Pseudoaffinity Chromatography Using a Convective Interaction Media®-Disk Monolithic Column
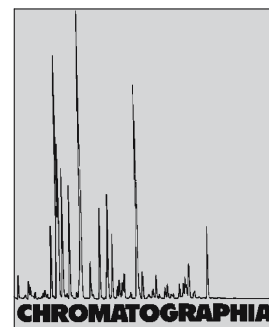
J. Champagne[1], C. Delattre[2,✉], C. Shanthi[2], B. Satheesh[2], L. Duverneuil[2], M. A. Vijayalakshmi[1,2]

[1] LIMTech.S, Centre de Recherches de Royallieu, B.P. 20 529, 60205, Compiègne Cedex, France
[2] CBST, Vellore Institute of Technology (VIT), Deemed University, Vellore 632014, Tamil Nadu, India; E-Mail: cedric_delattre@hotmail.com

Abstract

Monolithic convective interaction media-disk (CIM-disk) chromatography is one of the fastest liquid chromatographic methods for the separation and purification of biomolecules due to its high mass transfer rate. In this way, all separated molecules are transported by convection into the pores of the matrix, resulting in a very fast separation due to the low mass transfer resistance of the CIM-disk. Due to the advantages of monolith technique, in recent years, CIM-disk affinity chromatography has been developed and investigated for purification of peptides, restriction enzymes, antibodies, etc. In this review, applications of monolithic affinity chromatography are discussed. The purification of restriction enzymes, polyclonal and monoclonal antibodies using a new monolithic CIM-disk system with immobilized histidine affinity chromatography are presented.

Keywords

Introduction

Affinity chromatography (AC) is one of the most selective and rapid tools for the separation and purification of biomolecules such as antibodies, enzymes, antigens, hormones, receptors, etc. [1–4]. The success of affinity chromatography is essentially due to its high efficiency, which originates from the "specific" or "pseudo-specific" recognition between a solid phase-immobilized ligand and the molecule to be purified.

Affinity purification technology can be separated into two categories depending on whether the interaction mechanism of specific protein/ligand recognition is either biospecific or pseudo-biospecific [5]. Generally, proteins bind to biospecific ligands with high affinity in the range of 10^{-7} to 10^{-15} M. Consequently, protein desorption in the case of biospecific ligand affinity chromatography needs more drastic elution conditions which can elution cause ligand and/or protein denaturation. On the other hand, pseudo-biospecific ligand affinity chromatography leads to medium affinity interaction [1, 6] and methods have been devised to provide a fairly high selectivity allowing gentle methods for the purification of a large number of therapeutic proteins [2, 5].

The most crucial step of this method is to choose a good biospecific ligand for the product to be isolated and purified so that AC becomes an adsorption method, which exploits the recognition mechanism between a biomolecule and a specific ligand. Ligands such as amino acids and peptides, mimicking the active centres of proteins are the most widely used affinity sites [5, 7].

In biospecific ligand AC, all the interaction occur between the immobilized ligand and the solute molecules are based on their complementarity of hydrophobicity, charge, shape, etc. [7, 8]. Numerous kinetic and thermodynamic factors such as enthalpy and entropy (driving forces) related to electrostatic and hydrophobic interaction of ligands [9] are exploited to favor specific adsorption of proteins or glycoprotein onto the ligand-matrix. Van der Waals forces and hydrogen bonds are used to effect adsorption in pseudoaffinity systems as well as for biospecific binding. On the other hand, electrostatic distribution is an essential parameter to generate specific interactions. Therefore, introduction of positive charges on the pseudo-biospecific immobilized ligand by buffer modulation creates a cationic

environment that increases stability and biomoleculer adsorption [7]. Moreover, in liquid chromatography, the interaction and selectivity [10] between a sorbent matrix and a target biomolecule can be significantly influenced by the chemical nature of the support [11], the chelating properties of ligands [12–15], and the stationary phase density [16]. Furthermore, the feasibility of these interactions with the ligand depends on the protein surface amino acid accessibility, pH, temperature, ionic strength and support matrix.

A large amount of coupling chemistries have been proposed and used in affinity chromatography for linking protein affinity ligands to solid supports. The coupling reaction is known to affect the orientation of the polypeptide at the surface and hence, can influence the interactions with the target molecule. Despite this, the utilization of biospecific ligand for affinity separations can be counted as the most prefered way, considering the high cost of natural biomolecules, such as monoclonal antibodies, requiring purification.

For several years, pseudo-biospecific ligand such as metal chelates, dyes and amino acids, which can recognize proteins or others molecules through somewhat similar mechanism have been extensively used instead of biospecific ligands in AC [2, 5]. On the other hand, pseudo-biospecific ligands are usually smaller and simpler molecules with higher stability and low cost [2, 5, 7]. The amino acid L-histidine is attractive as a general ligand in affinity chromatography of proteins [5]. In fact, in the last two decades, pseudo-bioaffinity chromatography using L-histidines immobilized onto different matrices has been developed to separate and purify proteins and carbohydrates like oligouronides and then constitutes a high potential chromatographic tool for proteins and carbohydrates purification on a preparative scale [17–29].

In recent years, a new polymeric macroporous material based on radical co-polymerisation of glycidyl methacrylate and ethylene glycol dimethacrylate under the trademark CIM® (convective interaction media) has been introduced in bio-chromatographic processes. CIM monolithic supports represent a novel generation of stationary phase used for liquid chromatography and bioconversion [30–38]. Current studies involve the concept of new bioaffinity supports using monolith technology.

This present paper gives a review on the use and the development of affinity chromatography using monolithic supports made of poly-(glycidyl methalcrylate) for the separation and purification of biomolecules. The potential for further utilization and applications of L-histidine amino acid immobilized onto CIM-diskmonolith technology is presented and briefly discussed.

Hydrodynamic Parameters in Affinity/Adsorption: Importance and Comparison of Matrix Support

One of the most important factors in the concept of new tool for affinity chromatography and enzyme bioreactors is the development of original solid supports. For the accomplishment of desired affinity chromatography separation, a judicious choice of solid support and the chemical covalent coupling is essential for binding the matrix to the specific affinity ligand. Moreover, solid matrix supports play an important key role on the stability of immobilized affinity ligands. Separation efficiency is extremely dependent on the matrix structure, which determines the ideal surface accessibility and consequently binding capacity, as well as to the totality of the surface ligand, which influences the selectivity and recovery of biomolecules [39]. Therefore, to prepare new chromatographic matrixes with good reproducible properties, the control of optimum ligand density is crucial.

