FUNDAMENTAL AND PRACTICAL STUDIES ON HIGH PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY OF BIOPOLYMERS WITH NOVEL STATIONARY PHASES

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α	selectivity factor; slope of κ–κ plot
B°	permeability coefficient
d_p	particle diameter
ε_e	interstitial column porosity
ΔG°	Gibbs free energy
k'	retention factor
κ	logarithmic retention factor
L	length of the column
η	viscosity
ϕ	logarithmic of the phase ratio
ΔP	pressure drop
R	gas constant
S_{BET}	specific surface area
t_R	retention time
T	absolute temperature
υ	superficial flow velocity
V_e	frontal retention volume
V_o	void volume of the column
V'_R	adjusted retention volume
EDDA	ethylenediamine-*N*, *N'*-diacetic acid
EDTA	ethylenediaminetetraacetic acid
HPLC	high performance liquid chromatography

HPLAC	high performance liquid affinity chromatography
I.D.	internal diameter
IDA	iminodiacetic acid
IEC	ion-excange chromatography
MIC	metal interaction chromatography
PLRP	commercial poly(styrene-divinylbenzene)
PSDVB	poly(styrene-divinylbenzene)
RPC	reversed-phase chromatography
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
TED	tris(carboxymethyl) ethylenediamine

CHAPTER I

BACKGROUND AND RATIONALE

Introduction

High performance liquid affinity chromatography (HPLAC), which was first introduced by Ohlson and collaborators[1] in 1978, is now emerging as a powerful preparative as well as analytical separation method for a wide variety of biological substances.[2-5]

High performance metal interaction chromatography (MIC), a relatively new branch of HPLAC, is increasingly used in the separation and purification of proteins and peptides. It employs rigid microparticulate stationary phases in which a metal ion is immobilized to the stationary phase *via* chelating functions bound to the surface (e.g., iminodiacetic acid (IDA)). This technique which was initially introduced by Porath and collaborators in 1975[6], using low pressure chromatography on agarose-based sorbents, offers a selectivity different from that of other biopolymer HPLC techniques such as ion-exchange and hydrophobic interaction chromatography. The separation of solutes is based on their different affinities to the immobilized metal ions on the surface of the stationary phase.[7]

Metal interaction chromatography with soft metal chelate-gels such as iminodiacetic acid-agarose (i.e., low pressure chromatography) has found many applications. Typical examples include the purification and fractionation of various serum proteins,[8, 9] interferons,[10, 11] phosphoproteins,[12] and basic nuclear proteins of human sperm[13] as well as the determination of intact lactoferrin found in the urine of human milk-fed preterm

infants.[14] Other applications using agarose-based metal chelate sorbents have been well documented in recent reviews.[7, 15-19]

Recently, it has been shown that MIC is a suitable HPLC technique for the separation and purification of human erythrocyte glycophorins[20] where traditional approaches by size exclusion[21] and ion-exchange chromatography[22] were ineffective in purifying the different forms of these membrane proteins. In addition, MIC has been found to be more effective than hydroxyapatite chromatography for the separation of the site specific variants of subtilisin.[23] Another important application of MIC by HPLC has been the separation of synthetic peptide hormones.[24, 25]

Furthermore, the technique has proved useful in topological studies aimed at determining the number and location of histidine residues exposed on the surface of protein molecules[26, 27] as well as in characterizing and locating the metal ion-specific binding domains on the surface of the DNA-binding forms of the estrogen receptor protein.[28] Due to the avid affinity of some proteins to the immobilized metal, iminodiacetic acid-metal chelate sorbents have found applications in immobilization of enzymes (e.g. alkaline phosphatase, malate dehydrogenase and lactate dehydrogenase)[29] or lectin affinity ligands such as concanavalin A.[30] Also, Smith and collaborators[31] applied the principle of MIC to the purification of recombinant proteins from host organisms by cloning a specific chelating peptide onto the N-terminus of a recombinant protein.

The main objective of the present study was to further developing MIC. Systematic studies directed toward (i) the development and characterization of rigid porous and non-porous stationary phases having novel metal chelating ligands, (ii) the examination of the retention behavior of well characterized proteins over a wide range of elution conditions and (iii) the introduction of rapid HPLC separation schemes for proteins by MIC were plausibly the route to this objective.

In this chapter, a general background of metal interaction chromatography is

provided first and then the basic principles of rapid HPLC are discussed. Also, a detailed description of the rationale, significance and scope of the study is provided at the end of this chapter. For clarity, a more specific background on various aspects of packing materials will be given in some of the introductory parts of the remaining chapters.

Metal Interaction Chromatography

Protein-Metal Chelate Interactions

In MIC, the underlying principle of the binding of proteins to the metal chelate stationary phase is believed to be the result of the ability of electron-rich ligands on the surface of proteins to substitute weakly bonded ligands such as water or mobile phase ions in the metal complexes on the surface of the stationary phase.[6, 15, 19, 29, 32, 33] In this process, the chelated metal plays the role of accepting unshared pair of electrons from the side chains of the amino acids at the surface of the protein molecule, see Fig. 1.

According to Porath,[6, 33] the strength of interactions of proteins with the immobilized metals is largely influenced by the number of exposed histidine residues on the surface of the protein molecule and, to a lesser extent, by the number of cysteine or tryptophan residues. In fact, the results of several studies conducted by Porath's group using various model proteins have indicated that the affinity of the analytes increases with increasing histidine content.[27, 28, 34] This histidine-retention dependency has been further substantiated by Sulkowski's[35] findings that the adsorption of bovine serum albumin on Ni(II)-IDA column greatly decreased at pH < 6.5, indicating the involvement of imidazole group of histidine residues having pK_a of 6.5. Also, the sensitivity of protein retention to amine ions suggest that the primary binding site for the protein is a nitrogen containing group.[36] As a typical example, Figueroa and co-workers[37] have recently illustrated the histidine implication in MIC retention by studying the retention behavior of three types of

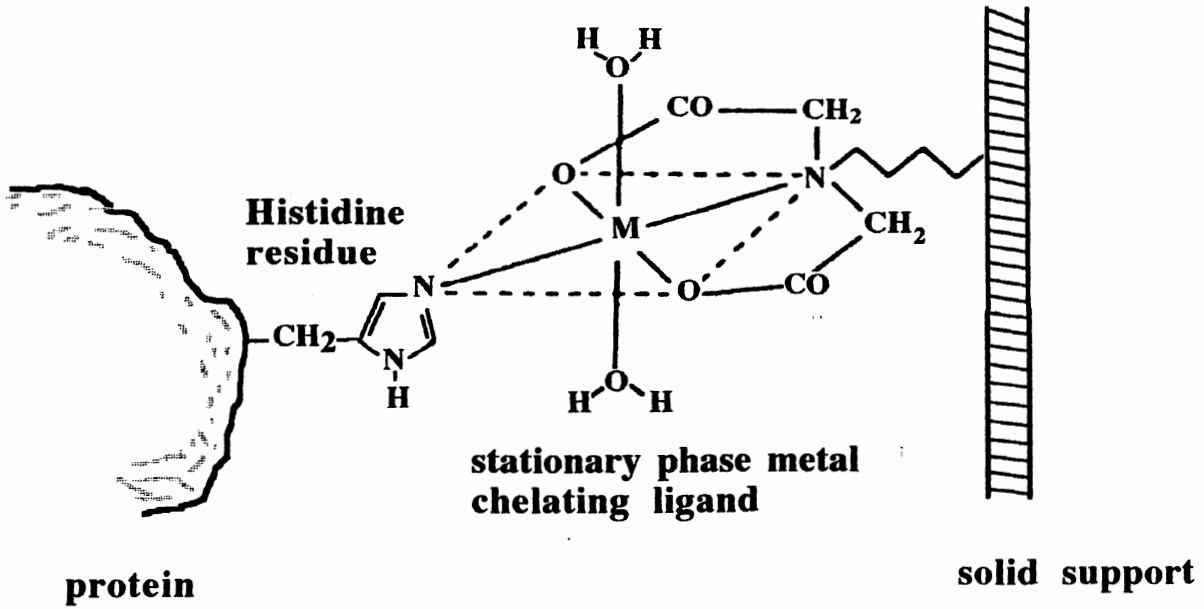


Figure 1. Protein Interaction with the Chelated Metal on the Stationary Phase.
M, immobilized metal.

myoglobin on a silica-based Cu(II)-IDA column. In this study, dog myoglobin (having one exposed histidine residue) eluted first followed by horse myoglobin (two exposed histidine residues), and sperm whale myoglobin (three exposed histidine residues) eluted last.

Although histidine implication in metal interaction chromatographic event has been well illustrated,[27, 28, 34-37] several other factors have been shown to influence the relative contributions of potential histidine residues on the surface of a protein toward the affinity of that protein for immobilized metal ions on the surface of the stationary phase.[38] In the absence of protein denaturation, geometrical constraints appear to demand that not all surface-exposed histidines may participate in protein-metal chelate associations.[38] In addition, because of variations in the local microenvironment of surface interaction sites (e.g. pK_a of histidine groups) caused by neighboring groups, not all 1-histidine or 2-histidines type of interaction may be of equal or even similar affinity.

Although it was reported that there was no evidence for any significant contribution of tryptophan or cysteine residues to the adsorption of several serum albumin on various IDA stationary phases with chelated Co(II), Ni(II), Cu(II) or Zn(II),[39] the binding of human β-interferon to the same metal chelate stationary phases was attributed to the presence of cysteine-17,[40] and the slight retention exhibited by a Peking duck lysozyme (no histidine residues) on Cu(II)-IDA at neutral pH was attributed to the presence of tryptophan residues.[15] Besides the fact that tryptophan is a weak ligand when present on the surface of a protein,[28] this hydrophobic residue is not an abundant amino acid constituent of proteins and is usually sequestered in the interior of a protein molecule.[41] On the other hand, the ruling out of the cysteine contribution to retention was explained by the possibility that the free thiol groups in the extracellular proteins may not be accessible to the metal-IDA function. This is not the case for intracellular proteins, the surface of which may frequently contain free thiol groups (i.e., cysteine).[28] Under these

circumstances, the histidine-retention dependency would fail since it has been postulated that a protein containing free thiol groups may scavenge the immobilized metal during chromatography.[28] The scavenged metal ions may occupy the protein's surface accessible metal binding sites thus rendering it unretained by the column.

Other groups on the surface of proteins have been shown to enhance or reduce protein-metal interactions. Phosphate groups have been shown to strengthen protein interactions with the immobilized metal. For instance phosvitin, a phosphorylated protein, was found to be retained avidly on Fe(III)-IDA, a borderline "hard" metal chelate stationary phase.[42] This was apparently caused by the high affinity of phosphate for ferric ions. This is in agreement with another report regarding the contribution of phosphates to an overall binding strength of differently phosphorylated ovalbumins on Fe(III)-IDA sorbents using an increasing pH gradient.[43] These ovalbumins were eluted and separated according to the number of phosphate residues, i.e., the ovalbumin with the greatest number of phosphate groups was retained the most.

On the other hand, glycosylated proteins have been shown to exhibit less affinity toward the chelated metal on the surface of the chelating stationary phase than their non-glyscosylated counterparts.[36] This was illustrated by the higher retention exhibited by ribonuclease A on Cu(II)-IDA column when compared with ribonuclease B. The glycan moiety or the high mannose oligosaccharide in the latter protein was suggested to cause the shielding of its two histidine residues from interacting with the metal chelate on the surface of the stationary phase. Also the finding that α_1-acid glycoprotein did not show a significant retention on Fe(III)-IDA was explained by the electrostatic repulsion caused by the presence of multiple sialic acid residues and the steric hindrances caused by the oligosaccharide envelope on the interacting sites of the glycoproteins.[42]

The above considerations regarding MIC retention are in some cases oversimplified. They do not take into account net charge and hydrophobic character of the

separated proteins.

Stationary Phases in MIC

Solid Support. The solid support for the preparation of metal chelate stationary phases for MIC of proteins must satisfy certain basic requirements such as hydrophilicity, rigidity, insolubility, inertness to the solutes and ease of functionalization.[44] Agarose, which fulfill most of these requirements has been the support of choice for low pressure MIC.[32] Due to the low mechanical strength of agarose, it can not withstand the high pressure used in HPLC.[32] Therefore, rigid microparticulate stationary phase supports were introduced for use in HPLC of proteins by metal interaction chromatography. They are based on silica gels[35, 45] or hydrophilic macroreticullar resin (TSK chelate).[46] Very recently, rigid polystyrene of relatively large particle size having a hydrophilic coating to which IDA ligands were attached has become commercially available[47] for rapid preparative MIC of proteins by HPLC; see below for more details.

Nature of the Chelating Ligands. The selection of the metal chelating functions for bonding to the solid support is another significant factor in the design of chemically bonded phases for protein separations by MIC. Generally, the metal chelating ligands fixed at the solid surface should hold the metal of interest sufficiently strongly so that it does not leach out under the operating conditions used in MIC, but not so strongly so as to shield the metal completely from interacting with the solutes.[19]

In both low and high pressure liquid chromatography, the most commonly used chelating ligand has been iminodiacetic acid (IDA).[18] It has always been the chelating ligand of choice because, when complexed with transition metals, the stability constants of the metal-IDA chelates are relatively high. The strong binding of metals to the IDA chelating groups aids in minimizing bleeding of the metal from the column. Despite the availability of other chelating ligands which can give higher stability constants when

chelated with metal, these ligands were only briefly explored on agarose supports. For example, a pentadentate tris(carboxymethyl) ethylenediamine (TED) has been successfully attached to an epoxy-activated agarose in order to immobilize various metal ions.[48, 49] Although they were prepared in the same manner, the properties of TED-gels were found to differ from those of IDA-agarose sorbents. In contrast with IDA-gel, TED-agarose exhibited stronger affinity to metal ions than to proteins. For this reason, columns packed with metal-free TED-gel have been used to trap metals during affinity elution from columns packed with immobilized metal-IDA-gel. Furthermore, owing to its strong affinity for metal ions, TED was able to remove zinc from the active site of carboxypeptidase, resulting in a loss of peptidase activity.[49] However, the pentadentate TED has lesser available sites for proteins compared to the tridentate IDA, hence carboxypeptidase A was found to be retained on Cu(II)-IDA agarose but not on Cu(II)-TED agarose.

Also, agarose-based stationary phases containing fixed mono-, di-, tri-, and hexahydroxamate metal chelating functions were explored using Fe(III) as the immobilized metal.[50-52] A novel metal chelate sorbent having surface bound nitriloacetic acid (NTA) was also introduced.[53] It is a quadridentate chelating ligand and especially suitable for metal ions with coordination number of six, since two coordination sites remain for the reversible binding of biopolymers. An attempt was also made to prepare a novel chelating ligand having a hybrid carboxyl/sulphonic acid function.[54] However, this chelating ligand met with only qualified success in view of the considerably weaker chelation with the metal ion Cu(II).

It is also believed that aside from the nature of the chelating ligands, the surface concentration seems to influence the adsorption of metal ions on the surface of the support.[16] The adsorption capacity is enhanced at higher concentration in most cases, but there is also a possibility of reducing the retention of analytes because a significant portion of the metal ions may be adsorbed *via* multipoint attachment.

Nature of the Chelated Metal. The nature of the immobilized metal is also an important parameter to be considered in MIC. The selection of metal ions to be used in MIC is dictated by the ability of the metal to form stable complexes with the chelating ligand and also to retain some adsorptivity toward the solutes.

Metal ions such as Zn(II), Cu(II), Ni(II) and Co(II) have been the most commonly used in MIC.[34, 55] In addition to the these metals, Andersson and Porath[12] also tested the following metals: Ce(III), Cr(III), La(III), Lu(III), Sc(III), Yb(III), Th(IV) and UO_2(II). With the exception of Cr(III) and UO_2(II), stable metal chelates were formed on the gel.

A study made with various metal chelates revealed that the adsorption capacity for serum albumin decreased in the following order: Cu(II) > Zn(II) > Ni(II) > Mn(II), with Cu(II) being very effective and Mn(II)-gel having almost negligible adsorption capacity.[42] The strong affinity of Cu(II) with serum albumin was also found to hold true for transferrin, carbonic anhydrase and ribonuclease A. However, Ni(II) was less interactive when compared with Zn(II), contrary to the results obtained for the serum albumin. In general, the extent of protein retention varies from one metal(II)-IDA column to another.[27]

Another metal which was investigated by many researchers is Fe(III). The unusual retention of acidic, neutral and basic proteins on Fe(III)-IDA, puts this sorbent in its own category.[42] It was suggested that both ionic interaction and coordinate covalent bonding are present in protein-Fe(III) interaction, but because of the concept of hard-soft ions which will be discussed later, the former is more important than the later.[50, 56, 57]

To facilitate the selection of the metal ion, as far as the protein-metal association is concerned, the concept of hard-soft ions of Pearson[58] based on the polarizability of interacting metal ions was employed by Porath and co-workers.[6,7] Accordingly, "hard" metal ions such as Mg(II), Ca(II), Fe(II), Al(III), Ga(III), and In(III) tend to coordinate with hard ligands, i.e., oxygen-containing ligands, whereas "soft" metals, such as Cd(II), Hg(II) and Tl(II), prefer to associate with sulfur-containing ligands.[59] Metals of the

intermediate type like Cu(II), Zn(II), Ni(II) and Co(II) preferentially coordinate with nitrogen but also interact with oxygen and sulfur. In fact, chelates of borderline "hard" metal ions, e.g., Fe(III)-IDA have been found to bind very strongly with phosphoproteins such as ovalbumin,[12] phosvitin and phosphorylated histones.[50] On the other hand, chelates of the intermediate type, such as Cu(II) or Zn(II)-IDA, bind protein rich in histidine such as sperm whale myoglobin[15] and lymphotoxin.[36] Thiol-containing human β-interferon was reported to bind specifically to intermediate or "soft" metal chelates.[40]

Elution Schemes in MIC.

The choice of appropriate elution conditions in MIC is rather tedious and more complicated than in any other type of interactive chromatography. For instance, in ion-exchange chromatography, a gradient of increasing salt concentration is the most efficient elution scheme[60] whereas in hydrophobic interaction chromatography a gradient of decreasing salt concentration is widely used.[61] On the other hand, in reversed-phase chromatography a gradient of increasing organic solvent concentration is usually employed to bring about elution and separation.[62] However, since in MIC metal interaction and to a lesser extent electrostatic and hydrophobic interactions are operating on the solute retention, the superimposition of several interactive forces may complicate the elution and separation of multicomponent mixtures. Therefore, the elution of proteins from a metal interaction column may necessitate a series of mobile phases varying in amount and nature of salts as well as in pH and competing agent content.

Only scarce data are available on the effects of the nature and concentration of neutral salts on MIC retention. For example, a study on the effect of sodium chloride concentration on protein retention performed on a Cu(II)-IDA column showed that high salt concentration enhanced the retention of calmodulin, an acidic protein, but decreased the retention of cytochrome c, a basic protein.[40] These observations could be explained by the

repulsion between the negatively charged protein (i.e., calmodulin) and the stationary phase surface at low salt concentration. However, at high salt concentration, the coulombic repulsion is screened, thereby resulting in an increased protein retention. Another study was conducted to investigate the effect of the salt concentration on the retention behavior of cytochrome c, lysozyme and β-lactoglobulin on a silica-based Fe(III)-IDA column.[45] The retention of these proteins first decreased and then increased with increasing ammonium sulfate concentration. These findings are indicative of the presence of different adsorptive forces, i.e., metal and/or electrostatic interactions at low salt concentrations and hydrophobic interactions at high salt concentrations. Due to the limited availability of retention data, no generalization regarding the effect of salt concentration on the retention behavior can be made.

Another parameter that can alter the retention of proteins in MIC is the eluent pH. The pH affects the ionization of side chain groups of proteins and consequently their associations with the metal on the surface of stationary phases. Since histidine groups are the most reactive metal binding sites, the use of low pH will cause the protonation of the histidine group thereby lessening its interaction with the metal.[18] Indeed, gradients of decreasing pH have been frequently employed in MIC.[14, 20, 63-65] However, with "hard" metals, such as Fe(III), Ce(III) or Al(III), proteins were found to exhibit higher interaction at slightly acidic medium, i.e., pH around 5.0.

Another way of achieving the selective elution of proteins would be the addition of ligands to the mobile phase which will effectively compete with the proteins for adsorption on the immobilized metal ions. The incorporation into the eluent of small amounts of competing agents, such as histidine,[66] histamine,[67] imidazole[68] or phosphoserine,[69] has been shown effective in affecting protein elution from MIC columns.

The use of strong displacers, however, could lead to metal stripping. When a small amount of glycine (a bidentate ligand) was added to the eluent a large change in the

UV absorbance during gradient elution was observed and it is believed to be due to the bleeding of metal ion from the Cu(II)-IDA column.[18] Likewise, the presence of tris(hydroxymethyl) aminomethane , a primary amine, was also found to cause metal leakage.[56] The loss of significant amounts of metal during chromatography requires that the column be reequilibrated with high concentrations of metal after each run. The reloading of the column with the metal can be troublesome, since it is not always possible to load the column with high concentrations of metal at the pH of the starting chromatographic conditions due to the precipitation of the metal hydroxide.[20] When reloading the metal at low pH to avoid its precipitation, many column volumes of mobile phase will be required to return to the starting conditions, thus increasing the time between runs.

Various effects could also be obtained by inclusion of denaturants in the eluent. Little or no adsorption was observed in 7 to 8 M urea.[31] Sodium dodecyl sulfate at concentrations above 0.1% abolished the adsorption of all serum proteins on a Ni(II)-IDA column. On the other hand, low concentrations of non-ionic detergent did not influence the adsorption of proteins.

Rapid High Performance Liquid Chromatography

Since its introduction, the aim of high performance liquid chromatography (HPLC) has been to achieve rapid separations on the time scale of minutes and seconds. This need has prompted researchers to introduce short columns packed with non-porous stationary phases in the particle size range of 1-2 μm. Indeed, non-porous silica-,[70-78] resin-[79-87] and agarose-based[88, 89] stationary phases were introduced for the rapid high performance ion-exchange,[79, 81, 85, 87] reversed-phase,[70, 73] hydrophobic interaction[74, 84] and affinity chromatography[75, 77, 78] of peptides, proteins, oligonucleotides and large DNA fragments.

These newly developed rapid analytical separation schemes have been the results of recent advances in particle technology. They have been engendered by the growing demand for rapid analytical techniques in monitoring clinical analyses[90] as well as industrial processes.[91]

Obviously, to achieve rapid separation by HPLC, high mobile phase flow velocities must be utilized. Under these conditions, with packed columns and porous sorbents, the limiting factor will be the increased mass transfer resistances in the stagnant mobile phase in the retentive material (i.e., intraparticle diffusional resistances), and concomitant decrease in column efficiency. To diminish diffusional resistance to mass transfer, the path length in the solid sorbent (i.e., in the porous structure) through which the solute undergo diffusion, was reduced by introducing small particle diameter supports (3-5 μm). This strategy worked well for the rapid HPLC of small molecules[92] due to their relatively high diffusivities. Conversely, due to the low diffusivities of large molecular weight substances such as proteins and large DNA fragments, reducing the particle diameter is essential but not sufficient. The slow diffusion of these macromolecules in the stagnant mobile phase is not favorable for rapid separation at high flow-rates. Although increasing the pore diameter may alleviate the restricted mass transfer in the porous interior of the column packings, small particle with macroporous structure (> 1500 Å) do not possess the required mechanical strength to be used at high flow velocities customarily used in rapid HPLC. Thus, in order to eliminate band broadening arising from intraparticle mass transfer resistances, the concept of pellicular sorbents,[93, 94] was revived by Unger and collaborators by introducing and evaluating non-porous monodispersed octadecyl-silica microparticles (1.5 μm) for rapid reversed-phase chromatography of proteins.[70, 71] With non-porous packing materials, the totally exposed retentive layer on the outer surface of the particle is readily accessible to the biomacromolecules, hence separations can be carried out at high flow-rates without sacrificing column efficiencies.[94]

In addition the absence of pores obviates the entrapment of large solutes, thus leading to high recoveries.[79, 96]

When utilizing non-porous sorbents, the specific surface area of the rigid support is relatively low (< 5 m^2/g) thereby resulting in a decrease in the linear sample capacity as well as the phase ratio, which is defined as the ratio of the volume of the stationary phase (i.e., the retentive layer) to that of the mobile phase. The reason is that less stationary phase is available per unit volume of column, and this available stationary phase volume is more quickly overloaded with the injected samples.

While the decrease in sample capacity would limit the system to microscale and analytical purposes, the decrease in phase ratio can be in many instances regarded as a gain in terms of bringing retention to practical range such as in affinity chromatography or in reversed phase chromatography, whereby, proteins can undergo multipoint attachment to the surface which would require harsh elution conditions or high organic solvent to bring about their elution, respectively. This can bring denaturation and loss of biological activity as well as low mass recovery.

The low sample capacity of non-porous microparticles can be alleviated by reducing the particle size to 1-2 μm. In fact, Kato and co-workers[80] have shown that the maximum loading capacity at the highest resolution for proteins with non-porous hydrophilic resin-based cation exchanger (2.5 μm particle size) was about 5 μg of pure sample. Also, the same authors have shown that with crude sample of proteins up to 100-200 μg could be applied to a short column of 75 x 7.5 mm I.D. with little decrease in resolution.[80]

Although decreasing the particle diameter of the non-porous stationary phases had alleviated the problem of sample capacity and had led to more efficient columns, problems associated with the use of particles smaller than 1.5-2.0 μm in diameter seem to arise. One very evident problem is the lowering of the column permeability since B°, which is the

specific permeability coefficient, is proportional to the particle diameter, d_p , and the interstitial column porosity, ε_e, by the Kozeny-Carman equation:[97]

$$B^\circ = \frac{d_p^2 \varepsilon_e^3}{180(1-\varepsilon_e)^2} \quad (1)$$

Lowering column permeability as a consequence of reducing the particle diameter leads to a high pressure drop, ΔP, across the column, as predicted by Darcy's Law:[98]

$$\upsilon = \frac{B^\circ}{\eta} \frac{\Delta P}{L} \quad (2)$$

where υ is the superficial flow velocity, η is the viscosity, L is the length of the column. In this regard, the length of the column must be decreased and/or less viscous mobile phases must be utilized. With aqueous buffers or hydro-organic eluents, 4.0 to 6.0 mL/min can be achieved with columns of 30 x 4.0 mm I.D. packed with 2.0 to 3.0 μm particles without exceeding the pressure limit of most modern pumping systems, which is 5000-6000 psi pressure drop across the column. Therefore, rapid separations with particles of less than 1 μm are awaiting further progress in HPLC hardware, as far as pumping technology, sampling valves and fittings are concerned.

Short columns packed with 2 to 3 μm non-porous supports have the merit of allowing rapid solute equilibration with the stationary phase and rapid column regeneration in gradient elution. These features would permit high speed analysis of biomolecules without significant loss in separation efficiency.

The above concept of non-porous stationary phase supports is purely for analytical and microscale rapid HPLC. To produce rapid preparative separations of large molecular weight substances at moderate sample loading capacity, reduced stagnant mobile phase

mass transfer and low pressure operation, flow-through particles having relatively large diameter (> 20 μm) have been introduced, and the technique has been termed perfusion chromartography.[47, 99, 100] This has been accomplished with 6000-8000 Å pores which transect the particle (these pores are called throughpores). The surface area of this very large pore diameter-material has been enhanced by the presence of 500-1500 Å pores or interconnecting pores at the wall or between the throughpores, respectively. This concept which combine the favorable mass transfer of non-porous supports to the high loading capacity of porous packing materials allowed the separation of an amount of protein equivalent to that of conventional HPLC columns as well as low performance agarose-based media at 10-100 times higher mobile phase velocity with no loss in resolution.[100]

Rationale, Significance and Scope of the Study

High perfomance metal interaction chromatography (MIC) is a relatively new branch of HPLC. Despite its increasing use in protein and peptide separations, many aspects of MIC still require further development. Prior to 1986 (the date at which the principle of metal interaction was adapted in HPLC by introducing rigid microparticulate metal chelate stationary phases), most of the fundamental studies were performed in low pressure chromatography with agarose-based sorbents. This lengthy process yielded only qualitative information regarding MIC selectivity and retention and the amount of systematic studies regarding the effects of various operating parameters is rather limited. With the advent of HPLC (i.e., precision instrumentation and advanced column technology), more quantitative retention data can be generated and thus, a better understanding of the physico-chemical phenomena underlying the MIC retention may be possible.

Thus far, most of the studies in both low and high pressure MIC have dealt virtually with a single chelating ligand, i.e., iminodiacetic acid (IDA), and other useful

chelating ligands were given little or no attention. Although IDA chelating sorbents have proved useful in many separations, the scope of applications of MIC can be certainly enlarged by evaluating and introducing sorbents bearing chelating ligands that would afford various degrees of analyte-metal chelate affinities. The conventional approach has favored affinity ligands with high avidity for the analytes. This has necessitated the use of strong eluents that are often denaturing to the analyte or harmful to the column, thus reducing its life span. In addition, harsh elution conditions lump closely related but different analytes into a single broad peak. Metal chelating ligands of various level of affinities are needed to suit the separation of a wider range of species. Affinity ligands with low association constant would allow high speed and higher separation efficiencies in a manner similar to other modalities of HPLC such as reversed-phase and ion-exchange chromatography.

The need for rigid microparticulate stationary phases of wider pH range stability than silica-based sorbents is widely recognized. Highly crosslinked poly(styrene-divinylbenzene) microparticles (PSDVB) with rigid structures are the most suitable supports in that regard. This organic support has found only few applications in non-denaturing protein HPLC, and its potential in MIC of proteins is yet to be explored. The major difficulty in adapting highly crosslinked PSDVB-based stationary phases to protein HPLC has been the hydrophobic nature of the support which may cause surface-induced denaturation of the analyte. Only scarce attempts have been made to overcome this limitation by introducing rather physically attached hydrophilic coatings to the surface of the polyaromatic support.[47, 99] These physically coated hydrophilic layers have the drawback of possible leakage from the surface of the column when harsh elution conditions are used.

Presently, there is a growing interest in rapid analytical high performance liquid affinity chromatography for studying the relationship between structure and function at the

molecular level and for understanding important interactions at the surface of biomacromolecules (i.e., molecular chromatography). Rapid HPLC with non-porous supports is well suited for such studies whereby pore mass transport is absent. Rapid high performance MIC of proteins has not yet been fully developed.

Although some of the underlying principles of MIC retention have been ilustrated, systematic studies concerning the effects of important variables such as the nature and concentration of salts in the eluent and the type of the chelating ligands on the surface of the stationary phases are lacking.

To further develop MIC , we have made the following contributions:

(i) Introduced and evaluated a novel tetradentate metal chelating ligand, the ethylenediamine-*N, N'*-diacetic acid (EDDA), over a wide range of conditions including eluent pH, nature and concentration of salts in the eluent, competing agents, type of chelated metal, and nature of the stationary phase matrices.

(ii) Developed and characterized non-porous highly crosslinked poly(styrene-divinylbenzene) microparticles in terms of their mechanical strength over a wide range of mobile phase flow-rates and composition customarily used in rapid HPLC.

(iii) Introduced simple and reproducible hydrophilic coatings on the surface of wide-pore and non-porous poly(styrene-divinylbenzene) by covalently attaching long polyether chains to the surface of the copolymeric organic packing materials. These hydrophilic surfaces could be readily activated with bifunctional groups for the subsequent attachment of metal chelating ligands such as IDA and EDDA as well as other affinity ligands or functional groups for biospecific interaction or general interactive HPLC, respectively.

(iv) Established chromatographic procedures for the characterization of the surface coverage of hydrophilic PSDVB with surface-bound IDA or EDDA metal chelating ligands and evaluated their retention properties *via* hydrophobic interaction toward small non-polar

probes, and *via* metal interaction with proteins.

(v) Introduced and tested empirical ways for ranking the eluting strength of various salts in MIC with different metals and metal chelating ligands.

(vi) Studied the retention behavior of well characterized and considerably differing proteins over a wide range of elution conditions.

(vii) Established novel elution schemes for rapid MIC of proteins on both non-porous silica- and PSDVB-based metal chelate stationary phases, on the time scale of seconds and minutes.

Some of above contributions may also find use in other modalities of chromatography and consequently improve biopolymer HPLC at large.

References

1. Ohlson, S.; Hansson, L.; Larsson, P. -O.; Mosbach, K. *FEBS Lett.* **1978**, *93*, 5.
2. Josic, D.; Hofmann, W.; Habermann, R.; Becker, A.; Reutter, W. *J. Chromatogr.* **1987**, *397*, 39.
3. El Rassi, Z.; Truei, Y.; Horvath, C. *Makromol. Chem., Macromol. Symp.* **1988**, *17*, 305.
4. Phillips, D.; Bell-Alden, B.; Cava, M.; Grover, E.; Mandeville, H.; Mastico, R.; Sawlivich, W.; Vella, G.; Weston, A. *J. Chromatogr.* **1991**, *536*, 95.
5. Wongyai, S.; Varga, J.; Bonn, G. *J. Chromatogr.* **1991**, *536*, 15.
6. Porath, J.; Carlsson, J.; Olsson, I.; Belfrange, G. *Nature (London)* **1975**, *258*, 598.
7. Lonnerdal, B.; Keen, C. *J. Appl. Biochem.* **1982**, *4*, 203.
8. Porath, J.; Olin, B.; Graustrand, B. *Arch. Biochem. Biophys.* **1983**, *225*, 543.
9. Andersson, L.; Sulkowski, E.; Porath, J. *J. Chromatogr.* **1987**, *421*, 141.
10. Bollin, E.; Sulkowski, E. *Arch. Virol.* **1978**, *58*, 149.
11. Edy, V. G.; Billiau, A.; DeSomer, P. *J. Biol. Chem.* **1977**, *252*, 5934.
12. Andersson, L.; Porath, J. *Anal. Biochem.* **1986**, *154*, 250.
13. Bianchi, F.; Rousseaux-Provost, R.; Sautiere, P.; Rousseaux, J. *J. Chromatogr.* **1990**, *578*, 123.
14. Hutchens, T. W.; Henry, J. F.; Yip, T. -T. *Clin. Chem.* **1989**, *35*, 1928.
15. Sulkowski, E. *Trends Biotechnol.* **1985**, *3*, 1.
16. Porath, J. in *Affinity Chromatography and Biological Recognition*, Chaiken, I.; Wilchek, M.; Parikh, I., Eds., Academic Press, Inc.: London, **1983**, 173.
17. Porath, J. *Biotechnol. Progr.* **1987**, *3*, 14.
18. Fatiadi, A.J.*Critical Reviews in Analytical Chemistry* ; C.R.C. Press: Boca Raton, FL, **1987**, *18*, 1.
19. El Rassi, Z.; Horvath, C. in *HPLC of Biological Macromolecules*, Gooding, K.; Regnier, F., Eds., Marcel Dekker, Inc.: New York, **1990**, Chapter 7.
20. Corradini, D.; El Rassi, Z.; Horvath, C.; Guerra, G.; Horne, W. *J. Chromatogr.* **1988**, *458*, 1.

21. Furthmayr, H.; Tomita, M.; Marchesi, V. T. *Biochem. Biophys. Res. Commun.* **1975**, *65*, 113.

22. Blanchard, D.; Dahr, W.; Hummel, M.; Latron, F.; Beyreuther, K.; Carton, J. -P. *J. Biol. Chem.* **1987**, *262*, 5808.

23. Chicz, R. M.; Regnier, F. E. *Anal. Chem.* **1989**, *61*, 1742.

24. Belew, M.; Porath, J. *J. Chromatogr.* **1990**, *516*, 333.

25. Nakagawa, Y.; Yip, T. -T.; Belew, M. Porath, J. *Anal. Biochem.* **1988**, *168*, 75.

26. Maisano, F.; Testori, S.; Gradi, G. *J. Chromatogr.* **1989**, *472*, 422.

27. Hemdan, E.S.; Zhao, Y.-J.; Sulkowski, E.; Porath, J. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1811.

28. Hutchens, T. W.; Li, C. M.; Sato, Y.; Yip, T. -T. *J. Biol. Chem.* **1989**, *264*, 17206.

29. Coulet, P. R.; Carlsson, J.; Porath, J. *Biotechnol. Bioeng.* **1981**, *23*, 663.

30. El Rassi, Z.; Truei, Y.; Maa, Y. -F.; Horvath, C. *Anal. Biochem.* **1988**, *169*, 172.

31. Smith, M.; Furmen, T.; Ingolia, T.; Pidgeon, C. *J. Biol. Chem.* **1988**, *263*, 7211.

32. Porath, J.; Olin, B. *Biochemistry* **1983**, *22*, 1621.

33. Porath, J. *Trends Anal. Chem.* **1988**, *7*, 25.

34. Belew, M.; Yip, T. -T.; Andersson, L.; Porath, J. *J. Chromatogr.* **1987**, *403*, 197.

35. Sulkowski, E. in *Protein Purification: Micro to Macro*, R. Burgess., Ed., Alan R. Liss: New York, **1987**, p. 149.

36. Gimpel, M.; Unger, K. *Chromatographia* **1983**, *17*, 200.

37. Figueroa, A.; Corradini, C.; Feibush, B.; Karger, B. L. *J. Chromatogr.* **1986**, *371*, 335.

38. Hutchens, T. W.; Yip, T. -T. *J. Chromatogr.* **1990**, *500*, 531.

39. Andersson, L.; Sulkowski, E.; Porath, J. *Bioseparation* **1991**, *2*, 15.

40. Sulkowski, E.; Vastola, K.; Oleszek, D.; Von Muenchhausen, W. in *Affinity Chromatography and Related Techniques*, Grabnau, T.; Visser, J.; Nivard, R., Eds., Veldhoven: The Netherlands, **1982**, 313.

41. Meirovitch, H.; Sheraga, H. A. *Macromolecules* **1980**, *13*, 1406.

42. Sulkowski, E. *Makromol. Chem. Macromol Symp.* **1988**, *17*, 335.

43. Andersson, L.; Porath, *Anal. Biochem.* **1986**, *154*, 250.

44. Porath, J.; Kristiansen, T. *Proteins* **1975**, *1*, 95.

45. El Rassi, Z.; Horvath, C. *J. Chromatogr.* **1986**, *359*, 241.

46. Kato, Y.; Nakamura, K.; Hashimoto, T. *J. Chromatogr.* **1986**, *354*, 511.

47. Afeyan, N. B.; Fulton, S. P.; Regnier, F. E. *J. Chromatogr.* **1991**, *544*, 267.

48. Yip, T. -T.; Tam, Y.; Koug, Y.; Belew, M.; Porath, J. *FEBS Lett.* **1985**, *187*, 345.

49. Muszynska, G.; Zhao, Y. -J.; Porath, J. *J. Inorg. Biochem.* **1986**, *26*, 127.

50. Ramadan, N.; Porath, J. *J. Chromatogr.* **1985**, *321*, 81.

51. Ramadan, N.; Porath, J. *J. Chromatogr.* **1985**, *321*, 93.

52. Ramadan, N.; Porath, J. *J. Chromatogr.* **1985**, *321*, 105.

53. Hochuli, E.; Dobeli, H.; Schacher, A. *J. Chromatogr.* **1987**, *411*, 177.

54. Hemdan, E. S.; Porath, J. *J. Chromatogr.* **1985**, *323*, 255.

55. Vallee, B. J.; Wacker, W. in *The Proteins*, Neurath, H., Ed., Vol 5.; Academic Press, Inc.: London, **1970**, 61.

56. Belew, M.; Yip, T. -T.; Andersson, L.; Ehrnstrom, R. *Anal. Biochem.* **1987**, *164*, 457.

57. Muszynska, G.; Andersson, L.; Porath, J. *Biochemistry* **1986**, *25*, 6850.

58. Pearson, R. G. *J. Am. Chem. Soc.* **1963**, *85*, 3533.

59. Pearson, R. G. *Science* **1966**, *151*, 172.

60. Regnier, F. E.; Chicz, R. M. in *HPLC of Biological Macromolecules*, Gooding, K.; Regnier, F., Eds., Marcel Dekker, Inc.: New York, **1990**, Chapter 4.

