receptor[47]	CNBr-Sepharose MAb	HEPES + NaCl + TRITON X-100 + 15-mer peptide
E.coli RNA Polymerase[48]	CNBr-Sepharose Mab	50mM Tris + 0.1M EDTA + 0.7M NaC1 + 30% ethylene glycol
Shewanella oneidensis RNA Polymerase[49]	CNBr-Sepharose Mab	50 mM Tris–HCl, pH 7.9 + 0.1 mM EDTA + 40% propylene glycol + 0.75 M ammonium sulfate
Flavocytochrome b (Cyt b)[50]	Protein A–Sepharose beads	0.25% DDM (Dodecylmaltoside) containing 1 mM elution peptide AC-PQVRPI-CONH2 (AC-, acetylation at the N-terminus of the elution peptide; -CONH2, amidation at the C-terminus of the elution peptide)
DNase II[51]	Formyl-cellulofine resin conjugated with a murine monoclonal anti-human DNase II antibody	PBS + 50% v/v ethylene glycol

Table 2: Application of IAC with direct detection

Analyte	Detection Method	Sample
Fibrinogen[52]	UV absorbance	Plasma
Granulocyte colony stimulating factor[53]	Fluorescence (after derivatization with o-phthaldialdehyde)	Plasma, cerebrospinal fluid (CSF)
Interferon[54]	UV absorbance	Plasma/ serum, urine, saliva, CSF
Linear alkylbenzenesulfonates (LASs)[55]	Fluorescence	Water
Transferrin bound iron[56]	UV absorbance	Serum
Gliadins[57]	Lighr Scattering Detector	Food and beverages

UV- Ultra-violet; CSF- Cerebrospinal fluid

Table 3: Application of Off-line immunoextraction

Analyte	Analytical Method		Samples
	Separation Method	Detection method	
Abscisic acid[61]	RPLC	ESI-MS	*Nicotiana tabacum* L. leaves
Aflatoxin[62]	RPLC	Fluorescence	Medicinal herbs
Aflatoxin B_1[63]	RPLC	Fluorescence	Animal liver
Albuterol [64]	RPLC	Fluorescence	Plasma
Epitestosterone[65]	RPLC	UV-Absorbance	Urine
Fumonisin B_1[66]	RPLC	Fluorescence	Animal tissues
Quinolone and sulfonamide antibiotics[67]	RPLC	MS/MS	Animal muscle tissues
Sudan dyes (I, II, III, IV)[68]	RPLC	UV-Absorbance	Food
Vitamin B_{12}[69]	RPLC	UV-Absorbance	Meat products

RPLC- reversed phase liquid chromatography; ESI-MS- electrospray ionization mass spectrometry; GC- gas chromatography; MS- mass spectrometry; ECD- electron capture detector; MS/MS- tandem mass spectrometry

Table 4: Application of On-line immunoextraction

Analyte	Analytical method		Samples
	Separation method	Detection method	
Immunoglobulin E[75]	Capillary electrophoresis	UV-absorbance	Serum
Insulin[76]	Capillary electrophoresis	UV-absorbance	Serum
Δ^9-tetrahydrocannabinol[77]	RPLC	UV-absorbance	Saliva
Triclopyr[78]	RPLC	UV-absorbance	Water samples
Human Serum Albumin[79]	RPLC	MS	Serum

RPLC- reversed phase liquid chromatography;UV- Ultra-violet; MS- mass spectrometry

Table 5: Application of Competitive binding immunoassays

Analyte	Label/detection method	Sample
Human chorionic gonadotropin[80]	HRP/ absorbance	Serum
Thyroid stimulating hormone[80]	HRP/ absorbance	Serum
Human serum albumin (HSA)[81]	HSA/ absorbance	Aqueous standards
Immunoglobulin G (human)[82]	GOD/ECD	Serum
Immunoglobulin G (human)[83]	Fluorescein/ fluorescence	Serum

HRP- Horse raddish peroxidase;HSA- Human Serum Albumin;
GOD- Glucose oxidase; ECD- Electrochemical detector

Table 6: Application of Sandwich immunoassays

Analyte	Label/ detection method	Sample
Anti-bovine IgG antibodies[84]	GOD/ ECD	Serum
Immunoglobulin G (human)[85]	Acridinium ester/ chemiluninescence	Serum
Parathyroid hormone[86, 87]	Acridinium ester/ chemiluninescence	Plasma

GOD- Glucose oxidase; ECD- Electrochemical detector

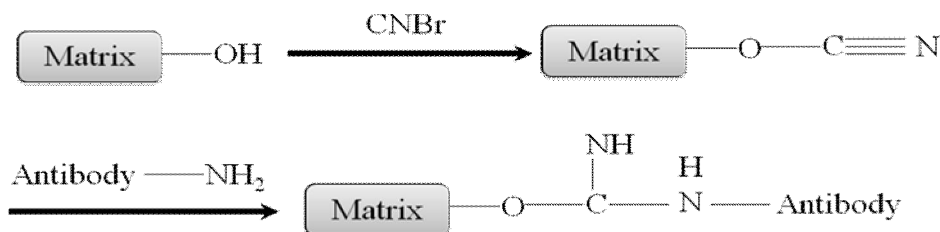

Fig. 1: Matrix Activation and Coupling using CNBR (Cyanogen Bromide)

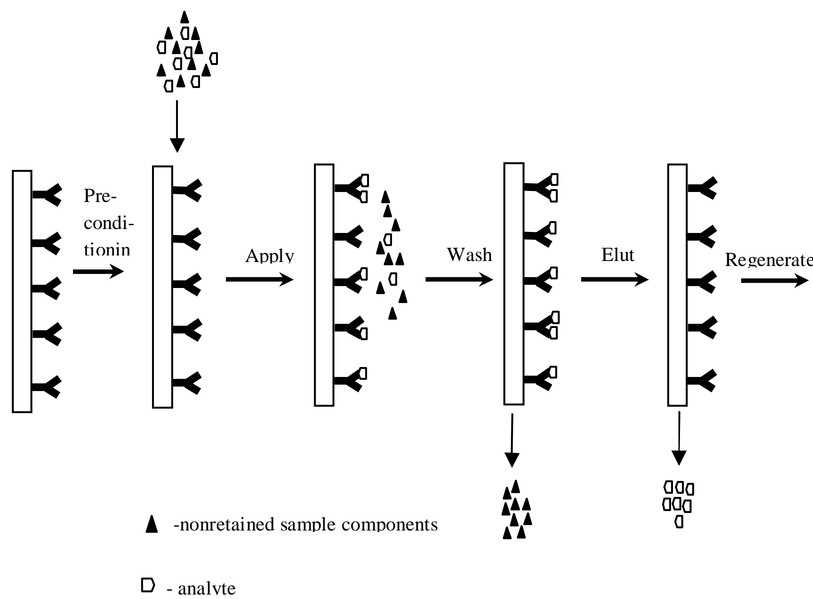

Fig. 2: General Scheme for IAC

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