For a successful application in bioaffinity chromatography and for the immobilization of enzymes, an ideal solid matrix should possess different following properties:

1. high rigidity and a suitable form of particles,
2. insolubility,
3. hydrophilic character,
4. sufficient permeability and a large specific area,
5. efficient chemical reactivity for the immobilization of affinity ligand or enzyme,
6. chemical stability for immobilization, adsorption, desorption and regeneration,
7. resistance to microbial and enzymatic cleavages,
8. long term stability and reusability.

New applications of affinity chromatography resulting in a changeover from soft gel supports to small rigid particles, used in high-performance liquid affinity chromatography (HPLAC) have been reviewed by Ohlson et al. [40]. A comparison of HPLAC with soft gel affinity chromatography (AC) showed that the porous rigid particles, with small and uniform sizes, provide acceptable flowrates giving them an overall high operational adsorption capacity. Nevertheless, the diffusion factor makes the elution peak broader and in scale-up the process, it could be limited by the time factor and dilution factor of sample. Furthermore, favourable mass transport and adsorption/desorption kinetic behaviour with non-porous supports has been demonstrated by Anspach et al. [41]. Monodisperse, solid particles (0.7, 1.5 and 2.1 µm) have relatively low surface areas compared to commonly used porous silica and they allow shorter contact times between the sample and the stationary phase surface during the chromatographic run.

Non-porous matrices are well known to exhibit greater accessibility of the immobilized ligand than equivalent porous affinity supports. The elution behaviour of proteins on a non-porous silica-based adsorbent was investigated both theoretically and experimentally using immunoglobulin G and immobilized protein A as the affinity ligands [42]. The desorption rate constant and equilibrium association constant under elution conditions were found to decrease elution time and improve the shape of the elution peak. However, the adsorption rate in column chromatography is limited by either slow intra particle diffusion for large beads or low axial velocities and high-pressure drops for small beads. Consequently, the main disadvantage is the limited access of the biomolecules to the small pores in the case of classical porous chromatographic supports, which is why the slow mass transport results in peak broadening and bad resolution.

In order to overcome all these limitations encountered by the difficulty of slow mass transfer, the development of new generation of monolithic solid supports [43, 44] were introduced and investigated in affinity chromatography for the effi-

cient separation of biomolecules. Instead of chromatographic supports consisting of macro porous particles, where the void volume between individual porous particles is unavoidable, CIM-disk technology is a monolithic macroporous material in which there is predominantly convective transport of the liquid within the pores and the transfer of molecules to active sites is not limited by diffusion. Monolithic supports are a single piece of continuous and highly porous material. They form a network of channels, which are highly interconnected. In this manner, all molecules to be separated are transported by convection into the pores of matrix, resulting in very fast separation (Fig. 1) due to the low mass transfer resistance (convection rather than diffusion) of the CIM-disk [34–38]. As the mobile phase is forced through the pores of monolithic support, the mass transfer is enhanced by convection contributing to minimization of the void volume and the peak broadening [44, 45].

The main advantage of CIM disc monolithic system versus traditional chromatographic supports such as membranes (limited by the thickness of their layers) or soft gel for the separation of biomolecules, takes account of these much better mass transfer properties, ease of use, the ability to be manufactured with a wide range of pore sizes and shapes, simple scaling up and scaling down, and the low back pressure even at very high flow rates (up to 10 mL min^{-1} for larger disk) without the loss of efficiency and capacity. One of the main advantages of monolithic supports is their capability to be used in miniaturized devices in chromatographic systems [39]. Monolithic supports can carry many specific ligands for affinity chromatography and the vast potential of monoliths for immobilization of affinity ligands has been slowly recognized, resulting in an increase in experience in applying this technology to bioanalytical and biotechnological activities.

Biotechnological Applications of CIM-Disk Monolithic Column

In recent years, a wide range of applications of the CIM® family of monolithic columns have been investigated (Table 1). Short monolithic CIM-disk columns are used for very fast separation

Table 1. Main applications of CIM-disk monolithic affinity chromatography

| CIM-disk ligands	Applications	References
Protein A/G	Purification of IgG	[83–86]
Protein A	Purification of monoclonal antibodies	[61]
Annexin	Isolation of monospecific antibodies	[97]
Bradykinin	Purification of antibradykinin polyclonal IgG	[84]
Monoclonal antibody	Purification of tissue plasminogen activator	[98]
Human IgG	Purification of recombinant Protein G	[99, 100]
Monoclonal antibody	Isolation and purification of antigen	[61]
	Purification of plasma inter-α inhibitor proteins	[62]
Concanavalin A	Purification of membrane protein	[61]

CIM®disk
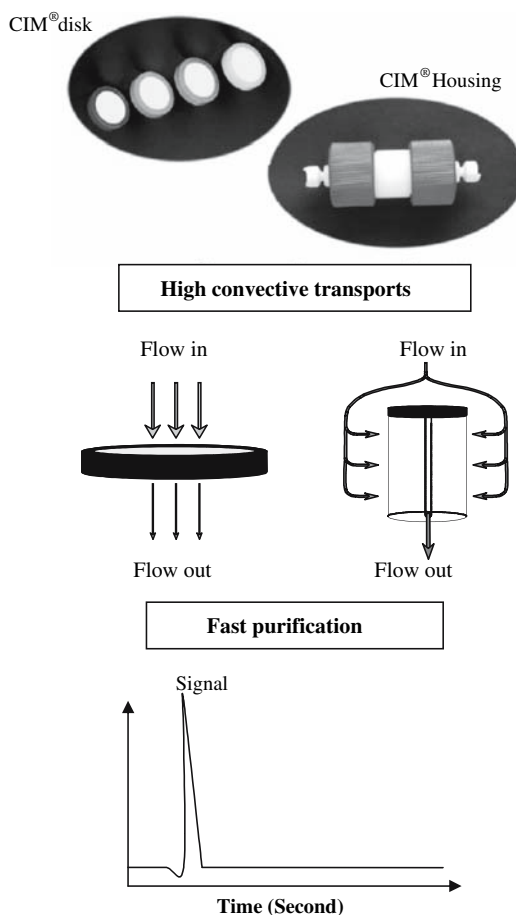
Fig. 1. Schematic conception of convective interaction media (CIM) monolithic column

and purification of proteins in different modes such as hydrophobic interaction, ion exchange, or affinity mode [30–34]. These processes have included separation and concentration of nucleic acids [45], peptides, proteins, recombinant proteins, monoclonal antibodies, enzymes, nanoparticles like plasmid DNA from *E. coli* cell lysate [46, 47]. Moreover, bacterial genomic DNA of size up to 200 kbp can be also separated on CIM monoliths [48]. Anion-exchange chromatography with CIM-disks allows the fractionation and purification of oligonucleotides of various sizes [49, 50]. Some data has also

reported about isolation of viruses [51]. Progress in immunoaffinity reactors can be envisaged in the future with CIM-disks since some publications describe the use of an immunoaffinity reactor for the qualitative analysis of cellular prion protein [52].