61. Shansky, R. E.; Wu, S. -L.; Figueroa, A.; Karger, B. L. in *HPLC of Biological Macromolecules*, Gooding, K.; Regnier, F., Eds., Marcel Dekker, Inc.: New York, **1990**, Chapter 5.

62. Frenz, J.; Hancock, W.S.; Henzel, W.J.; Horvath, C. in *HPLC of Biological Macromolecules*, Gooding, K.; Regnier, F., Eds., Marcel Dekker, Inc.: New York, **1990**, Chapter 6.

63. Heine, J. W.; Van Dame, J.; De Ley, M.; Billian, A.; DeSomer, P. *J. Gen. Virol.* **1981**, *54*, 47.

64. Torres, A. R.; Peterson, E. A.; Evans, W. H.; Mage, M. G.; Wilson, S. M. *Biochim. Biophys. Acta* **1979**, *576*, 385.

65. Bollin, E., Jr.; Sulkowski, E. *J. Gen. Virol.* **1981**, *52*, 227.

66. Ohkubo, I.; Kondo, T.; Taniguchi, N. *Biochim Biophys. Acta* **1980**, *616*, 89.

67. Kikuchi, H.; Watanabe, M. *Anal. Biochem.* **1981**, *115*, 109.

68. Rijken, D. C.; Wijngaards, G.; Zaal-de Jong, M; Welbergen, *J. Biochim. Biophys. Acta* ,**1979**, *580*, 140.

69. Boyer, R. F.; Generous, S. M.; Nieuwenhuis, T. J.; Ettinger, R. A. *Biotech. Appl. Biochem.* **1990**, *12*, 79.

70. Unger, K. K.; Jilge, G.; Kinkel, J. N.; Hearn, M. T. W. *J. Chromatogr.* **1986**, *359*, 61.

71. Unger, K. K.; Jilge, G.; Janzen, R.; Giesche, J.; Kinkel, J. N. *Chromatographia* **1986**, *22*, 379.

72. Colwell, Jr. L. F.; Hartwick, R. A.; *J. High Resol. Chromatogr. Chromatogr. Commun.* **1986**, *9*, 304.

73. Kalghatgi, K.; Horvath, C. *J. Chromatogr.* **1987**, *398*, 335.

74. Janzen, R.; Unger, K. K.; Giesche, H.; Kinkel, J. N.; Hearn, M. T. W. *J. Chromatogr.* **1987**, *397*, 9.

75. Varady, L.; Kalghatgi, K.; Horvath, C. *J. Chromatogr.* **1988**, *458*, 207.

76. Sczerba, T. J.; Baehr, D. N.; Glunz, L. J.; Perry, J. A.; Holdoway, M. J. *J. Chromatogr.* **1988**, *458*, 281.

77. Anspach, F. B.; Hans-Jurgen, W.; Unger, K. K.; Stanton, P.; Davies, J. R. *Anal. Biochem.* **1989**, *179*, 171.

78. Bonn, G. K.; Kalghatgi, K.; Horne, W.; Horvath, C. *Chromatographia* **1990**, *30*, 484.

79. Burke, D. J.; Duncan, J. K.; Dunn, L. C.; Cummings, L.; Siebert, C. J.; Ott, G. S.*J. Chromatogr.* **1986**, *353*, 425.

80. Kato, Y.; Kitamura, T.; Mitsui, A.; Hashimoto, T. *J. Chromatogr.* **1987**, *398*, 327.

81. Mazaroff, I. Rounds, M. A.; Regnier, F. E. *J. Chromatogr.* **1987**, *411*, 452.

82. Maa, Y. -F.; Horvath, C. *J. Chromatogr.* **1988**, *445*, 71.

83. Yamasaki, Y.; Kitamura, T.; Nakatani, S.; Kato, Y. *J. Chromatogr.* **1989**, *481*, 391.

84. Kato, Y.; Kitamura, T.; Nakatani, S.; Hashimoto, T. *J. Chromatogr.* **1989**, *483*, 401.

85. Maa, Y. -F.; Lin, S. -C.; Horvath, C.; Yang, U. -C.; Crothers, D. M. *J. Chromatogr.* **1990**, *508*, 61.

86. Hashimoto, T. *J. Chromatogr.* **1991**, *544*, 257.

87. Ludwig, R. C. *J. Chromatogr.* **1992**, *592*, 101.

88. Hjerten, S.; Liao, J. -L. *J. Chromatogr.* **1988**, *457*, 175.

89. Hjerten, S.; Li, J. -P. *J. Chromatogr.* **1990**, *500*, 543.

90. Stevens, A.; Morrill, T.; Parlante, S. *BioChromatography* **1986**, *1*, 50.

91. Danielson, N.; Kirkland, J. *Anal. Chem.* **1987**, *59*, 2501.

92. DiCesare J. L.; Dong, M. W.; Ettre, L. S. *Introduction to High-Speed Liquid Chromatography*; The Perkin-Elmer Corporation: U.S.A., 1981, Chapter 5.

93. Horvath, C.; Preiss, B. A.; Lipsky, S. R. *Anal. Chem.* **1967**, *39*, 1422.

94. Horvath, C.; Lipsky, S. R. *J. Chromatogr. Sci.* **1969**, *7*, 109.

95. Duncan, J.; Chen, A.; Siebert, C. *J. Chromatogr.* **1987**, *397*, 3.

96. Burke, D. J.; Duncan, J. K.; Siebert, C. J.; Ott, G. S. *J. Chromatogr.* **1986**, *359*, 533.

97. Horvath, Cs.; Melander, W. in *Chromatography* 22A, Heftmann, E., Ed., Elsevier Scientific Publishing Company: The Netherlands, **1983**, Chapter 3.

98. Karger, B. L.; Snyder, L. R.; Horvath, C. *Introduction to Separation Science*; John Wiley and Sons: New York, **1973**, Chapter 3.

99. Afeyan, N. B.; Gordon, N. F.; Mazsaroff, L.; Varady, L.; Fulton, S. P. Yang, Y. B.; Regnier, F. E. *J. Chromatogr.* **1990**, *519*, 1.

100. Fulton, S. P.; Afeyan, N. B.; Gordon, N. F.; Regnier, F. E. *J. Chromatogr.* **1991**, *547*, 452.

CHAPTER II

HIGH-PERFORMANCE METAL CHELATE INTERACTION CHROMATOGRAPHY OF PROTEINS WITH SILICA-BOUND ETHYLENEDIAMINE-*N,N'*-DIACETIC ACID

Introduction

As discussed in Chapter I, the selectivity and retention in MIC can be conveniently modulated and adjusted (i) by changing the type of the metal on the stationary phase,[1-3] (ii) by varying the eluent pH,[2, 4] (iii) by the nature and concentration of the salt,[1, 5-7] and (iv) by the nature and concentration of the competing agent in the eluent.[1, 8] Thus far, little attempt has been made to manipulate retention and selectivity by the nature of the chelating functions on the surface of the stationary phase.[2, 9, 10] Indeed, with agarose or rigid microparticulate stationary phases, iminodiacetic acid chelating functions (IDA) have been the most widely used. This indicates that the potential of MIC has not been fully explored so that the technique will have a common place in a wider range of applications.

This study addresses the need for investigating the potential of new chelating functions in MIC of proteins. In this regard, large-pore silica-bound ethylenediamine-*N,N'*-diacetic acid (EDDA) was developed in our laboratory. The chromatographic behavior of standard proteins and glycosylated proteins was evaluated with metal-EDDA chelates over a wide range of elution conditions including eluent pH, salts, and competing agents. A comparison with iminodiacetic acid (IDA) stationary phases is also presented. This work was published in *J. Chromatogr.* **1990**, *512*, 237.

Experimental

Instrumentation

The chromatograph was assembled from an ISCO (Lincoln, NE, U.S.A.) Model 2350 solvent delivery pump and a Model 2360 gradient programer, with a variable-wavelength detector Model V^4. A Rheodyne (Cotati, CA, U.S.A.) Model 7010 sampling valve with a 100-µl sample loop was used for injection. Chromatograms were recorded with a Shimadzu (Columbia, MD, U.S.A.) Model C-R5A integrator.

Reagents and Materials

The following materials were purchased from Sigma Chemical (St. Louis, MO, U.S.A.): cytochrome c from horse heart, lysozyme from chicken egg white, iron-free (approx. 90% substantially iron-free) and holo (approx. 98% iron saturated) transferrins from human, lactoferrin from bovine colostrum, β–casein from bovine milk and ethylenediamine-*N, N'*-diacetic acid. Iminodiacetic acid (IDA) was obtained from W.R. Grace (Nashua, NH, U.S.A.). Reagent grade sodium hydroxide, ethylenediaminetetraacetic acid (EDTA) disodium salt, acetic acid, ferric chloride, zinc chloride, cupric chloride, cobalt chloride, nickelous nitrate, phosphoric acid, sodium acetate, sodium formate, monobasic sodium phosphate, methanol, and acetonitrile (both HPLC grade) were obtained from Fisher (Pittsburgh, PA, U.S.A.). γ-Glycidoxypropyltrimethoxysilane was obtained from Aldrich (Milwaukee, WI, U.S.A.).

Columns

Zorbax PSM 300, a spherical silica, having mean particle and pore diameters of 7.5-µm and 300 Å, respectively, was obtained from DuPont (Wilmington, DE, U.S.A.). The EDDA and IDA stationary phases were made by first reacting the silica gel with γ–

glycidoxypropyltrimethoxysilane in aqueous solution, pH 6.0, for 2 hours at 95°C.[11] The products thus obtained were allowed to react with EDDA or IDA using well established procedures.[1] The surface coverage with EDDA or IDA functions was found to be approximately 2.0-2.2 μmoles/m^2, which correspond to 1.2-1.3 μmoles/mL of packed silica gel, as calculated from the nitrogen content measured by elemental analysis at Galbraith Laboratories, Inc (Knoxville, TN, U.S.A.). The approximate structures of both stationary phases in metal chelate forms are shown in Fig. 2. The stationary phases thus obtained were packed from an aqueous sucrose-NaCl slurry containing 50% sucrose (w/v) at 8000 psi with 1.0 *M* NaCl solution and using Shandon column packer instrument (Keystone Scientific, Bellefonte, PA, U.S.A.). All columns were made of 100 x 4.6 mm No. 316 stainless steel tubes (Alltech Associates, Inc., Deerfield, IL, U.S.A.).

Procedures

Freshly packed columns with IDA or EDDA siliceous stationary phases were first conditioned with water and then loaded with the appropriate metal by injecting 10 ml of 50 mM metal salt solution using a sampling valve equipped with 10 ml sample loop. This amount was enough to saturate the column with the desired metal since we have found that the retention of standard proteins did not change for concentration above 50 m*M*. After loading the column with a given metal, the excess unchelated metal was subsequently removed from the column by washing it with an ample amount of water followed by the equilibrating mobile phase in order to ensure reproducible results during the ensuing chromatographic separation. The column was unloaded from the metal by washing it with 20 ml of 50 m*M* EDTA disodium salt. After regeneration with water, the column regained its naked form (without chelated metal), and was ready for reloading with a different metal.

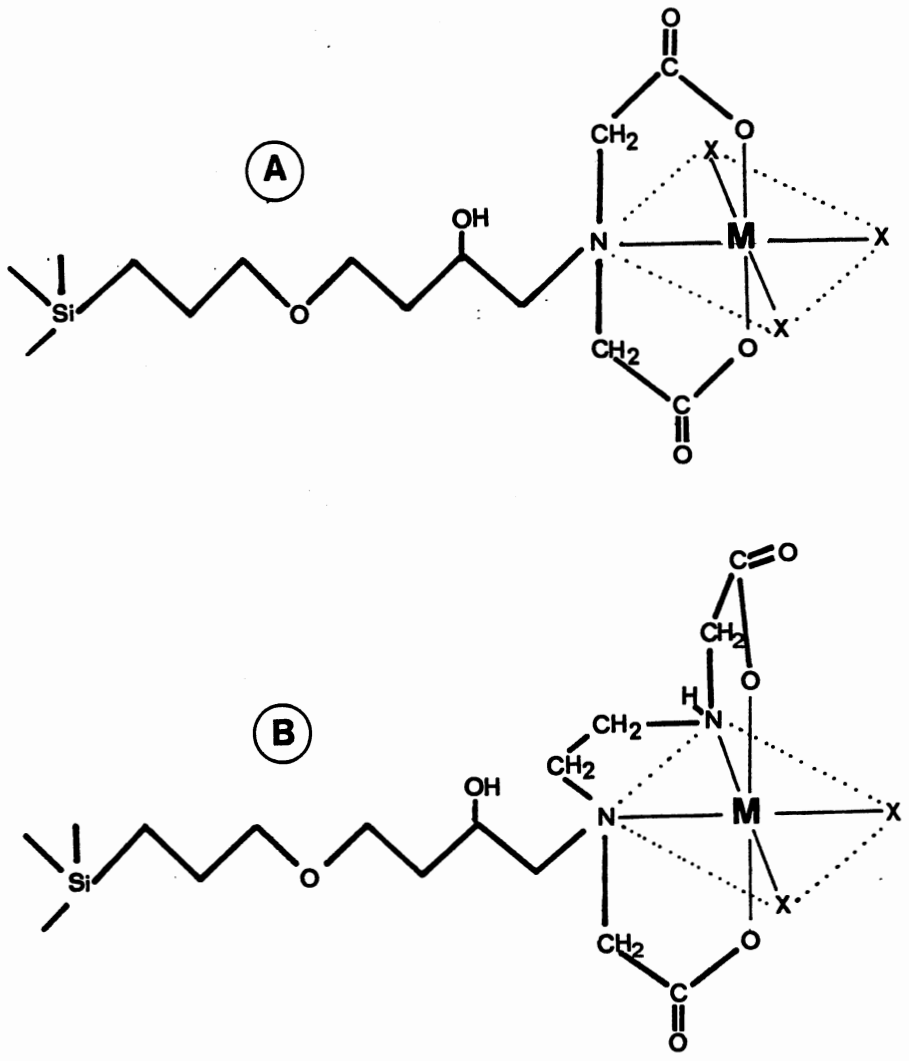


Figure 2. Schematic Illustration of the Surface-Bound Metal Chelates.
(A) tridentate IDA and (B) tetradentate EDDA. M, metal; X, protein molecule, salt ion, water molecule, or any competing agent.

Results and Discussion

Comparison of Metal-EDDA and Metal-IDA Columns

Columns packed with silica-bound EDDA functions chelated with Cu(II), Co(II), Ni(II), Zn(II) or Fe(III) metal ions were evaluated in protein HPLC over a wide range of elution conditions. These metal-EDDA stationary phases were also compared to metal-IDA columns. Phosphorylated and dephosphorylated β–caseins, lactoferrin, iron-free and holo transferrins, cytochrome c, and lysozyme were used as model solutes. These proteins which are well characterized in many aspects form an attractive set of solutes for elucidation of protein-metal chelate stationary phase associations. Horse heart cytochrome c and egg white lysozyme, each having one surface exposed histidine residue, may serve to evaluate the extent to which proteins that have the same histidine content but are slightly different in molecular weights and pI values (cytochrome c: MW = 12 200, pI = 10.6; lysozyme: MW =14 314, pI = 11.0) will interact with different metal chelate sorbents. Phosphorylated and dephosphorylated β–caseins may be useful to ascertain the involvement of phosphate groups in metal interaction chromatography. Human serum transferrins and bovine lactoferrin, which are iron-binding proteins[12] (two ferric ions per 1 molecule of protein) of similar molecular weights (about 80 000 for lactoferrin and 75 000 for transferrin), yet differing in their isoelectric points (about 6.0 for human transferrin and 10.0 for bovine lactoferrin), may be regarded as model proteins to assess the implication of the net charge of protein in MIC.

In this study, the chromatographic behavior of two chelating ligands, namely, IDA and EDDA were evaluated. As expected, metal-EDDA columns exhibited weaker interactions with the proteins investigated than their counterparts metal-IDA columns under otherwise identical elution conditions. As illustrated in Fig. 2 metal-IDA columns provide more coordination sites for interaction with the protein molecule, than do metal-EDDA

columns. On the other hand, as a result of one fewer donor atom in IDA molecule, metal-IDA complexes are less stable than metal-EDDA chelates; *cf.* Table I, which compiles the logarithmic stability constants of both metal complexes as measured in free solution.[13] Indeed, certain metal-IDA stationary phases such as Zn(II)-IDA[4] and Cu(II)-IDA[14] are unstable under most elution conditions and change in retention time from run to run is common. In order to circumvent this problem, Corradini *et al.*[14] added small amount of salt of the chelated metal to the mobile phase.

Table I. Stability Constants, log K, of Metal-EDDA and Metal-IDA Complexes in Free Solutions. Data taken from Ref. 13.

	Logarithm of the stability constants	
Metal	EDDA	IDA
Zn(II)	11.22	7.24
Ni(II)	13.65	8.13
Co(II)	11.25	6.94
Cu(II)	16.20	10.57
Fe(III)	--	10.72

Particularly noticeable is the strong binding of most proteins to Cu(II)-IDA column.[1, 2] The elution of proteins from the cupric-IDA column necessitated the use of either a linear gradient at increasing both salt and glycine (a bidentate competing agent) in the eluent or a gradient at decreasing pH and increasing imidazole (a monodentate competing agent), which often lead to leaching out of the metal and contamination of the separated proteins. It has been demonstrated that the contamination of the protein with metal ions removed by the competing agent from the stationary phase may be avoided by

loading the first two-thirds of the column with the metal only.[15] Another alternative may be the use of a naked IDA post column. However, in both cases a metalloprotein may lose its metal to the naked IDA and consequently its biological activity may be reduced as it has been found for holocarboxypeptidase A.[16] In contrast, with Cu(II)-EDDA column the proteins under investigation were readily eluted and separated with gradient at increasing sodium chloride concentration in the eluent. On the other hand, the proteins studied could be eluted from both Co(II)-IDA and Co(II)-EDDA columns using the same salt gradient. The retention data obtained on both stationary phases are shown in Table II in terms of selectivity. As can be seen in Table II, Co(II)-EDDA column exhibited higher selectivity than Co(II)-IDA column and in particular toward holo and iron free transferrins. However, Co(II)-IDA stationary phases yielded higher retention for the proteins than did

Table II. Comparison of α–Values of Proteins Measured with Porous Co(II)-IDA— and Co(II)-EDDA—Silica Columns.
Columns, 100 x 4.6 mm I.D.; flow-rate, 1.0 mL/min; temp., 25°C. Linear gradient in 15 min from 0 to 1.0 *M* NaCl in 10 m*M* acetate buffer, pH 5.5, followed by 5 min isocratic elution with 1.0 *M* NaCl in 10 m*M* sodium acetate.

	Selectivity, α	
Pairs of proteins	Co(II)-EDDA	Co(II)-IDA
Iron-free transferrin/holo transferrin	8.79	1.00
Cytochrome c/iron-free transferrin	1.23	1.06*
Lysozyme/cytochrome c	1.15	1.12
Lactoferrin/lysozyme	1.32	1.45

* Reversal in elution order

Co(II)-EDDA column. As a result , peaks were broader on the former column than on the latter.

As stated above, the elution of the different proteins from the various metal-EDDA columns was carried out using linear salt gradient at increasing sodium chloride concentration in the eluent. Different selectivities were obtained when going from one metal chelate column to another under otherwise identical elution conditions. Figure 3, which depicts the separation of five proteins on Co(II)-EDDA column, demonstrates the high selectivity and high efficiency that can be obtained with such columns.

Figure 4 illustrates the separation of phospho- and dephosphorylated β-caseins on Fe(III)-EDDA column using linear gradient at increasing sodium chloride concentration in the eluent. Whereas dephosphorylated casein eluted from the ferric-EDDA column with practically little or no retention, phosphorylated casein was retained by the metal chelate column. This is not unexpected since it is well known that phosphate forms complex with ferric ions.

In another set of experiments phosphoserine, a phosphorylated amino acid, was added to the eluent and the retention of cytochrome c, lysozyme, and iron-free transferrin on Fe(III)-EDDA column was measured using isocratic elution. As can be seen in Fig. 5, a few mM of this amino acid were a useful adjunct for modulating protein retention. Indeed, when adding 20 m*M* phosphoserine to the eluent, the retention factors of iron-free transferrin, lysozyme, and cytochrome c decreased by a factor of 0.2, 0.5 and 0.7, respectively, from their values obtained in the absence of phosphoserine. This is another indication of the predominance of metal interaction with ferric-EDDA stationary phase.

Effects of the Eluent pH

The effect of eluent pH on MIC retention was investigated with various metal-EDDA columns using gradient elution with linearly increasing sodium chloride

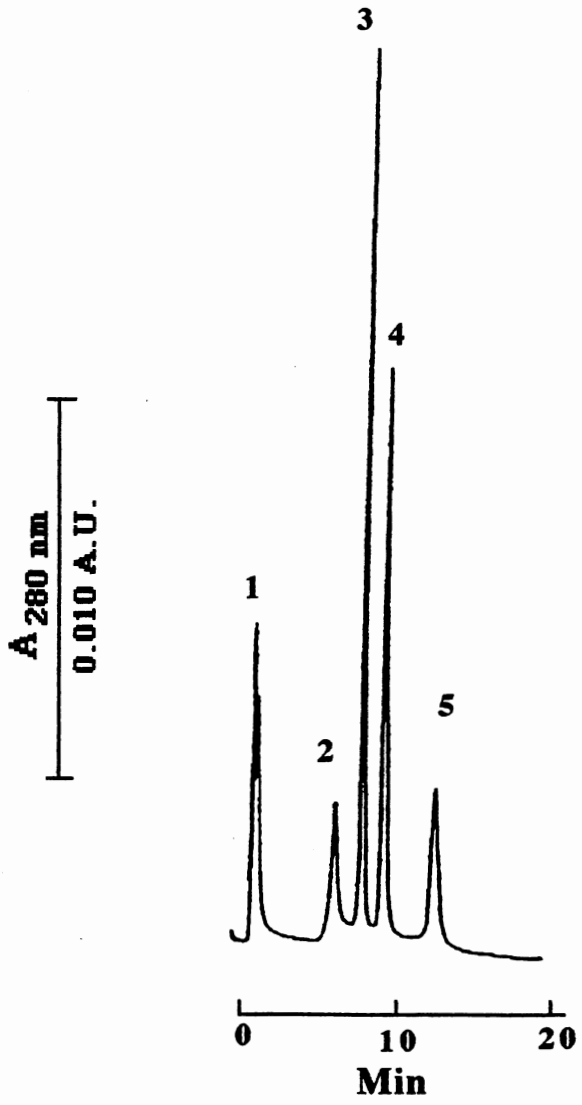


Figure 3. Chromatogram of Standard Proteins Obtained on Porous Co(II)-EDDA—Silica Column.
Column, 100 x 4.6 mm I.D. ; flow-rate, 1.0 mL/min; temp., 25°C. Linear gradient in 15 min from 0.02 to 1.0 *M* NaCl in 10 m*M* sodium acetate buffer, pH 5.5, followed by 5 min isocratic elution with 1.0 *M* sodium chloride in 10 m*M* sodium acetate. Proteins: 1, holo transferrin; 2, iron-free transferrin; 3, cytochrome c; 4, lysozyme; 5, lactoferrin.

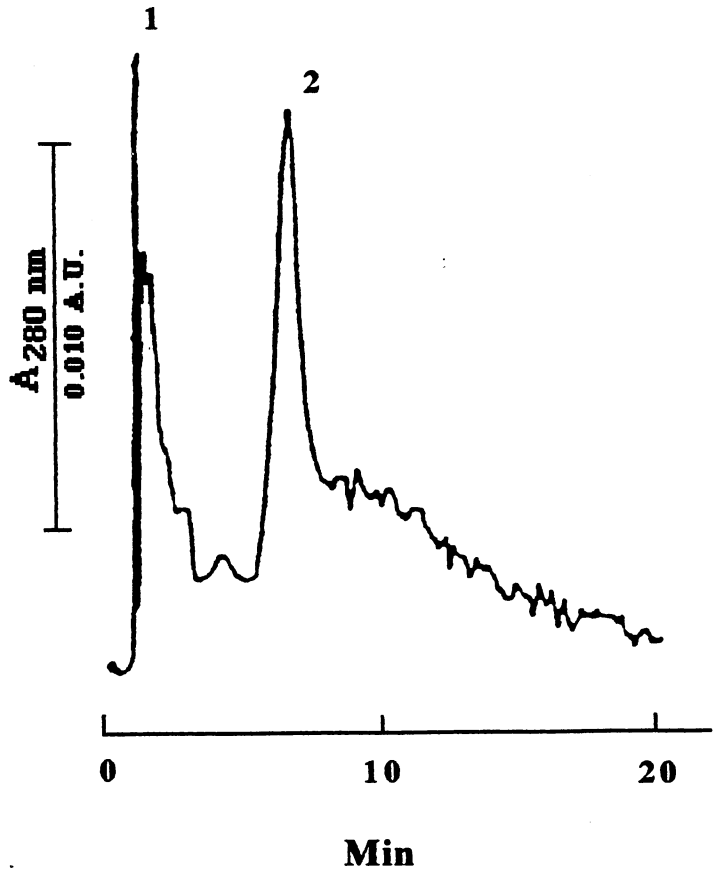


Figure 4. Chromatogram of Phosphorylated and Dephosphorylated β-Caseins. Column, porous Fe(III)-EDDA—silica, 100 x 4.6 mm I.D.; flow-rate, 1.0 mL/min; temp., 25°C. Linear gradient in 15 min from 0 to 1.0 *M* NaCl in 10 m*M* sodium acetate, pH 5.0, followed by 5 min isocratic elution with 1.0 *M* sodium chloride in 10 m*M* sodium acetate. Proteins: 1, dephosphorylated β-casein; 2, β-casein.

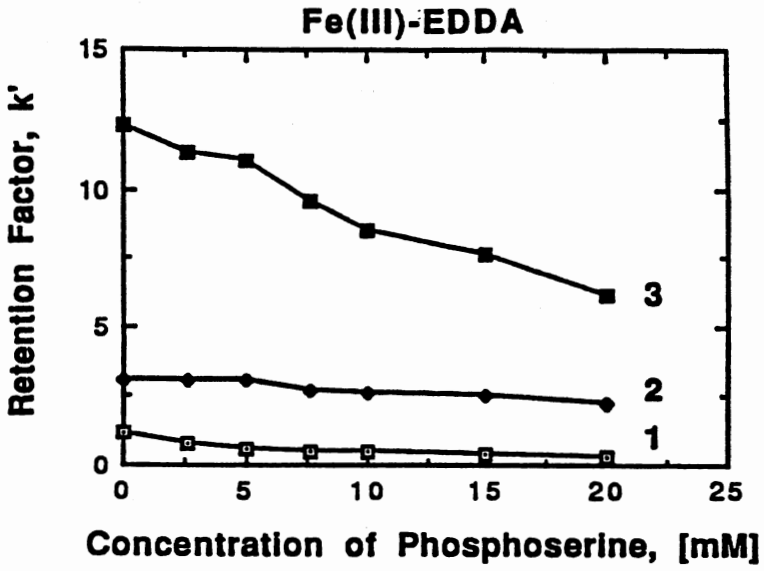


Figure 5. Plots of Retention Factor of Proteins Against Phosphoserine Concentration in the Eluent.
Column, porous Fe(III)-EDDA—silica, 100 x 4.6 mm I.D.; flow-rate, 1.0 mL/min; temp., 25°C. Isocratic elution with 10 m*M* acetate containing 0.15 *M* NaCl at different phosphoserine concentration, pH 5.0. Proteins: 1, iron-free transferrin; 2, cytochrome c; 3, lysozyme.

concentration in the eluent. The results are depicted in Fig. 6 by plots of adjusted retention volume *versus* eluent pH. The adjusted retention volume (V'_R) of a solute is by definition the difference between the retention volume of that solute and the void volume of the column. As expected, this effect varies from one metal chelate column to another for a given set of proteins.[1] With the exception of Ni(II)-EDDA column, which did not exhibit an affinity toward holo and iron-free transferrins in the pH range studied, the retention of these two proteins decreased with increasing pH on all other metal-EDDA columns and reached zero at pH values which were different from one column to another. Both transferrins were retained to the same extent on Zn(II)-EDDA at pH 5.0 but eluted with no retention at pH 5.5 and above. On the other hand, on Fe(III)-EDDA column the holo and iron-free transferrins were separated at only pH 6.25, whereas on Co(II)-EDDA they could be separated at pH ranging from *ca.* 5.2 to 6.0 (*cf* Fig. 6). The decrease in affinity of transferrins toward the chelated metals on the surface of the stationary phase with increasing pH may be explained by the increase in net negative charge of the proteins leading to electrostatic repulsion from the sorbent having the same net charge. The equal affinity of holo and iron-free transferrins toward some of the metal chelate columns at low pH (below or equal to 5.0) may be explained by the dissociation of the iron-protein complex at that pH[12] so that the holo transferrin will lose its iron and become iron-free protein. Therefore, we believe that MIC will find use in studying protein-metal complexes provided that the metal-binding site in the protein molecule is also involved in the interaction process between the protein and the chelated metal on the surface of the stationary phase. The monotonic increase in the retention of lactoferrin with eluent pH on the various metal-EDDA columns (Fig. 6) may reflect the presence of imidazole groups on the surface of the protein molecule. Indeed, with the high histidine content of such molecule[17] and the high pI value (net positive charge over a wide range of pH) an interaction of that kind may be favored. In contrast, due to their net negative charge at pH

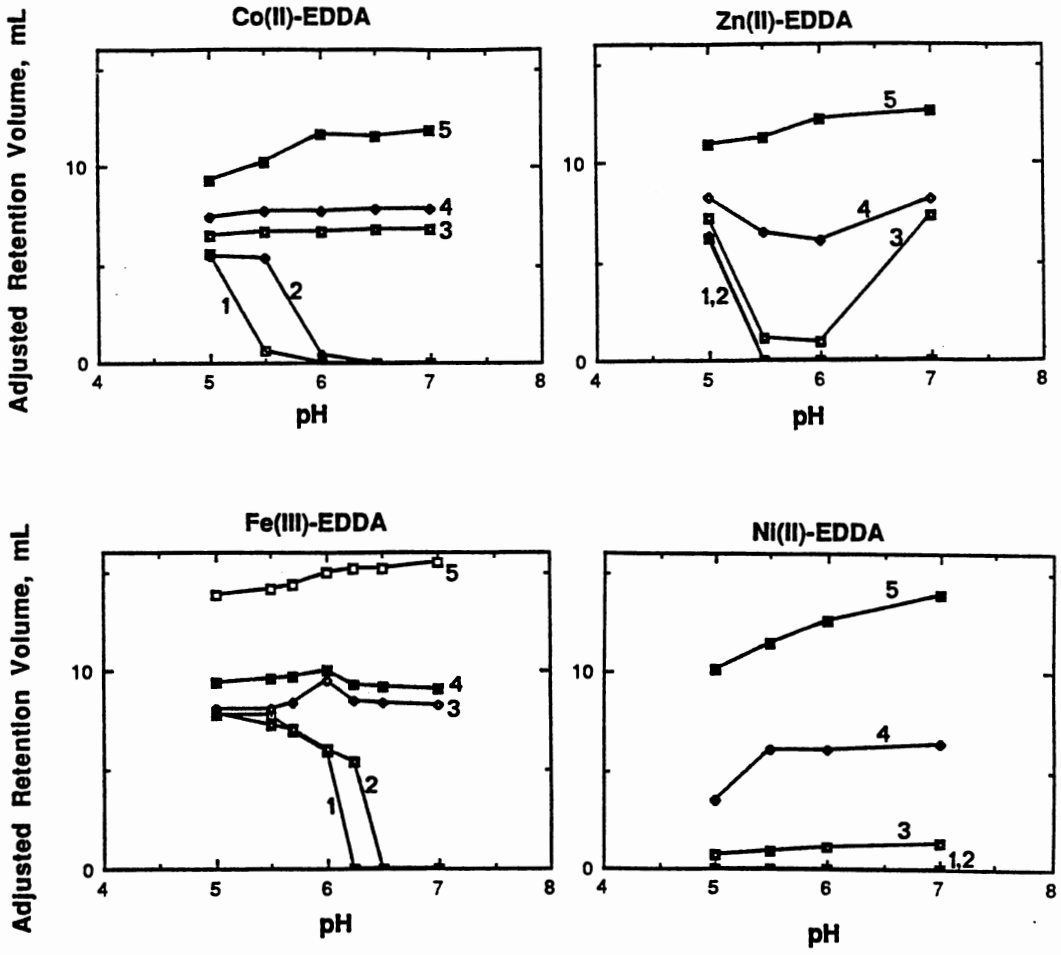


Figure 6. Plots of Adjusted Retention Volume *versus* pH, Measured with Different Porous Metal-EDDA—Silica Columns.
Column, 100 x 4.6 mm I.D.; flow-rate, 1.0 mL/min; temp., 25°C. Linear gradient in 15 min from 0 to 1.0 *M* NaCl in 10 m*M* acetate or phosphate buffer at different pH, followed by 5 min isocratic elution with 1.0 *M* sodium chloride in 10 m*M* acetate or phosphate buffer. Proteins: 1, holo transferrin; 2,iron-free transferrin; 3, cytochrome c; 4, lysozyme; 5, lactoferrin.

values above 5.5-6.0, acidic transferrins may be hindered from interacting with the chelated metals of the stationary phases despite their high content of histidine. The plots of retention *vs* eluent pH for cytochrome c and lysozyme on Zn(II)-EDDA column are U-shaped curves (see Fig. 6). This may be explained by the presence of both carboxyl and imidazole groups in the binding site to the Zn(II)-EDDA column. These same proteins showed little or no change in retention on other columns when varying the eluent pH. It has been advocated that a cluster of groups rather than a single group[18,19] may be involved in the binding of proteins to the chelated metal on the surface of the stationary phases.

Effects of the Nature and Concentration of Salt

To study the effect of the nature and concentration of the salt in the eluent on MIC retention and selectivity, isocratic measurements were carried out with various salts at pH 5.5. The salts studied were Na-chloride, Na-formate, Na-acetate, and Na-phosphate, and the columns examined were Co(II)- and Fe(III)-EDDA. Cobalt is a representative of "soft" metal ions, whereas Fe(III) is somewhat on the borderline of "hard" metal ions.[17] In all cases retention decreased with increasing salt concentration in the eluent in the concentration ranging from 0 to 0.5 *M*. Typical results are shown in Fig. 6 by plots of logarithmic retention factor *versus* the logarithmic salt concentration in the eluent. As shown in Fig. 7 straight lines were obtained for all the proteins under investigation. The intercepts of these lines with the ordinate (y-intercept), which are summarized in Table III and IV, were used to rank the eluting strength of the different salts. A greater negative intercept reflects a stronger eluting salt. According to this empirical consideration the eluent strength with Fe(III)-EDDA column increased in the order of $Cl^- < CH_3COO^- \leq HCOO^- < H_2PO_4^-$ (except for lysozyme), whereas with Co(II)-EDDA column it increased in the order $CH_3COO^- < H_2PO_4^- < Cl^-$. These results are in agreement with the observation that "hard" metals such as Fe(III) coordinate preferably with oxygen

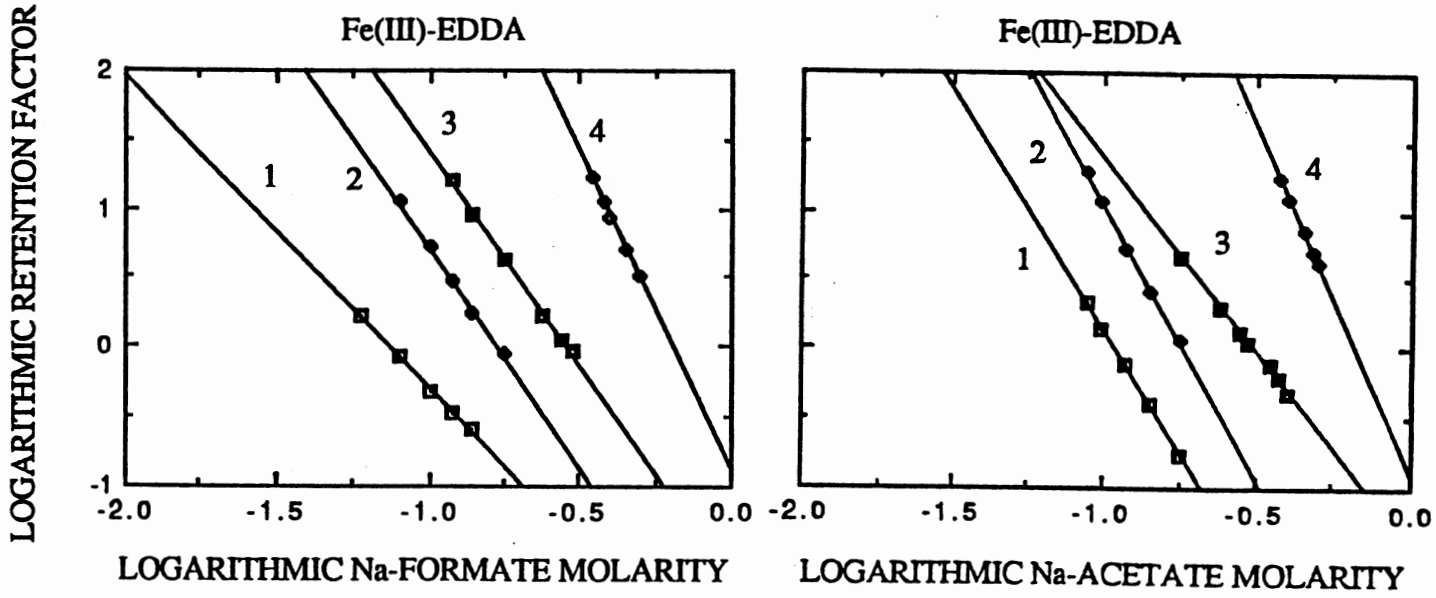


Figure 7. Plots of Logarithmic Retention Factor of Proteins *versus* Logarithmic Salt Molarity.
Column, porous Fe(III)-EDDA—silica, 100 x 4.6 mm I.D.; flow-rate, 1.0 mL/min; temp., 25 °C. Isocratic elution with 10 mM acetate buffer, pH 5.5, at different salt concentration. Proteins: 1, iron-free transferrin; 2, cytochrome c; 3, lysozyme; 4, lactoferrin.

containing ions (e.g. phosphate, acetate, formate), whereas "soft" metal ions such as cobalt have preference for large donor atoms, e.g. chloride ions in this study.