Recently, numerous applications have been investigated using CIM-disk material as a support for the immobilisation of enzymes in enzyme bioreactor processes, for example glucose oxidase (GOD) [53]. In comparison with particulate silica supports, these processes were possible because of the fast conversion of enzyme
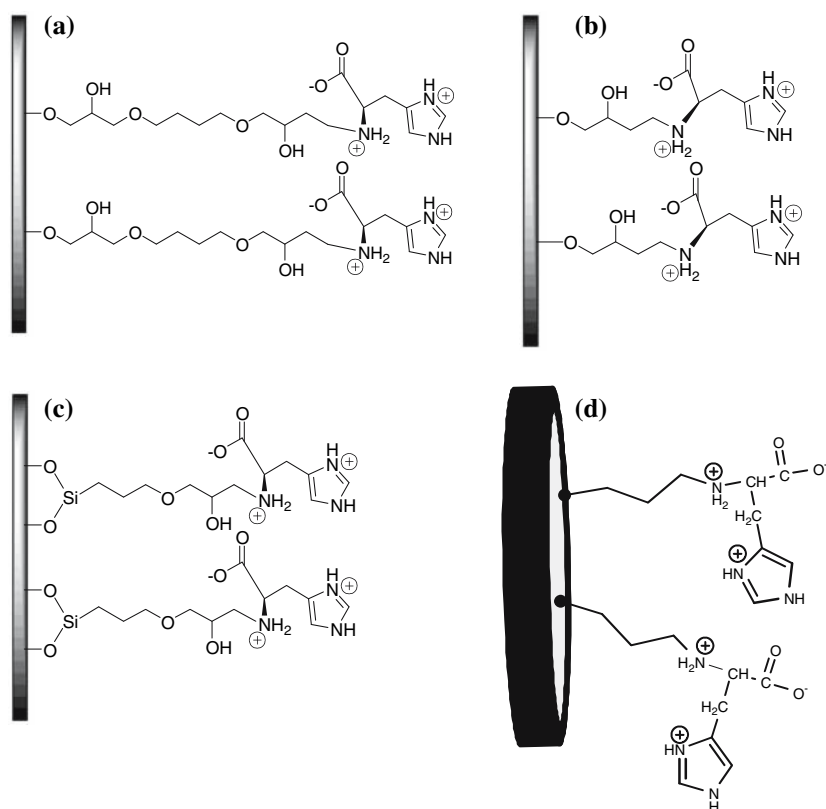
Fig. 2. Proposed schematic structure of various pseudobioaffinity supports. **a** Sepharose 4B-bisoxyran-histidine. **b** Sepharose 4B-epoxy-histidine. **c** Silica-oxyran-histidine. **d** CIM-disk-EDA-histidine

substrates due to the total lack of diffusion resistance during the mass transfer [54, 55].

"Conjoint liquid chromatography" (CLC) has also been described where it is possible to stack several monolithic CIM-disks with different ligands into one cartridge [56]. Finally, a number of examples of biological applications of modern affinity separations using macroporous monoliths have been summarized [57–60]. Here the most important and attractive data on affinity chromatography are presented and briefly discussed in order to consider new applications of CIM-disk support with L-histidine pseudospecific ligand.

Affinity Chromatography

Immobilized Antibodies on CIM-Disk

Antibodies can be directly immobilized on CIM-disk in order to isolate and purify their corresponding antigens [61]. Using the same efficient procedure, others authors [62] had isolated and purified a plasma inter-alpha inhibitor protein.

This protein was then digested by elastase, also immobilized on monolithic CIM-disk and the corresponding peptides were mapped by SDS-PAGE. The immobilized antibodies and enzymes remain stable and still active after repeated runs.

Immobilized Lectin on CIM-Disks

Progress has been made in glycomic and proteomic field using immobilized lectins like affinity ligands in order to separate and purify glycoproteins before structural analysis of glycosylation [63, 64]. In this domain some data described the potential of monolithic affinity chromatography. Much work was carried out on the glycosylation pattern analysis of certain glycoproteins, specific to cancer cells in order to propose new biospecific markers [65, 66]. In this context, several strategies were developed as for example first, the immobilization of Concanavalin A on CIM-disk to separate and purify soluble and membrane-bound proteins [61] and second, the efficient and fast separation of glycopeptides and glycoproteins with

specific lectin immobilized onto monolith matrix [67].

Monolithic CIM-Disk Immobilized Histidine Affinity Chromatography

L-histidine amino acid has been studied for more than 20 years and investigated as a pseudospecific ligand in affinity chromatography [5, 7]. Histidine as a ligand presents many advantages for the recovery of active and stable enzymes: ease of immobilization, affinity by multimodal interaction, and mild adsorption/elution conditions. In a more general way, according to the choice of the matrix, the spacer and the method of immobilization of histidine, the ligand will have a particular specificity. Histidine interacts with the target molecules with the imidazol core, but also with residual charges carried by the free remaining functional groups [7]. Biomolecules such as proteins, peptides and carbohydrates has been separated and purified using histidine ligand affinity chromatography (HLAC) on a preparative scale [5, 7, 17–29].

Because of all advantages of monolithic supports and the efficiency of L-histidine amino acid as a pseudobiospecific ligand, we propose an interesting monolithic chromatographic support. With a rational combination of "pseudobiospecific" affinity ligands and the CIM-disk/tubes support matrixes, we have been able to exploit immobilized histidine affinity chromatography (IHAC) systems for the efficient purification of several proteins such as restriction enzymes, immunoglobulins, etc., from crude extract or complex biological media, in a minimum number of steps. We have studied this process using L-histidine immobilized onto CIM-disk (Fig. 2d) for the fast purification of the model restriction enzyme *Bam*HI and monoclonal antibodies toward malaria.

Purification of Restriction Enzyme Using CIM-Disk-EDA-Histidine

Restriction endonucleases (RE's) were discovered about 30 years ago during investigations into the phenomenon of host-specific restriction and modification of bacterial viruses. Bacteria initially resist infections by new viruses, and this "restriction" of viral growth stems

from endonucleases within the cells that destroy foreign DNA molecules. These endonucleases recognize specific base sequences in double-helical DNA and cleave both strands at specific places. Restriction endonucleases usually occur in combination with a modification enzyme (DNA-methyltransferases) that protects the cell's own DNA from cleavage by the restriction enzyme. Modification enzymes recognize the same DNA sequence as the restriction enzyme that they accompany, but instead of cleaving the sequence, they methylate one of the bases in each of the DNA strands. Although primarily found in bacteria, restriction endonucleases also exist in archea, viruses, and eukaryotes. Including all types, more than 3,500 restriction endonucleases that recognize 259 different DNA sequences have been isolated so far [68]. Restriction endonucleases have become essential tools for molecular biologists. More than 3,500 enzymes have been isolated and new restriction endonucleases with new specificity are still found regularly. They have proved to be indispensable tools for analyzing chromosome structure, sequencing very long DNA molecules, isolating genes, and creating new DNA molecules that can be cloned. The development of standard purification protocols for all restriction enzymes still remains a challenge. Conventional chromatographic methods that are used to purify restriction endonucleases at a large scale involve long and laborious processes to obtain homogenous enzymes in poor yields.

Conventional protocols usually applied for the purification of restriction endonucleases combine chromatographic methods with different media such as phosphocellulose [69–73], hydroxyapatite [70, 71], heparine-agarose [74–76], ion exchangers [77] and size exclusion gels [78]. Most of these methods involve several steps and hence, required relatively long processing times. Long dialysis is required to remove high salt concentrations or to change the buffer between steps. This usually results in low yield because of the low stability of restriction enzymes (for review see [79, 80]). Since these enzymes are able to bind DNA molecules, immobilized DNA sequences have been used for purification. Restriction enzymes EcoRI and SphI can be purified near to homogeneity in one or two steps using sequence-specific DNA affinity chromatography [81]. The rela-
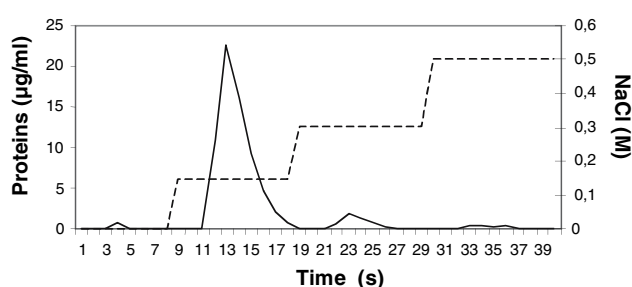

Fig. 3. Purification of *Bam*HI with EDA-histidine CIM-disk

1. Crude extract
2. Not retained
3. PC 0,1 M KCl
4. PC 0,3 M KCl
5. PC 1 M KCl
6. His not retained
7. His 0,15 M NaCl
8. His 0,3 M NaCl
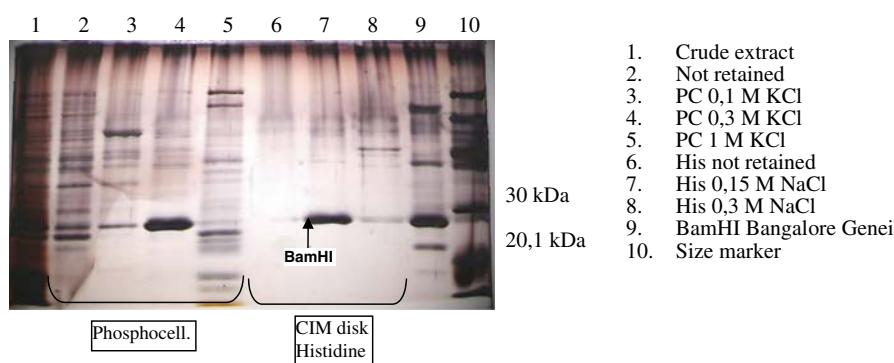9. BamHI Bangalore Genei
10. Size marker

Fig. 4. SDS-PAGE from fractions after phosphocellulose and EDA-histidine CIM-disk purification steps of restriction enzyme *Bam*HI (12% acrylamide, reducing conditions, silver nitrate staining)

tively high cost of such affinity columns can be reduced by designing DNA ligands containing the recognized sequences for a number of different restriction endonucleases. However, biospecific ligands, DNA in the case of restriction endonucleases, are quite fragile and expensive. These ligands would be difficult to implement in commercial applications and in large-scale production.

The purification of restriction enzymes using histidine has not reported in the literature. We have investigated the specific purification of the model restriction enzyme BamHI using L-histidine as biospecific ligand immobilized on traditional Sepharose-based gel and on a monolithic support CIM-disk. Thus, we have tested the affinity of histidyl-ethylenediamine for the purification of the model restriction enzyme *Bam*HI. An eda-histidine CIM-disk was first used in a single step procedure using crude recombinant *E. coli* crude extract as starting material. Elution was also carried out by a discontinuous gradient of 0.15, 0.3 and 0.5 M NaCl but in contrast to soft gel columns, the flow applied was 5 mL min^{-1} and therfore the separation was faster. Active fractions obtained after

this one-step procedure contained other endonucleases contaminants also. Hence, the restriction enzyme quality is not sufficient as was previously observed in the case of Sepharose-aminohexyl-histidine used as one-step separation. We thus followed the same strategy by adding a preliminary separation step with phosphocellulose. The two-step protocol combining phosphocellulose and histidine was applied using the same crude extract and the same adsorption and elution conditions for the histidine column step. The chromatogram from the second step is shown in Fig. 3. The biospecific L-histidine ligand was immobilized on a monolithic CIM-disk, which has many advantages in terms of speed and handling and proved to be very efficient for enzyme purification.

The fractions obtained in the two-step procedure were analyzed by SDS-PAGE (Fig. 4) and a test of ligation/digestion was carried out on the fractions obtained. The profiles of the bands obtained (Fig. 5, lanes 2, 3, 4) are comparable with those of the commercial enzyme (Fig. 5, lanes 5, 6, 7). Thus the CIM-disk system makes it possible to obtain the same result as gel chromatography but with an important gain of time in the second step
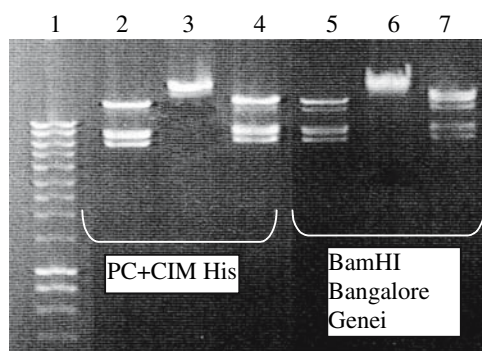
1.	Marker
2.	BamHI PC+CIM His digestion 1
3.	BamHI PC+CIM His ligation
4.	BamHI PC+CIM His digestion 2
5.	BamHI B. Genei digestion 1
6.	BamHI B. Genei ligation
7.	BamHI B. Genei digestion 2

Fig. 5. Endonuclease activity digestion/ligation test after two steps procedure (PC + EDA-His CIM-disk) for purification of BamHI

Table 2. Recovered activity for *Bam*HI with the different protocols integrating immobilized-histidine affinity supports.