Table III. Values of y-Intercept of Plots of Logarithmic Retention Factor of Proteins *versus* the Logarithmic Salt Molarity in the Eluent.
Column, porous Co(II)-EDDA—silica, 100 x 4.6 mm I.D.; flow-rate, 1.0 mL/min; temp., 25°C. Isocratic elution with 10 m*M* acetate buffer, pH 5.5, at different salt concentration.

	y-Intercept		
PROTEIN	Na-ACETATE	Na-PHOSPHATE	Na-CHLORIDE
Iron-free transferrin	-3.54	-3.03	-4.73
Cytochrome c	-2.05	-2.81	-3.11
Lysozyme	-1.29	-2.10	-2.53
Lactoferrin	-0.97	-1.69	-2.01

Table IV. Values of y-Intercept of Plots of Logarithmic Retention Factor of Proteins *versus* the Logarithmic Salt Molarity in the Eluent.
Column, porous Fe(III)-EDDA—silica, 100 x 4.6 mm I.D.; flow-rate, 1.0 mL/min; temp., 25°C. Isocratic elution with 10 m*M* acetate buffer, pH 5.5, at different salt concentration.

	y-Intercept			
PROTEIN	Na-CHLORIDE	Na-FORMATE	Na-ACETATE	Na-PHOSPHATE
Iron-free transferrin	-2.07	-2.55	-3.36	-4.00
Cytochrome c	-2.04	-2.44	-2.84	-2.78
Lysozyme	-1.94	-1.68	-1.41	-2.15
Lactoferrin	-1.06	-0.88	-0.89	-1.51

Table V. Selectivity, α, Measured with Two Salts of Comparable Eluting Strength. Column, porous Fe(III)-EDDA—silica, 100 x 4.6 mm I.D.; flow-rate, 1.0 mL/min; temp., 25°C. Isocratic elution with 0.12 *M* sodium acetate or formate buffer, pH 5.5.

	Selectivity, α	
Pairs of proteins	Na-acetate	Na-formate
Cytochrome c/transferrin	6.75	8.85
Lysozyme/cytochrome c	2.66	5.29

Based on the above results, MIC selectivity can be varied by keeping the eluting strength of the salt roughly the same while changing the nature of the salt in the eluent. As shown in Fig. 7 and Table V, different selectivities were achieved on Fe(III)-EDDA column by exchanging sodium acetate for sodium formate; both salts are about the same eluting strength.

The slopes of the plots of log k' (i.e.; logarithm of the retention factor) *vs* log salt molarity, which measure the magnitude of interaction between the protein and the metal chelate stationary phases,[1] were also calculated. A greater negative slope reflects a stronger interaction. As determined from these slopes, lactoferrin exhibited the strongest interaction with the Co(II)- and Fe(III)-EDDA columns, while all other proteins, i.e. cytochrome c, lysozyme, and iron-free transferrin, interacted with the metal chelate columns to a lesser extent than lactoferrin and at slightly different magnitude among each others.

Conclusions

The utilization of wide-pore silica with surface-bound chelating ligands was found to be very useful in the separation of proteins by high performance metal interaction chromatography. The EDDA stationary phases in an appropriate metal form are very

suitable for the separation and determination of proteins. Such stationary phases, can be viewed as complementary to IDA stationary phases, since they afford different selectivity and retentivity toward proteins. In addition, metal-EDDA stationary phases are stable as manifested by the constancy of the retention of proteins under elution conditions used in this study.

References

1. El Rassi, Z.; Horvath, C. *J.Chromatogr.* **1986**, *359*, 241.
2. Porath, J.; Carlsson, J.; Olsson, I.; Belfrage, G. *Nature (London)* **1975**, *258*, 598.
3. Porath, J.; Olin, B.; Granstrand, B. *Arch. Biochem. Biophys.* **1983**, *225*, 543.
4. Kato, Y.; Nakamura, K.; Hashimoto, T. *J. Chromatogr.* **1989**, *354*, 511.
5. Corradini, C.; El Rassi, Z.; Horvath, C.; Guerra , G.; Horne, W. *J. Chromatogr.* **1988**, *458*, 1.
6. Porath, *J. Biotechnol. Progr.* **1987**, *3*, 14.
7. Sulkowski, E. in *Protein Purification: Micro to Macro, UCLA Symposium on Molecular and Cellular Biology, New Series*, Burgers, R., Ed., Vol. 68; Alan R. Liss Inc.: New York, **1987**, 149.
8. Muszynska, G.; Anderson, L.; Porath, J. *Biochemistry* **1986**, *25*, 6850.
9. Hemdan, E. S.; Porath, J. *J. Chromatogr.* **1985**, *323*, 255.
10. Hochuli, E.; Dobeli, H.; Schacher, S. *J. Chromatogr.* **1987**, *411*, 177.
11. Chang, S. H.; Gooding, K. M.; Regnier, F. E. *J. Chromatogr.* **1976**, *125*, 114.
12. Putnam, F. W. in *The Plasma Proteins,* Putnam, F.W., Ed., 2nd edition, Vol. IV; Academic Press Inc.: Orlando, FL, **1984**, pp. 96-98.
13. Martell, A. E.; Smith, R. M. *Critical Stability Constants*, Vol. 1; Plenum Press: New York, **1974**, pp. 86-87 and pp. 116-117.
14. Figueroa, A.; Corradini, C.; Karger, B. L. *J. Chromatogr.*, **1986**, *371*, 335.
15. Kikuchi, H.; Watanabe, M. *Anal. Biochem.* **1981**, *115*, 109.
16. Muszynska, G.; Zhao, Y. J.; Porath, J. *J. Inorg. Biochem.* **1986**, *26*, 127.
17. Pearson, R. G. *J. Am. Chem. Soc.* **1963**, *85*, 3533.
18. Sulkowski, E. *Makromol. Chem., Macromol. Symp.* **1988**, *17*, 335.
19. Gurd, F. R. N.; Wilcox, P. E. *Adv. Protein Chem.* **1956**, *11*, 311.

CHAPTER III

RAPID HIGH PERFORMANCE METAL CHELATE INTERACTION CHROMATOGRAPHY WITH NON-POROUS SILICA-BASED STATIONARY PHASES

Introduction

In the present study, the potentials of non-porous silica-based metal chelate stationary phases are demonstrated for the rapid separation of proteins. These sorbents having an average particle diameter of 1.8 μm were packed in short columns of 30 x 4.6 mm I.D. This column configuration permitted the utilization of high flow velocity without sacrificing column efficiency due to the absence of mobile phase intraparticle mass transfer resistances. These stationary phases whose ligands are on the outer surface of the rigid non-porous particles afforded rapid sorption kinetics and column equilibration. As a result, separation on the time scale of few minutes and seconds were obtained.

Experimental

Instrumentation

The chromatograph was assembled from an LDC/Milton Roy (Riviera Beach, FL, U.S.A.) Model CM4000 solvent delivery pump, and an LDC/Milton Roy variable-wavelength detector Model SpectroMonitor 3100. A Rheodyne (Cotati, CA, U.S.A.) Model 7125 sampling valve with a 20-μL sample loop was used for injection.

Chromatograms were recorded with a Shimadzu (Columbia, MD, U.S.A.) Model C-R6A integrator interfaced with a single floppy disk drive and a CRT monitor.

Reagents and Materials

The following materials were purchased from Sigma Chemical (St. Louis, MO, U.S.A.): β-lactoglobulin A from bovine milk, carbonic anhydrase from bovine erythrocytes, carboxypeptidase A, α-chymotrypsinogen A and ribonuclease A from bovine pancreas, α-lactalbumin from bovine milk, myoglobin from horse skeletal muscle, lipoxidase from soy bean, ovalbumin from chicken egg white, imidazole and sodium dodecyl sulfate (SDS). All other proteins and reagents used in this study were described in Chapter II.

Columns

Microspherical monodispersed non-porous silica particles in the 1.8-μm particle diameter were prepared in our laboratory by precipitating tetraethylorthosilicate (TEOS) under alkaline conditions according to well established procedures.[1-3] The preparation was carried out as follows: anhydrous ammonia was bubbled into 45.8 mL of absolute ethyl alcohol placed in a 500 mL three-neck round bottom flask until saturation at room temperature. Thereafter, 19.5 mL of saturated ammonium hydroxide and 4.7 mL of TEOS were added to the content of the flask. The solution was continuously stirred with a paddle stirrer. To increase the solid weight fractions and to achieve larger particles than what can be obtained with a one step growth process, a seeded growth technique was implemented.[3] After 8 hours, the above seed suspension was supplemented with 9.4 mL of TEOS and 14 mL of water every 8-hour interval. The reaction was stopped after 10 additions.

The product thus obtained was used for the preparation of the metal chelate interaction stationary phases. The EDDA and IDA chelating ligands were covalently

attached to the non-porous silica by employing the same procedures outlined in the experimental section of Chapter II.

The non-porous silica with surface-bound EDDA or IDA were packed into columns made of 30 x 4.6 mm No. 316 stainless steel tubes (Alltech Associates, Inc., Deerfield, IL, U.S.A.) in the same manner as described in the experimental section of Chapter II. The freshly packed columns with non-porous silica-IDA or -EDDA were first equilibrated with water and then loaded with the appropriate metal by following the same method described earlier (see Chapter II).

Results and Discussion

Chromatographic Evaluation of Non-porous Silica-Based Metal Chelate Stationary Phases

Table VI summarizes the adjusted retention volumes of widely different proteins obtained on various non-porous IDA and EDDA metal chelate stationary phases at pH 6.0, using gradients of increasing sodium chloride or imidazole concentrations in the eluent. α-Lactalbumin showed little or no retention on the different metal chelate sorbents. It was slightly retained on Cu(II)-IDA column, and its retention on Zn(II)-IDA column was significantly enhanced by the presence of 1.0 *M* NaCl in the imidazole gradient. This can be attributed to salting-out effect, a process in which the protein solute is repulsed from the aqueous mobile phase into the proximity of the interacting sites on the surface of the stationary phase.

On the other hand, some proteins could not be eluted from Cu(II)-IDA and Zn(II)-IDA columns by simply increasing the ionic strength of the eluent. Their elution and separation were brought about by a gradient of increasing imidazole concentration, a monodentate competing ligand. As can be seen in Table VI, Zn(II)-IDA column is slightly less retentive than Cu(II)-IDA column toward the proteins studied under otherwise identical elution conditions.

Table VI. Adjusted Retention Volume of Different Proteins on Various Metal-EDDA or -IDA Columns. Column, 30 x 4.6 mm I.D.; flow-rate, 4.0 mL/min.

Proteins	Cu(II)-IDA[a]	Cu(II)-IDA[b]	Zn(II)-IDA[a]	Zn(II)-IDA[b]	Zn(II)-EDDA[a]	Cu(II)-EDDA[a]	Fe(III)-EDDA[a]
α-Lactalbumin	0.43	NM	0.0	1.05	0.0	0.0	0.0
α-Chymotrypsinogen A	1.24	NM	1.23	NM	1.03	1.31	1.28
Carboxypeptidase A	1.39	NM	1.03	0.0	0.0/0.94	1.20	1.10
Ribonuclease A	1.57/1.70	NM	1.20	0.20	0.91	1.26	1.40
Cytochrome c	1.86	NM	1.54	NM	1.07	1.39	1.49
Lysozyme	2.59	NM	1.78	0.16	1.24	1.47	1.38
Lactoferrin	NE	1.03	2.18	1.02	1.55	2.38	2.45
Iron-free transferrin	NE	1.86	NE	1.64	0.0	1.30	1.26
Myoglobin	NE	1.63	NE	1.43	0.0	0.10	1.13

[a] 3.0 min linear gradient from 0 to 1.0 *M* NaCl in 10 m*M* phosphate, pH 6.0

[b] 3.0 min linear gradient from 0 to 50 m*M* imidazole in 10 m*M* phosphate and 1.0 *M* NaCl, pH 6.0

NE, no elution

NM, not measured

As we reported in Chapter II, under the same elution conditions, EDDA metal chelate stationary phases are less retentive than their counterpart IDA metal chelate sorbents (see Table VI). Proteins are usually eluted and separated by an increasing salt gradient as in ion-exchange chromatography. This may be due to one fewer binding site on the chelated metal with EDDA stationary phases. Indeed, IDA ligands are tridentate, whereas EDDA ligands are tetradentate. Zn(II)-EDDA column is the least retentive toward the proteins when compared to Cu(II)-EDDA and Fe(III)-EDDA stationary phases. The coordination number of Zn(II) is 4, that of Cu(II) is either 4 or 6 and that of Fe(III) is 6. This means that in Zn(II)-EDDA coordination sphere, the central metal ions would not have sites to which other ligands or solutes can attach themselves. This may explain the low retentivity of Zn(II)-EDDA towards the proteins under investigation.

An inherent property of non-porous stationary phases is their relatively low phase ratio, which leads to milder elution conditions for the separation of biopolymers when compared to porous stationary phases having identical immobilized metal chelating ligands. For instance, ribonuclease A, cytochrome c and lysozyme were eluted and separated on non-porous Cu(II)-IDA column by increasing salt gradient (see Table VI). The elution and separation of these proteins from wide-pore Cu(II)-IDA microparticulate stationary phases would require the use of gradients of increasing concentrations of competing agents in the eluent and/or at decreasing pH.[4]

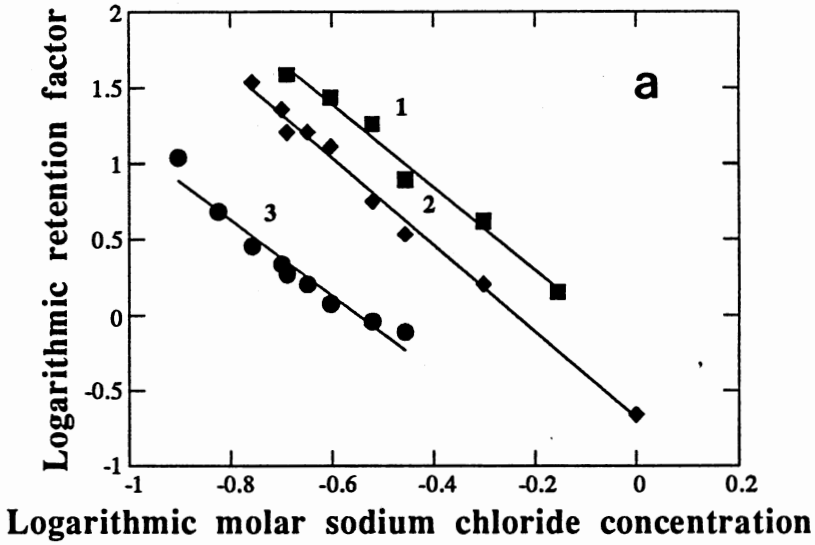
As mentioned in Chapter I, the lower phase ratio of the non-porous stationary phases represents an advantage in analytical high performance affinity chromatography at large as well as in metal affinity chromatography of biopolymers. Indeed, in many instances high phase ratio lead to avid binding of some species to the stationary phase and as a result harsh elution conditions would be required for the separation of biopolymer solutes. Under these circumstances, the strong eluent could be viewed as a levelling eluent in the sense that solute-stationary phase dissociations will occur to the same extent for

otherwise different species, thus producing less resolution and decreased selectivity. Lower phase ratio may be more useful in analytical affinity chromatography whereby milder elution conditions could bring about elution and separation. The mild eluent may be regarded as differentiating eluent that permits solute-stationary phase dissociations to be different for closely related but different biopolymers, thus producing widely different selectivity.

Effects of the Nature and Concentration of Salt on Retention. Case of IDA columns

As discussed above, wide-pore IDA metal chelate stationary phases exhibited in general high retention towards proteins. Due to their low phase ratio, with non-porous stationary phases, solute retention falls within the range of practical value, thus providing the opportunity to evaluate the effect of salts on MIC retention in more quantitative term.

To study such effects, a non-porous Co(II)-IDA—silica column was chosen as a typical intermediate "soft" metal chelate column. The retention of three typical proteins was measured under isocratic elution conditions with mobile phases at various concentrations of NaCl and NaH_2PO_4 in 10 m*M* phosphate containing 10 m*M* imidazole at pH 6.0. Imidazole was added to the eluent to further decrease the strong affinity of Co(II)-IDA towards the proteins under investigation. The results are depicted in Fig. 8a and b in terms of logarithmic retention factor *versus* logarithm of the molar salt concentration in the eluent. In all cases the retention of proteins decreased first and then approached zero at high salt concentrations. An exception, however, is the case of lysozyme when sodium phosphate was used as the eluent. In this case the retention of lysozyme first decreased, reached a minimum and then increased at high salt concentration in the eluent. Similar behavior for lysozyme in the presence of sodium sulfate in the eluent was reported.[5] This behavior is explained by the interplay of two kinds of interactions: metal and/or electrostatic interactions which dominate the retention process at low salt concentrations,



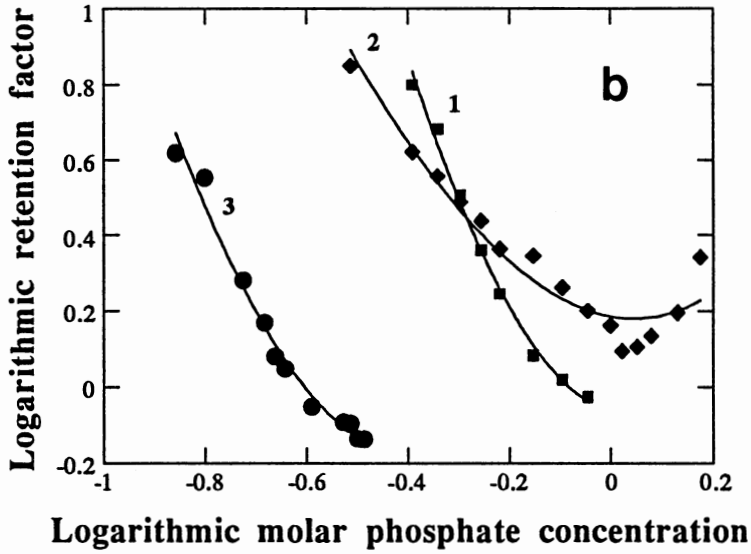


Figure 8. Plots of Logarithmic Retention Factor of Proteins *versus* Logarithmic Sodium Chloride Concentration.in (a) and Sodium Phosphate Concentration in (b) on a Non-porous Co(II)-IDA—Silica Column.
Column, 30 x 4.6 mm I.D; flow-rate, 1.0 mL/min. Isocratic elution using 10 m*M* phosphate containing 10 m*M* imidazole at various salt concentration, pH 6.0. Proteins: 1, lactoferrin; 2, lysozyme; 3, cytochrome c.

and hydrophobic interactions which are responsible for the retention at high salt concentrations.

Table VII summarizes the y-intercept of the plots of logarithmic retention factor of the proteins *versus* the logarithmic salt concentration. As can be noticed in Table VII, with the exception of lysozyme, the y-intercepts obtained with sodium phosphate salt are more negative than those calculated with sodium chloride salt. As defined previously in Chapter II, the more negative the y-intercept is, the higher the eluting strength of the salt. It follows then that sodium phosphate is a stronger eluting salt than sodium chloride. This is found to be a deviation from the earlier findings that "soft" metal ions such as cobalt have preference for large donor atoms like chloride ions (refer to Chapter II). These differences may be due to the effect of the nature of the metal chelating ligands of the stationary phase. In the earlier study, the Co(II) metal was chelated by the tetradentate EDDA ligand whereas in the present study, it was chelated by the tridentate IDA ligand.

Table VII. Values of the y-Intercept of Plots of Logarithmic Retention Factor of Proteins *versus* the Logarithmic Salt Molarity in the Eluent.
Isocratic elution using 10 m*M* phosphate containing 10 m*M* imidazole at various salt concentration, pH 6.0. Column, non-porous Co(II)-IDA—silica, 30 x 4.6 mm I.D.; flow rate, 1.0 mL/min.

PROTEIN	y-Intercepts	
	Na-Chloride	Na-Phosphate
Lactoferrin	-0.25	-0.42
Lysozyme	-0.68	0.13
Cytochrome c	-1.38	-1.68

Novel Rapid Separation Schemes- Illustrative Examples

Non-porous silica-based stationary phases with surface-bound IDA or EDDA metal chelating ligands were evaluated in the rapid MIC of proteins. Novel elution schemes were devised by taking into account the effects of various operational parameters on retention and selectivity in MIC, e.g., salt, competing agents and pH.

EDDA Columns. Figures 9-11 illustrate typical chromatograms obtained on various EDDA metal chelate columns. Figure 9 portrays the separation of three different transferrins in less than three minute on a Co(II)-EDDA column. Holo-transferrin eluted with little or no retention, whereas iron-free transferrin and lactoferrin eluted and separated in the salt gradient. On Cu(II)-EDDA column, base line resolution for myoglobin, iron-free transferrin and lactoferrin was obtained in less than 90 seconds (see to Fig. 10). Figure 11a and b shows the separation of four proteins, namely, α-lactalbumin, carboxypeptidase A, cytochrome c and lactoferrin on an Fe(III)-EDDA column at two different flow-rates. As can be seen, the resolution between the proteins is almost unaffected by the flow velocity, and the analysis time is substantially reduced when going from a flow-rate of 4.0 to 6.0 mL/min. In all cases linear gradients at increasing sodium chloride concentrations in the eluent was sufficient to bring about elution and separation of widely different proteins from the various EDDA metal chelate columns.

IDA Columns. Although the relatively low phase ratio of non-porous IDA stationary phases favored the elution of some proteins under mild conditions, the elution and separation of some other proteins still necessitated the use of competing agents in the eluent. Lactoferrin, iron-free transferrin and carbonic anhydrase, as will be seen later, are among those proteins which exhibited strong retention on metal-IDA columns, and hence, were eluted only in the presence of competing agents. The use of imidazole or sodium dodecyl sulfate have been reported to cause a decrease in protein retention because of the

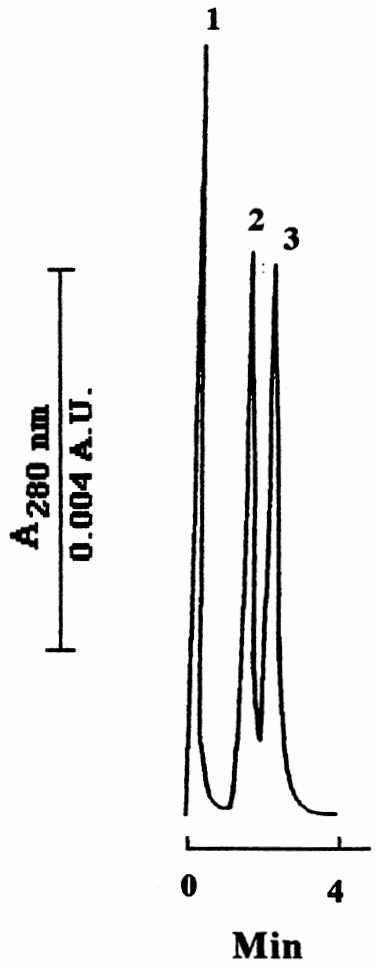


Figure 9. Rapid Separation of Proteins on Non-porous Co(II)-EDDA—Silica Column. Column, 30 x 4.6 mm I.D.; flow-rate, 4.0 mL/min. Fast linear gradient in 3.0 min from 0 to 1.0 *M* NaCl in 10 m*M* acetate, pH 5.5. Proteins: 1, holo-transferrin; 2, iron-free transferrin; 3, lactoferrin.

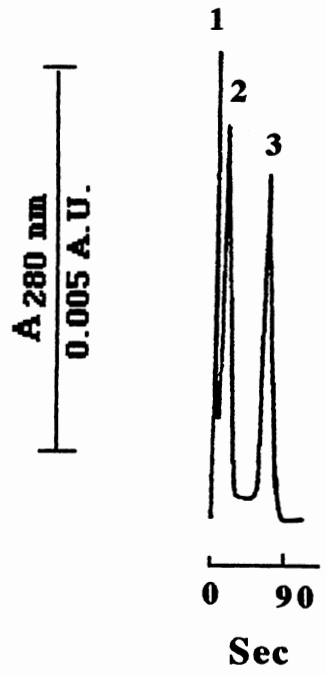


Figure 10. Rapid Separation of Proteins on Non-porous Cu(II)-EDDA—Silica Column. Column, 30 x 4.6 mm I.D; flow-rate, 2.0 mL/min. Fast linear gradient in 2.0 min from 0 to 1.0 *M* NaCl in 10 m*M* phosphate, pH 6.0, injection was delayed for 0.4 min. Proteins: 1, myoglobin; 2, iron-free transferrin; 3, lactoferrin.

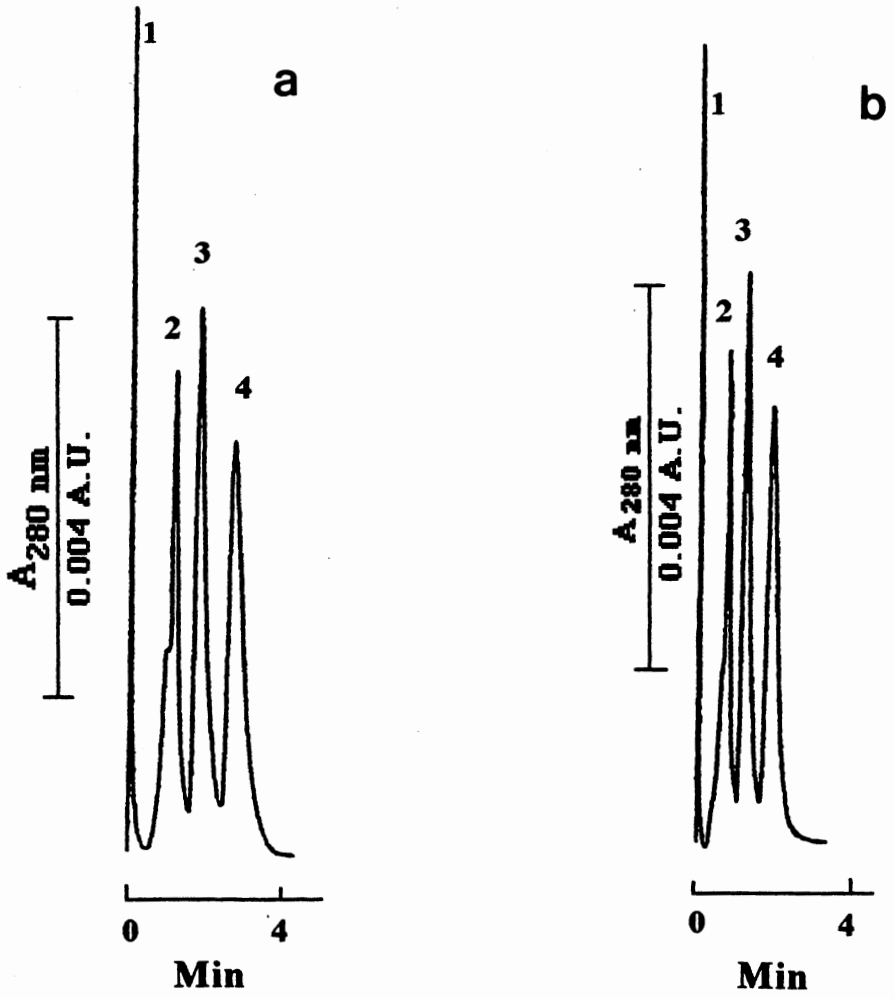


Figure 11. Rapid Separation of Proteins on Non-porous Fe(III)-EDDA—Silica Column. (a) 3.0 min linear gradient from 0 to 1.0 *M* NaCl in 10 m*M* phosphate, pH 6.0; flow-rate, 4.0 mL/min and (b) 2.0 min linear gradient from 0 to 1.0 *M* NaCl in 10 m*M* phosphate, pH 6.0; flow-rate, 6.0 mL/min. Column, 30 x 4.6 mm I.D. Proteins: 1, α-lactalbumin; 2, carboxypeptidase A; 3, cytochrome c; 4, lactoferrin.

competition for the immobilized metal on the surface of the stationary phase or for the metal chelate binding sites on the protein molecule, respectively.[6, 7]

Figure 12a shows the rapid separation of three proteins, carboxypeptidase A, lactoferrin and iron free transferrin on non-porous Cu(II)-IDA—silica column using a decreasing salt gradient at constant SDS concentration in the eluent. At low salt and in the presence of SDS in the eluent, the proteins under investigation did not exhibit any significant binding to the chelated metal. Sodium dodecyl sulfate binds to most proteins and therefore compete with the metal chelate stationary phase to the binding sites on the surface of the protein molecule. Under these circumstances and to bring about protein binding to the metal chelate column, the proteins are first adsorbed to the stationary phase at high salt concentration in the presence of SDS. The high salt decreases the binding of SDS to the protein and strengthen the protein-metal chelate associations. The proteins are then eluted by decreasing salt gradient at constant SDS concentration. As the amount of salt is lowered the SDS would bind to the protein and therefore facilitates its elution from the column. This novel elution scheme can be viewed as quite gentle as far as the stability of the immobilized metal is concerned. Indeed, the column exhibited constant retention from run-to-run and day-to-day when operated under these conditions. However, the SDS obliterates the difference between the proteins for their affinity to the metal chelate sorbents.

The peak capacity (defined as the number of peaks that can be separated with a resolution of unity in a chromatographic run) of the Cu(II)-IDA column can be further increased by combining two consecutive gradients. The results are illustrated in Figure 12b, whereby three proteins that exhibit relatively weak affinity toward the metal are first eluted and separated in increasing salt gradient and the other proteins that are avidly bound to the metal chelate column are eluted with a second linear gradient at increasing SDS and decreasing sodium chloride concentrations in the eluent.

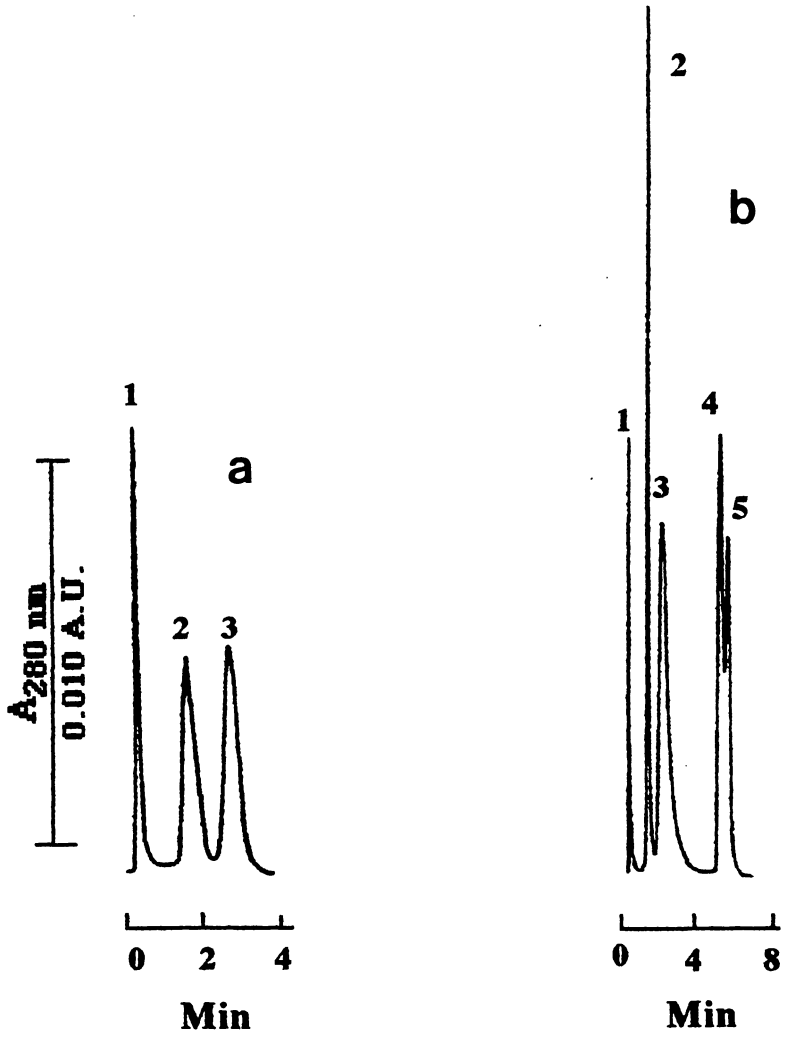


Figure 12. Rapid Separation of Proteins on Non-porous Cu(II)-IDA—Silica Column. Column, 30 x 4.6 mm I.D; flow-rate, 3.0 mL/min. (a) 3.0 min linear gradient from 0.7 to 0 *M* NaCl in 10 m*M* phosphate containing 0.1% SDS, in 10 m*M* phosphate, pH 6.0. Proteins: 1, carboxypeptidase A; 2, lactoferrin; 3, iron-free transferrin. (b) two consecutive linear gradients: 3.0 min from 0 to 1.0 *M* NaCl and 3.0 min from 1.0 to 0 *M* NaCl and from 0 to 0.1% SDS in 10 m*M* phosphate, pH 6.0. Proteins: 1, α-lactalbumin; 2, α-chymotrypsinogen A; 3, cytochrome c; 4, iron-free transferrin; 5, carbonic anhydrase.

To further enhance the resolving power of the metal chelate column another approach was introduced. It involved the use of a dual linear gradient consisting of an increasing salt gradient followed by an increasing imidazole gradient at constant salt concentration. Imidazole is a monodentate competing ligand known to exhibit affinity toward the immobilized metal. Figure 13 represents the separation of 8 standard proteins on a Cu(II)-IDA column by employing the salt-imidazole consecutive linear gradient system. Whereas lactoferrin, iron-free transferrin and carbonic anhydrase were strongly retained, β-lactoglobulin A, α-lactalbumin, α-chymotrypsinogen A and cytochrome c exhibited lower affinity toward the chelated metal and eluted with increasing salt concentration gradient. As explained in Chapter II and as will be discussed in great details in Chapter VI, the high retention of iron-free transferrin and carbonic anhydrase can be attributed to their high histidine content whereas in the case of lactoferrin both the high histidine content and basic pI value of the protein analyte are responsible for its chromatographic behavior.[8] On the other hand, the lower retention of the other four proteins maybe due primarily to their lower content in metal binding sites (mostly histidine).

The above separation scheme was also applied to the elution of proteins from a Zn(II)-IDA column. Figure 14a and b represents the rapid separation of β-lactoglobulin A, carboxypeptidase A, lysozyme and iron-free transferrin using a relatively high flow-rate and a rapid dual linear gradient consisting of increasing NaCl concentration followed by an increasing imidazole concentration at constant salt concentration. Besides effecting much faster separation by increasing the flow-rate from 4.0 to 6.0 mL/min, the sharpness of protein peaks had increased (see Figure 14b).

The Zn(II)-IDA column was also found to be particularly important in recognizing different isoforms of biopolymers. Figure 15 shows the high selectivity obtained on that

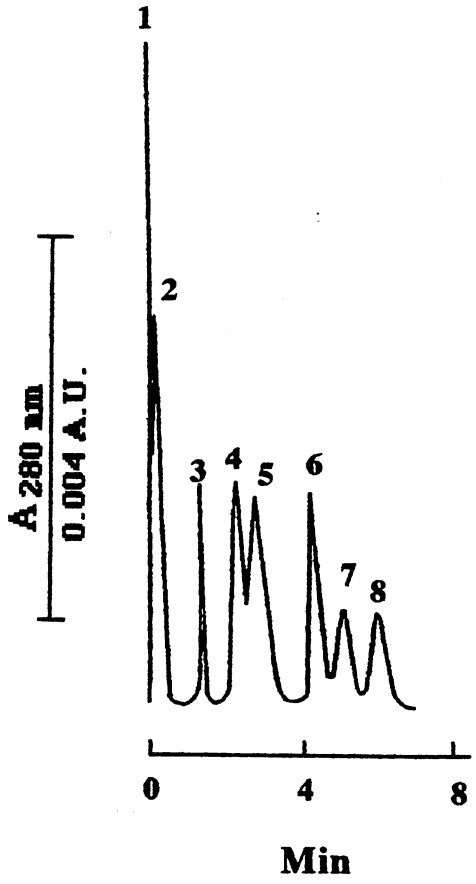


Figure 13. Rapid Separation of Proteins on Non-porous Cu(II)-IDA—Silica Column. Column, 30 x 4.6 mm I.D.; flow-rate, 3.0 mL/min. Two consecutive linear gradients: 3.0 min from 0 to 1.0 *M* NaCl in 10 m*M* phosphate, pH 6.0 and 3.0 min from 0 to 50 m*M* imidazole in 10 m*M* phosphate containing 1.0 *M* NaCl, pH 6.0. Proteins: 1, β-lactoglobulin A; 2, α-lactalbumin; 3, α-chymotrypsinogen A; 4, cytochrome c; 5, lysozyme; 6, lactoferrin; 7, iron-free transferrin; 8, carbonic anhydrase.

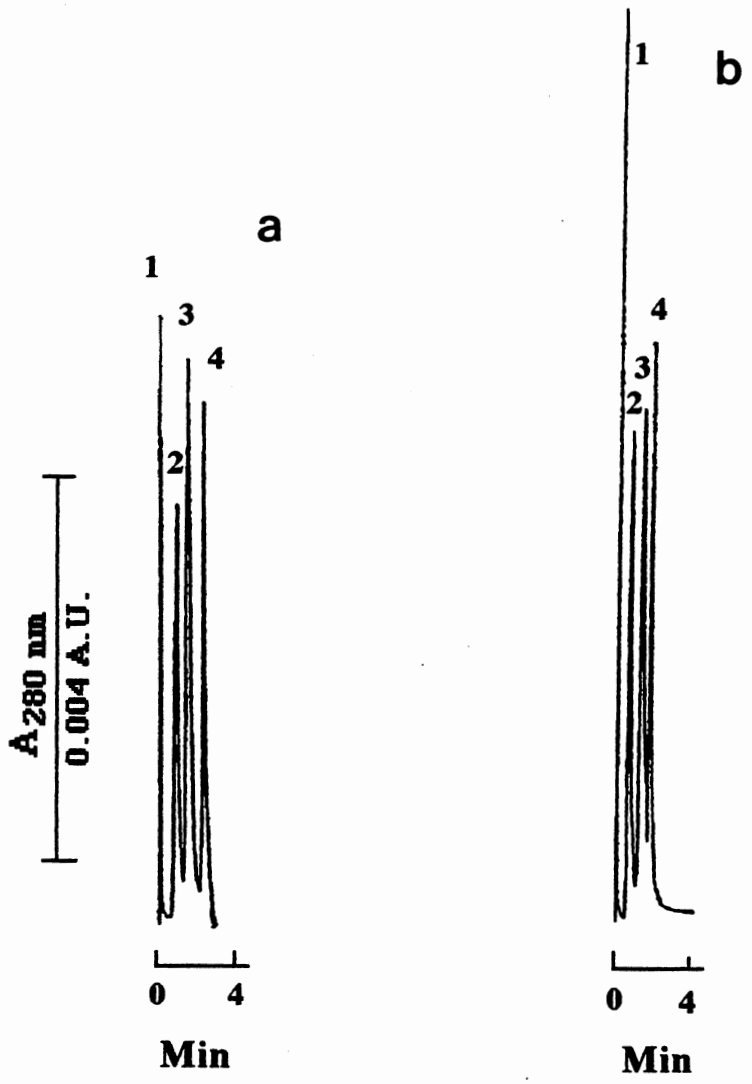


Figure 14. Rapid Separation of Proteins on Non-porous Zn(II)-IDA—Silica Column. (a) two consecutive linear gradients: 2.0 min from 0 to 1.0 *M* NaCl in 10 m*M* phosphate, pH 6.0 and 1.0 min from 0 to 50 mM imidazole in 10 m*M* phosphate containing 1.0 *M* NaCl; flow-rate, 4.0 mL/min and (b) two consecutive linear gradients: 1.5 min from 0 to 1.0 *M* NaCl in 10 m*M* phosphate, pH 6.0 and 0.5 min from 0 to 50 m*M* imidazole in 10 m*M* phosphate containing 1.0 *M* NaCl, pH 6.0; flow-rate, 6.0 mL/min. Column, 30 x 4.6 mm I.D. Proteins: 1, β-lactoglobulin A; 2, carboxypeptidase A; 3, lysozyme; and 4, iron-free transferrin.

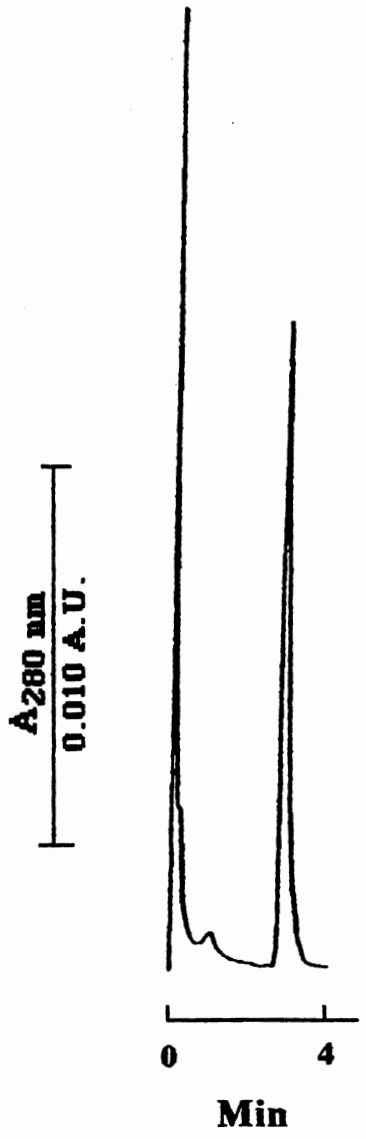


Figure 15. Rapid Separation of Commercial Lipoxidase on Non-porous Zn(II)-IDA—Silica Column.
Column, 30 x 4.6 mm I.D.; flow-rate, 4.0 mL/min. Two consecutive linear gradients: 2.0 min from 0 to 1.0 *M* NaCl in 10 m*M* phosphate, pH 6.0 and 0.1 min from 0 to 50 m*M* imidazole in 10 m*M* phosphate containing 1.0 *M* NaCl, pH 6.0, and isocratic elution for 1.0 min at 50 m*M* imidazole in 10 m*M* phosphate containing 1.0 *M* NaCl in 10 m*M* phosphate, pH 6.0.

column toward the different isoforms of lipoxidase when the sodium chloride-imidazole consecutive linear gradients was employed.

It has to be noted that unlike with SDS, the chelated metal leached slowly from the column when imidazole was used. This necessitated the reloading of the column with the metal after prolonged use. However, these elution schemes represent selective elution of widely differing proteins and are expected to find general use in the field of rapid metal interaction chromatography of proteins. In general, multiple gradient elution has the virtue of increasing peak capacity and therefore allowing the rapid separation of a wider range of proteins.

Conclusions

Rigid non-porous microparticulate siliceous stationary phases with surface-bound metal chelating functions, EDDA or IDA, were developed for rapid high performance metal interaction chromatography of proteins. The non-porous metal chelate sorbents allowed rapid separation on the time scale of seconds and minutes instead of minutes and hours; exhibited rapid sorption kinetics since the stationary phase ligands are totally exposed and homogeneously distributed on the surface; yielded high separation efficiencies at elevated flow velocities due to the absence of stagnant mobile phase intraparticle diffusional mass transfer resistances; and permitted rapid column equilibration. In addition, the development of a multiple gradient system resulted in a higher column peak capacity, thereby enabling the separation of greater number of proteins in a single chromatographic run.

References

1. Stober, W.; Fink, A.; Bohn, E. J. *J. Colloid Interface Sci.* **1968**, *26*, 62.

2. Tan, C. G.; Bowen, B. D.; Epstein, N. *J. Colloid Interface Sci.* **1987**, *118*, 290.

3. Bogush, G. H.; Tracy, M. A.; Zukoski IV, C. F. *J. Non-Cryst. Solids* **1988**, *104*, 95.

4. El Rassi, Z.; Horvath, C. in *HPLC of Biological Macromolecules*, Gooding, K.; Regnier, F., Eds., Marcel Dekker, Inc.: New York, **1990**, 179.

5. El Rassi, Z.; Horvath, C. *J. Chormatogr.* **1986**, *359*, 241.

6. Rijken, D. C.; Wijngaards, G.; Zall-de Jong, M.; Welbergen, J. *Biochim. Biophys. Acta* **1979**, *850*, 140.

7. Porath, J.; Olin, B. *Biochemistry* **1983**, *22*, 1621.

8. Muzynska, G.; Andersson, L.; Porath, J. *Biochemistry* **1986**, *26*, 6850.

CHAPTER IV

PREPARATION AND CHARACTERIZATION OF HIGHLY CROSSLINKED POLY(STYRENE-DIVINYLBENZENE) MICROPARTICLES

Introduction

Silica-based stationary phases have been the most popular adsorbents in high performance liquid chromatography due to their excellent mechanical strength and nearly perfect pore and particle size distributions. However, silica packing materials have been found to be unstable in alkaline solutions, and the Si-O-Si bond anchoring the stationary phase ligands to the support cleaves in acidic media.[1] These limitations make the silica columns inadequate for certain separations. As a consequence, the development of new packing materials have been the subject of many recent studies.[2-6]

Ever since Sober and Peterson[7] introduced diethylaminoethyl (DEAE) derivatized cellulose as polymeric anion-exchange sorbents for the liquid chromatography of proteins, several organic sorbents such as carboxymethyl (CM) cellulose derivative,[8] highly cross-linked polydextran gel,[9] polysaccharide and polysaccharide-acrylamide[10] have been also developed. Although all of these materials are very useful for the preparative fractionations of biomacromolecules in traditional low pressure liquid chromatography, they were not suitable for high performance liquid chromatography where high pressures are usually involved. In addition to their poor mechanical stability, the problems of non-specific interactions, i.e., ionic-exchange and/or hydrophobic interactions have always been encountered.[11, 12]

Early attempts with poly(styrene-divinylbenzene) supports as alternatives to silica gels failed because of the lack of rigidity and concomitant swelling and shrinking. For this reason, a number of studies have focused on improving the mechanical strength and solvent compatibility of these supports by introducing highly crosslinked particles.[13-17]

Highly crosslinked polymeric particles can be conveniently prepared by employing one of the many available polymerization processes. The traditional method used to prepare polymeric particles is suspension polymerization.[18] However, this procedure yields a broad particle size distribution because of the inherent size distribution in the mechanical homogenization step. These particles, if used as chromatographic stationary phases will give poor performance.

Another approach to preparing polymeric particles is based on dispersion polymerization. It is a very simple and yet an efficient method of preparing micron size particles.[19]

Emulsion polymerization is another method being used in the preparation of polymeric particles. However, this type of polymerization usually produces submicron particles with a rather broad size distribution.[20] Ugelstad and co-workers[21] developed an improved method for the preparation of monodisperse particles through emulsion polymerization. It involves two-step swelling polymerization and is applicable for a wide range of monomers and monomer binary mixtures. This process was reported to yield polymeric particles which are much bigger than those obtained from an ordinary emulsion polymerization.

In all these processes, either porous or non-porous particles can be produced. For the HPLC of biomolecules, it is well documented that the support materials must be monodispersed and possess relatively wide pores (> 300 Å) and small particle sizes in order to allow fast mass transfer in and out of the pores. It is even more ideal to have totally exposed surface for the rapid equilibration of the solute between the mobile and

stationary phases. Hence, the introduction of non-porous packing materials made considerable contributions in improving the separation efficiencies at high flow velocities.[22-24] One of the apparent drawbacks, however, of non-porous beads is the reduced surface area which leads to lower sample capacity. To alleviate this problem, the particle size of these materials must be decreased to the 1-2 μm level.[25, 26]

The goal of this study is to prepare non-porous spherical poly(styrene-divinylbenzene) microparticles which will exhibit the mechanical strength of silica gel and an excellent chemical stability over a wide range of pH in order to achieve chromatographic separations with high performance.

Experimental

Instrumentation

The liquid chromatograph was assembled from an ISCO (Lincoln, NE, U.S.A.) Model 2350 solvent delivery pump and a Model 2360 gradient programmer with a variable wavelength detector Model V[4]. A Rheodyne (Cotati, CA, U.S.A.) Model 7010 sampling valve with a 20-μL sample loop was used for injection. Chromatograms were recorded with a computing integrator Model C-R5A from Shimadzu (Columbia, MD, U.S.A.).

Reagents and Materials

Reagent grade styrene, 1,2-dichloroethane, acetone, acetonitrile and ethanol were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Divinylbenzene was a gift from Dow Chemical Company (Midland, MI, U.S.A.). Hydroxypropyl cellulose, methyl cellosolve (2-methoxyethanol), benzoyl peroxide and potassium thiosulfate, were from Aldrich (Milwaukee, WI, U.S.A.). Prepurified nitrogen gas was from Union Carbide Corp. (Somerset, NJ, U.S.A.). Reagent grade 1-bromodecane was purchased from Alfa Products (Danvers, MA, U.S.A.). PLRP-S, a spherical poly(styrene-divinylbenzene)

copolymers with 300 Å and 15-20 μm mean pore and particle diameters, respectively, was from Polymer Laboratories Limited (Amherst, MA, U.S.A.).

Columns

A slurry of poly(styrene-divinylbenzene) (PSDVB) particles was prepared and packed at 6000 p.s.i. with a 50:50 (v/v) solution of acetonitrile-deionized water and using a Shandon column packer instrument (Keystone Scientific, Bellefonte, PA, U.S.A.). All columns were No. 316 stainless steel tubes obtained from Alltech Assoc. (Deerfield, IL, U.S.A.) of dimensions 30 x 4.6 mm I.D.

Polymerization by Dispersion Method

The procedure developed by Lok and Ober,[27] which was originally described for the polymerization of styrene, was adapted for the preparation of poly(styrene-divinylbenzene) copolymers. In a three-necked round bottom flask containing 96.8 g ethanol and 73.2 g methyl cellosolve, 3.0 g hydroxypropyl cellulose were dissolved. This reaction mixture was stirred for 30 minutes at 65°C under nitrogen atmosphere. Then, a solution containing 1.2 g benzoyl peroxide, 21 mL styrene and 9 mL divinylbenzene was added. After two hours, the temperature was raised to 75-80°C and allowed to react for 22-24 hours. The mixture was cooled and centrifuged. After washing the polymers with ethanol, they were redispersed in water and later freeze dried using a Labconco freeze dryer (Kansas City, MO, U.S.A.) Model Lyph-Lock 6.

Polymerization by Two-Step Swelling Method

The two-step swelling polymerization method developed by Ugelstad[21] was adapted for the synthesis of poly(styrene-divinylbenzene) microparticles. The first step was the preparation of the monodispersed seed latex. This was carried out by dissolving

72 mg of potassium persulfate ($K_2S_2O_8$) in 92 mL of deionized water. Then 10 mL solution of styrene:divinylbenzene was added. Instead of using a high pressure homogenizer, the reaction mixture was homogenized at 5000 rpm for 90 seconds using a Brinkmann homogenizing unit (Westbury, NY, U.S.A.). Finally, the solution was stirred at room temperature for several hours. In a separate reaction vessel, an emulsion of 1-bromodecane was prepared as follows: 8.0 mL of 1-bromodecane were added to 46 mL of deionized water containing 1.15 g of sodium dodecyl sulfate. To this emulsion 1.15 g of benzoyl peroxide dissolved in 5.75 mL 1,2-dichloroethane were added. Following, the reaction mixture was homogenized.

The monodispersed seed particles which were prepared in the first step were then swollen with the 1-bromodecane emulsion by mixing 30 mL of the monodispersed seed latex with 60 mL of 1-bromodecane emulsion. Acetone in the amount of 7.4 mL was added to help in facilitating the transport of the emulsion. The solution was again homogenized and stirred at 35°C for 10 hours.

In a second polymerization reaction, the previously swollen particles were further swollen with the monomer as follows: to a homogenized mixture of 200 mL of deionized water and 0.3 g of SDS, 20 mL of styrene:divinylbenzene and 30 mL of the swollen particles were added. The solution was again homogenized and allowed to polymerize for 22 hours at 60°C.

Specific Surface Area, S_{BET}, of PSDVB-3

The specific surface area of the poly(styrene-divinylbenzene) microparticles prepared from 60:40 styrene:divinylbenzene polymerization reaction (denoted PSDVB-3 throughout the remainder of this dissertation) was measured at Leeds and Northrup (St. Petersburg, FL, U.S.A.) using the nitrogen adsorption method. The S_{BET} of the product was found to be 0.35 m^2/g. This surface area is an indication of the non-porosity of the

material and is in accordance with the literature values reported for non-porous materials of similar particle size;[28] see below for more details.

Results and Discussion

Synthesis of Poly(styrene-divinylbenzene) by Dispersion Polymerization

Since the early stages of the development of HPLC, the major goal has been to reduce the particle size of the stationary phases to below 5 μm to favor rapid mass transfers and consequently increase the separation efficiencies. In this regard, our goal was to produce rigid and highly-crosslinked poly(styrene-divinylbenzene) copolymers of less than 5 μm mean particle diameter. First, we adapted the dispersion polymerization introduced by Ober and Lok[27] for the synthesis of microspherical polystyrene particles.

Returning to the procedure of Ober and Lok[27] outlined in the experimental section in which we have kept their original binary solvent mixture (methyl cellosolve-ethanol), stabilizer (hydroxypropyl cellulose), initiator (benzoyl peroxide), and polymerization temperature (75°C), the reaction mixture did not lead to the desired polymerization. This is not unexpected since in addition to the styrene monomer we also have used the divinylbenzene as crosslinking agent. Therefore, we have explored the possibility of higher polymerization temperature while keeping all the other conditions the same. Indeed, at 80°C (5°C higher than the original polymerization temperature utilized by Ober and Lok[27]), polymerization occurred as denoted by the appearance of an opaque-colored reaction mixture. Although, increasing the reaction temperature was reported to increase the particle sizes,[29] the polymer produced was less than 1 μm as revealed by scanning electron microscopy.

Because of these findings, we have attempted to increase the polymerization time from 22 hours to 24 hours at 80°C. Under these new conditions, the polymers precipitated into very large particles which were not useful for our purposes.

Since increasing both the reaction time and temperature failed to produce the desired polymer, we have undertaken the possibility of using the seeds produced in the reaction for 22 hours to grow them into larger particles. Using 75°C and 50 hours polymerization temperature and time, respectively, but keeping all other conditions the same, yellowish plastic-like coagulates were formed. In another attempt, the polymerization temperature was decreased by 15°C but keeping other conditions the same. The resulting products were too big for our studies. By using the same polymerization temperature (60°C) but decreasing the polymerization time from 50 to 24 hours, polymers of a very broad particle size distribution were obtained.

This procedure which yielded either too small or too big particles, plastic-like coagulates and particles of very broad particle size distribution was abandoned. Instead, the two-step swelling polymerization method which will be discussed next was used as an alternative for the preparation of the desired materials.

Synthesis of Poly(styrene-divinylbenzene) Copolymers by Two-Step Swelling Polymerization

Unlike the above method, the two-step polymerization method described in the experimental part yielded rigid poly(styrene-divinylbenzene) copolymers in the particle size range desired. This is because the two-step swelling polymerization carried out here and originally described by Ugelstad and co-workers in 1979,[21] is a seeded growth polymerization process of styrene and divinylbenzene in an oil droplet whose size decides the particle size of the poly(styrene-divinylbenzene) product. As can be noticed from the experimental section, the first step was the swelling of the monodispersed submicron particles prepared by ordinary emulsion polymerization with an insoluble compound (1-bromodecane) containing an oil soluble initiator (benzoyl peroxide). This was followed by the addition of water, emulsifier and monomers (styrene and divinylbenzene) and the seed particles were allowed to swell with the monomers. This second swelling gave

monodisperse oil droplets with the same amount of initiator in each droplet and the subsequent polymerization led to quasi-monodispersed particles. Furthermore, the use of oil soluble initiator reduced drastically the tendency of formation of new particles in the aqueous phase.

Figures 16, 17 and 18 portray the scanning electron micrographs (SEM) of the poly(styrene-divinylbenzene) microparticles at various degrees of crosslinking in order to find the best composition for high rigidities. In the following discussion, PSDVB-1, PSDVB-2 and PSDVB-3 denote the copolymers at 30%, 35% and 40% divinylbenzene, respectively.

As can be in Figures 16, 17 and 18, by adapting the above method spherical microparticles could be obtained in relatively high yield. The particle size ranged from 0.5-4.0, 3.9-4.2 and 2.5-3.9 μm for PSDVB-1, PSDVB-2 and PSDVB-3, respectively. These results are in good agreement with those obtained by Ugelstad and co-workers,[21] who have demonstrated that polymers with particle sizes in the range of 2-20 μm could be easily produced. The relatively large particle size of the polymer is due to the fact that the seed particles produced by the traditional emulsion polymerization[20] were further swollen with a water-insoluble compound (1-bromodecane, in our case) which resulted in oil droplets containing much larger amount of monomers than ordinary particles which were not previously swollen.

Besides controlling the reaction conditions as described in the experimental part to produce particles with high solid content, an important step in the polymerization reaction is the production of homogeneous droplets that will lead to monodispersed particles. This is usually achieved by using a high pressure homogenizer. In our studies we have utilized an ordinary low pressure homogenizer. Simple comparison of the scanning electron micrographs of Figures 17 and 18 with that of Figure 16 reveals the importance of the homogenization step. The particles shown in Figure 16 were obtained by omitting the

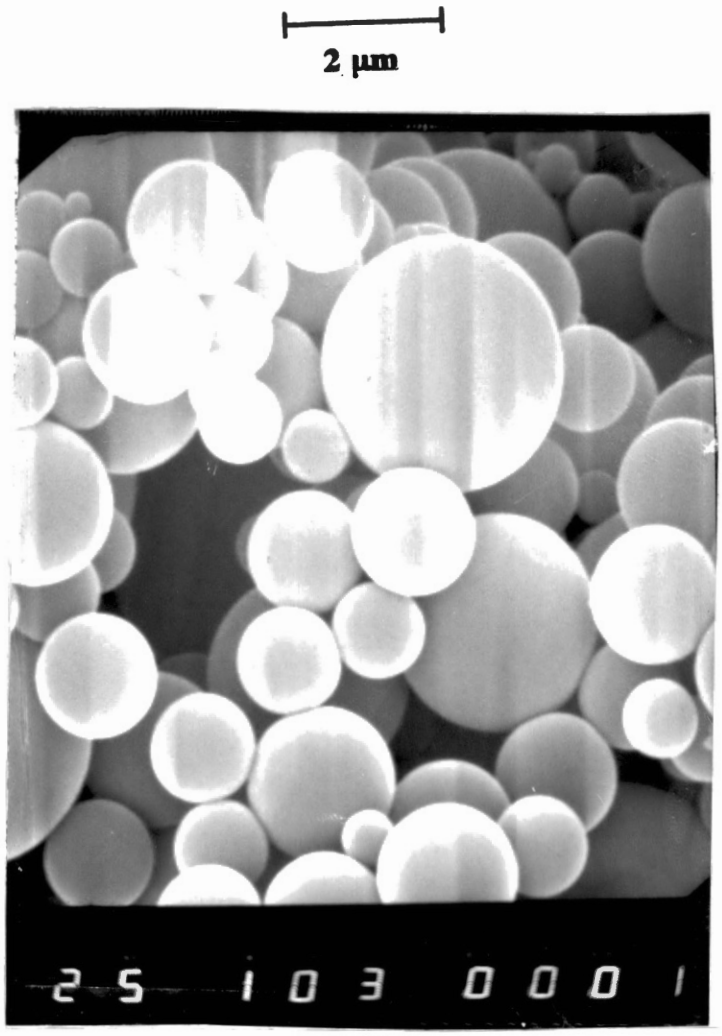


Figure 16. Scanning Electron Micrograph (SEM) of PSDVB-1.
PSDVB-1 was prepared from 70:30 (v/v) styrene:divinylbenzene using two-step swelling polymerization. Homogenization step was omitted.

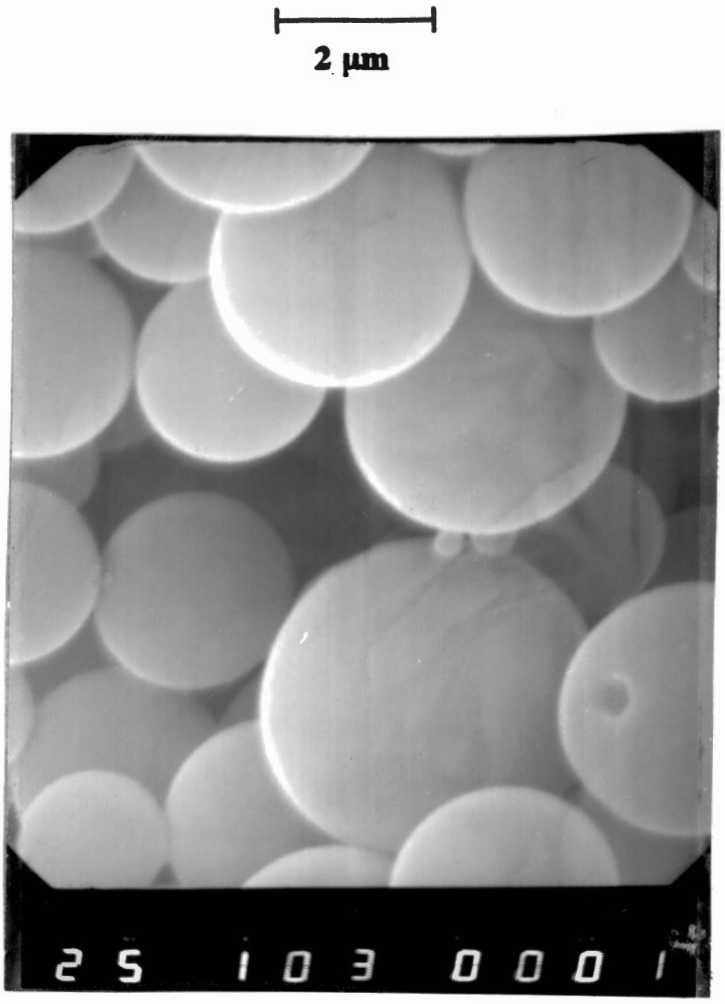


Figure 17. Scanning Electron Micrograph (SEM) of PSDVB-2.
PSDVB-2 was prepared from 65:35 (v/v) styrene:divinylbenzene using two-step swelling polymerization.

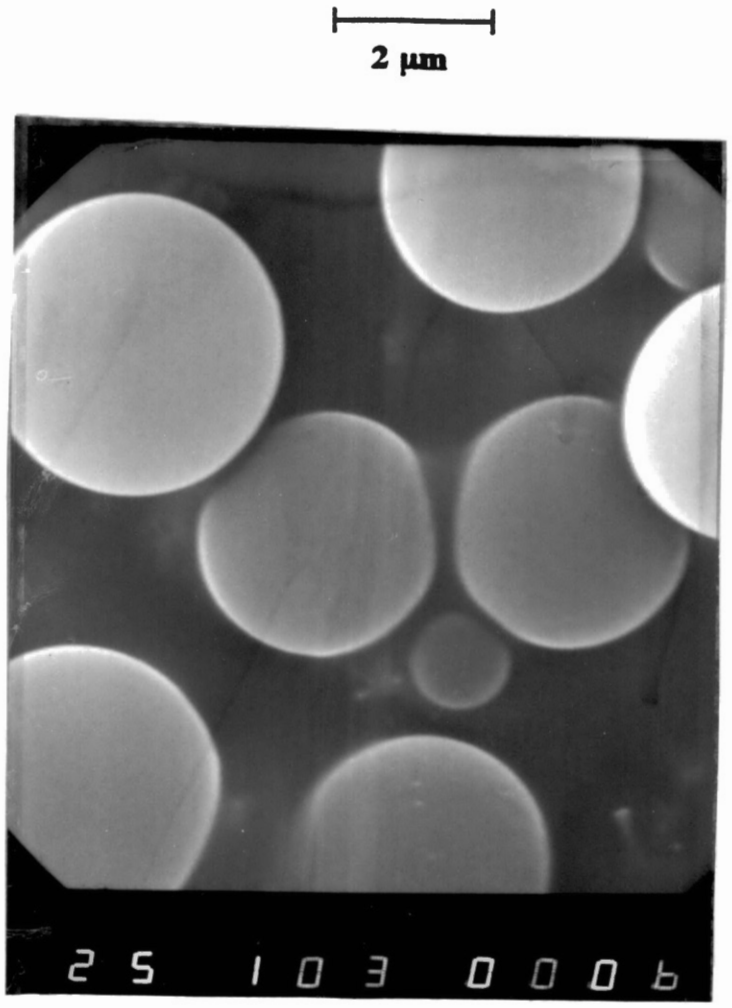


Figure 18. Scanning Electron Micrograph (SEM) of PSDVB-3.
PSDVB-3 was prepared from 60:40 (v/v) styrene:divinylbenzene using two-step swelling polymerization.

homogenization step. It is clear that the particle size distribution is rather broad. It seems wise to say that low pressure homogenizer used in our study is a good substitute for an expensive high pressure homogenizing unit. Indeed, a simple inspection of Figures 17 and 18 shows relatively narrow size distribution.

Mechanical Stability

Before conducting any type of modern chromatography on these packing materials, it was necessary to examine their mechanical strength at high flow-rates customarily used in HPLC. The mechanical stability of the microspherical PSDVB were monitored through the dependence of column pressure on eluent flow-rate. The PSDVB microparticles at various degrees of crosslinking were packed in short columns (see experimental part) and evaluated with different eluents at various flow velocities.

The PSDVB-1 which is 30% crosslinked did not withstand pressure and was not amenable to packing into HPLC columns. This illustrates its weak mechanical strength and the necessity of higher degree of crosslinking. In fact, both PSDVB-2 and PSDVB-3 of higher degree of crosslinking could be packed without difficulty even at 4000-6000 psi. By operating the PSDVB-2 columns with pure organic solvent such as acetonitrile, the packing underwent tremendous swelling as indicated by the occurrence of a very high pressure even at a very low flow-rate. However, when these packing materials were evaluated with aqueous solvents that are normally used in high performance metal interaction chromatography and non-denaturing biopolymer HPLC, i.e., without organic solvent, the column was found to be extremely stable as shown in Figure 19. As can be seen, these materials are stable in pure water and in 10 m*M* phosphate solution at pH 6.0 containing 1.0 *M* NaCl over a wide range of flow-rates.

Similarly, the mechanical strength of the PSDVB-3 was also evaluated in terms of pressure drop *versus* mobile phase flow-rates. The results are shown in Figure 20.

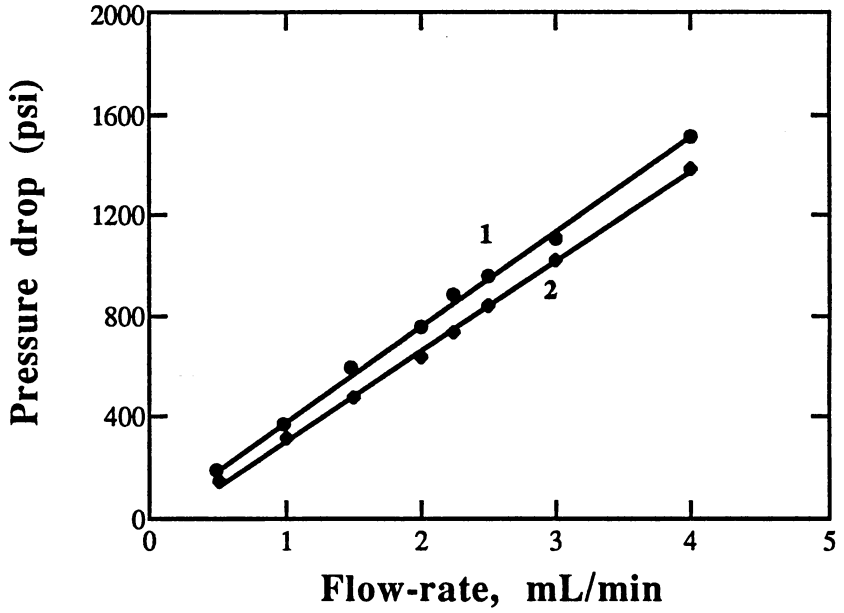


Figure 19. Plots of Pressure Drop Obtained on PSDVB-2 *versus* Flow-rate. Column, 30 x 4.6 mm I.D. Mobile phases: (1) 10 m*M* phosphate containing 1.0 *M* NaCl, pH 6.0 and (2) deionized water; temp., 25°C.

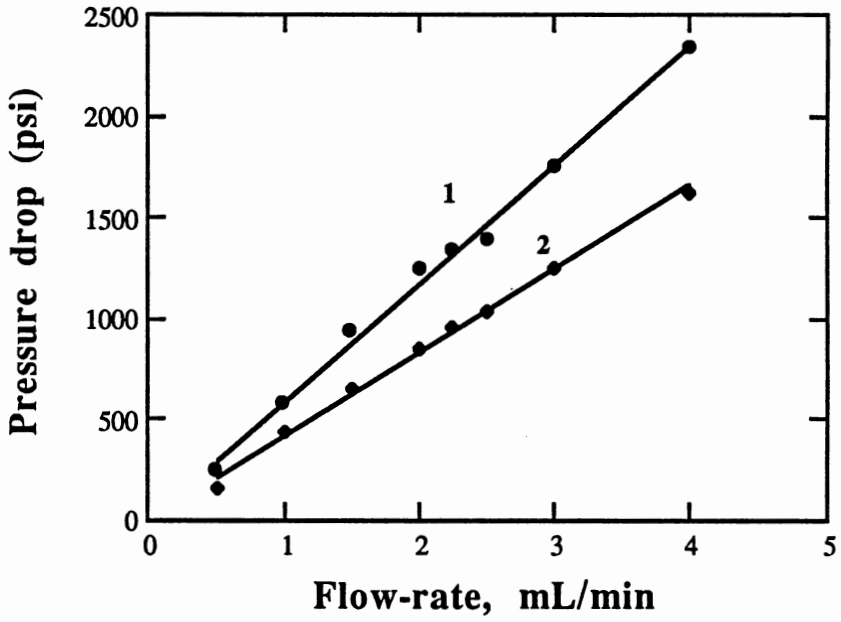


Figure 20. Plots of Pressure Drop Obtained on PSDVB-3 *versus* Flow-rate. Column, 30 x 4.6 mm I.D. Mobile phases: (1) acetonitrile and (2) deionized water; temp., 25°C.

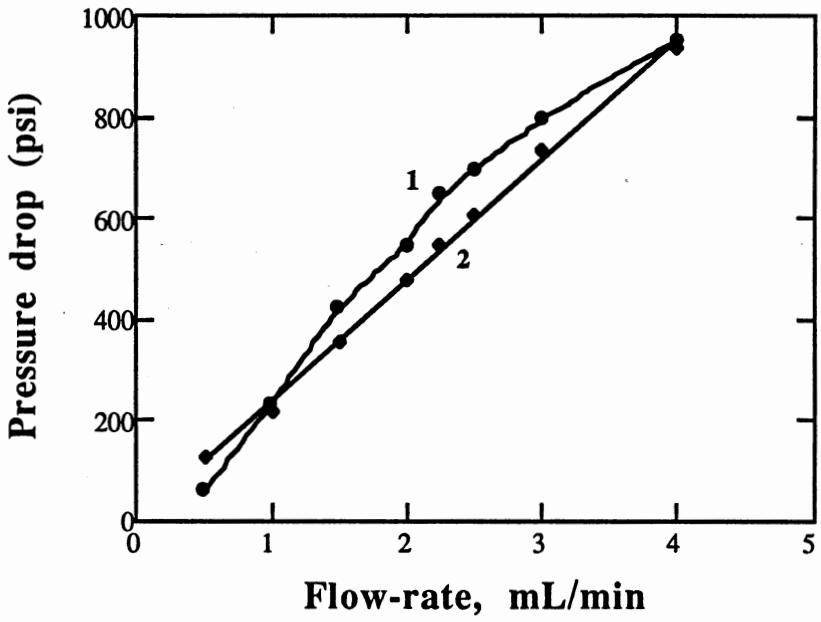


Figure 21. Plots of Pressure Drop Obtained on PLRP *versus* Flow-rate. Column, 30 x 4.6 mm I.D. Mobile phases: (1) acetonitrile and (2) deionized water; temp., 25°C.

Straight lines were obtained over the flow rate range studied with both pure water and organic solvents. These linear curves are an indication of the excellent mechanical stability of these packing materials.

To further assess the mechanical strength of the packing materials developed in our laboratory, similar experiments were performed on the commercially available PLRP 300Å. Plots of the pressure drop at various flow-rates using water and acetonitrile are shown in Figure 21. Using water as a solvent, a linear curve was obtained. However, with acetonitrile, a non-linear curve resulted . This indicates the swelling of this polymeric packing materials in the presence of organic solvents. This may be due to its porous network that is more freely accessible to the solvent than the non-porous matrices developed in this study.

The different rigidities exhibited by the polymeric supports with various degrees of crosslinking were in agreement with the reported results,[30] that is, a high degree of crosslinking is necessary for good mechanical stability. A low degree of crosslinking leads to substantial swelling and to collapse of the macroreticular network.

Conclusions

Highly crosslinked poly(styrene-divinylbenzene) microparticles were prepared *via* a modified two-step swelling polymerization. The crosslinking of styrene monomer with divinylbenzene monomer improved the mechanical strength of the resulting copolymers. Based on the specific surface area measurement of the PSDVB developed in this study, the supports can be classified as non-porous column packing materials. As will be shown in the following chapters, the surface of these organic supports can be modified with suitable functionalities to yield rigid stationary phases for the rapid HPLC of biomacromolecules.

References

1. Unger. K.K. in *HPLC of Biological Macromolecules*; Gooding, K. M.; Regnier, F. E., Eds., Marcel Dekker, Inc.: New York, **1990**, 3.

2. Wongyai, S.; Varga, J. M.; Bonn, G. K. *J. Chromatogr.* **1991**, *536*, 155.

3. Mikes, O.; Coupek, J. in *HPLC of Biological Macromolecules*; Gooding, K.; Regnier, F., Eds., Marcel Dekker, Inc.: New York, **1990**, 25.

4. Kato, J.; Komiya, K.; Sasaki, H.; Hashimoto, T. *J. Chromatogr.* **1980**, *193*, 311.

5. Germershausen, J.; Karsas, J. D. *Biochem. Biophys. Res. Commun.* **1981**, *99*, 1020.

6. Wada, H.; Makino, K.; Takeuchi, T.; Hirouki, H.; Noguchi, K. *J. Chromatogr.* **1985**, *320*, 369.

7. Sober, H. A.; Peterson, E. A. *J. Am. Chem. Soc.* **1954**, *76*, 1711.

8. Peterson, E. A. *Cellulosic Ion- Exchanger*; Elsevier: Amsterdam, **1970**, Chapter 2.

9. Porath, J.; Flodi, P. *Nature (London)* **1959**, *183*, 1657.

10. Wilchek, M.; Miron, T.; Kohn, J. in Jakoby, W. B., Ed., *Methods in Enzym.* **1984**, *104*, 3.

11. Kohn, J.; Wilchek, M. *Enzyme Microbiol. Technol.* **1982**, *4*, 161.

12. Wilchek, M.; Miron, T. *Biochem. Biophys. Res. Commun.* **1976**, *72*, 108.

13. Lee, D. P.; Kindsvater, J. H. *Anal. Chem.* **1980**, *52*, 2425.

14. Smith, R. M. *J. Chromatogr.* **1984**, *291*, 372.

15. Beinert, G.; Chaumont, P.; Rempp, P.; Herz, J. *Eur. Polym. J.* **1984**, *20*, 837.

16. Heffernan, J. G.; Sherrington, D. C. *J. Appl. Polym. Sci.* **1984**, *29*, 3013.

17. Benson, J. R.; Woo, D. J., *J. Chromatogr. Sci.* **1984**, *22*, 386.

18. Almog, Y.; Levy, M. *J. Polym. Sci. Polym. Chem. Ed.* **1982**, *20*, 417.

19. Barrett, K. E. *Dispersion Polymerization in Organic Media*; Wiley: London, **1975**, Chapter 1.

20. Ugelstad, J.; Mork, P. C. *Adv. Colloid and Interface Sci.*, **1980**, *13*, 101.

21. Ugelstad, J.; Kaggerud, K. H.; Hansen, F. K.; Berge, A. *Makromol. Chem.* **1979**, *180*, 737.

22. Burke, D. J.; Duncan, J. K.; Siebert, C.; Ott, G. S. *J. Chromatogr.* **1986**, *359*, 533.

23. Duncan, J. K., Chen, A.; Siebert, C. J. *J. Chromatogr.* **1987**, *397*, 3.

24. Kato, Y.; Kitamura, T.; Mitsin, A.; Hashimoto, T. *J. Chromatogr.* **1987**, *398*, 327.

25. Unger, K. K.; Jilge, G., Kinkel, J. N.; Hearn, M. T. W. *J. Chromatogr.* **1986**, *359*, 61.

26. Dewaele, C.; Verzele, M. *J. Chromatogr.* **1983**, *282*, 341.

27. Lok, K.; Ober, C. *Can. J. Chem.* **1985**, *63*, 209.

28. Unger, K. K.; Jilge, G.; Janzen, R.; Giesche, H. *Chromatographia* **1985**, *22*, 379.

29. Ober, C.; Hair, M. *J. Polym. Sci. Part A: Polym. Chem.* **1987**, *25*, 1395.

30. Nevejans, F.; Verzele, M. *J. Chromatogr.* **1987**, *406*, 325.

CHAPTER V

HYDROPHILIC COATINGS OF RIGID POLY(STYRENE-DIVINYLBENZENE) MICROPARTICLES

Introduction

Several organic supports are currently utilized in HPLC of biopolymers.[1-3] They can be classified into two main categories, hydrophilic and hydrophobic supports. The most hydrophilic supports are the polysaccharide types. Although these supports were recently developed in a form suitable for HPLC,[4,5] these new materials did not achieve the hardness of other synthetic copolymers such as polymethacrylate-based hydrophilic supports,[6] hydroxylated polyether copolymers,[7] hydroxylated polyvinyl polymers[8] and polyvinyl alcohols.[9] In general, hydrophilic copolymeric supports have been less mechanically stable than their hydrophobic counterparts which are essentially poly(styrene-divinylbenzene). In fact, poly(styrene-divinylbenzene)-based stationary phases being the most rigid organic copolymers have found wide use in HPLC.[10-15] Their most extensive applications have been in size exclusion chromatography (SEC) of non-polar species[16, 17] and reversed-phase liquid chromatography (RPC) of polar and non-polar compounds including biopolymers.[18-21] However, the potentials of PSDVB as chromatographic supports for the separation of proteins under non-denaturing conditions such as ion-exchange and affinity chromatography have only been briefly explored[21-25] due to their strong hydrophobic backbone, which if not covered well by a hydrophilic spacer would give rise to low recovery and denaturation of the protein analyte.