Protocol	Activity recovered (U g^{-1} bacteria)	Ligation/digestion test (enzyme quality)
AH (2 mL)	6,000	–
PC (2 mL)	40,000	–
PC + AH (2 mL)	30,000	+
CIM-EDA-His disk (0,34 mL)	6,000	–
PC + CIM-EDA-His disk (0,34 mL)	20,000	+

Recovered activity is expressed in unit per gram bacteria has activity in crude extract is difficult to measure and the endonuclease activity test is semi-quantitative
AH aminohexyl histidine sepharose, *PC* phosphocellulose

Table 3. Main comparison of IgG separated and purified by histidine ligand affinity chromatography (HLAC)

Antibodies	Maximum adsorption capacity on different matrices		
	Histidyl sepharose (mg mL^{-1} gel)	Histidyl PEVA (mg g^{-1} support)	Hystidyl CIM-disk (mg 0.34 mL^{-1})
IgG	0.5	17	1.5

PEVA poly-(ethylene vinyl alcohol)

with the histidine ligand column. In addition, investigations were carried out to add the preliminary purification step before the histidine CIM-disk in an integrated way by combination of two CIM-disks in the same chromatographic module in order to reduce the purification steps. In fact, in recent work, different CIM-disks was associated to reduce fractionation steps and "conjoint liquid chromatography" was introduced. A quaternary amine disk (anion exchanger) and a Protein A disk were associated to purify monoclonal IgG in only one-step [82]. We thus tried to replace the column of phosphocellulose used until now by a carboxymethyl CIM-disk (weak cation exchanger like phosphocellulose). However the two supports are not equivalent and we did not observe the adsorption of

*Bam*HI enzyme on this disk. The results obtained for the purification of *Bam*HI with the different protocols proposed are summarized below (Table 2).

The two-step protocol combining a phosphocellulose column and a histidine ligand matrix allowed us to efficiently purify the restriction enzyme *Bam*HI from crude extract. The combination of two separation steps was critical for the elimination of contaminating endonucleases from the enzyme sample. The use of the histidine CIM-disk combined with a preliminary phosphocellulose stage allowed us to obtain results equivalent to those obtained with the histidine–Sepharose columns but the procedure was less time consuming due to the separation speed of the CIM-disk. The global binding capacity should be taken into account

for the difference in terms of recovered activity as the Sepharose column used was 2 mL and the CIM-disk has a volume of 0.34 mL. Consequently, we have developed a protocol integrating CIM-disk support derivatized with histidine ligand, which allows a significant gain of time. Although, the first separation step still involves conventional chromatographic support (phosphocellulose), the second step with a histidine CIM-disk considerably decreases the time of the procedure, which becomes less than 1 min. Both affinity supports associated with a preliminary phosphocellulose separation steps, allow the effective purification of *Bam*HI in only two steps. The enzyme obtained was active without non-specific nucleases.

Purification of Monoclonal Antibodies Using CIM-Disk-EDA-Histidine

The majority of affinity chromatography separations of antibodies from human serum or of monoclonal type has been performed using conventional ligands such as protein A and protein G. In recent years, the macroporous ultra-short methacrylate-based monoliths with corresponding immobilized ligands have been used for the separation and the purification of antibodies. Indeed, many applications using Protein A or G CIM-disks for IgG purification have been described in the literature [61, 83–89]. It is also possible to stack two monolithic disks with different surface properties into one housing. This type of chromatography was introduced by Strancar et al. [33] and Josic et al. [61] and named conjoint liquid chromatography (CLC).

Recently, a multi-fractionation approach for the fast purification of IgGs and Serum albumin (SA) from mammalian blood media was envisaged by using the combination of highly selective and specific recombinant G proteins having unique IgG-binding properties [87]. Thus, in this high-performance monolith chromatography process, one IgG-binding protein G disk and one SA binding Protein G disk were installed into the same cartridge. Unlike a pseudobiospecific ligand such as L-histidine, acid pH is needed for protein desorption and this could be lead to protein denaturation. The pseudobioaffinity ligand L-histidine can be easily used to purify polyclonal and

monoclonal immunoglobulin from human plasma and other sources [7, 17, 18, 28, 29]. The L-histidine ligand has been grafted onto different matrices (gel, membrane and silica particles) to purify IgG (Fig. 2) and presents similar binding capacities as protein A and protein G using mild elution conditions. Consequently, for purification of IgG using monolithic column, we propose an alternative to the conventional utilization of a biospecific ligand like Protein A. We have studied the purification of pure polyclonal IgG (From Sigma) using a monolithic CIM-disk. The efficiency of the monolithic system compared to other histidyl-immobilized matrixes (Table 3) allows this new chromatographic tool for specific purification of antibodies to be used for fast scale up (Fig. 6).

In this context, the possibility of separation and purification of monoclonal antibodies from mouse serum directed towards glycophorin erythrocytes in malaria was investigated using CIM-disk IHAC (immobilised histidine affinity chromatography). Malaria remains a major public health problem in many regions of the tropical world (2 billions of people, 40% of world population in 90 country, are exposed) [88]. There is an urgent need to develop vaccines that can provide protection against *Plasmodium* malaria. The rapid nature of erythrocyte invasion by merozoites (blood stage) indicates that it is a tightly controlled process, which involve specific receptor-ligand interactions between host and parasite molecules. Whereas *Plasmodium falciparum* uses sialic acid residues of glycophorin A as receptors to invade human erythrocytes [89–91], *Plasmodium vivax* and *Plasmodium knowlesi* use the Duffy blood group antigen [92–94]. Parasite ligands that bind these receptors belong to a family of erythrocyte-binding proteins (EBP). Parasite proteins bind with erythrocyte receptors to mediate invasion. They are attractive candidates for malaria vaccine development since antibodies are directed against erythrocyte ligands, which may block erythrocyte invasion, reducing parasite multiplication and providing protection against malaria. The production and purification of monoclonal antibodies (MAbs) are directed towards glycophorin of human red blood cells, which have been developed according to Koehler and Milstein [95]. They are poorly produced in cell culture supernatants and have been
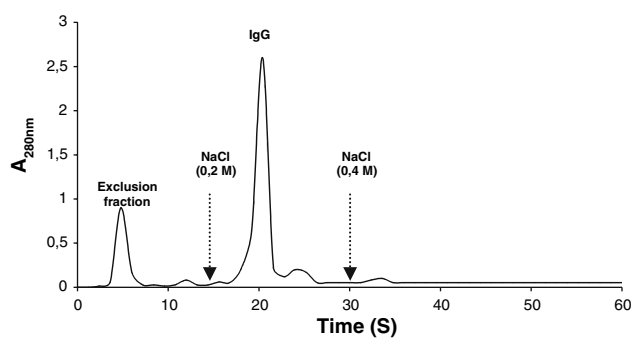

Fig. 6. Adsorption of pure IgG (from Sigma) on CIM-disk-EDA-histidine. IgG were eluted at a flow rate of 5 mL min⁻¹ with increasing the ionic strength of Tris–HCl (25 mM, pH 7,4) buffer (NaCl 0.2 and 0.4 M in starting buffer)
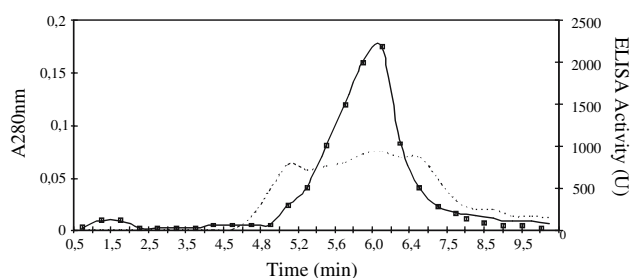

Fig. 7. Separation of monoclonal antibody (MAb) on CIM® disk EDA-histidine monolithic column. Experimental conditions: injection volume: 20 μL, sample: 5.46 mg of total protein; flow rate: 5 mL min⁻¹; wavelength: 280 nm; binding buffer: 25 mM Tris–HCl buffer pH 7.4; elution buffer: 25 mM Tris–HCl buffer pH 7.4, 1 M NaCl (*continued dots*) $A_{280\,nm}$. (*Continued lines*) ELISA activity

partially characterized in our laboratory. One of our studies was to efficiently separate Mabs, produced in cell culture supernatants, which many contain contaminating proteins.

The monolithic CIM® disk EDA-histidine was used for fast biospecific separation in order to isolate and separate glycophorin erythrocytes specific monoclonal antibodies from cell culture supernatants. Column having a volume of 0.34 mL was used and experiments were performed on a HPLC system equipped with UV detector. Before the purification on the CIM® disk monolithic column, proteins in the medium were precipitated by ammonium sulphate, desalting and concentrated by ultrafiltration on a membrane (column Centricon, Amicon® Ultra-4, 10,000 Da). From the data shown in Fig. 7, we can observe one large peak in CIM-disk EDA-histidine eluent. However, according to qualitative ELISA test, it is obvious that a family of Mabs are eluted with 0.3 M of NaCl. In this present study, ELISA test (Sandwich Type) was done with malaria antigen from glycoprotein of human eythrocytes.

Table 4 summarizes the purification characters obtained from CIM-disk EDA-histidine separation and the small quantity of Mabs that was produced and separated. Identification of the high-molecular-mass protein in the eluted peaks was done by SDS-PAGE analysis, which gave the confirmation of IgM. Then, monoclonal IgM from cell culture supernatant was purified with a purification factor of 578.30 using the CIM-disk EDA-histidine monolithic column. Nevertheless, the adsorption of monoclonal antibodies was not important on CIM® disk EDA-histidine comparatively to CIM-disk DEAE. The results obtained are similar to those reported by Buenos et al. [17, 18] where it was described that IgM had lower selective retention on IHAC than others immunoglobulins.

According to previous studies purification of immunoglobulin on the using, pseudobioaffinity chromatography histidine [7] is a new approach. Consequently, monolithic CIM-disk IHAC systems offer advantages of fast separation and purification of Mabs and to obtain this, a promising way is to develop a miniatur-

Table 4. Purification characters of monoclonal antibody (MAb) on CIM® disk EDA-histidine monolithic column

Steps	Protein quantity (mg)[a]	Activity[b]	Specific activity[c]	Protein yield[d]	Purification factor[e]
Cell culture supernatant before ammonium sulfate precipitation	1,130	1820	1.61	100	1
Cell culture supernatant after ammonium sulfate precipitation	546	1628	2.98	48.32%	1.85
Cell culture supernatant after purification on CIM® disk monolithic column	2.35	2188	931.06	0.21%	578.30

[a]Determined by Bradford assay
[b]Determined by ELISA test
[c]Activity/protein quantity
[d]Protein quantity after purification step/total protein quantity before purification step
[e]Specific activity (after purification)/specific activity (before purification)
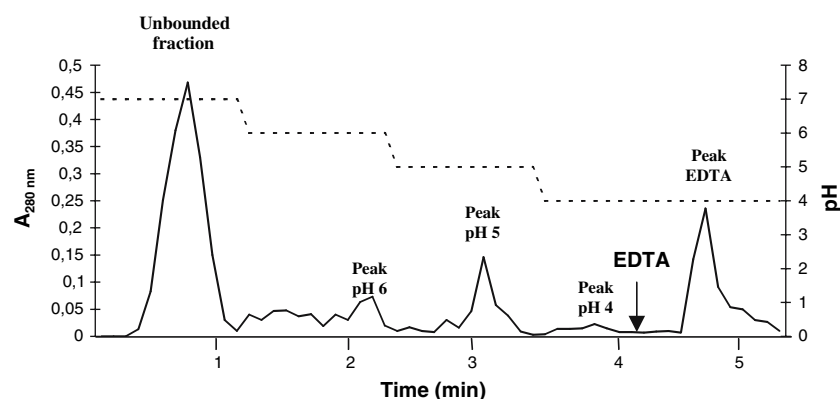
Fig. 8. Purification of soybean lectins using CIM-disk IMAC-8 monolithic support. Experimental conditions: buffer composition: 25 mM phosphate, 0.5 M NaCl for pH 7.0 and 6.0 and 100 mM acetate, 0.5 M NaCl for pH 5.0 and 4.0. Regeneration is done with 50 mM EDTA
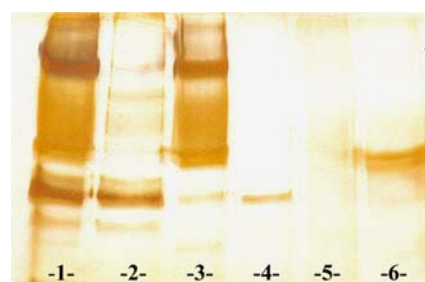
Fig. 9. SDS-PAGE under non-reducing conditions of the fractions from chromatography of soybean extract on CIM IMAC-8 tube monolithic support. Lane 1: crude extract (load), Lane 2: pH 7.0 elution peak: unbounded fraction, Lane 3: elution peak pH 6.0, Lane 4: elution peak pH 5.0, Lane 5: elution peak pH 4.0, Lane 6: peak EDTA

ised model for screening and preparative scale separation.

Purification of Soybean Lectin Using CIM-Disk-IMAC

There are only few reports about application of monoliths for IMAC. About three decades have elapsed since Porath first introduced IMAC, a technique that takes advantage of the property of a few amino acids to bind chelated transition metals, as a purification method for biological molecules such as amino acids, nucleotides or, aromatic amines and a large variety of proteins [2, 7]. In this context, the purification of lectins by IMAC was investigated using an IMAC monolith column. Lectins are a group of glycoproteins characterized by their ability to bind carbohydrates with considerable specificity. There has been increased interest in the potential health benefits of soybean lectin, which is being exploited for many applications from therapy to industry. Soybean lectins are well known to have anti-cancer activity and this compound may contribute to the role of soy in the prevention and/or treatment of cancer [96].