The functionalization of organic polymeric supports can be carried out either by

direct copolymerization of monomers already carrying the desired functionalities or by subsequent modifications of the polymer beads.[26, 27] In the former approach, since polymerization occurs by a random process the amount of the functional groups may vary from one batch to another. Therefore, subsequent modifications of the support is more attractive because it allows a better control of the amount of functional groups available for further modifications. The subsequent surface modifications of the polymeric supports can be carried out by either physical adsorption or by a chemical reaction that links the desired ligand to the matrix of the polymers. However, covalent surface modification is far superior and should be used whenever possible.

Chemical modification of the polystyrene supports is rather tedious and entails carefully chosen reaction conditions. Several chemical reactions have been reported for the functionalization of polystyrene-based materials.[27-30] Among these reactions, chloromethylation and sulfonation have been particularly useful in preparing strong ion-exchange resins for small molecules including amino acids, small peptides, constituents of nucleic acids, and sugars. However, these stationary phases are not suitable for the separations of proteins because their largely uncovered hydrophobic matrix can cause denaturation. Therefore, the addition of hydrophilic functionalities which can shield the polymeric backbone from the solutes is necessary prior to the attachment of the interactive ligands. Various types of hydrophilic functionalities can be readily attached to polymeric supports when the surfaces of which are rich in hydroxylic or amino groups.[31] Alcoholic functionalities are, however, preferred because they would not introduce non-specific interactions with the separated analytes.

Thus far, only a few reports have appeared on the hydrophilic coatings of PSDVB. In one approach, the PSDVB was first functionalized with sulfonic groups and thereafter polyethyleneimine coating was adsorbed to the negatively charged surface through ion-pair formation to yield a hydrophilic strong anion exchanger.[10] In another approach, polyvinyl

alcohol was adsorbed to the surface of PSDVB[4, 32] and the hydroxyl groups were utilized for covalent attachment of affinity ligand through bifunctional catalysts. Very recently, a rigid and porous PSDVB with hydrophilic coating was introduced. The proprietary procedure which was not disclosed entails the adsorption of a monomer to the PSDVB resin followed by a polymerization reaction that led to a highly crosslinked hydrophilic coating containing free hydroxyl groups.[23]

In this study, it is our intention to (i) introduce covalently attached hydrophilic functionalities to the rigid poly(styrene-divinylbenzene) microparticles; (ii) to develop and evaluate reaction schemes that involve the least number of steps while providing functional groups for subsequent attachment of affinity ligands; and (iii) to develop stable stationary phases over a wide range of elution conditions having surface-bound metal chelating functions for high performance liquid affinity chromatography of proteins.

Experimental

Instrumentation

The HPLC system used in this study resembles that described in the previous chapter. Likewise, the poly(styrene-divinylbenzene) stationary phases with surface-bound IDA or EDDA were slurry packed using the same procedures and column packer instrument described in Chapter IV. The infrared (IR) spectra of the polymeric samples dispersed in KBr pellets were recorded using a Perkin Elmer Model 681 spectrophotometer (Norwalk, CT, U.S.A.).

Reagents and Materials

In addition to the reagent grade chemicals and materials described in previous chapters, the following chemicals were used. Horse γ-globulin and human liver ferritin were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.), *canavalia ensiformis*

agglutinin (concanavalin A) was from United States Biochemical Corporation (Cleveland, OH, U.S.A.). Reagent grade ethylbenzene, propylbenzene, butylbenzene, pentylbenzene, 1-phenylheptane, 1-phenylnonane, epichlorohydrin, anhydrous aluminum chloride, α,α-dichloromethyl methyl ether, 1,4-butanediol diglycidyl ether, sodium borohydride, sodium cyanoborohydride, glutaraldehyde, infrared grade potassium bromide, polyethylene glycol of M.W. 200 (PEG 200), methyl-α-*D*-mannopyranoside and boron trifluoride etherate were from Aldrich (Milwaukee, WI, U.S A.). Reagent grade *N, N'*-dimethylformamide (DMF), isopropanol and granulated tin were from EM Science (Cherry Hill NJ, U.S.A.). Methylene chloride, benzene, toluene, naphthalene, sodium nitrite and nitrate were from Fisher Scientific (Pittsburgh, PA, U.S.A.). Technical grade polyethylene glycol diglycidyl ether of M.W. 600 (diepoxy PEG 600) was purchased from Polysciences Inc. (Warrington, PA, U.S.A.).

Hydroxylation of Polystyrene Supports *via* Nitration

Commercially available and home made rigid poly(styrene-divinylbenzene) supports were hydroxylated *via* nitration by adapting and modifying reaction schemes which are typically used in free solutions of benzene-containing compounds.[32] This method which involves nitration, amination, diazotization and hydrolysis, has been recently introduced for the surface hydroxylation of rigid PSDVB.[34]

In the nitration step, 3.0 g of the microspherical PSDVB particles were dispersed in 15.0 mL of DMF by sonication. To this mixture, a solution (24.0 mL) of 3:1 (v/v) concentrated nitric acid:sulfuric acid was added in portions. The reaction mixture was stirred for 3 hours at 5-10°C. Thereafter, the temperature was raised to 60°C and the mixture was allowed to react while stirring for 3 hours. The nitrated product was washed with water, 0.1 *N* NaOH, water and finally DMF.

The product thus obtained was dispersed in a three-neck round bottom flask

containing 18.0 mL of DMF while cooling in an ice bath. To this mixture 1.2 g of granulated tin were added. Following, 30.0 mL of concentrated hydrochloric acid were added in portions. The reaction mixture was heated to 90°C for 9 hours. Finally, the amino-PSDVB was washed with water, 0.1 *N* NaOH and water.

Twenty milliliters of an aqueous suspension of the amino-PSDVB were introduced into a 3-neck round bottom flask containing 72.0 mL of 16 % (v/v) aqueous sulfuric acid. Then, 80.0 mL of cold deionized water were added while stirring which continued for about 15 minutes. Subsequently, a cold solution of 14.0 mL of water and 7.2 g of sodium nitrite were added very slowly. After stirring for another 20 minutes in an ice bath, the temperature was slowly raised to 45°C. After 2 hours the hydroxylated-PSDVB was washed with water.

Hydroxylation of Polystyrene Supports *via* Formylation

Again, slightly modified reaction schemes normally used in free solution for the hydroxylation of benzene rings[35] were employed for the hydroxylation of the polyaromatic rigid supports, i.e., PSDVB

The microspherical PSDVB particles (3.0 g) were washed and suspended in 30 mL of methylene chloride and then cooled to 0°C. To this suspension, 2.25 g of α,α-dichloromethyl methyl ether were added and stirred for about 30 minutes. This was immediately followed by the addition of 2.61 g of anhydrous aluminum chloride. The reaction mixture was heated at 35°C for 12 hours. The aldehyde containing product thus obtained was poured into an ice bath, and was washed with water and isopropanol. This product was then dispersed in 30.0 mL of isopropanol. After adding 2.0 g of sodium borohydride, the reduction reaction was allowed to proceed at room temperature for 2 hours. The hydroxylated product thus obtained was washed with water.

Hydrophilic Coating of Polystyrene Supports with PEG 200 and Attachment of Metal Chelating Ligands

The hydroxylated PSDVB microparticles were first washed with 0.1 *N* NaOH and then dispersed in 3.0 ml of the same NaOH solution. To this suspension, 2.0 mL of 1,4-butanediol diglycidyl ether and 6.0 g of sodium borohydride were added. The reaction mixture was stirred at room temperature for 8 hours.[36] The epoxy-activated support was then washed with water and dioxane and subsequently dispersed in 15.0 mL of dioxane. To this reaction mixture, 3.0 mL of PEG 200 and 300 μL of boron trifluoride etherate were added. The reaction mixture was allowed to react overnight at room temperature with stirring. The PEG 200-modified support was washed with dioxane and then suspended into a solution of 15.0 mL of dioxane, 2.0 mL of epichlorohydrin and 200 μL of boron trifluoride etherate which was maintained at 55°C for 4 hours. To form an epoxy-activated-PEG 200-PSDVB, the product of the reaction with epichlorohydrin was first washed with water and then dispersed in 30.0 mL of 2 *M* sodium carbonate solution.[37] Thereafter, 0.5 g of 1,4-butanediol diglycidyl ether was added 10 minutes prior to the addition of 3.0 g of IDA or EDDA. Then, the reaction mixture was heated at 60°C overnight. The metal-chelate stationary phases thus obtained were washed with water and methanol and finally air dried.

Hydrophilic Coating of Polystyrene Supports with Diepoxy PEG 600 and Attachment of Metal Chelating Ligands and Lectin

After dispersing the hydroxylated microspherical PSDVB particles in 30 mL ofdioxane, 1.5 mL of diepoxy-PEG 600 and 300 μL of boron trifluoride etherate were introduced into the round bottom flask containing the suspension. This mixture was allowed to react at room temperature for 45 minutes. The product was finally washed with dioxane, then water. The chelating ligands were attached to the hydrophilic support as outlined above.

Concanavalin A (Con A), a lectin affinity ligand, was also attached to the surface of the diepoxy PEG 600-activated PSDVB.[38] The epoxy-activated PSDVB (2.0 g) was first suspended in 50 mL of 0.01 *N* HCl then heated to 90°C for one hour to yield a diol-containing surface. The product was washed with distilled water until the pH became neutral. The washed diol-PSDVB was then suspended in 50 mL of 90% (v/v) aqueous acetic acid. To this suspension 1.0 g of sodium periodate was added. After the reaction has been allowed to proceed for 2 hours at room temperature, the aldehyde-activated product thus obtained was washed with 0.1 *M* phosphate buffer, pH 6.0. Thereafter, the aldehyde-activated product was suspended in the same buffer containing 80 mg of Con A, 5 mg methyl-α-*D*-mannopyranoside and 20 mg of sodium cyanoborohydride. The reaction mixture was allowed to react at room temperature for 4 hours and then continued to react at 0°C overnight. The Con A-PSDVB product was first washed with distilled water and then dispersed in 20 mL of 0.1 *M* phosphate buffer, pH 7.0, containing 100 mg of sodium borohydride to scavenge the unreacted aldehyde groups. The Con A-PSDVB was washed thoroughly with distilled water after one hour.

To increase the Con A surface concentration and to ensure that no leakage of lectin will occur in the ensuing chromatographic step, additional Con A was adsorbed to the Con A-PSDVB and thereafter crosslinked with glutaraldehyde. This was done by suspending the Con A-PSDVB in 10 m*M* phosphate solution, pH 7.0, containing 50 mg of Con A. To this suspension, 5 mL glutaraldehyde solution (1% v/v) were added. After allowing the reaction to proceed for 3 hours, the product was first washed with water and then suspended in 0.1 *M* tris buffer to scavenge the remaining aldehyde groups. Finally the crosslinked Con A-PSDVB was washed with water.

Measurement of the Cu(II) uptake

The Cu(II) uptake of the various polystyrene-based metal chelate stationary phases

was determined by frontal chromatography using the HPLC system described in Chapter II. In these experiments, extreme care was undertaken to ensure that the solvent delivery lines were filled with the Cu(II) solution used in the experiment. Thereafter, the column was connected to the HPLC system and the Cu(II) solution was fed continuously through the column until the breakthrough was observed. The absorbance of the effluent was monitored at 280 nm. The void volume of the column was measured by injecting an aqueous solution of sodium nitrate.

Results and Discussion

Hydroxylation of the PSDVB-Based Microparticles

In order to attach a hydrophilic coating on the surface of the rigid poly(styrene-divinylbenzene) microparticles, hydroxyl functional groups were first introduced as described under the Experimental. As outlined above, the hydroxylation of the polymeric supports was carried out using two different methods.

The first method involved three steps, nitration, reduction and diazotization/ hydrolysis as shown in the reaction schemes provided in Fig. 22. As can be seen in Fig. 22, this method involved three well established reactions frequently used in free solution for the functionalization of aromatic materials.[33] The only difference is that the reactions between insoluble materials, i.e., the poly(styrene-divinylbenzene) microparticles, and soluble chemicals are heterogeneous, and are therefore more difficult to control. In addition, the reactions are more sluggish than in free solutions due to the porous structure of the support especially in the case of the commercial PLRP. This porosity slows down the reaction rate because of the presence of mass transfer resistances, i.e., diffusion-controlled reactions.

Due to the multiplicity of the steps in the above surface-modifications, we have

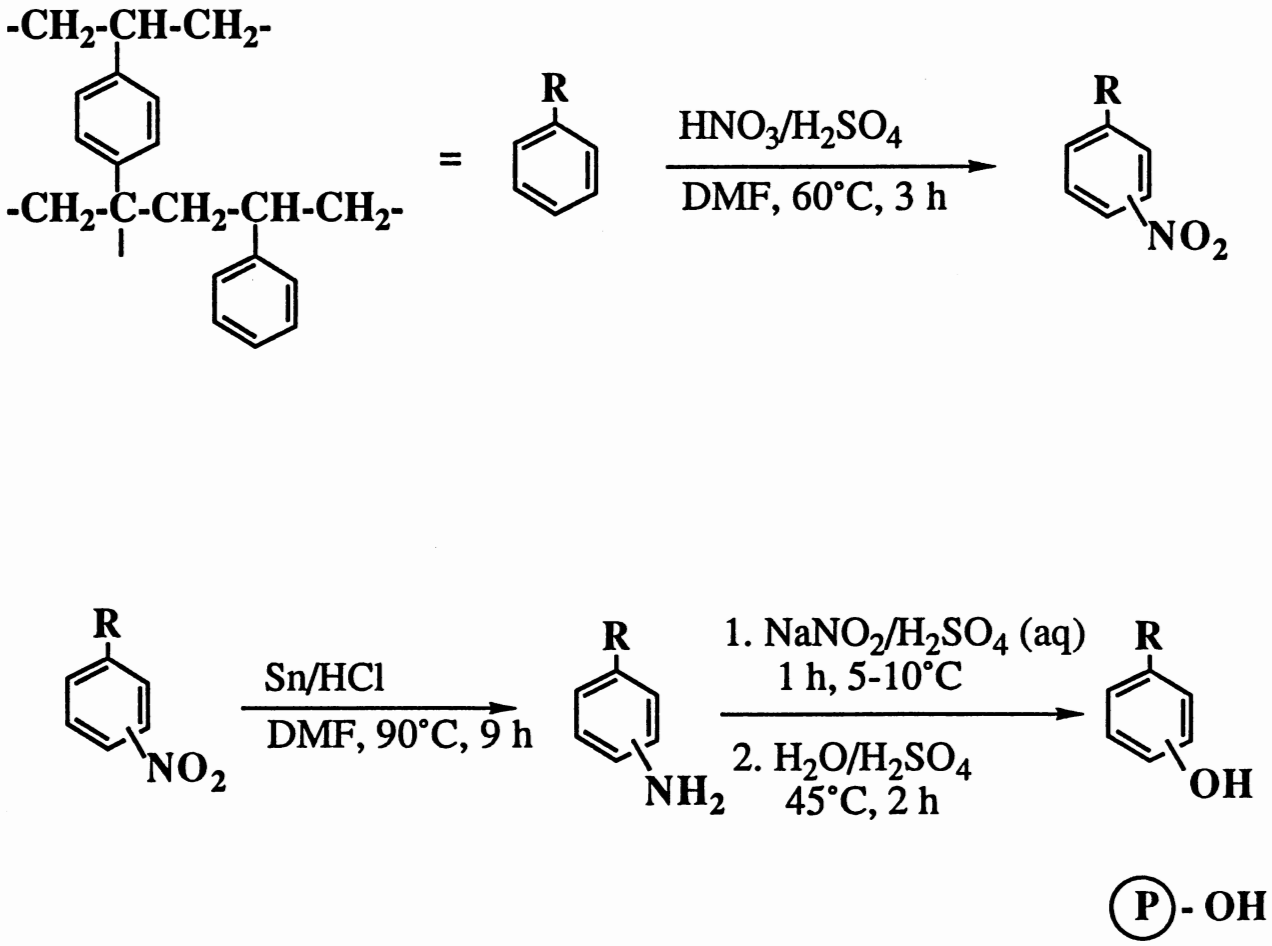


Figure 22. Reaction Schemes of the Hydroxylation of PSDVB *via* Nitration. P, poly(styrene-divinylbenzene) copolymer.

introduced and evaluated a simpler approach for the attachment of hydroxyl groups to the polyaromatic support matrix. As can be seen in Fig. 23, this method is simpler and easier to perform when compared to the nitration method. It involved the formylation of the PSDVB surface and subsequent reduction (see reaction schemes in Fig. 23). The introduction of the formyl groups as substituents for the aromatic ring usually involves the use of Lewis acid and Friedel-Crafts catalysts such as boron trifluoride etherate,[26] stannic chloride,[28] aluminum chloride[35] and zinc chloride.[39] They are all equally efficient catalysts. In our case we used aluminum chloride. The second step involved the reduction of the carbonyl groups into hydroxyl groups using sodium borohydride in the presence of isopropanol. Although sodium borohydride has been utilized as a reducing agent primarily in water or methanol, isopropanol was used instead because it has been reported that the reducing agent reacts with the former solvents thereby complicating the reactions.[40]

Hydrophilic Coatings and Attachment of Ligands

The substitution of the aromatic rings of the PSDVB beads with primary hydroxyl groups is the first step in converting the support into a matrix that would be useful in HPLC of proteins under non-denaturing conditions, i.e., aqueous mobile phases and hydrophilic stationary phases. These primary hydroxyl groups constituted the sites for the covalent attachment of hydrophilic coatings. The hydrophilic layer should be thick enough and water-loving to keep the incoming protein solutes from interacting with the support proper. Having this in mind, we have investigated the potentials of PEG 200 and diepoxy-PEG 600 as the inert spacers. As noticed in the experimental section, the functionalization with PEG 200 involved more reaction steps than the diepoxy-PEG 600.

The reaction schemes involving the hydrophilic coatings and the attachment of the interacting sites are presented in Figures 24-26. As can be seen in Figure 24, the surface

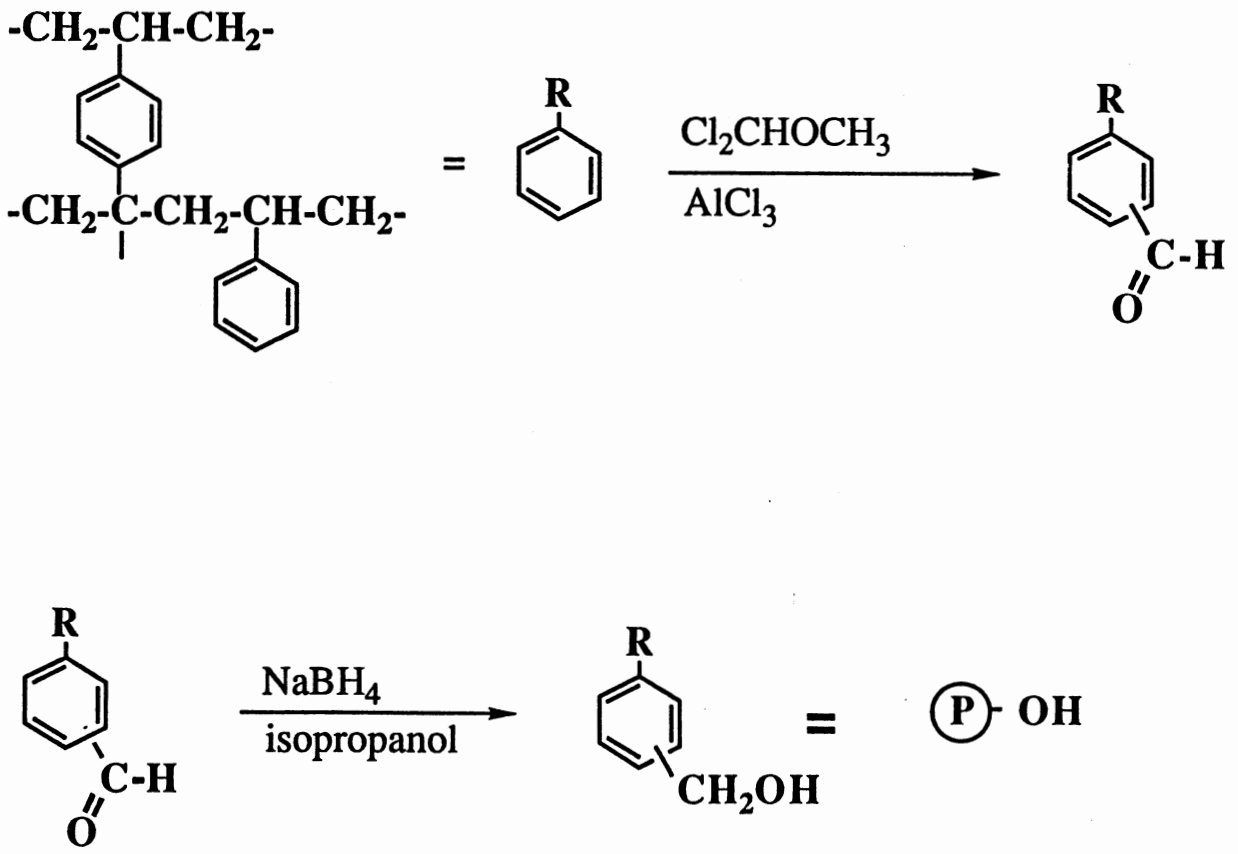


Figure 23. Reaction Schemes of the Hydroxylation of PSDVB *via* Formylation.

was first epoxylated with 1,4-butanediol diglycidyl ether. Although the epoxylated polymer is now ready for the attachment of the ligand, an even larger spacer arm is needed to render the surface highly hydrated when in contact with aqueous phases and to effectively shield the polyaromatic network of the support. The PEG 200 with its long polyether chain has provided the shielding of the hydrophobic matrix toward the solutes. The conversion of the PSDVB-PEG 200 to an epoxylated support was achieved by epichlorohydrin activation.

To investigate the effect of the spacer arm length and to reduce the number of reaction steps, we have evaluated the hydrophilic coating based on diepoxy-PEG 600. The diepoxy-PEG 600 provided a longer polyether chain than that of PEG 200, and with its two epoxide groups, has led to a shorter pathway for attaining the final product (see Figure 25). In addition, in order to provide high surface concentration of metal chelating ligands we have added 1,4-butanediol diglycidyl ether during the covalent attachment of the metal chelate sites.

In both cases, the epoxy-activated hydrophilic polystyrene were reacted with IDA or EDDA metal chelating ligands. The reaction schemes are shown in Fig. 26.

Spectral Analysis of the Hydroxylated-PSDVB by IR Spectroscopy

Although chromatographic evaluations of surface modified supports provide direct information on the performance of sorbents as far as the quality of separation and utility of the stationary phases are concerned, extra chromatographic means are also useful in monitoring the various steps involved in the chemical modifications of chromatographic supports. We have selected infrared spectroscopy to assess the efficiency of the hydroxylation steps of the various supports. Besides the simplicity of the infrared spectroscopic technique, the spectrum provides a reliable means of identifying functional groups based on their characteristic vibrational frequency within well-defined region.[41, 42]

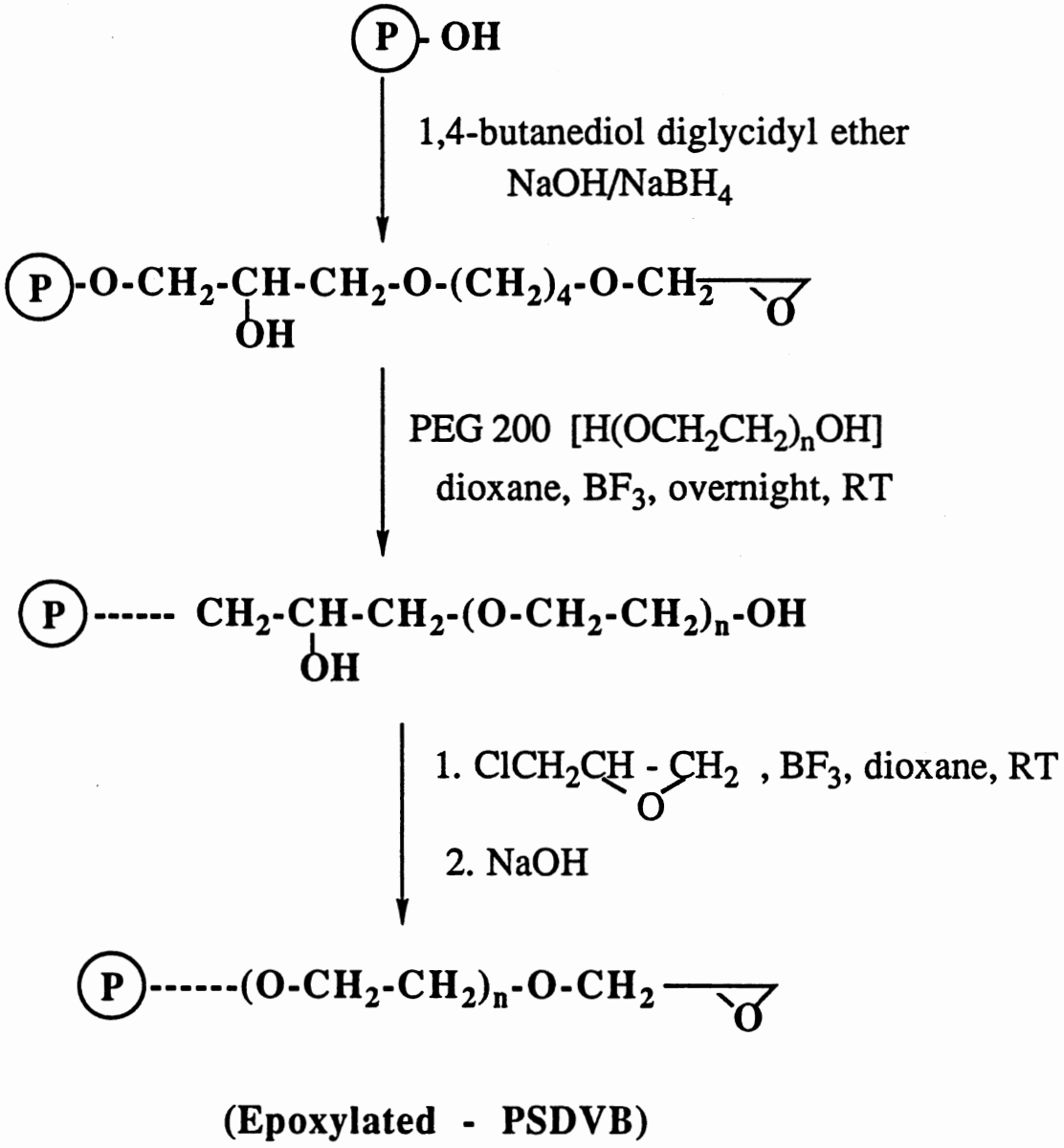


Figure 24. Reaction Schemes of the hydrophilic coating of PSDVB *via* PEG 200. RT, room temperature.

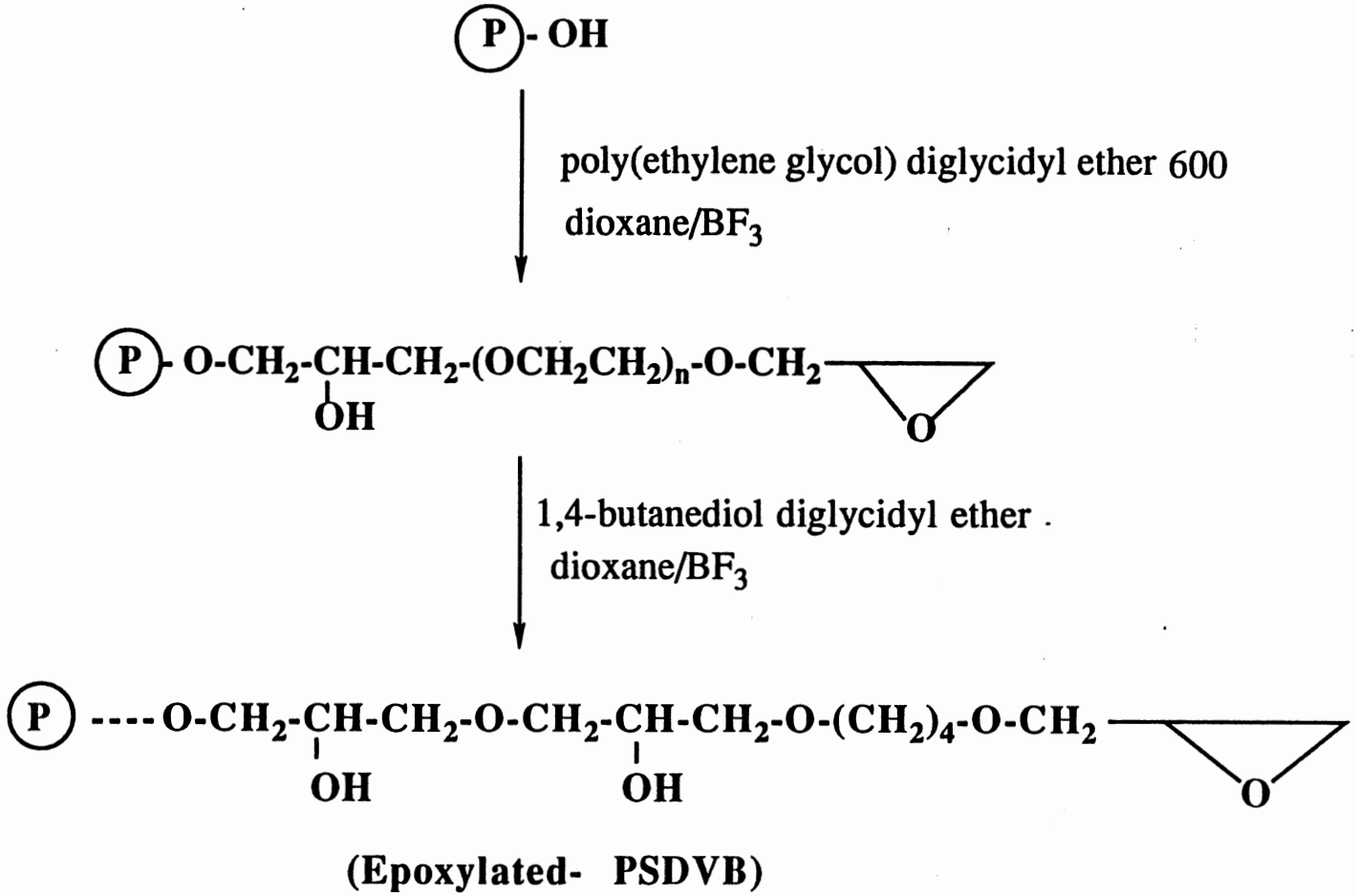


Figure 25. Reaction Schemes of the Hydrophilic Coating of PSDVB *via* Diepoxy-PEG 600.

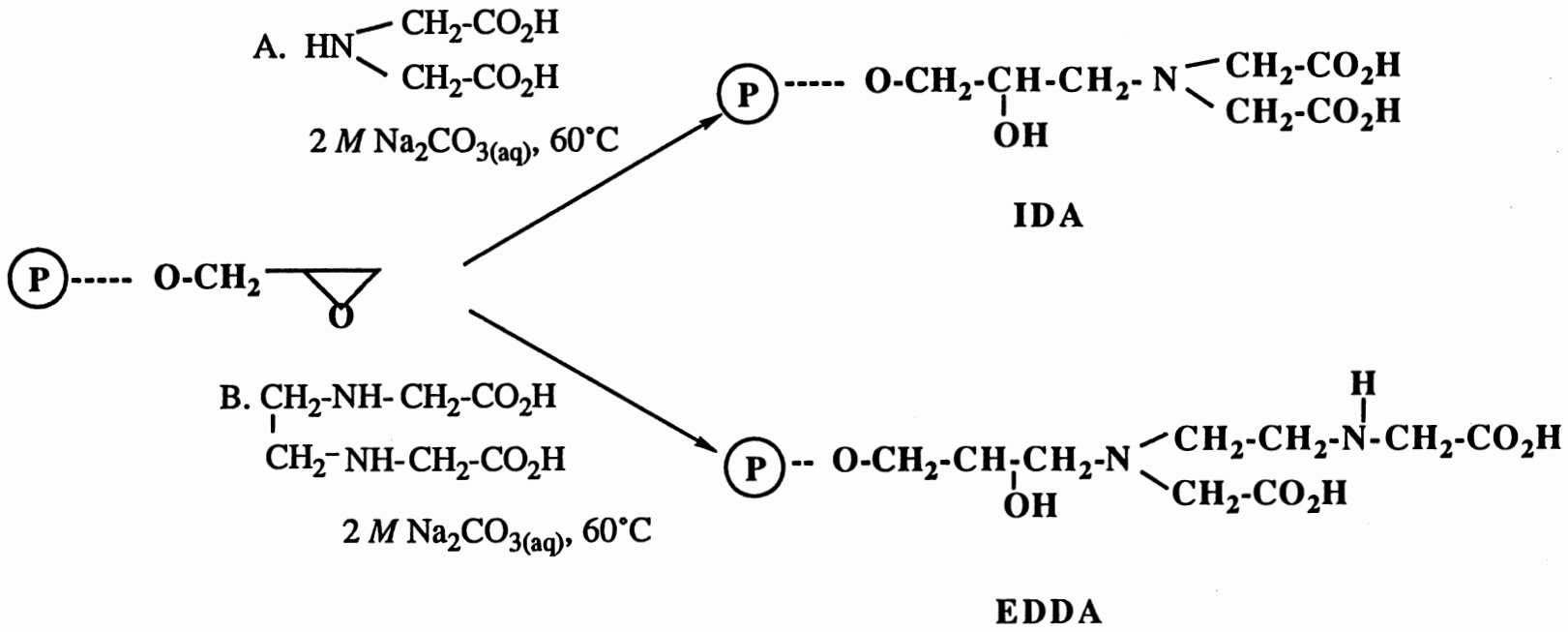


Figure 26. Attachment of the Metal Chelating Ligands to the Hydrophilic PSDVB.

Figure 27-29 represent the infrared spectra of the untreated-PSDVB and hydroxylated-PSDVB through nitration and formylation, respectively. Despite the fact that the spectral analysis of PSDVB is complicated by the amorphous nature of the support, useful information regarding surface modification can still be obtained. All the spectra displayed weak bands at about 3030 cm^{-1} and strong bands at 1600 and 1500 cm^{-1} for C-H stretchings characteristic of aromatic compounds. Strong bands at about 2940 cm^{-1} were also observed in the three samples suggesting the presence of saturated C-H stretching. One important point in these spectra is the presence of broad bands at about 3500 cm^{-1} in the spectra of the two hydroxylated-PSDVB. These bands correspond to the O-H stretching. As expected this band was found to be absent in the untreated-PSDVB. In addition, the presence of a weak band at 1350 cm^{-1} in the PSDVB-OH via nitration can be attributed to the asymmetrical and symmetrical stretching of the NO group, thereby suggesting that not all of the nitro groups were converted into amino groups. These findings would support that hydroxylation *via* formylation is not only a shorter reaction pathway but also yield a more uniform surface as far as the subsequent hydrophilic coating and ligand attachment are concerned.

Metal Uptake- Adsorption Isotherms

Multidentate chelating functions such as IDA and EDDA on the surface of the stationary phases can form 1:1 complexes with metal ions.[43] Thus, the metal uptake measured by frontal analysis (see Experimental) can be utilized as means to compare the extent of surface coverage with immobilized metal chelating ligands from column-to-column.

Figure 30 shows a typical frontal chromatogram that was obtained with Cu(II) as the metal solution. The amount of the chelated Cu(II) was calculated according to the following equation:

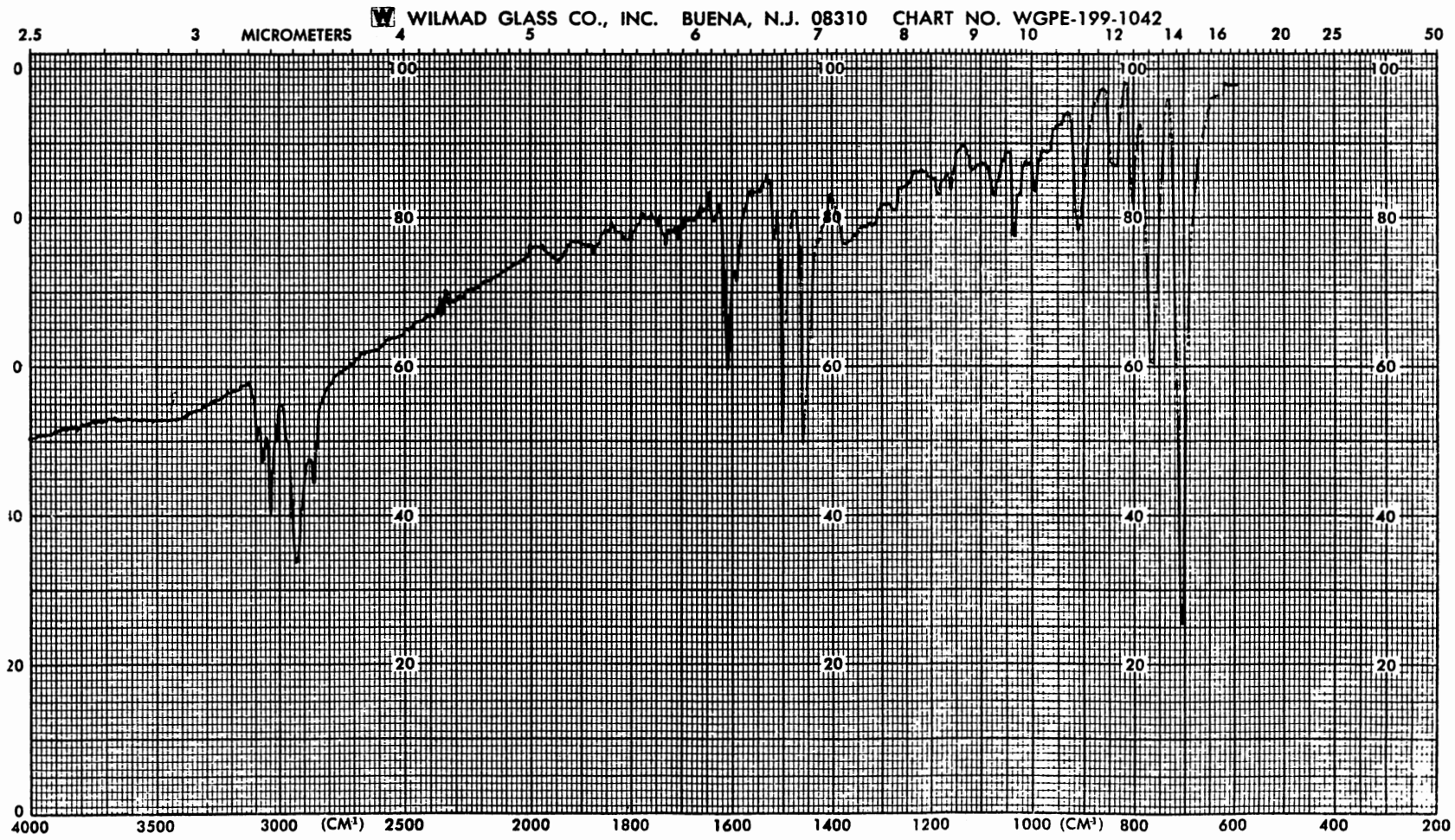


Figure 27. Infrared Spectrum of Untreated-PSDVB-3.

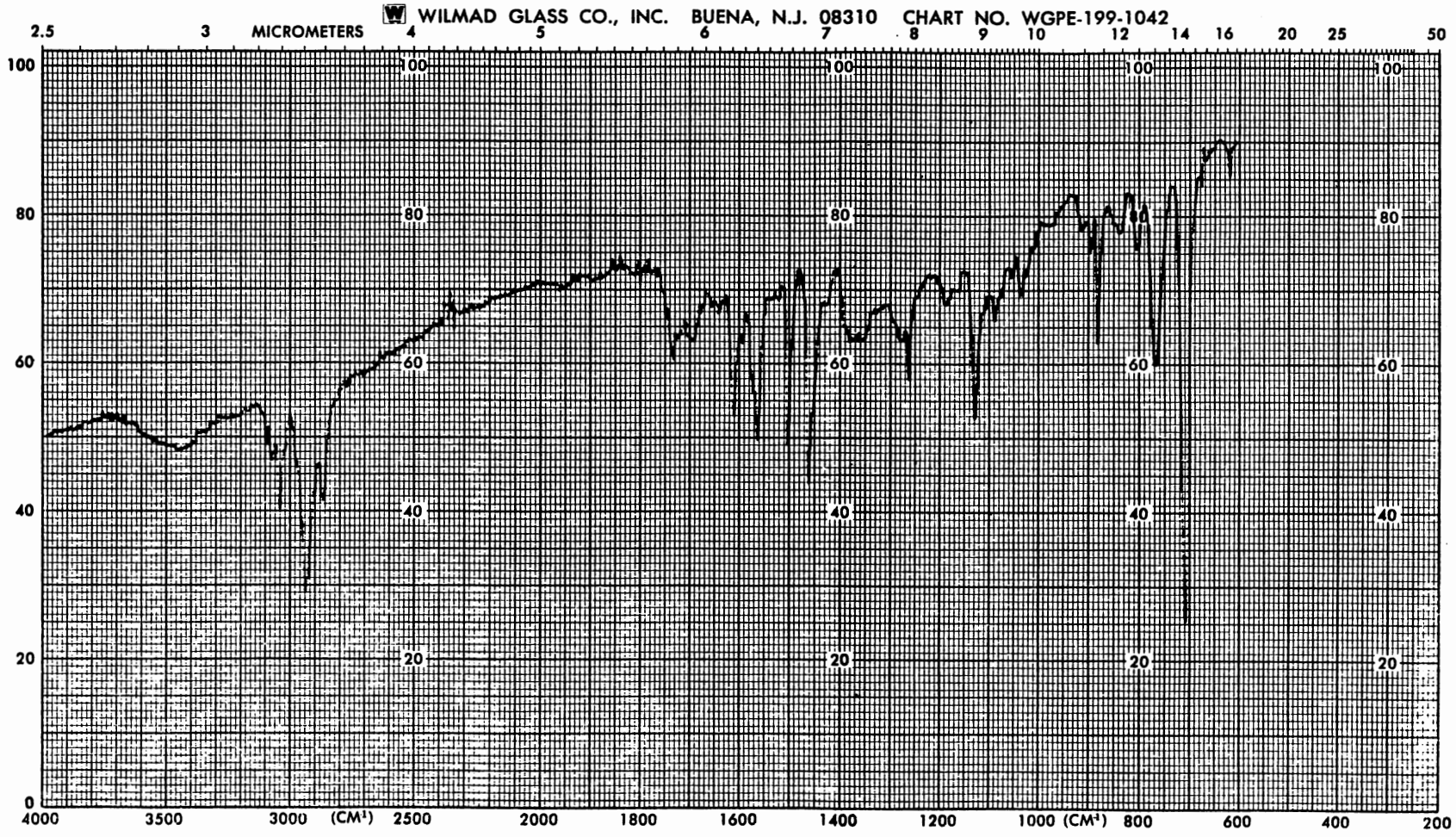


Figure 28. Infrared Spectrum of Hydroxylated PSDVB-3 *via* Nitration Method.

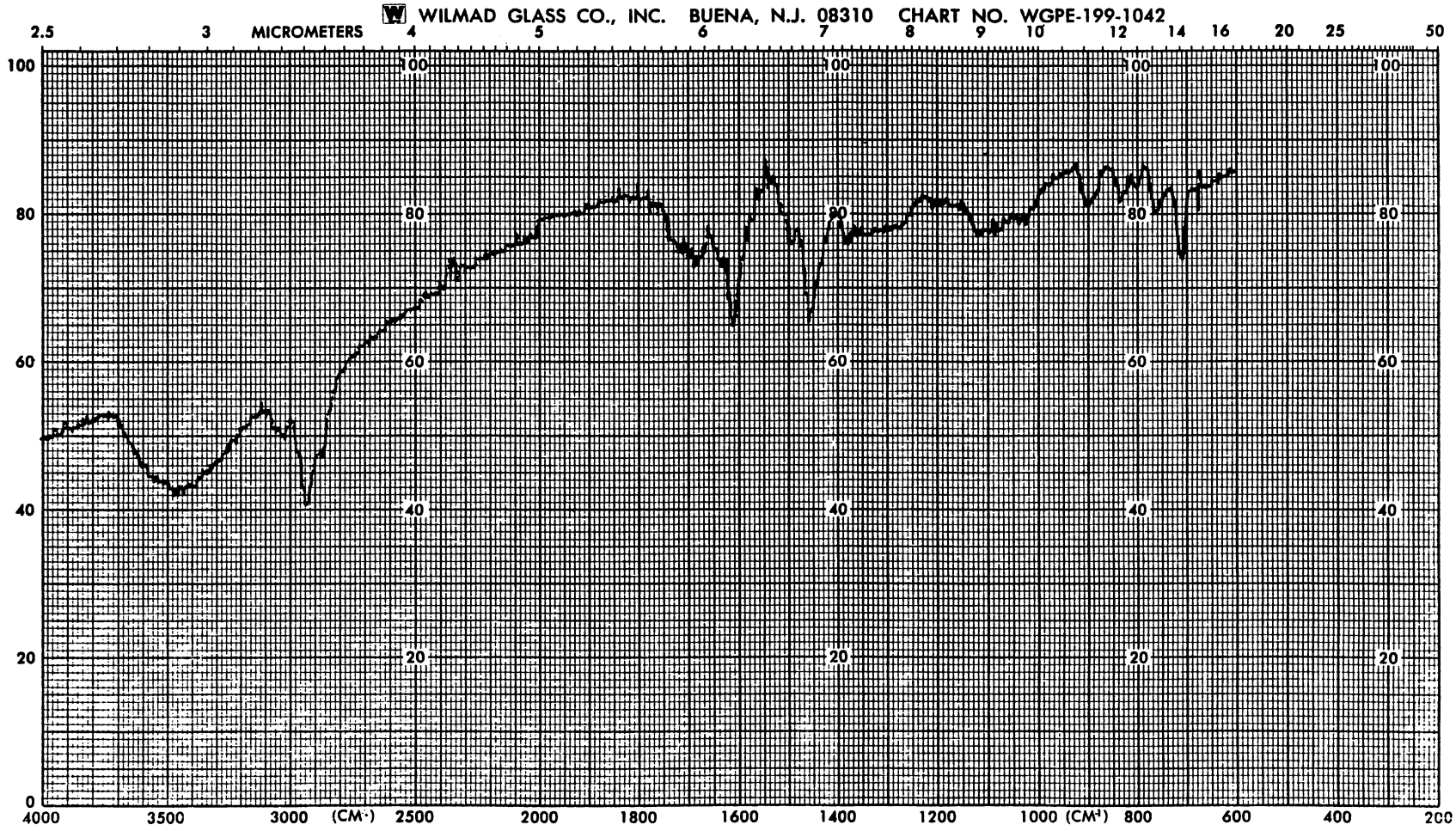


Figure 29. Infrared Spectrum of Hydroxylated PSDVB-3 *via* Formylation Method.

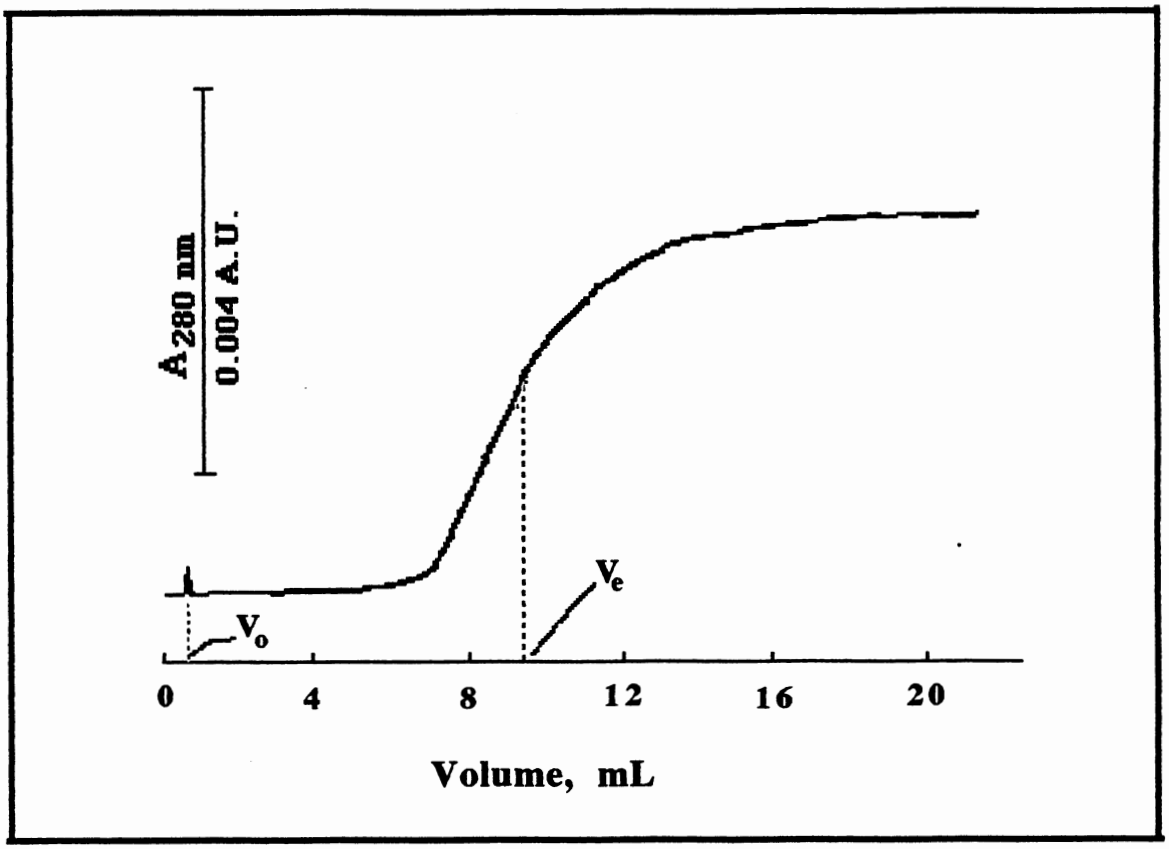


Figure 30. Typical Frontal Chromatogram.

$$\text{amount of Cu(II)} = (V_e - V_o)\, C \qquad (1)$$

where V_e is the frontal retention volume of the Cu(II) solution, V_o is the void volume of the column, i.e., retention volume of an unretained solute, and C is the molar concentration of Cu(II) in the feeding solution. V_e was determined from the breakthrough curve at the median bisector of the frontal curve as shown in Figure 30. As will be demonstrated below, this method yielded accurate data and was useful in comparing the various chemistry utilized to achieve the desired coatings.

Table VIII summarizes the Cu(II) uptake of seven polystyrene-based columns obtained by frontal analysis using 5 mM Cu(II) solution as described in the experimental part. It can be seen that the commercial IDA-PLRP showed the highest uptake. This can be explained by the greater surface area of the commercial PLRP-S (384 m^2/g)[21] when compared to the home made non-porous polymeric supports (0.35 m^2/g). It can also be noted that both IDA-PSDVB obtained *via* the formylation method exhibited higher Cu(II) uptake when compared to their counterparts which were hydroxylated *via* the nitration method. This may indicate that the formylation method yielded higher surface coverage with hydroxyl groups which may be attributed in part to the lesser number of reaction steps involved as compared to the nitration method. In all cases, the stationary phases which were coated with diepoxy-PEG 600 gave higher Cu(II) uptake with respect to those columns coated with PEG 200. This implies that diepoxy-PEG 600 provided more active sites for the attachment of the IDA or EDDA ligands and this may be due to the fact that the polyether chains were already epoxylated and therefore reacted with the hydroxypolymeric support to a greater extent.

Figure 31 shows the adsorption isotherms obtained for both IDA and EDDA-PSDVB stationary phases. As can be seen in Figure 31, the IDA-PSDVB column adsorbed less Cu(II) when compared to EDDA-PSDVB column. The EDDA-PSDVB

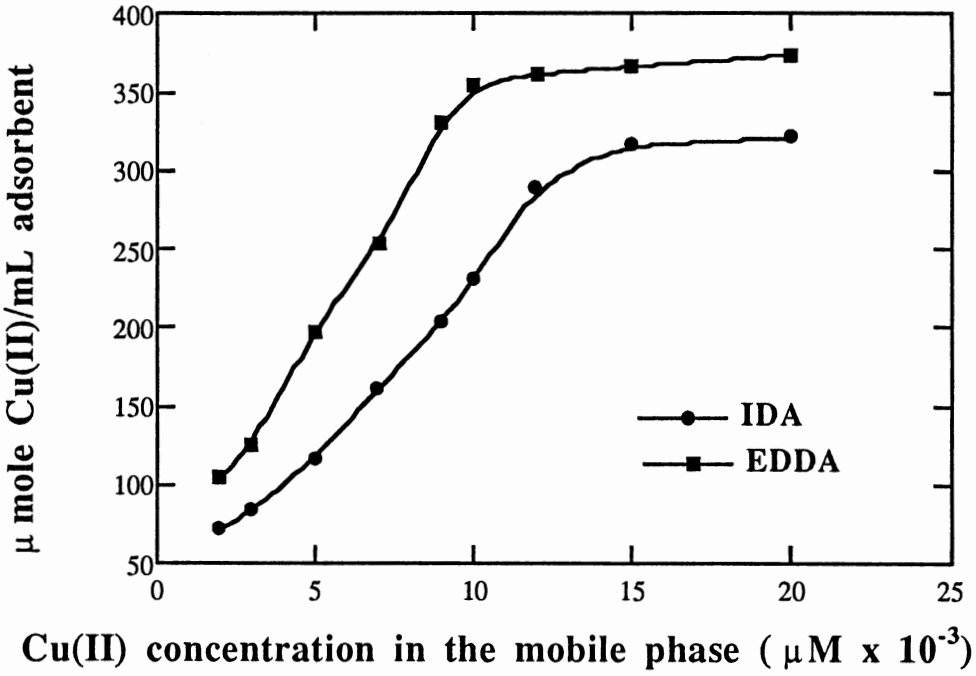


Figure 31. Adsorption Isotherms of IDA-PSDVB-3 and EDDA-PSDVB-3. Column, 30 x 4.6 mm I.D.; flow-rate, 1.0 mL/min.

column started to show its maximum adsorption at about 10 μ*M* Cu(II) in the mobile phase whereas for IDA-PSDVB column it was at about 15 μ*M* Cu(II).

Table VIII. Cu(II) Uptake of the Various Surface-Modified Polymeric Columns.

Stationary Phases	Hydroxylation method	Spacer arm	Cu(II) uptake, μmole/column
PLRP-IDA	nitration	PEG 200	141.735
PSDVB-IDA	nitration	PEG 200	10.010
PSDVB-IDA	nitration	PEG 600	15.665
PSDVB-IDA	formylation	PEG 200	10.885
PSDVB-IDA	formylation	PEG 600	31.810
PSDVB-EDDA	formylation	PEG 200	12.320
PSDVB-EDDA	formylation	PEG 600	50.235

Evaluation of the Surface Coverage Using Small Nonpolar Probes

The highly crosslinked PSDVB-3 was shown to undergo little or no swelling when in contact with organic solvent, e.g., acetonitrile, see Chapter IV. To illustrate its potential as a reversed-phase chromatography packing material, the retention behavior of benzene and seven alkylbenzene homologs, namely, toluene, ethylbenzene, propylbenzene, butylbenzene, pentylbenzene, 1-phenylheptane and 1-phenylnonane was examined using native PSDVB as the stationary phase and hydro-organic solvent as the mobile phase. The results are depicted in Figure 32 in terms of logarithmic retention factor (log k') *versus* the number of carbon atoms in the homologs. The linear curve obtained in Figure 32 is typical

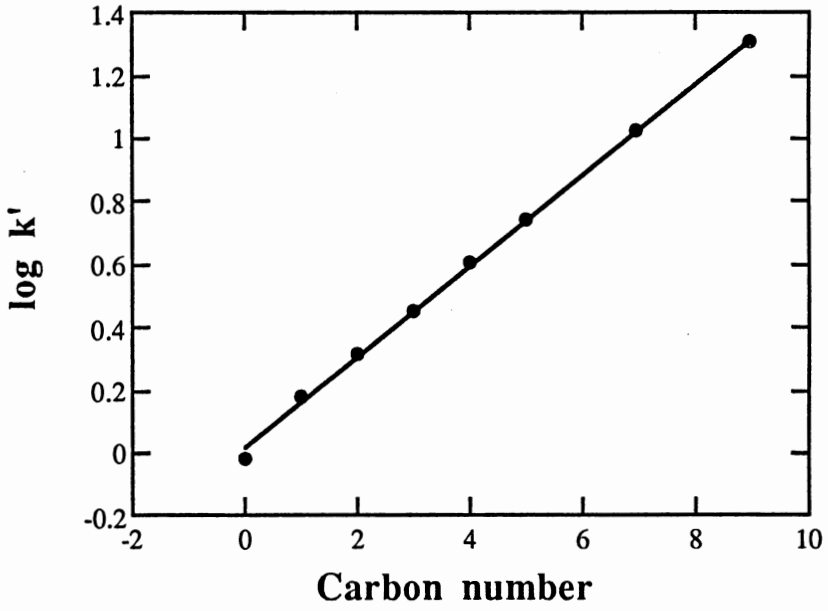


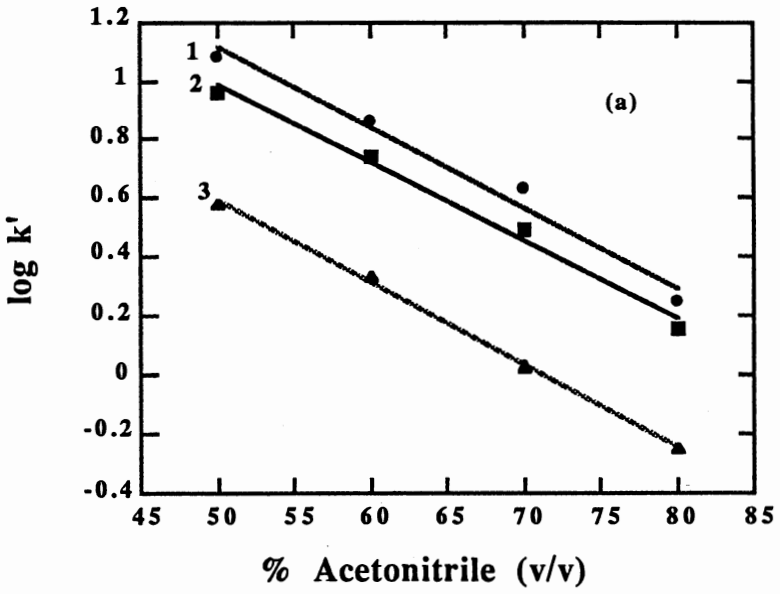
Figure 32. Plot of Logarithmic Retention Factor of Alkylbenzene Homologous Series *versus* Carbon Number of the Side Chains.
Isocratic elution with 80:20 (v/v) acetonitrile:water. Column, 30 x 4.6 mm I.D.; flow-rate, 1.0 mL/ml; temp., 25°C.

of reversed-phase chromatography retention mechanism. Thus, native PSDVB-3 could be utilized in rapid RPC of proteins.

To evaluate the effectiveness of the hydrophilic coatings that we have developed and described above in shielding the surface of the support proper, and to determine the extent of surface hydroxylation through nitration and formylation procedures, small hydrophobic molecules such as benzene and naphthalene were chromatographed under reversed-phase chromatography conditions on the various metal chelate stationary phases. The results are illustrated in Figure 33a and b by plots of log k' *versus* the % of acetonitrilre (v/v) in the mobile phase. In all cases, these plots were linear indicating a reversed-phase chromatography retention mechanism. As expected, both solutes exhibited higher retentions on the untreated-PSDVB than on the two IDA-PSDVB stationary phases. This shows that both hydroxylation methods and subsequent attachment of hydrophilic spacers and metal chelating functions were quite successful since the hydrophobic character of the surface decreased substantially. It has to be noted that the retention of both probes were much less on the surface which was modified *via* formylation (see curves 3 in Fig. 33a and b). These results further support the above findings (i.e., metal uptake) that the metal-chelate stationary phases obtained *via* formylation had higher surface coverage in metal chelating functions and consequently higher metal uptake.

Figure 34 illustrates the plots of log k' against the % of acetonitrile (v/v) obtained with naphthalene on IDA- and untreated-PLRP. Again, the retention of the hydrophobic probe decreased substantially when going from untreated-PLRP to IDA-PLRP stationary phases. These results have proved that the hydrophilic coatings developed in this work were equally effective in shielding the surface of the commercial wide-pore PLRP and home made non-porous polystyrene-based materials.

To shed light on the energetics of retention *via* hydrophobic interaction of small hydrophobic solutes on the native and surface modified polystyrene stationary phases, the



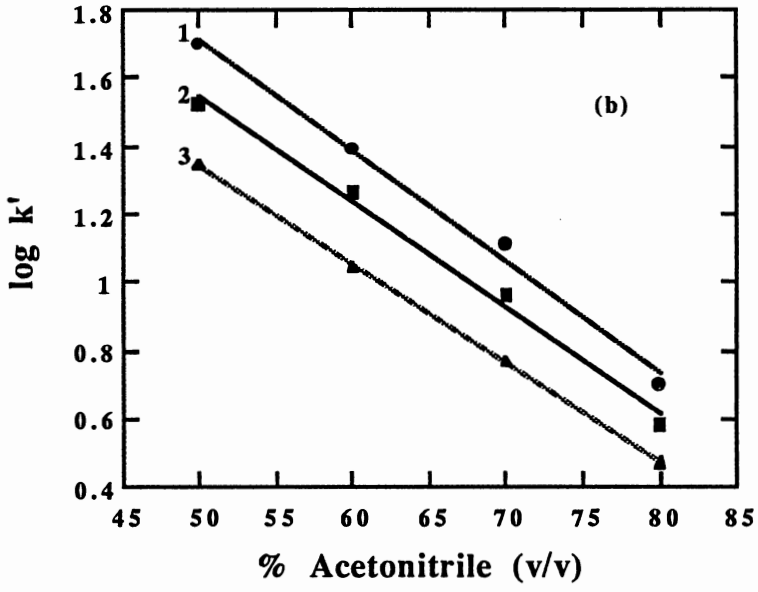


Figure 33. Plots of Logarithmic k' *versus* % of Acetonitrile (v/v) Obtained With Benzene (a) and Naphthalene in (b).
Stationary phases: (1) untreated-PSDVB; (2), IDA-PSDVB-3 hydroxylated *via* nitration and coated with diepoxy-PEG 600; (3), IDA-PSDVB-3 hydroxylated *via* formylation and coated with diepoxy-PEG 600. Column, 30 x 4.6 mm I.D.; flow rate, 1.0 mL/min. Isocratic elution at various acetonitrile:water concentration.

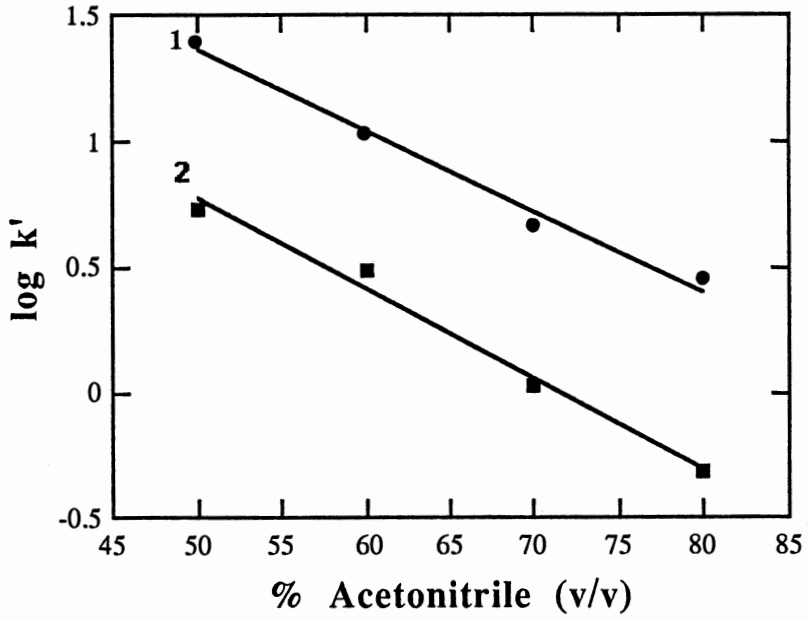


Figure 34. Plots of Logarithmic k' *versus* % Acetonitrile Obtained on (1) Untreated-PLRP and (2) IDA-PLRP Hydroxylated *via* Nitration and Coated with Diepoxy-PEG 600.
Column, 30 x 4.6 mm I.D.; flow rate, 1.0 mL/min. Isocratic elution at various acetonitrile:water concentration. Sample, naphthalene.

logarithm of the retention factor (log k', denoted κ) of naphthalene obtained on one stationary phase, A, was plotted *versus* that obtained on another stationary phase, B, under the same elution conditions. A typical example of κ-κ plots is shown in Figure 35.

For two columns A and B, the logarithmic retention factors, κ, are written as:[44-46]

$$\kappa_A = \phi_A - \Delta G^\circ_A/2.3\ RT \quad (3)$$

$$\kappa_B = \phi_B - \Delta G^\circ_B/2.3\ RT \quad (4)$$

where R, T, ϕ and ΔG° are the gas constant, absolute temperature, logarithmic phase ratio and Gibbs free energy, respectively. Subtraction and subsequent rearrangements of the above equations give:

$$\kappa_A = \kappa_B + (\phi_A - \phi_B) + (\Delta G^\circ_B - \Delta G^\circ_A)/2.3\ RT \quad (4)$$

This equation expresses the relationship between the retention factors obtained on two different columns (A and B). For any solute, the second term on the right hand side of eqn. 4 is constant because it is the quotient of the phase ratios for two columns A and B. The third term is also constant, but its value may depend on the solute used and will depend systematically on κ_A or κ_B if the Gibbs retention energies for the two columns are not identical for all solutes when for each solute the retention energy is the same for both stationary phases, although the energies need not be the same for all solutes. If the difference in the Gibbs retention energies of the two stationary phases is zero for all solutes, equation 4 can be further simplified to:

$$\kappa_B = \kappa_A - \phi_A + \phi_B \quad (5)$$

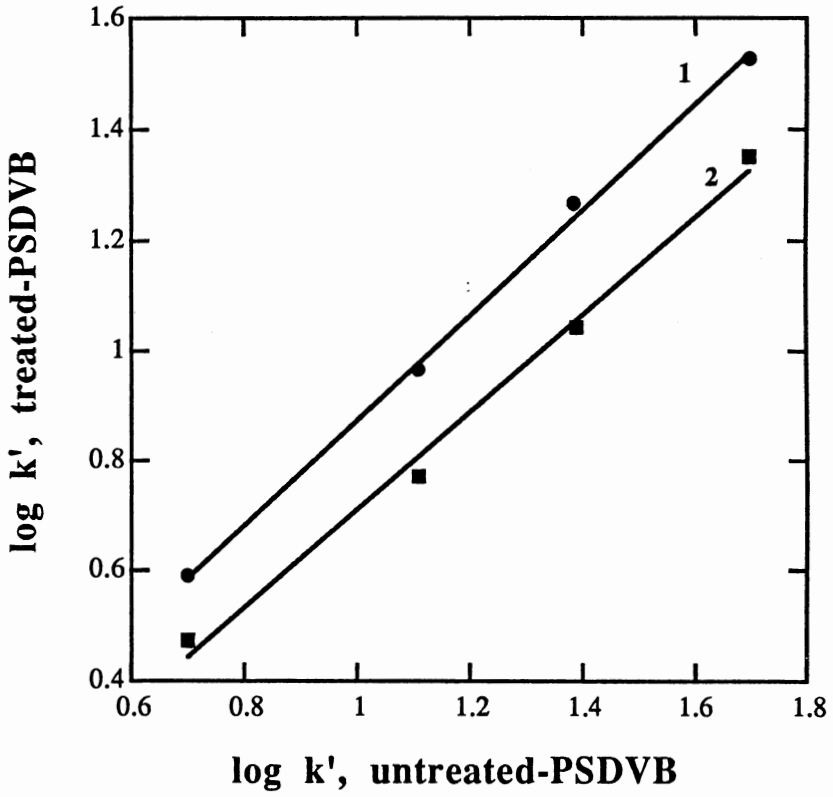


Figure 35. Plots of Logarithmic k' of Naphthalene Obtained on Treated-PSDVB-3 *versus* Logarithmic k' of the Same Solute Obtained on Untreated-PSDVB-3. Stationary phases: (1), IDA-PSDVB-3 hydroxylated via nitration and coated with diepoxy PEG 600; (2), IDA-PSDVB-3 hydroxylated via formylation and coated with diepoxy PEG 600. Column, 30 x 4.6 mm I. D.; flow rate, 1.0 mL/min. Isocratic elution at various acetonitrile:water concentration (50:50, 60:40, 70:30 and 80:20 v/v).

According to equation 5, in the case of homoenergetic retention, a κ-κ plot should give a straight line with a unit slope and an intercept equal to the logarithm of the quotient of the two phase ratios.[47] If the corresponding Gibbs energies for the two chromatographic phases are not identical at a fixed temperature, they may be proportional so that:[46]

$$\Delta G^{\circ}_{A} = \alpha \Delta G^{\circ}_{B} \qquad (6)$$

where α is a constant. Equation 6 can be combined with eqns. 2 and 3 to yield:

$$\kappa_A = \alpha\kappa_B + \phi_A - \alpha\phi_B \qquad (7)$$

Equation 7 shows that when the ratio of the Gibbs retention energies in the two stationary phases are constant, linear κ-κ plots with slope of α are obtained. In this case, the retention on a column pair can be termed homeoenergetic[47] (i.e., like). It can be seen that eqn. 5 is a special case of eqn. 7 when α is unity.

Table IX summarizes the results of the κ-κ plots obtained on the various sets of stationary phases. In all cases, the correlation coefficients and the slopes were very close to unity, hence, suggesting a quasi-homoenergetic retention. Also, Table IX lists the quotient of the phase ratios which is the phase ratio of a column relative to that of the reference column. They were evaluated as the antilog of the intercept of the κ-κ plots. It can be seen from Table IX that the hydrophobic phase ratio toward naphthalene of IDA-PLRP was reduced by a factor of 0.18 with respect to the untreated-PLRP. The residual hydrophobicity of the IDA-PSDVB-3 which was hydroxylated *via* nitration was less than the hydrophobic surface of untreated-PSDVB-3 by a factor of 0.83, see Table IX. On the other hand, the surface coverage of IDA-PSDVB-3 obtained by formylation was much

Table IX. Results obtained from the κ-κ plots of the various polystyrene columns. Column, 30 x 4.6 mm I.D. Isocratic elution with 75:25 (v/v) acetonitrile:water at a flow rate of 1.0 mL/min. Sample, naphthalene.

Column A	Column B	Slope	r	Quotient of phase ratio ϕ_B/ϕ_A
PLRP-untreated	PLRP-IDA	1.11	0.986	0.18
PSDVB-untreated	PSDVB-IDA-nitration	0.95	0.999	0.83
PSDVB-untreated	PSDVB-IDA-formylation	0.88	0.997	0.67
PSDVB-IDA-formylation	PSDVB-IDA-nitration	1.10	0.996	1.30

higher than that obtained *via* nitration as indicated by its lower hydrophobic phase ratio relative to the untreated-PSDVB-3. In fact, IDA-PSDVB-3 obtained *via* nitration has a hydrophobic surface which is 1.30 times higher than that of IDA-PSDVB obtained *via* formylation, see Table IX.

Evaluation of the Porosity of PSDVB by Size Exclusion Chromatography

To further confirm the non-porosity of the home made PSDVB microparticles toward biomacromolecules, PSDVB coated with hydrophilic diepoxy-PEG 600 was packed into a 100 x 4.6 mm I.D. column and was used in the size exclusion chromatography (SEC) of five proteins of widely differing molecular weights (see Table X). In SEC, it is necessary that the support matrix is neutral as well as hydrophilic in order to prevent the non-size exclusion partitioning between the support material and the proteins.

Table X. Molecular Weights of the Five Model Proteins and their Corresponding Retention Time.
Column, 100 x 4.6 mm I.D.; flow-rate, 1.0/mL; mobile phase, 50 m*M* phosphate containing 0.1 *M* NaCl, pH 7.0.

Proteins	MW[a]	t_R (min)
Myoglobin	17,400	1.9
α-Chymotrypsinogen A	25,500	2.0
γ-Globulin	33,500	1.9
Lactoferrin	80,000	1.9
Ferritin	149,900	1.9

[a]Data from reference 48.

Ideally, if the existing mechanism is size exclusion, the proteins would elute in the order of decreasing molecular weight, i.e., the protein having the highest molecular weight (ferritin) will be the first to elute whereas the protein having the lowest molecular weight (myoglobin) will be the last to elute. As can be seen in Table X, the retention time (t_R) of the various proteins was almost the same (within the experimental error), indicating the absence of pores that can be accessible for the penetration of biomacromolecules into the PSDVB matrix. These results also indicate that the hydrophilic coating had no residual adsorptivity and efficiently shielded the hydrophobic matrix toward proteins.

Chromatographic Evaluation of the Naked PSDVB-IDA and EDDA Stationary Phases with Proteins

To assess the hydrophilicity as well as the presence of metal chelating functions on the surface of the stationary phases, EDDA- or IDA-PSDVB columns were evaluated in their naked forms (i.e., absence of chelated metal) as cation exchangers. For both EDDA and IDA columns (see Figure 36a and b), the 3 basic proteins tested eluted in the order of increasing pI value (see Table XI), i.e., myoglobin, α-chymotrypsinogen A and lactoferrin, when a linear gradient of increasing NaCl concentration in 10 m*M* phosphate, pH 6.0, was employed. These proteins were more retained on EDDA-PSDVB column than on IDA-PSDVB column indicating that the ligand surface coverage of the former stationary phase is larger than that of the latter. This has also been confirmed by the metal uptake, see Table VIII.

Furthermore, the above results have confirmed the effectiveness of the hydrophilic coatings developed in this study. The three test solutes are hydrophobic proteins and would not elute from the column with aqueous phases even if the hydrophobic surface proper of the support would have still been slightly exposed to the biomacromolecule solute.