As compared with experiments done with soft gel, the quality of lectin from soybean crude extract did not change with the speed of the separation. The working flow rate of the monolithic column was maintained at 10 mL min^{-1} and the total time, starting from the injection of the sample to regeneration with EDTA, took only 5 min (Fig. 8). The same experiment done with the soft gel took 100 min because the system was operated at low speed having flow rate of 0.5 mL min^{-1}.

The hemagglutination activity of the soybean lectin purified by CIM-IMAC was only recovered in the fraction eluted at pH 5. The purity of this pH 5 lectin-containing fraction was confirmed by SDS-PAGE (Fig. 9). CIM-IMAC-8 tube monolithic support was used as an ideal method to purify lectins from soybean extract. It took seconds to set up or change the configuration of a column, and the column have fast equilibration and regeneration times.

Conclusion

In the last decade, much work has shown the great potential application of short monolithic columns in semi-preparative purification of therapeutic biomolecules. Because of high separation speed, CIM-disk monolithic columns are a good tools for scaling up the separation and the purification of proteins from larger extract amounts by affinity chromatography. Compared to conventional affinity chromatographic system, the monolithic disk chromatography is compatible with high flow rates without slowing down the separation and purification by protein/ligand affinity interactions. This is due to the superior fluid dynamics of the CIM-disk monoliths. Due to their high ability for very fast fractionation of biomolecules and/or nanoparticles, CIM-disk monoliths present a large potential for application in biotechnology. The development of new monolithic CIM-disk affinity support using L-histidine as pseudobiospecific ligand opens the way to new IHAC processes for the fast purification of biomolecules.

References

1. Cuatrecasas P, Wilchek M, Anfinsen (1968) CB Proc Natl Acad Sci USA 61:636
2. Porath J, Carlsson J, Olsson I, Belfrage G (1975) Nature 258:598
3. Wilchek M (1974) Methods Enzymol 34:182
4. Clonis YD (1987) Biotechnol 5:1290
5. Vijayalakshmi MA (1989) Trends in Biotechnol 3:71
6. Scopes RK (1978) Eur J Biochem 91:119
7. Vijayalakshmi MA (2002) Biochromatography: theory and practice. Taylor & Francis, London, pp 526
8. Katzir Katchalsky E (1985) In: Chaiken I et al. (eds) Affinity chromatography and biological recognition. Academic, New York, pp 7–26
9. Neuman D, Lehr CM, Lenhof HP, Kohlbacher O (2004) Adv Drug Deliver Rev 56:437
10. Tanaka N, Hashizume H, Araki M (1987) J Chromatogr 400:33
11. Gaberc-Porekar V, Menart V (2001) J Biochem Biophys Methods 49:335
12. Bacolod MD, El RZ (1990) J Chromatogr 512:237
13. Zachariou M, Hearn MT (1992) J Chromatogr 599:171
14. Millot MC, Herve F, Sebille B (1995) J Immunol Methods 181:225
15. Chaouk H, Hearn MT (1999) J Chromatogr A 852:105
16. Liesiene J, Racaityte K, Morkeviciene M, Valancius P, Bumelis V (1997) J Chromatogr A 764:27
17. Bueno SMA, Haupt K, Vijayalakshmi MA (1995) J Chromatogr B 667:57
18. Bueno SMA, Legallais C, Haupt K, Vijayalakshmi MA (1996) J Membrane Sci 117:45
19. Pirlet AS, Pitiot O, Guentas L, Heyraud A, Courtois B, Courtois J, Vijalayakshmi MA (1998) J Chromatogr A 826:157
20. Delattre C, Michaud P, Hamze K, Courtois B, Courtois J, Vijayalakshmi MA (2005) J Chromatogr A 1099:121
21. Amourache L, Vijayalakshmi MA (1984) J Chromatogr A 303:285
22. Kanoun S, Amourache L, Krishnan S, Vijayalakshmi MA (1986) J Chromatogr A 376:259
23. Mdiba A, Kanoun S, Vijayalakshmi MA (1989) In: Stolz JF, Rivat C (eds) Biotechnology of blood proteins, vol 175, p 237
24. El Kak A, Vijayalaksmi MA (1991) J Chromatogr Biomed Appl 570:29
25. Ezzedine M, Lawny F, Vijayalakshmi MA (1993) In: Stolz JF, Rivat C (eds) Biotechnology of blood proteins, vol 227, p 115
26. Acconci C, Legallais C, Vijayalakshmi MA, Bueno SMA (2000) J Membrane Sci 173:235
27. Pitiot O, Nedonchelle E, Legallais C, Vijayalakshmi MA (2001) J Chromatogr B 758:173
28. El-Kak A, Mandjiny S, Vijayalakshmi MA (1992) J Chromatogr B 604:29
29. Haupt K, Bueno SMA, Vijayalakshmi MA (1995) J Chromatogr B 667:57
30. Tennikova T, Reusch J (2005) J Chromatogr A 1065:13
31. Abou-Rebyeh H, Koerber F, Schubert-Rehberg K, Reusch J, Josic D (1991) J Chromatogr B 566:341
32. Strancar A, Koselj P, Schwinn H, Josic D (1996) Anal Chem 68:3483
33. Strancar A, Barut M, Podgornik A, Koselj P, Josic D, Buchacher A (1998) LC–GC Int 10:660
34. Tennikova TB, Belenkii BG, Svec F (1990) J Liq Chromatogr 473:63
35. Iberer G, Hahn R, Jungbauer A (1999) LC–GC 17:998
36. Mihelic I, Koloini T, Podgornik A, Strancar A (2000) J H R C 23:39
37. Vodopivec M, Podgornik A, Berovic M, Strancar A (2000) J Chromatogr Sci 38:489
38. Tennikova TB, Bleha M, Svec F, Almazova TV, Belenkii BG (1991) J Chromatogr 555:97
39. Barut M, Podgornik A, Merhar M, Strancar A (2003) In: Svec F, Tennikova TB, Deyl Z (eds) Monolithic materials: preparation, properties and applications (Chapt 3), vol 3. Elsevier, Amsterdam
40. Ohlson L (1989) Am J Physiol 256:29
41. Anspach FB, Johnston A, Wirth HJ, Unger KK, Hearn MT (1989) J Chromatogr A 476:205
42. Lee CT, Chuang FR, Hsu KT, Lam KK, Liao SC, Liu CC, Chen JB, Jang SW, Chien YS, Pan HH (1996) Changgeng Yi Xue Za Zhi 19:313
43. Svec F, Tennikova TB, Deyl Z (eds) (2003) Monolithic materials: preparation, properties and applications, J Chromatogr Libr 67
44. Mihelic I, Koloini T, Podgornik A, Barut M, Strancar A (2001) Acta Chim Slov 48:551
45. Strancar A, Podgornik A, Barut M, Necina R (2002) Adv Biochem Eng Biotechnol 76:49
46. Giovannini R, Freitag R, Tennikova T (1998) Anal Chem 70:3348
47. Branovic K, Forcic D, Ivancic J, Strancar A, Barut M, KosuticGulija T, Zgorelec R, Mazuran R (2004) J Chromatogr B 801:331
48. Bencina M, Podgornik A, Strancar A (2004) J Sep Sci 27:801
49. Podgornik A, Barut M, Jancar J, Strancar A (1999) J Chromatogr A 848:51
50. Podgornik A, Barut M, Jancar J, Strancar A, Tennikova T (1999) Anal Chem 71:2986
51. Barut M, Podgornik A, Brne P, Strancar A (2005) J Sep Sci 28:1876
52. Bilkova Z, Castagna A, Zanusso G, Farinazzo A, Monaco S, Damoc E, Przybylski M, Benes M, Lenfeld J, Viovy JL, Righetti PG (2005) Proteomics 5:639
53. Vodopivec M, Berovic M, Jancar J, Podgornik A, Strancar A (2000) Anal Chim Acta 407:105
54. Jungbauer A, Buchacher A, Josic D (2001) J Chromatogr B: Biomed Sci Appl 752:191
55. Meyer JJ, Liapis AI (1999) J Chromatogr A 852:3
56. Branovic K, Lattner G, Barut M, Strancar A, Josic D, Buchacher A (2002) J Immunol Methods 271:47
57. Tennikova TB, Freitag R (2000) J HRC 23:27
58. Tennikova T, Freitag R (1999) In: Aboul-Enein HY (ed) Analytical and preparative separation methods of macromolecules. Marcel Dekker, New York, p 255
59. Tennikova T, Strancar A (2002) Labplus Int 10
60. Platonova GA, Tennikova TB (2003) In: Svec F, Tennikova T, Deyl Z (eds) Monolithic materials: preparation, properties, and applications. Elsevier, Amsterdam, p 601
61. Josic D, Schwinn H, Strancar A, Podgornik A, Barut M, Lim YP, Vodopivec M (1998) J Chromatogr A 803:61
62. Lim YP, Josic DJ, Callanan H, Brown J, Hixson DC (2005) J Chromatogr A 1065:39
63. Demelbauer UM, Plematl A, Josic DJ, Allmaier G, Rizzi A (2005) J Chromatogr A 1080:15
64. Yang Z, Hancock WS (2005) J Chromatogr A 1070:57
65. Comunale MA, Lowman M, Long RE, Krakower J, Philip R, Seeholzer S, Evans AA, Hann HWL, Block TM, Mehta AS (2006) J Proteome Res 5:308
66. Xiong L, Regnier FE (2002) J Chromatogr B 782:405
67. Okanda FM, El Rassi Z (2006) Electrophoresis 27:1020
68. Williams RJ (2003) Mol Biotechnol 23:225
69. Smith DL, Blattner FR, Davies J (1976) Nucleic Acids Res 3:343
70. Greene PJ, Heyneker HL, Bolivar F, Rodriguez RL, Betlach MC, Covarrubias AA, Backman K, Russel DJ, Tait R, Boyer HW (1978) Nucleic Acids Res 5:2373
71. Rubin RA, Modrich P (1980) Methods Enzymol 65:96
72. Fuchs LY, Covarrubias L, Escalante L, Sanchez S, Bolivar F (1980) Gene 10:39
73. Ward JM, Wallace LJ, Cowan D, Shadbolt P, Levison PR (1991) Anal Chim Acta 249:195
74. Bickle TA, Pirrotta V, Imber R (1977) Nucleic Acids Res 4:2561
75. Pirrotta V, Bickle TA (1980) Methods Enzymol 65:89
76. Farooqui AA (1980) J Chromatogr 184:335
77. Bouriotis V, Zafeiropoulos A, Clonis YD (1987) Anal Biochem 160:127
78. Baksi K, Rushizky GW (1979) Anal Biochem 99:207
79. Dubey AK, Mukhopadhyay SN, Bisaria VS, Ghose TK (1987) Process Biochem 22:25
80. Gadgil H, Oak SA, Jarrett HW (2001) J Biochem Bioph Meth 49:607
81. Vlatakis G, Bouriotis V (1991) Anal Biochem 195:352
82. Tennikova TB, Blagodatskikh IV, Svec F, Tennikov MB (1990) J Chromatogr 509:233
83. Langlotz P, Kroner KH (1992) J Chromatogr 591:107
84. Platonova GA, Pankova GA, Ilina IY, Vlasov GP, Tennikova TB (1999) J Chromatogr A 852:129
85. Zhou D, Zou H, Ni J, Wang H, Yang L, Zhang Y (1999) Chromatographia 50:23
86. Zou H, Luo Q, Zhou D (2001) J Biochem Biophys Methods 49:199
87. Gupalova TV, Lojkina OV, Palagnuk VG, Totolian AA, Tennikova TB (2002) J Chromatogr A 949:185

88. Boutin JP, Pradines B, Pages F, Legros F, Rogier C, Migliani R (2005) Rev Prat 55(8):833
89. Miller LH, Haynes JO, McAuliffe FM, Shiroishi T, Durocher J, McGinnis MH (1977) J Exp Med 146:277
90. Pasvol G, Wainscoat JS, Weatherall DJ (1982) Nature 297:64
91. Friedman MJ, Blankenburt T, Sensabaugh G, Tenforde TS (1984) J Cell Biol 98:1682
92. Sim BKL, Orlandi PA, Haynes JD, Klotz FW, Carter JM, Camus D (1990) J Cell Biol 111:1877
93. Adams JH, Sim BKL, Dolan SA, Fang X, Kaslow DC, Miller LH (1992) Proc Natl Acad Sci (USA) 89:7085
94. Chitnis CE, Miller LH (1994) J Exp Med 180:497
95. Koehler G, Milstein C (1975) Nature 256:495
96. Mejia D, Gonzalez E, Traliece B (2003) Nutr Rev 61:239
97. Josic DJ, Lim YP, Strancar A, Reutter W (1994) J Chromatogr B 662:217
98. Vlakh EG, Platonova GA, Vlasov GP, Kasper C, Tappe A, Kretzmer G, Tennikova T (2003) J Chromatogr A 992:109
99. Hagedorn J, Kasper C, Freitag R, Tennikova T (1999) J Biotechnol 69:1
100. Kasper C, Meringova L, Freitag R, Tennikova T (1998) J Chromatogr A 798:65