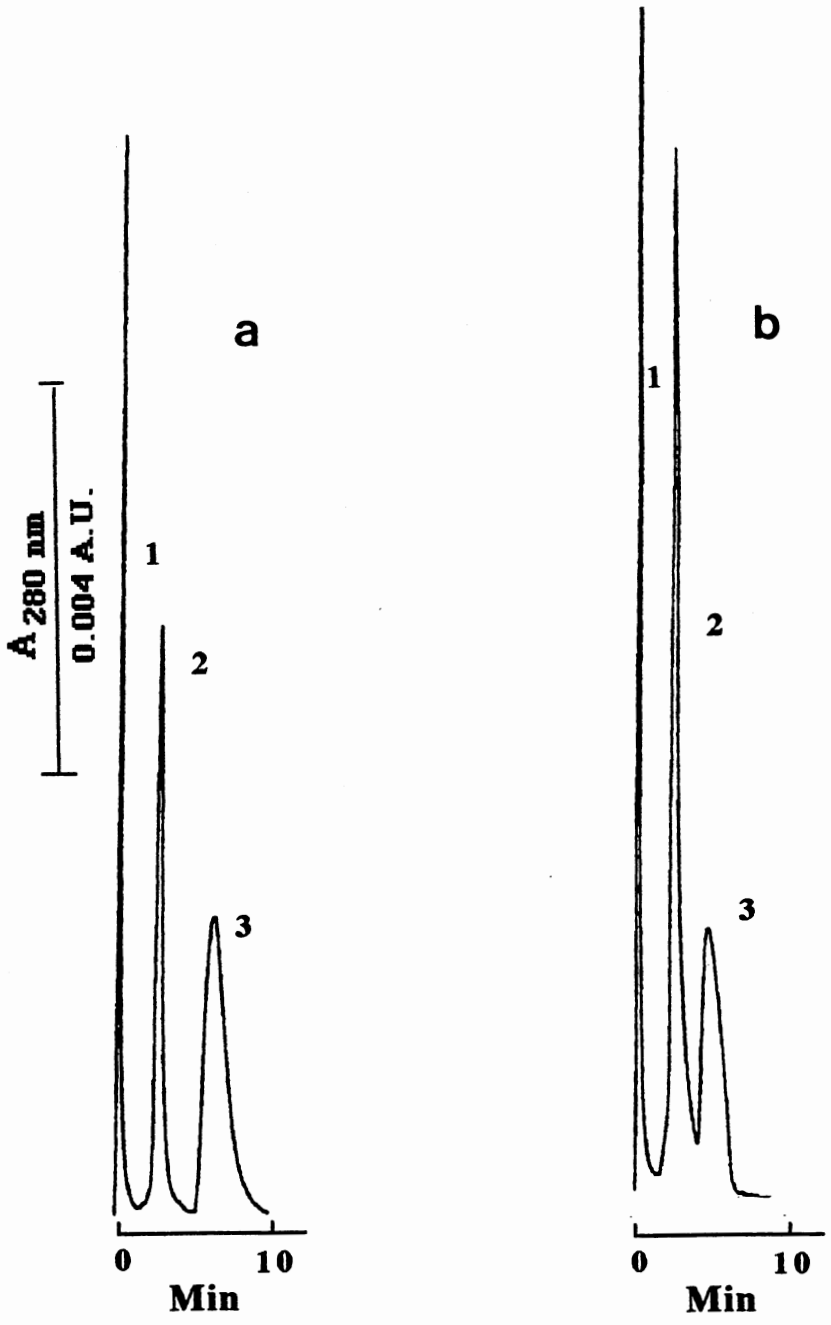


Figure 36. Chromatograms Obtained on Naked (a) EDDA-PSDVB-3 and (b) IDA-PSDVB-3.
Column, 30 x 4.6 mm I.D.; flow rate, 2.0 mL/min.
6.0 min linear gradient from 0 to 1.0 *M* NaCl in 10 m*M* phosphate, pH 6.0.
Proteins: 1, myoglobin; 2, α-chymotrypsinogen A; 3, lactoferrin.

Table XI. Molecular Weight and pI values of the Three Model Proteins.

Proteins	pI[a]
myoglobin	7.1
α-chymotrypsinogen A	9.5
lactoferrin	10

[a] Data from Reference 40.

Comparison of PSDVB-IDA and Silica-IDA Columns

To further demonstrate the hydrophilicity of the PSDVB-based metal chelate stationary phases toward proteins, the chromatographic behavior of test proteins obtained on the PSDVB packing materials was compared to that obtained on silica metal chelate sorbent, a support known for its hydrophilic nature. Figure 37a and b represents the separation of six proteins, namely, α-lactalbumin, cytochrome c, lysozyme, lactoferrin, iron-free transferrin and carbonic anhydrase obtained on non-porous Zn(II)-IDA—silica and Zn(II)-IDA—PSDVB columns, respectively, using two consecutive linear gradients of increasing NaCl concentration followed by a linear gradient of increasing imidazole concentration at constant salt concentration. It can be seen that despite the large difference between the two columns in terms of the support matrix these two columns having the same chelating ligands and chelated metal exhibited comparable behavior towards the six model proteins. However, slight differences in retentivities and selectivities can be noticed. These can be attributed to the matrix effect on the properties of the metal chelate proper, which in turn, might affect their affinities towards proteins.

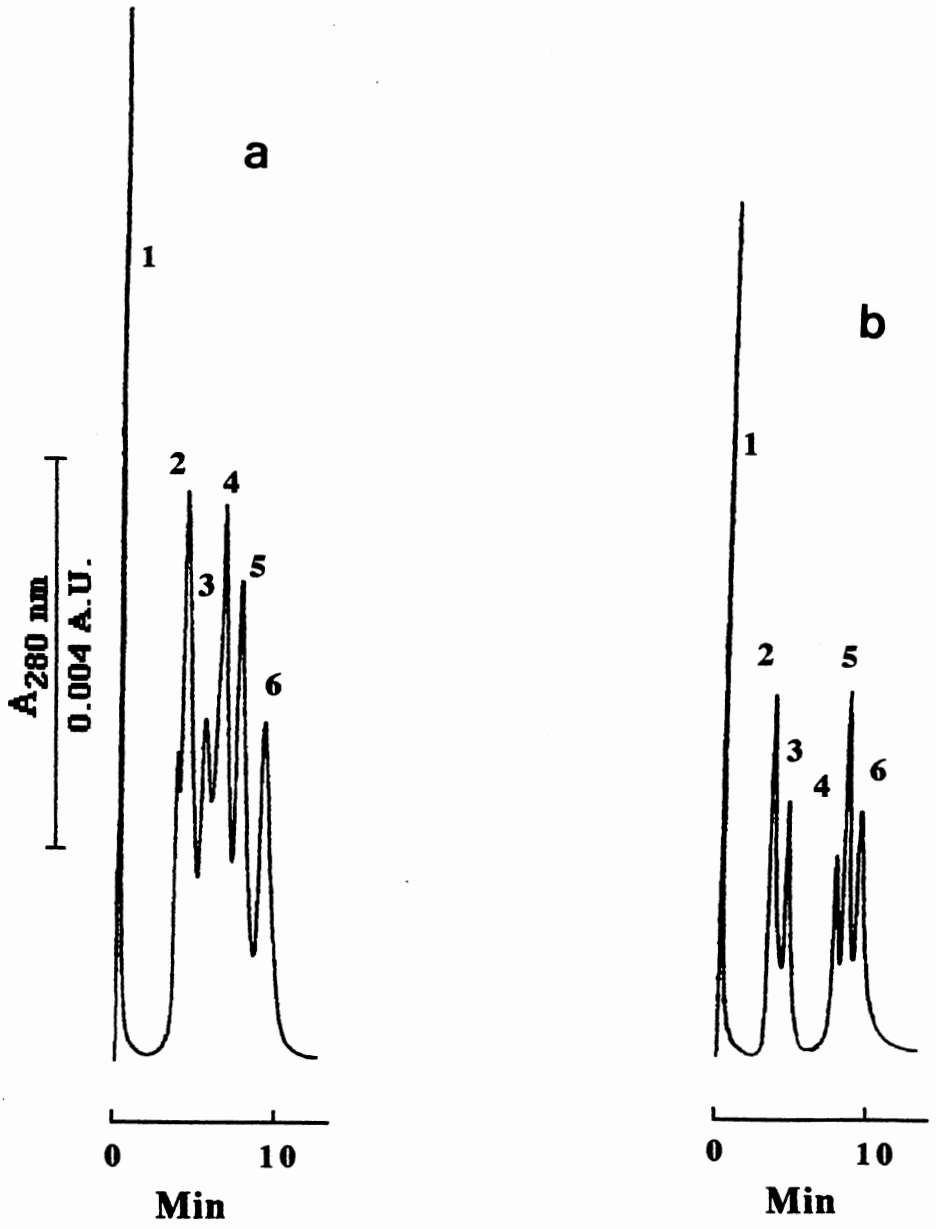


Figure 37. Chromatograms Obtained on (a) Zn(II)-IDA—Silica and (b) Zn(II)-IDA—PSDVB-2.
Column, 30 x 4.6 mm I.D.; flow rate, 2.0 mL/min. 6.0 min linear gradient from 0 to 1.0 *M* NaCl followed by a 6.0 min linear gradient from 0 to 50 m*M* imidazole in 10 m*M* phosphate containing 1.0 *M* NaCl, pH 6.0. Proteins: 1, α-lactalbumin; 2, cytochrome c; 3, lysozyme; 4, lactoferrin; 5, iron-free transferrin; 6, carbonic anhydrase.

Conclusions

The development of new and efficient hydrophilic coatings for the rigid poly(styrene-divinylbenzene) supports proved to be useful in providing a solution to their most predominant limitation, which is, their strong hydrophobic character. As we have demonstrated the hydrophilic poly(styrene-divinylbenzene) stationary phases can be bonded with metal chelating functions, namely, iminodiacetic acid (IDA) and ethylenediamine-*N,N'*-diacetic acid (EDDA) for high performance metal interaction chromatography of biomolecules. It is interesting to note that non-porous PSDVB and silica with surface-bound metal chelating ligands exhibited similar chromatographic behavior toward proteins. It is anticipated that the hydrophilic coatings we have developed will find its applications in the preparation of sorbents for other interactive chromatography of biopolymers such as hydrophobic interaction, ion-exchange and thiophilic interaction chromatography.

References

1. Tanaka, N.; Araki, M. in *Advances in Chromatography*, Giddings, C.; Grushka, E.; Brown, P., Eds., Marcel Dekker, Inc.: New York, **1989**, 81.

2. Mikes, O.; Coupek, J.; in *HPLC of Biological Macromolecules*, Gooding, K.; Regnier, F., Eds., Marcel Dekker, Inc.: New York, **1990**, 25.

3. LC Packings *J. Chromatogr.* **1991**, *544*, 1.

4. Hjerten, S; Liu, Z.-Q.; Yang, D. *J. Chromatogr.* **1984**, *296*, 115.

5. Hjerten, S.; Li, J.-P. *J. Chromatogr.* **1990**, *500*, 543.

6. Coupek, J.; Krivakova, M.; Pokorny, S. *J. Polym. Sci., Polymer Symp.* **1973**, *42*, 185.

7. Kato, J.; Komiya, K.; Sasaki, H.; Hashimoto, T. *J. Chromatogr.* **1980**, *193*, 311.

8. Germershausen, J.; Karsas, J. D. *Biochem. Biophys. Res. Commun.* **1981**, *99*, 1020.

9. Wada, H.; Makino, K.; Takeuchi, T.; Hirouki, H.; Noguchi, K. *J. Chromatogr.* **1985**, *320*, 369.

10. Rounds, M. A.; Rounds, W. D.; Regnier, F. *J. Chromatogr.*, **1987**, *397*, 25.

11. Swadesh, J. K. *J. Chromatogr.* **1990**, *512*, 315.

12. Thomas, A. H.; Newland, P. *J. Chromatogr.* **1985**, *326*, 3.

13. Isobe, T.; Kuroso, Y.; Fang, Y.; Ishioka, N.; Kawasaki, H.; Takai, N.; Okuyama, T. *J. Liq. Chromatogr.* **1984**, *7*, 1101.

14. Letourner, D.; Migonney, V.; Muller, D.; Jozefowics, M. *J. Chromatogr.* **1992**, *589*, 87.

15. Jacquot-Dourges, M. A.; Muller, D.; Barritault, D.; Josefonvicz, J. *J. Chromatogr.* **1990**, *510*, 141.

16. Vivelecchia, R. V.; Lightbody, B. G.; Thimot, N. Z.; Quinn, H. M. *J. Chromatogr. Sci.* **1977**, *15*, 424.

17. Ozaki, E.; Saitoh, K.; Suzuki, N. *J. Chromatogr.* **1979**, *177*, 122.

18. Yang, B; Verzele, M. *J. Chromatogr.* **1987**, *387*, 197.

19. Tweeten, K. A.; Tweeten, T. N. *J. Chromatogr.* **1986**, *359*, 111.

20. Sasagawa, T.; Ericsson, L. H.; Teller, D. C.; Titani, K.; Walsh, K. A. *J. Chromatogr.* **1984**, *307*, 29.

21. Lloyd, L. L. *J. Chromatogr.* **1991**, *544*, 201.

22. Wonayai, S.; Varga, J. M.; Bonn, G.K. *J. Chromatogr.* **1991**, *356*, 155.

23. Yang, Y.-B.; Regnier, F. E. *J. Chromatogr.* **1991**, *544*, 233.

24. Afeyan, N. B. A.; Gordon, N. F.; Mazsaroff, I.; Varady, L.; Fulton, S. D.; Yang, Y.-B.; Regnier, F. E. *J. Chromatogr.* **1990**, *519*, 1.

25. Afeyan, N. B. A.; Fulton, S. P.; Regnier, F. E. *J. Chromatogr.* **1991**, *544*, 267.

26. Hodge, P. in *Syntheses and Separations Using Functional Polymers*, Sherrington, D. C.; Hodge, P., Eds., John Wiley and Sons Ltd.: New York, **1988**, 43.

27. Frechet, J. M. *Tetrahedron.* **1981**, *37*, 663.

28. Pepper, K. W.; Paisley, H. M.; Young, M. A. *J. Chem. Soc.* **1953**, 4097.

29. Blossey, E. C.; Ford. W. T. in *Comprehensive Polymer Science*, Eastmond, G. C.; Ledwith, A.; Russo, S.; Sigwalt, P., Eds., Pergamon Press: New York, **1989**, 81.

30. Goldstein, L. *J. Chromatogr.* **1981**, *215*, 31.

31. Porath, J.; Sundberg, L. *Protides Biol. Fluids Colloq.* **1970**, *18*, 401.

32. Chibowski, J. S. *J. Colloid and Interface Sci.* **1990**, *134*, 174.

33. Furniss, B. S.; Hannaford, A. J.; Smith, P. W. G.; Tatchell, A. R. *Vogel's Textbook of Organic Chemistry,* 5th ed.; John Wiley and Sons, Inc.: New York, **1989**, Chapter 6.

34. Maa, Y. F.; Lin, S.; Horvath, C.; Yang, U.; Crothers, D. *J. Chromatogr.* **1990**, *508*, 61.

35. March, J. *Advanced Organic Chemistry,* 3rd ed.; John Wiley and Sons, Inc.: New York, **1985**, Chapter 11.

36. Hemdan, E. S.; Porath, J. *J. Chromatogr.* **1985**, *323*, 247.

37. El Rassi, Z.; Horvath, C. *J. Chromatogr.* **1986**, *359*, 241.

38. Borchert, A.; Larsson, P.; Mosbach, K. *J. Chromatogr.* **1982**, *244*, 49.

39. Feinberg, R. S.; Merrifield, R. B. *Tetrahedron.* **1974**, *30*, 3209.

40. Brown, H. C.; Wheeler, O. H.; Ichikawa, K. *Tetrahedron.* **1957**, *1*, 214.

41. Silverstein, R. M.; Bassler, G. C.; Merrill, T. C. *Spectrophotometric Identification of Organic Compounds*, 4th ed.; John Wiley and Sons: New York, **1981**, Chapter 3.

42. Williams, D. H.; Fleming, I. *Spectroscopic Methods in Organic Chemistry*, 4th ed.; McGraw-Hill Book Company (UK) Limited: London, **1987**, Chapter 2.

43. El Rassi, Z.; Horvath, C. in *HPLC of Biological Macromolecules*, Gooding, K; Regnier, F., Eds., Marcel Dekker, Inc.: New York, **1990**, 179.

44. Melander, W. R.; Chen, B. K.; Horvath, C. *J. Chromatogr.* **1979**, *185*, 99.

45. Chen, B. K.; Horvath, C., *J. Chromatogr.*, **1979**, *171*, 15.

46. El Rassi, Z.; Horvath, C. *Chromatographia* **1984**, *19*, 1.

47. Melander, W.; Stoveken, J.; Horvath, C. *J. Chromatogr.* **1980**, *199*, 35.

48. Righetti, P. G.; Tudor, G.*J. Chromatogr.* **1981**, *220*, 115.

CHAPTER VI

RAPID HIGH PERFORMANCE METAL INTERACTION CHROMATOGRAPHY OF PROTEINS USING POLY(STYRENE-DIVINYLBENZENE) MICROPARTICLES

Introduction

In this present study, it was our objective to evaluate the home made non-porous poly(styrene-divinylbenzene) microparticles with surface-bound chelating functions in the rapid MIC of proteins. The chelating ligands were attached to the non-porous polystyrene packing materials using the surface modifications developed and described in Chapter V. The effects of several operational parameters on MIC retention and selectivity such as eluent pH, salt concentration and nature of salt have been investigated. In addition, the chromatographic performance of non-porous PSDVB under various conditions have been assessed using a commercially available and well characterized macroporous support as reference.

Experimental

Instrumentation

Chromatographic measurements concerning the investigation of the effects of the operational parameters were carried out either isocratically or by gradient elution using the instrumental set-up described in Chapter II. On the other hand, all rapid separation studies

were conducted by using the liquid chromatograph described in Chapter III. In the rapid MIC, the chromatographic instrument must be appropriately designed in order to avoid extra column effects (connection, sample introduction and detection) on band broadening. The pre- and post-column connections, as well as the sampling loop and the detection cell were of relatively low volumes. The pH of the eluents were adjusted by using a digital pH/mV meter Model 701A from Orion Research Incorporated (Boston, MA, U.S.A.).

Reagents and Materials

In addition to the list of proteins and reagents given in the earlier chapters, peroxidase from horseradish was purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Potassium thiocyanate and sodium perchlorate were from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Columns

The non-porous PSDVB or macroporous PLRP-S with surface-bound IDA or EDDA were packed into 30 x 4.6 mm I.D. No. 316 stainless steel tubes from Alltech Associates, Inc. (Deerfield, IL, U.S.A.) in the same manner as described in the experimental section of Chapter V. The surface modifications of the polystyrene supports were carried out by using the procedures described in the experimental section of Chapter V.

Other Procedures

The columns were loaded and unloaded with the desired metals by following the procedures described in Chapter II. This involved the washing of the columns packed with IDA- or EDDA- PSDVB stationary phases with water and then loading with the appropriate metal by injecting 10 ml of 50 m*M* metal salt solution using a sampling valve

equipped with 10 ml sample loop. After loading the column with a given metal, the excess unchelated metal was subsequently removed from the column by washing it with an ample amount of water followed by the mobile phase used in the ensuing chromatographic separations. The unloading of the column with the metal was carried out by washing it with 20 ml of 50 m*M* of EDTA disodium salt. After regeneration with water, the column in its naked form, i.e., without chelated metal, was ready for reloading with a different metal.

Results and Discussion

To assess the chromatographic performance of the home made non-porous metal chelate stationary phases, the results obtained on these phases were compared to those generated on a wide pore and commercially available PSDVB support, the surface of which was modified in-house using the same reaction schemes as with the non-porous PSDVB. Ideally, this comparison would be more meaningful with well characterized polymeric non-porous sorbents, but such supports are not yet commercially available.

Effects of Eluent pH

Although linear gradients of decreasing pH in conjunction with linear gradients of increasing salt and /or competing agent concentrations have been frequently applied in order to bring about the elution and separation of most proteins,[1-4] systematic studies on the effect of pH with various proteins and different metal chelate stationary phases are lacking.

The present study on the effect of pH on retention is an extension of the previous investigations performed on wide-pore siliceous metal-EDDA columns (see, Chapter II). Polymeric metal chelate sorbents have the advantage of permitting the study of pH's greater than 7.0 which could not be explored with silica-based stationary phases owing to

their chemical instability at pH above 7-8.

To evaluate the effect of pH, six widely differing proteins, namely, α-chymotrypsinogen A, carboxypeptidase A, lysozyme, iron-free transferrin, lactoferrin and carbonic anhydrase were utilized as model solutes. These well characterized proteins can be considered as an ideal set of solutes for the pH mapping of MIC retention. Table XII provides information on the number of histidine residues (most reactive), for each protein as well as their corresponding pI value and molecular weight. Carboxypeptidase A and iron-free transferrin are slightly acidic proteins whereas carbonic anhydrase is nearly neutral protein. On the other hand, α-chymotrypsinogen, lactoferrin and lysozyme are basic proteins. α-Chymotrypsinogen A and lysozyme with approximately the same number of histidine residues but with different pI values may serve as model proteins to assess the influence of the net charge of proteins in MIC. Likewise, human iron-free transferrin and bovine lactoferrin have about the same molecular weights but different isoelectric points, hence, may again be used in evaluating the effect of the net charge of protein in MIC. Iron-free transferrin and carbonic anhydrase may be regarded as model proteins to determine the extent to which retention may depend on pH when both protein solutes would have similar pI values but different number of interacting sites.

The above model proteins were utilized to evaluate Co(II)-EDDA and Fe(III)-EDDA columns as well as Co(II)-IDA and Zn(II)-IDA columns. The former set of columns was chosen to assess the retention dependency on pH on an intermediate "soft" metal (Co^{+2}) and a borderline "hard" metal (Fe^{+3}) chelated by the same ligand. On the other hand, the second set of columns represents a pair of sorbents with two different intermediate "soft" metals, i.e., Zn(II) and Co(II). Also, the effect of the pH of the eluent on the retention of proteins on the same metal, i.e., Co(II), but different chelating ligands (EDDA or IDA) were evaluated.

TableXII. Numbers of the Histidine Residues[a], Molecular Weights[b] and pI[b] Values of the Six Model Proteins.
[a, b] Data taken from references, 5 and 6, respectively.

PROTEINS	Histidine	pI	Molecular Weight
Lysozyme	1	11	14,314
α-Chymotrypsinogen A	2	9.5	25,666
Carbonic Anhydrase	11	6.9-7.2	29,182
Carboxypeptidase A	8	5.2	34,409
Iron-Free Transferrin	19	6	75,000
Lactoferrin		10	80,000

EDDA Columns. All proteins listed in Table XII except carbonic anhydrase were chromatographed on Co(II)-EDDA— and Fe(III)-EDDA—PSDVB columns using a 12 min linear gradient from 0 to 1.0 *M* NaCl at various pH over the range 4-10. Carbonic anhydrase was eliminated in this particular study because it yielded two peaks in all cases. Table XIII and XIV summarize the adjusted retention volumes obtained on Co(II)-EDDA and Fe(III)-EDDA columns, respectively. With Co(II)-EDDA column, the retention of lysozyme and lactoferrin increased with increasing pH whereas the retention of iron-free transferrin increased slightly in the pH range 4-6 and then approached zero starting from pH 7.0. As expected, these results were in good agreement with those obtained on the Co(II)-EDDA attached on silica support, see Chapter II.

With the Fe(III)-EDDA column, lysozyme and iron-free transferrin showed retention behavior that followed the same trends as those obtained on the silica-based sorbents (see Chapter II) whereas the retention of lactoferrin decreased with increasing pH, a behavior that did not conform with that obtained on Fe(III)-EDDA-silica stationary phases. This behavior, however, follows the generally observed trend in that "hard"

Table XIII. Adjusted Retention Volume at Various pH on Co(II)-EDDA—PSDVB-3. 12.0 min linear gradient from 0 to 1.0 *M* NaCl in 10 m*M* phosphate. Column, 30 x 4.6 mm I.D.; flow-rate, 1.0 mL/min.

	pH						
PROTEINS	4.0	5.0	6.0	7.0	8.0	9.0	10.0
Iron-free transferrin	4.63	4.77	**4.77**	0.00	0.00	0.00	0.00
Carboxypeptidase A	4.85	4.74	4.70	**0.00**	**0.00**	***4.62***	**0.00**
α–Chymotrypsinogen A	4.30	4.69	4.76	4.66	4.34	4.83	0.00
Lactoferrin	5.90	7.77	8.47	8.89	9.40	9.57	9.73
Lysozyme	4.60	4.76	5.29	5.46	5.41	5.80	5.63

Table XIV. Adjusted Retention Volume at Various pH on Fe(III)-EDDA—PSDVB-3. 12.0 min linear gradient from 0 to 1.0 *M* NaCl in 10 m*M* phosphate. Column, 30 x 4.6 mm I.D.; flow-rate, 1.0 mL/min.

	pH						
PROTEINS	4.0	5.0	6.0	7.0	8.0	9.0	10.0
Iron-free transferrin	4.63	5.91	4.57	**4.42**	0.00	0.00	0.00
Carboxypeptidase A	5.63	6.11	3.55	4.59	**0.00**	***5.23***	**0.00**
α-Chymotrypsinogen A	4.93	5.92	5.89	5.35	4.99	5.35	4.31
Lactoferrin	9.64	12.00	11.72	11.27	10.86	10.16	9.64
Lysozyme	4.86	5.63	6.22	5.90	5.69	5.82	5.57

metals like Fe(III) bind proteins at low pH stronger than at high pH whereas soft metal like Co(II) bind proteins at high pH stronger than at low pH.[7] The probable explanation for the discrepant behavior between silica-based stationary phases and PSDVB-based chelate sorbents is the absence of silanol groups in the latter supports. The residual silanol groups make the silica support more negative at high pH thereby inducing greater retention by mixed-mode mechanism, i.e., electrostatic and metal interaction.

The retention of α-chymotrypsinogen A on both Co(II)-EDDA and Fe(III)-EDDA columns were found to fluctuate with pH. Carboxypeptidase A displayed an interesting behavior in the sense that its retention first dropped to 0 at pH 8.0, increased at pH 9.0 and became unretained again at pH 10. One possible explanation for this peculiar trend would be the possibility that carboxypeptidase A may have undergone some conformational changes such that at pH 8.0 most of its metal interacting sites got buried and were reexposed at pH 9.0.

In general, for both metal-EDDA columns, i.e., Co(II) and Fe(III) loaded columns, lactoferrin exhibited the highest retention followed by lysozyme, α-chymotrypsinogen A, iron-free transferrin and carboxypeptidase A. The stronger affinity exhibited by lactoferrin toward the metal chelate sorbent with respect to that of iron-free transferrin is apparently caused by the basic pI of the former. As explained in the Chapter II, the net charge of the acidic iron-free transferrin would become negative at pH above its pI value and this may induce electrostatic repulsion from the negatively charged sorbent with concomitant decrease in retention. In addition, the steric hindrance caused by the oligosaccharide envelope of iron-free transferrin is apparently sufficient to prevent its binding with the metal although it has a very high histidine content (19 His/mole); see Table XII.[8] Likewise, the difference in isoelectric points of lysozyme (pI = 11) and α-chymotrypsinogen A (pI = 9.5) resulted in the higher retention of the former over the latter although they possess about the same number of histidine residues. Despite its high

number of histidine groups, the low retention of carboxypeptidase A on the two metal-EDDA columns can again be explained by its acidic nature which caused the protein analyte to be electrostatically repulsed from the sorbent at high pH.

IDA Columns. In another set of experiments, the effect of eluent pH on the retention of the six model proteins on Co(II)-IDA and Zn(II)-IDA columns was investigated. Tables XV and XVI summarize the adjusted retention volumes of the proteins over the pH range 4-10 obtained on Co(II)-IDA and Zn(II)-EDDA columns. It can be clearly observed that in most cases, the six model proteins exhibited stronger affinities toward the Co(II)-IDA column than Zn(II)-IDA column in the pH range studied. In fact, lactoferrin did not show any elution with a linear gradient of increasing NaCl concentration at pH 6-8 on Co(II)-IDA column. As mentioned earlier (see Chapter III), Zn(II) has a coordination number of four whereas Co(II) has a maximum of six. Therefore, in the metal-IDA coordination sphere, the Zn(II) would have only one available site for the proteins *versus* at least three sites in the case of Co(II). Iron-free transferrin, α-chymotrypsinogen A, carbonic anhydrase and lysozyme exhibited almost the same retention trends on both columns. Carboxypeptidase A was also found to behave similarly except that with Zn(II)-IDA column it did not have retention at 3 pH's, 7.0, 8.0 and 10.0 whereas with Co(II)-IDA column, the absence of affinity toward the immobilized metal was only observed at pH 8.0 and 10.0. These results indicate that it would require more hydroxide ions in the mobile phase to dissociate the protein from Co(II) column than from a Zn(II) column since as mentioned above, there are three available sites with the former metal chelate while there is only one site with the latter metal complex.

Co(II)-EDDA *versus* Co(II)-IDA Columns. In almost all cases, the six model proteins were more retained on the Co(II)-IDA column (Table XV) when compared with Co(II)-EDDA column (Table XIII). Besides higher retention, some of the proteins under

Table XV. Adjusted Retention Volume at Various pH on Co(II)-IDA—PSDVB-3. 12.0 min linear gradient from 0 to 1.0 *M* NaCl in 10 m*M* phosphate. Column, 30 x 4.6 mm I.D.; flow-rate, 1.0 mL/min.

	pH						
PROTEINS	4.0	5.0	6.0	7.0	8.0	9.0	10.0
Iron-free transferrin	4.69	4.83	**4.92**	0.00	0.00	0.00	0.00
Carboxypeptidase A	5.37	5.59	5.98	5.20	**0.96**	***5.18***	**0.00**
Carbonic anhydrase	5.00	6.26	**5.43**	0.00	0.00	0.00	0.00
α-Chymotrypsinogen A	5.09	5.07	5.60	5.76	4.69	5.08	5.32
Lactoferrin	7.68	11.30	**NE**	**NE**	**NE**	10.37	8.43
Lysozyme	5.27	6.59	6.82	6.95	6.49	6.28	6.84

NE, no elution.

Table XVI. Adjusted Retention Volume at Various pH on Zn(II)-IDA—PSDVB-3. 12.0 min linear gradient from 0 to 1.0 *M* NaCl in 10 m*M* phosphate. Column, 30 x 4.6 mm I.D.; flow-rate, 1.0 mL/min.

	pH						
PROTEINS	4.0	5.0	6.0	7.0	8.0	9.0	10.0
Iron-free transferrin	4.69	4.67	**5.80**	0.00	0.00	0.00	0.00
Carboxypeptidase A	5.07	4.83	4.72	**0.00**	**0.00**	***4.74***	**0.00**
Carbonic anhydrase	4.75	5.07	**4.72**	0.00	0.00	0.00	0.00
α-Chymotrypsinogen A	4.52	4.60	5.06	4.90	4.59	4.84	4.33
Lactoferrin	5.06	7.37	7.98	8.50	9.48	9.07	9.72
Lysozyme	4.60	5.93	6.43	6.36	6.12	6.31	6.19

investigation exhibited different retention patterns on the two columns. In particular, the elution of lactoferrin from Co(II)-IDA column required the addition of imidazole to the eluent in the pH range 6-8. Furthermore, the monotonic increase in the retention of lactoferrin with pH observed with the Co(II)-EDDA was found to be missing with the Co(II)-IDA column. Instead, after showing no elution from pH 6-8, the retention started to decrease. Although iron-free transferrin, lysozyme, α-chymotrypsinogen A, carboxypeptidase A and carbonic anhydrase were more retained on Co(II)-IDA than on Co(II)-EDDA, these proteins exhibited more or less the same retention behavior on both columns.

Under all conditions employed using metal-IDA columns, the elution order was found to be almost identical with that of the metal-EDDA column, i.e., lactoferrin > lysozyme > α-chymotrypsinogen A > carboxypeptidase A, iron-free transferrin, carbonic anhydrase. In most cases, carbonic anhydrase A was more retained that iron-free transferrin. It is clear then that with carbonic anhydrase and iron-free transferrin, the more predominant factor in determining the retention with the metal chelates is the isoelectric point and not the histidine content.

Macroporous *versus* Non-porous Columns. The retention data obtained on porous stationary phases are summarized in Tables XVII-XX. They were obtained under the same elution conditions as those with non-porous metal-chelate columns. Normally, one would expect that the effect of the eluent pH will result in the same trend for the two types of polymeric supports, while the magnitude of retention will be higher on the wide pore sorbent considering the fact that the surface concentration in metal chelating ligands is higher. However, based on the data obtained, the eluent pH affected the retention of proteins on the porous support in a manner that was significantly different from that of the non-porous material, depending on the proteins used.

As expected, in all cases, the retention of the six model proteins were significantly higher on the wide pore PLRP than on the non-porous home made PSDVB (see Tables XIII-XX), which indicates the presence of higher surface ligand density in the porous material (see Chapter V). In fact, with the non-porous stationary phases the model proteins eluted with a linear gradient of increasing sodium chloride concentration, and did not require the inclusion of a competing ligand such as imidazole in the eluent. The only exception was lactoferrin on Co(II)-IDA column which showed no elution at pH 6-8 when a gradient of increasing NaCl concentration was used. On the other hand, with the wide pore PLRP-S, lactoferrin, iron-free transferrin and carbonic anhydrase did not elute unless a relatively large amount of imidazole was added to the eluent at high concentration of salt.

To further assess the differences and similarities between the two types of sorbents (i.e. porous *versus* non-porous) the results obtained on the two types of Co(II)-EDDA polymeric supports (see Tables XIII and XVII) were compared. Lactoferrin, α-chymotrypsinogen A, lysozyme and carboxypeptidase A exhibited similar retention behavior on both sorbents. A significant difference was observed with iron-free transferrin which did not elute at pH 4.0 from the macroporous Co(II)-EDDA column. However, at higher pH the retention of this solute on the wide pore resembled that obtained on non-porous Co(II)-EDDA, in that, its retention decreased sharply and approached zero at high pH. Another difference between the two stationary phases is that with Co(II)-EDDA—PLRP, iron-free transferrin was found to be more retained than lactoferrin at pH 4.0. Likewise, carbonic anhydrase showed similar behavior. The higher retention of iron-free transferrin and carbonic anhydrase at this pH may be attributed to some residual hydrophobic adsorptivities with uncovered and accessible non-polar patches of the polymeric supports. Hydrophobic interactions are favored at low pH because the protein surface is less charged.

Table XVII. Adjusted Retention Volume at Various pH on Co(II)-EDDA—PLRP. 12.0 min linear gradient from 0 to 1.0 *M* NaCl in 10 m*M* phosphate. Column, 30 x 4.6 mm I.D.; flow-rate, 1.0 mL/min.

	pH						
PROTEINS	4.0	5.0	6.0	7.0	8.0	9.0	10.0
Iron-free transferrin	NE	6.86	**4.70**	0.00	0.00	0.00	0.00
Carboxypeptidase A	6.92	6.65	5.58	5.12	**4.67**	***5.60***	**0.00**
Carbonic anhydrase	NE	**7.14**	0.00	0.00	0.00	0.00	0.00
α-Chymotrypsinogen A	6.75	5.82	5.66	5.81	5.46	5.99	1.68
Lactoferrin	9.74	9.92	11.08	12.00	**NE**	**NE**	**NE**
Lysozyme	7.41	7.75	7.93	7.90	7.89	7.77	7.87

NE, no elution.

Table XVIII. Adjusted Retention Volume at Various pH on Fe(III)-EDDA—PLRP . 12.0 min linear gradient from 0 to 1.0 *M* NaCl in 10 m*M* phosphate. Column, 30 x 4.6 mm I.D.; flow-rate, 1.0 mL/min.

	pH						
PROTEINS	4.0	5.0	6.0	7.0	8.0	9.0	10.0
Iron-free transferrin	**NE**	**NE**	**5.69**	0.00	0.00	0.00	0.00
Carboxypeptidase A	7.58	7.63	6.59	5.63	**5.53**	***6.71***	**0.00**
Carbonic anhydrase	**NE**	**NE**	**7.11**	0.00	0.00	0.00	0.00
α-Chymotrypsinogen A	6.21	5.31	6.72	6.51	5.89	6.26	5.39
Lactoferrin	10.14	12.00	12.00	**NE**	**NE**	**NE**	**NE**
Lysozyme	7.74	9.05	9.07	8.64	8.99	9.47	9.12

NE, no elution.

Table XIX Adjusted Retention Volume at Various pH Obtained on Co(II)-IDA—PLRP Column.
(a) 12.0 min linear gradient from 0 to 1.0 *M* NaCl in 10 m*M* phosphate at various pH and (b) 12 min gradient from 0 to 50 m*M* imidazole in 10 m*M* phosphate and 1.0 *M* NaCl. Column, 30 x 4.6 mm I.D.; flow rate, 1.0 mL/min.

PROTEINS	4.0		5.0		6.0		7.0		8.0		9.0		10.0	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b
α-Chymotrypsinogen A	0.71	NM	0.33	NM	6.31	NM	5.88	NM	6.32	NM	6.32	NM	1.29	NM
Carboxypeptidase A	10.10	NM	8.50	NM	7.86	NM	5.57	NM	6.04	NM	6.98	NM	0.00	NM
Lysozyme	9.21	NM	8.45	NM	9.46	NM	8.33	NM	9.11	NM	8.23	NM	7.83	NM
Lactoferrin	10.55	NM	10.19	NM	NE	5.60	NE	5.26	NE	7.59	NE	NE	NE	NE
Iron-free transferrin	NE	NE	NE	5.38	NE	6.70	NE	4.59	NE	4.17	NE	0.00	NE	0.00
Carbonic anhydrase	NE	NE	NE	9.25	NE	8.21	NE	5.68	NE	4.69	NE	4.24	NE	4.23

NM, not measured.
NE, no elution.

Table XX. Adjusted Retention Volume at Various pH Obtained on Zn(II)-IDA—PLRP Column.
(a) 12.0 min linear gradient from 0 to 1.0 *M* NaCl in 10 m*M* phosphate at various pH and (b) 12 min gradient from 0 to 50 m*M* imidazole in 10 m*M* phosphate and 1.0 *M* NaCl. Column, 30 x 4.6 mm I.D.; flow rate, 1.0 mL/min.

PROTEINS	4.0		5.0		6.0		7.0		8.0		9.0		10.0	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b
α-Chymotrypsinogen A	0.60	NM	0.36	NM	5.82	NM	6.20	NM	6.85	NM	7.16	NM	5.97	NM
Carboxypeptidase A	11.25	NM	9.71	NM	6.66	NM	5.93	NM	0.49	NM	0.00	NM	0.00	NM
Lysozyme	10.03	NM	9.63	NM	8.25	NM	8.51	NM	0.04	NM	9.73	NM	9.75	NM
Lactoferrin	11.60	NM	12.00	NM	NE	4.51	NE	5.83	NE	7.79	NE	4.01	NE	4.12
Iron-free transferrin	NE	NE	NE	6.38	NE	5.39	NE	4.40	NE	4.07	NE	4.11	NE	4.05
Carbonic anhydrase	NE	NE	NE	7.89	NE	5.64	NE	5.43	NE	5.14	NE	4.33	NE	4.19

NM, not measured.
NE, no elution.

With the two types of polymeric Fe(III)-EDDA stationary phases (i.e. porous *vs* non-porous, see Tables XIV and XVIII), the retention trends exhibited by lysozyme, carboxypeptidase A and α-chymotrypsinogen A were more or less the same. On both columns, i.e., PLRP and PSDVB, iron-free transferrin displayed no retention at high pH, i.e., starting from pH 7.0 and 8.0. Again, iron-free transferrin and carbonic anhydrase were found to be more retained than lactoferrin at pH 4.0 and 5.0. This can be explained by the same reasoning as with Co(II)-EDDA columns; see above. The findings that non-porous Fe(III)-EDDA stationary phases were more retentive than the Co(II)-EDDA columns (see Tables XIV and XIII, respectively) were also found to hold true in the case of wide pore PLRP (see Tables XVII and XVIII).

By comparing the two types of Co(II)- and Zn(II)-IDA columns, i.e., non-porous and porous (see Tables XV and XVIII; Tables XVI and XX, respectively), it can be noticed that the retention trends exhibited by lysozyme, α-chymotrypsinogen A and carboxypeptidase A were more or less identical. As with EDDA metal chelate columns, with Co(II)- and Zn(II)-IDA macroporous columns, both iron-free transferrin and carbonic anhydrase were more retained than lactoferrin at pH 4-6 but less retained at higher pH.

With few exception, retention data obtained on PSDVB and PLRP metal chelate sorbents had more or less the same energetics with the difference that retentions are higher in magnitude on the latter than on the former columns. These data give confidence in the reliability of the home made sorbents as far as their chromatographic behavior is concerned.

Rapid Separation Schemes With Wide Pore Metal Chelate Columns

To illustrate the potentials of wide pore PLRP microparticles with surface-bound metal chelating ligands in protein chromatography, and to evaluate the effectiveness of the surface modifications that we have developed (Chapter V) several proteins were

chromatographed on the macroporous poly(styrene-divinylbenzene) stationary phases under a wide range of elution conditions.

Figure 38a-c shows the rapid separation of 3 model proteins, namely, iron-free transferrin, α-chymotrypsinogen A and lysozyme on Fe(III)-EDDA—PLRP column. Figure 38a illustrates the separation obtained when a 12.0 minute linear gradient of increasing sodium chloride at pH 9.0 was used. Figure 38b represents the separation obtained when the gradient time was decreased to 6.0 min and the flow-rate was doubled, i.e., the steepness of the gradient was conserved but the analysis time was decreased. In fact, under these conditions, the separation was accomplished in 4.6 minutes instead of 9.7 minutes when compared to Fig. 38a without any loss in column efficiency. Fig. 38c shows the resulting chromatogram when the injection time was delayed by 2.5 minutes. Hence, proper manipulation of the operating parameters can lead to the desired rapid separation.

In another set of experiments, the same polystyrene support was used, but having surface-bound IDA chelating ligands. Four model proteins, α-lactalbumin, cytochrome c, lysozyme and iron-free transferrin were eluted and separated in about 14.7 minutes by using two consecutive linear gradients consisting of 9.0 minutes linear gradient from 0 to 0.75 *M* NaCl and 12.0 minutes linear gradient of increasing imidazole concentration at constant NaCl concentration, pH 6.0, and a flow-rate of 1.0 mL/min (Fig. 39a). Chromatogram 39b illustrates the rapid separation of the same set of proteins using the same set of mobile phases but reducing the time of the first and second gradients to 4.5 and 6.0 minutes, respectively, with a concurrent doubling of the flow-rate. As can be clearly seen, under these conditions the separation of the protein mixture was obtained in a shorter time. Delaying the injection for 2.5 minutes has further reduced the retention without affecting the separation efficiency of the chromatographic column.

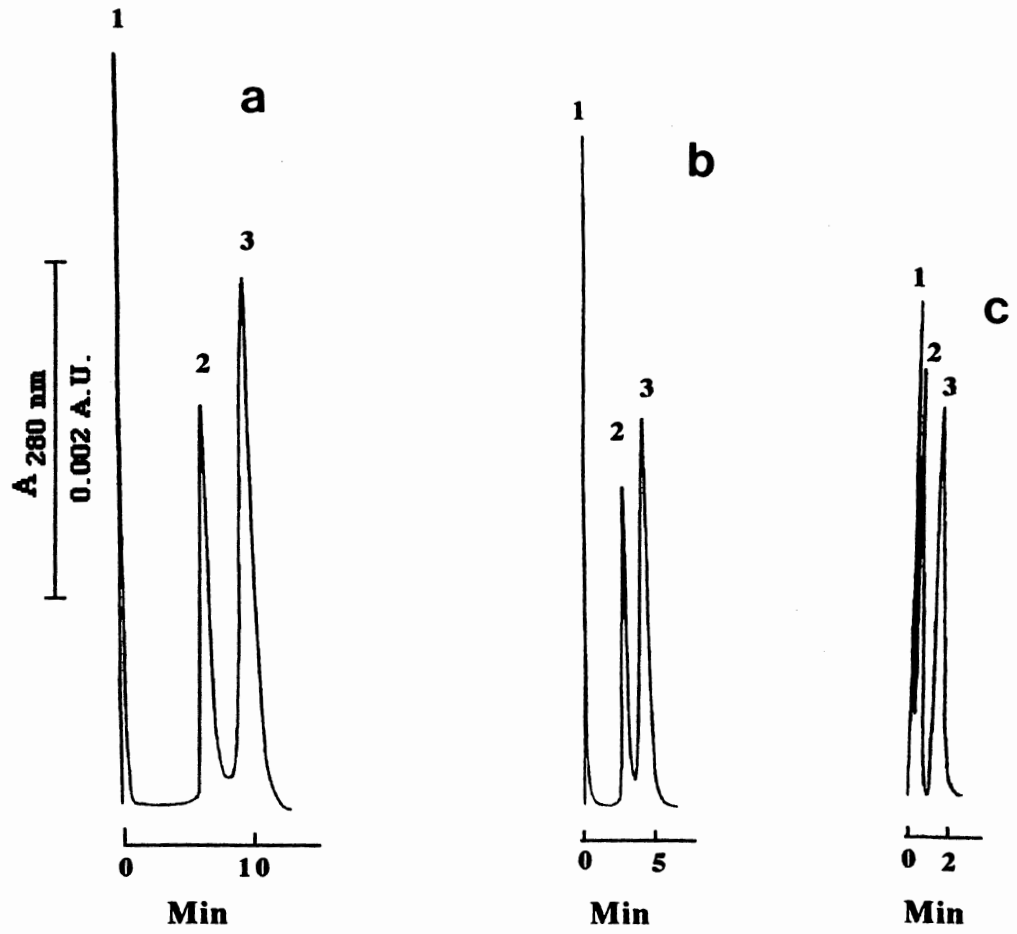


Figure 38. Rapid Separation of Proteins on Fe(III)-EDDA—PLRP Column.
Column, 30 x 4.6 mm I.D. (a) 12.0 min linear gradient from 0 to 1.0 M NaCl in 10 mM boric acid, pH 9.0; flow-rate, 1.0 mL/min and (b) 6.0 min linear gradient from 0 to 1.0 M NaCl in 10 mM boric acid, pH 9.0; flow-rate, 2.0 mL/min and (c) gradient as in (b) but the injection was delayed for 2.5 min. Proteins: 1, iron-free transferrin; 2, α-chymotrypsinogen A; 3, lysozyme.

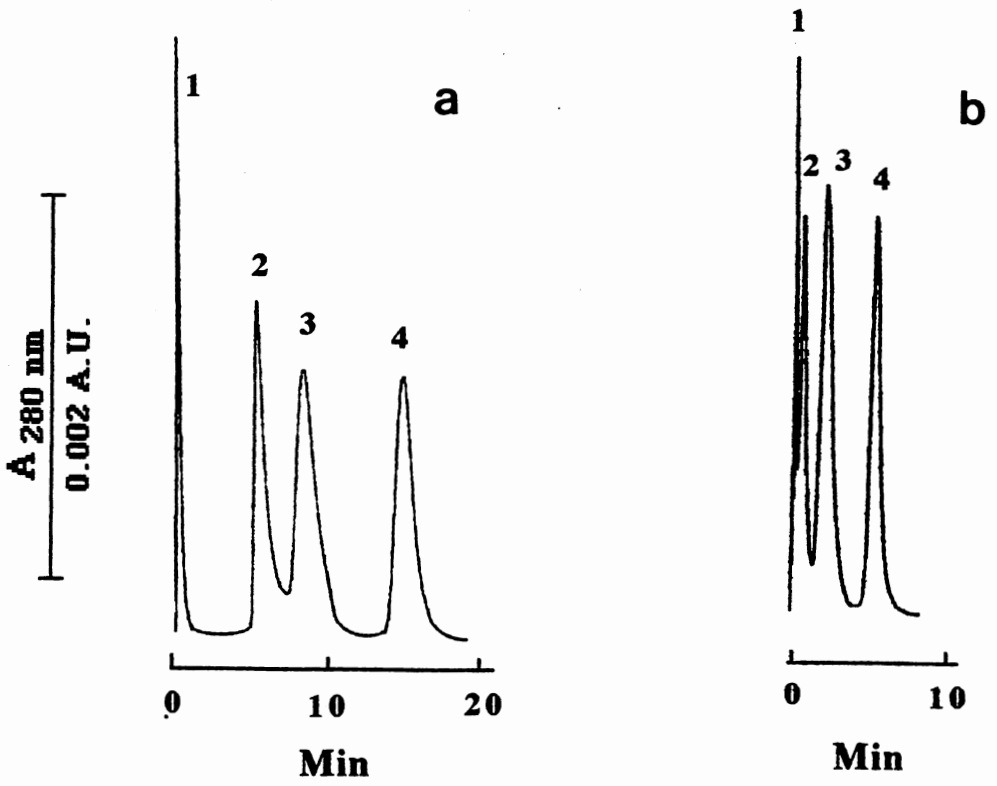


Figure 39. Rapid Separation of Proteins on Zn(II)-IDA—PLRP Column. Column, 30 x 4.6 mm I.D. (a) 9.0 min linear gradient from 0 to 0.75 *M* NaCl followed by 12.0 min linear gradient from 0 to 50 m*M* imidazole in 10 m*M* phosphate containing 1.0 *M* NaCl, pH 6.0; flow-rate, 1.0 mL/min and (b) 4.5 min linear gradient from 0 to 0.75 *M* NaCl followed by 6.0 min linear gradient from 0 to 50 m*M* imidazole in 10 m*M* phosphate and 1.0 *M* NaCl at pH 6.0; injection delayed for 2.5 min; flow-rate, 2.0 mL/min. Proteins: 1, α-lactalbumin; 2, cytochrome c; 3, lysozyme; 4, iron-free transferrin.

Using the same column, Zn(II)-IDA-PLRP, a different set of model proteins comprising of holo-transferrin, α-chymotrypsinogen A, lysozyme and iron-free transferrin was separated at pH 8.0 (Figure 40). Again, two consecutive linear gradients were utilized. A linear gradient of increasing NaCl concentration was followed by a linear gradient of increasing imidazole at constant NaCl concentration with gradient times of 4.5 minutes and 6.0 minutes, respectively, at a flow-rate of 1.0 mL/min. As can be seen, even when a relatively low flow velocity, i.e., 1.0 mL/min was used, a rapid separation of the above-mentioned proteins was still accomplished. In addition, the stability of this polymeric sorbent at high pH was clearly illustrated.

Effects of the Nature and Concentration of Salt

The magnitude of retention of proteins in MIC depends among other things on the nature and concentration of salts in the eluent.[9-11] Thus, a systematic study on its effect on protein retention with various metal chelates and chelating ligands would provide an insight into the dynamic behavior of proteins in MIC.

In this study, the salts were selected as to represent antichaotropic and chaotropic types. According to Hofmeister series,[12] salts containing ions such as phosphates, sulfates, acetates and chlorides are called antichaotropic, i.e., water-structuring salts, whereas salts containing ions such as thiocyanates and perchlorates are called chaotropic, i.e., water-destructuring agents. The former has the tendency of salting-out while the latter has the tendency to bind to the proteins and consequently promote salting-in.

The studies concerning the effects of the nature and concentration of salt on MIC retention was carried out using isocratic elution at pH 6.0 and employing lysozyme and lactoferrin as model proteins. The salts studied on Fe(III)-EDDA and Co(II)-EDDA columns were NaCl, Na_2SO_4, KSCN and $NaClO_4$. In another set of experiments, NaCl, NaH_2PO_4, KSCN and $NaClO_4$ were investigated on Co(II)-IDA column. The first two

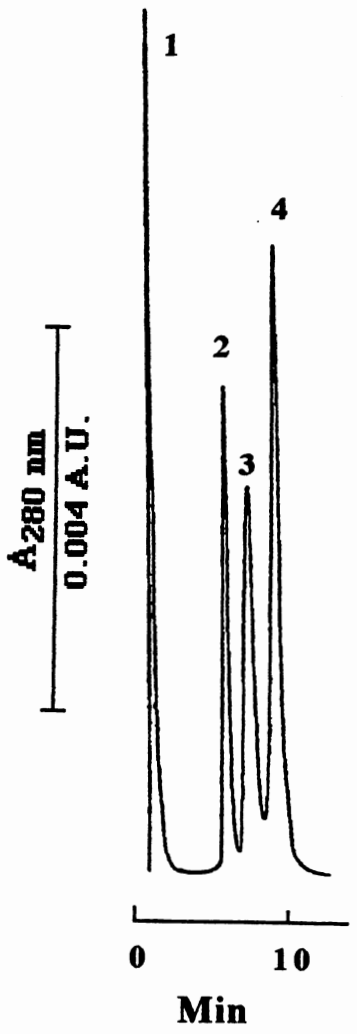


Figure 40. Rapid Separation of Proteins on Zn(II)-IDA—PLRP Column. Column, 30 x 4.6 mm I.D.; flow-rate, 1.0 mL/min. 4.5 min linear gradient from 0 to 0.75 *M* NaCl followed by 6.0 min linear gradient from 0 to 50 m*M* imidazole in 10 m*M* phosphate containing 1.0 *M* NaCl, pH 8.0. Proteins: 1, holo-transferrin; 2, α-chymotrypsinogen A; 3, lysozyme; 4, iron-free transferrin.

salts are classified as antichaotropic while the last two salts are known as chaotropic salts. On the other hand, Co(II) and Zn(II) are representatives of intermediate "soft" metals whereas Fe(III) is in the borderline of "hard" metal ions. Typical plots of the logarithm of the retention factor (log k') of lysozyme *versus* logarithm of the molar salt concentration obtained on Co(II)-EDDA, Fe(III)-EDDA and Co(II)-IDA columns are shown in Figures 41, 42 and 43, respectively.

As expected, in all cases, the log k' decreased linearly with log [salt] at relatively low salt content in the eluent. The two model proteins showed no interaction with the metal chelate stationary phases at relatively high salt concentration in the eluent. An exception is the case of lysozyme with NaCl on Fe(III)-EDDA column whereby after the initial decrease in retention, it passed through a minimum and then increased at high salt concentration. It is hardly surprising that all metal chelate columns studied showed little or no hydrophobic interactions since metals are known to bind water extensively, thus rendering the stationary phase more hydrated than when naked (i.e. no chelated metal) metal chelating sorbents are used.

At low concentration of salt, increasing the concentration of the counterions would saturate the metal sites with electron-donor groups, hence, less binding sites are available for the solutes, which result in decrease in protein retention. The exception observed with lysozyme, where the retention first decreased and then increased at high salt concentration can be attributed to the interplay of two kinds of interactions: metal and/ or electrostatic interactions which dominate the retention at low salt concentrations and hydrophobic interactions at high salt concentrations. The same behavior was also reported for lysozyme when ammonium sulfate was used as an eluting salt.[13]

The intercepts of the linear plots of log k' vs log salt molarity with the ordinate (y-intercept) are summarized in Tables XXI-XXIII, and were used to rank the eluting strength

of various salts. It can be seen from these tables that the eluting strengths of the salts depends on the type of the solute, chelated metal and chelating ligand.

As explained in Chapter II, a greater negative intercept will reflect a stronger eluting salt. Based on this consideration, with Co(II)-EDDA the eluting strength of the salt for lysozyme was in the following order: Na_2SO_4 > NaCl > KSCN > $NaClO_4$ whereas for lactoferrin, the order was: KSCN > $NaClO_4$ > Na_2SO_4 > NaCl. With Fe(III)-EDDA, the trend was found to be NaCl > Na_2SO_4 > KSCN > $NaClO_4$ for lysozyme and KSCN > NaCl > $NaClO_4$ > Na_2SO_4 for lactoferrin. With Co(II)-IDA, the eluting strength was arranged as NaCl > KSCN > NaH_2PO_4 > $NaClO_4$ for lysozyme and KSCN > $NaClO_4$ > NaH_2PO_4 > NaCl for lactoferrin. In order to provide a brief comparison of the eluting strengths of the salts under investigation on various metal chelate columns Tables XXIV-XXV summarize their ranking with lysozyme and lactoferrin, respectively.

It can be noticed that with lactoferrin, the chaotropic salt KSCN was found to be the strongest eluting salt on all the MIC columns tested. In addition, on both Co(II)-EDDA and IDA, $NaClO_4$ was ranked as second to KSCN in terms of eluting strength. From Hofmeister series KSCN is more chaotropic than $NaClO_4$. Chaotropic salts promote salting-in by virtue of their tendency to bind to proteins, which in this case may be with the metal binding sites of the proteins, thereby reducing metal-protein interaction. Another possible explanation is that chaotropic salts are known to disrupt the native states of the proteins,[12] hence, there is a possibility that in the presence of these salts lactoferrin underwent a conformational change which might led to the exposure of lesser amount of metal interacting sites.

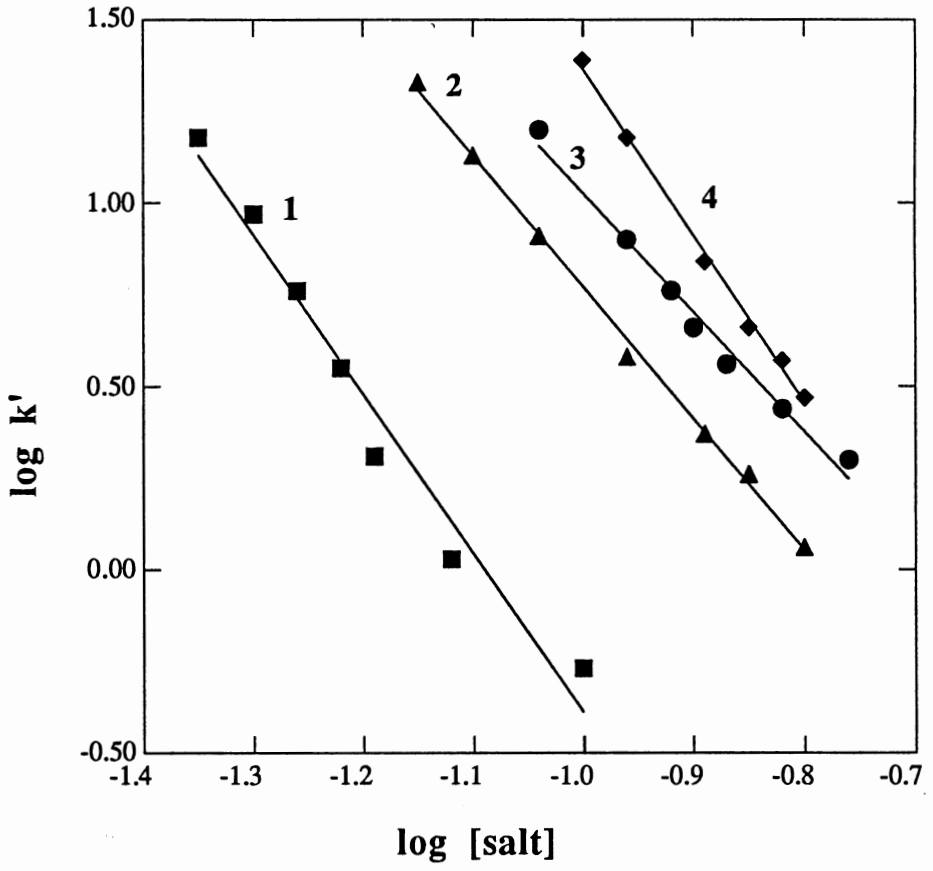


Figure 41. Plots of the Logarithm of the Retention Factor of Lysozyme *versus* Logarithm of the Salt concentration on Co(II)-EDDA—PSDVB-3.
Column, 30 x 4.6 mm I.D.; flow-rate, 1.0 mL/min. Salts: 1, Na_2SO_4; 2, KSCN; 3, $NaClO_4$; 4, NaCl.

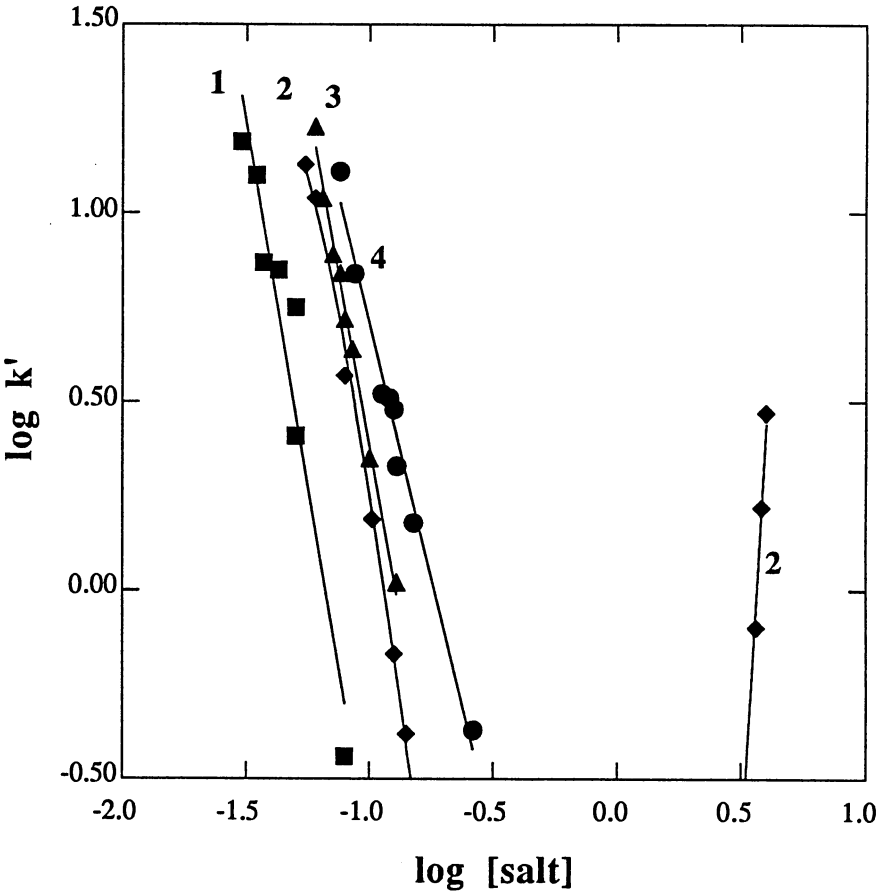


Figure 42. Plots of the Logarithm of the Retention Factor of Lysozyme *versus* Logarithm of the Salt Concentration on Fe(III)-EDDA—PSDVB-3. Column, 30 x 4.6 mm I.D.; flow-rate, 1.0 mL/min. Salts: 1, Na_2SO_4; 2, NaCl; 3, KSCN; 4, $NaClO_4$.

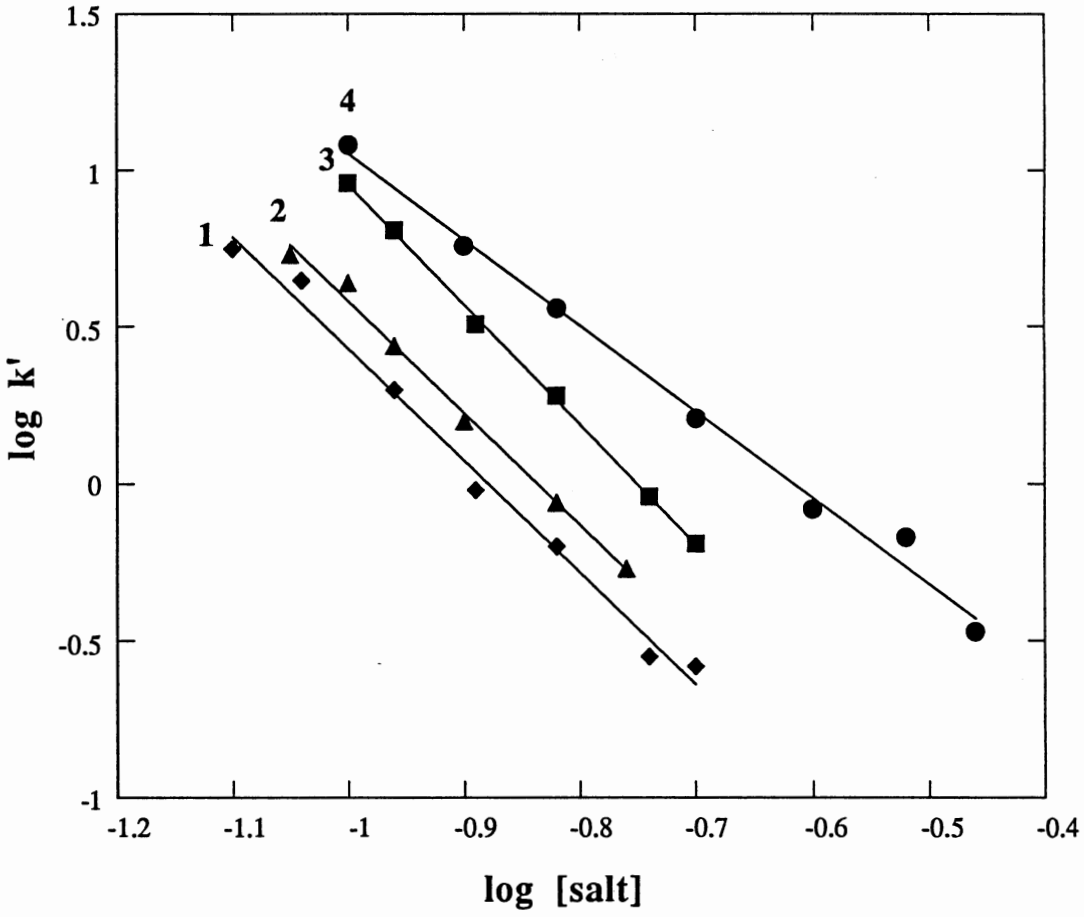


Figure 43. Plots of the Logarithm of the Retention Factor of Lysozyme *versus* Logarithm of the Salt Concentration on Co(II)-IDA—PSDVB-3. Column, 30 x 4.6 mm I.D.; flow-rate, 1.0 mL/min. Salts: 1, NaCl; 2. KSCN; 3, NaH_2PO_4; 4, $NaClO_4$.

Table XXI. Values of the y-Intercept of Plots of Logarithm of the Retention Factor *versus* Logarithm of the Salt Concentration Obtained on Co(II)-EDDA—PSDVB-3 Column.
Isocratic measurements in 10 m*M* phosphate buffer, pH 6.0. Column, 30 x 4.6 mm I.D; flow-rate, 1.0 mL/min.

SALTS	y-Intercepts	
	Lysozyme	Lactoferrin
Na-SULFATE	-4.72	-2.81
Na-CHLORIDE	-3.20	-1.80
K-THIOCYANATE	-2.82	-4.17
Na-PERCHLORATE	-2.22	-3.14

Table XXII. Values of the y-Intercept of Plots of Logarithm of the Retention Factor *versus* Logarithm of the Salt Concentration Obtained on Fe(III)-EDDA—PSDVB-3 Column.
Isocratic measurements in 10 m*M* phosphate buffer, pH 6.0. Column, 30 x 4.6 mm I.D; flow-rate, 1.0 mL/min.

SALTS	y-Intercepts	
	Lysozyme	Lactoferrin
Na-CHLORIDE	-6.20	-3.04
Na-SULFATE	-4.52	-1.84
K-THIOCYANATE	-3.21	-3.74
Na-PERCHLORATE	-1.98	-2.84

Table XXIII. Values of the y-Intercept of Plots of Logarithm of the Retention Factor *versus* Logarithm of the Salt Concentration Obtained on Co(II)-IDA—PSDVB-3 Column. Isocratic measurements in 10 m*M* phosphate buffer, pH 6.0. Column, 30 x 4.6 mm I.D; flow-rate, 1.0 mL/min.

SALTS	y-Intercepts	
	Lysozyme	Lactoferrin
Na-CHLORIDE	-3.14	-1.06
K-THIOCYANATE	-2.99	-2.57
Na-PHOSPHATE	-2.87	-1.84
Na-PERCHLORATE	-1.69	-2.30

Table XXIV. Ranking of Salts on Various Metal Chelates Using Lysozyme as the Model Protein.
Isocratic measurements in 10 m*M* phosphate buffer, pH 6.0. Column, 30 x 4.6 mm I.D.; flow-rate, 1.0 mL/min.

COLUMNS	Na_2SO_4	NaCl	KSCN	$NaClO_4$	NaH_2PO_4
Co(II)-EDDA	++++	+++	++	+	NM
Co(II)-IDA	NM	++++	+++	+	++
Fe(III)-EDDA	+++	++++	++	+	NM

(++++, strongest eluting salt; +++, second; ++, third; +, least; NM, not measured)

Table XXV. Ranking of Salts on Various Metal Chelates Using Lactoferrin as the Model Protein.
Isocratic measurements in 10 m*M* phosphate buffer, pH 6.0. Column, 30 x 4.6 mm I.D.; flow-rate, 1.0 mL/min.

COLUMNS	KSCN	$NaClO_4$	Na_2SO_4	NaCl	NaH_2PO_4
Co(II)-EDDA	++++	+++	++	+	NM
Co(II)-IDA	++++	+++	NM	+	++
Fe(III)-EDDA	++++	++	+	+++	NM

(++++, strongest eluting salt; +++, second; ++, third; +, fourth; NM, not measured)

In the case of lysozyme with the two metal-EDDA columns, the two antichaotropic salts were found to be stronger eluting salts when compared with the two chaotropic salts. It is also very interesting to point out that with Co(II)-EDDA, Na_2SO_4 is a stronger eluting salt when compared with NaCl but the reversal holds true with the Fe(III)-EDDA column. Pearson[14] classified SO_4^{2-} as a soft Lewis base whereas Cl^- is a hard Lewis base. On the other hand, Co(II) is classified as a soft Lewis acid whereas Fe(III) is a hard Lewis acid. Hard acids are assumed to bind bases with primarily ionic forces and the soft acids bind bases by covalent bonds.[15] Because of the different nature of the two types of interactions, soft acids prefer to combine with soft bases and hard acids with hard bases. Hence, it is not surprising that the hard base Cl^- exhibited greater affinity towards the hard acid Fe(III) and the soft SO_4^{2-} ions interacted more with the soft immobilized Co(II).

To compare with silica-based sorbents (see Chapter III), lactoferrin was chromatographed with sodium phosphate and sodium chloride on Co(II)-IDA—PSDVB-2. The results indicate that sodium phosphate is a stronger eluting salt on both types of

sorbents, i.e., silica and PSDVB. However, with lysozyme, NaCl was found to possess a greater eluting strength on the PSDVB-based Co(II)-IDA. This is in agreement with the results obtained when similar study was conducted on Co(II)-IDA—silica (see Chapter III).

From the above results the following conclusions can be made: (i) regardless of the nature of the salt, protein-metal associations decrease with increasing salt concentration in the eluent, (ii) the eluting strength of the salt is enhanced when the salt induces changes in the conformation of the solute or when the salt anionic counterions bind to the protein metal binding sites, (iii) with "hard" metal ions, retention decreases with increasing hardness of the salt anionic counterions whereas with "soft" metal ions, solute-metal interaction decreases with increasing softness of the anions of the salts and (iv) for a given metal, the eluting strength of the salts varies with the nature of the chelating ligand.

Rapid MIC with Non-porous Metal Chelate-PSDVB

In rapid HPLC of biomacromolecules, an ideal stationary phase should satisfy the following major criteria: (i) small particle diameter, (ii) absence of intraparticle mass transfer resistances, i.e., non-porous, and (iii) rigid structure. The highly crosslinked and non-porous PSDVB with their excellent mechanical strength and small particle diameter allowed the rapid separation of proteins at high flow velocity.

As demonstrated in Chapter IV, the PSDVB microparticles having 35% divinylbenzene (PSDVB-2) proved to be stable at high flow velocities with aqueous solvents only. This poses some limitations for PSDVB-2 since some studies have demonstrated that the retention and selectivity of proteins in MIC can be modulated by the addition of small amounts of organic modifiers to the eluent.[3, 13] Nevertheless, as can be seen in Fig. 44, PSDVB-2 having Zn(II)-IDA ligands permitted the rapid separation of

four different proteins in less than 4.0 minutes using 6.0 minutes linear gradient of increasing imidazole concentration at pH 8.0 and at a flow-rate of 2.0 mL/min.

On the other hand, PSDVB-3 column having 40% divinylbenzene was found to be mechanically stable in both organic and aqueous eluents (see Chapter IV). This excellent behavior has allowed the extensive use of PSDVB-3 in most of our studies. Figure 45a and b illustrates the rapid separation of four different proteins on two Zn(II)-IDA—PSDVB-3 columns having PEG 200 or diepoxy-PEG 600 as hydrophilic coatings, respectively. Although these two columns exhibited comparable retention toward the proteins, the analytes were better resolved on the column with diepoxy-PEG 600 coating (compare Fig. 45a and b). As explained in Chapter V, this may be due to the fact that diepoxy-PEG 600 coating provided higher metal chelating ligand concentration on the surface of the stationary phase. Nevertheless, in both cases, sharp peaks were obtained when two consecutive linear gradients consisting of 2.0 min gradient of increasing NaCl concentration and 2.0 min gradient of increasing imidazole concentration in 1.0 *M* NaCl were utilized at a flow-rate of 4.0 mL/min.

It has been demonstrated above that the wide pore PLRP microparticles can yield relatively fast separations. However, it was clear that its performance can not surpass the excellent performance of the home made non-porous metal chelating sorbents. Although high flow velocities can be utilized on macroporous PSDVB owing to their high permeability, rapid separation of proteins having low selectivity factor, i.e., relative retention, can not be performed. This is due to the fact that with macroporous stationary phases, stagnant mobile phase mass transfer resistances are present, hence lowering the efficiency of separation. A very good illustration would be the result obtained in Figure 46a and b when six model proteins, α-lactalbumin, cytochrome c, lysozyme, iron-free transferrin, carbonic anhydrase and lactoferrin were separated on Zn(II)-IDA—PSDVB-2

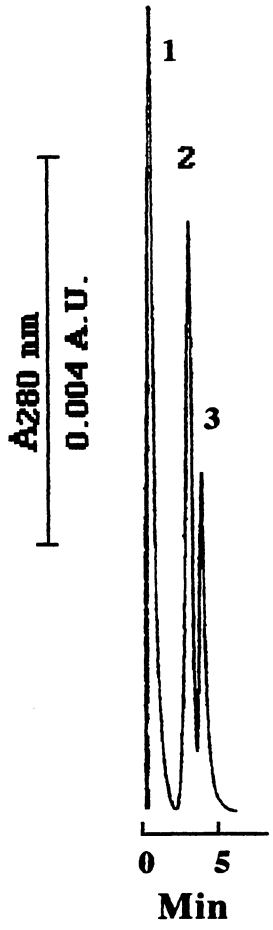


Figure 44. Rapid separation of proteins on Zn(II)-IDA—PSDVB-2.
Column, 30 x 4.6 mm I.D.; flow-rate, 2.0 mL/min. 6.0 min linear gradient from 0 to 50 m*M* imidazole in 10 m*M* phosphate, pH 8.0. Proteins: 1, lactoferrin; 2, iron-free transferrin; 3, carbonic anhydrase.

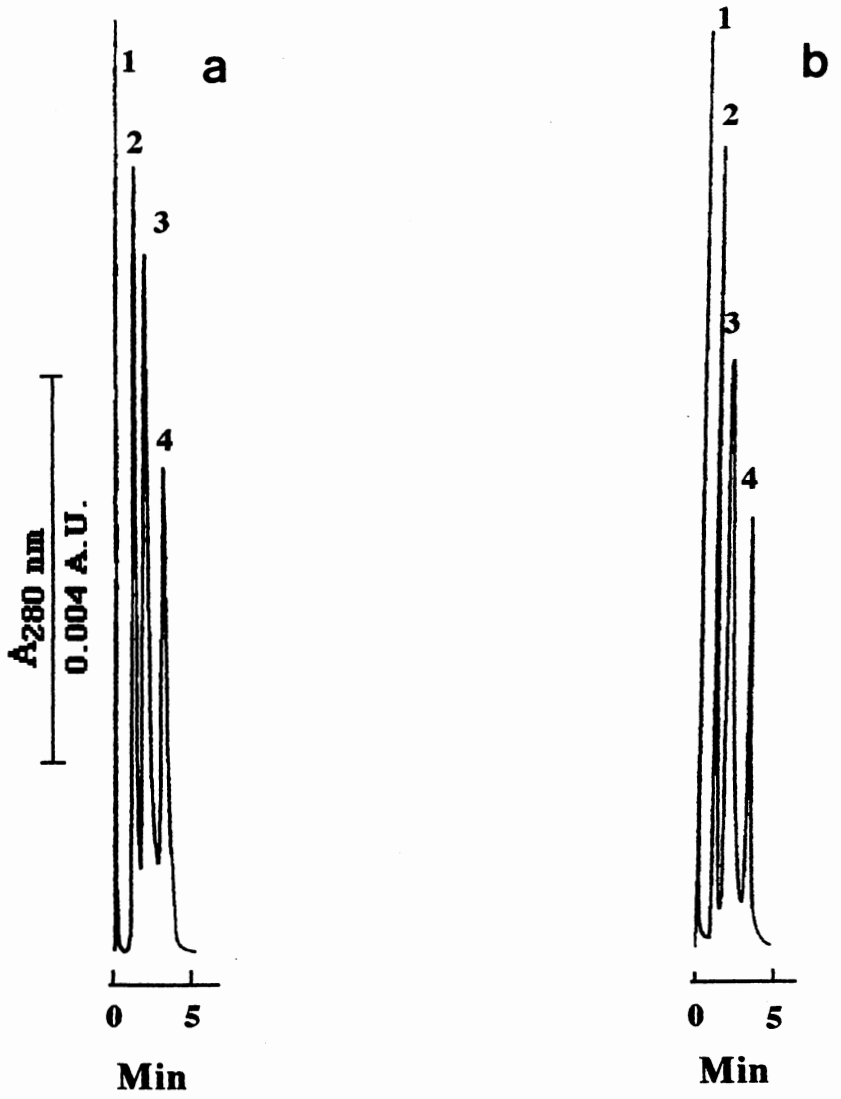


Figure 45. Rapid separation of proteins on Zn(II)-IDA—PSDVB-3 Column. Column, 30 x 4.6 mm I.D.; flow-rate, 4.0 mL/min. The attachment of the ligand was carried out *via* (a) PEG 200 and (b) diepoxy-PEG 600. 2.0 min linear gradient from 0 to 1.0 *M* NaCl followed by a 2.0 min linear gradient from 0 to 50 m*M* imidazole in 10 m*M* phosphate containing 1.0 *M* NaCl, pH 6.0. Proteins: 1, α-lactalbumin; 2, cytochrome c; 3, lactoferrin; 4, carbonic anhydrase.

and Zn(II)-IDA—PLRP columns, respectively. Comparison of Fig. 46a and b clearly demonstrated that iron-free transferrin, carbonic anhydrase and lactoferrin were much better resolved on the non-porous stationary phase under otherwise identical elution conditions. This proved that the presence of pores in the PLRP-S stationary phases will indeed lower the separation efficiency of biomolecules at high flow rates.

Lectin-PSDVB for High Performance Liquid Affinity Chromatography of Glycoproteins

To demonstrate the usefulness of the hydrophilic PSDVB developed in this study as a chromatographic support for a different mode of HPLC, a lectin affinity ligand, concanavalin A (Con A) was immobilized on the surface of the hydrophilic support. Concanavalin A is a ligand which has a specific affinity towards glycoproteins.[16] Figure 47a and b show the elution of two glycoproteins, ovalbumin and peroxidase from Con A-PSDVB column using a 7.0 minute linear gradient from 0 to 50 m*M* methyl-α-*D*-mannopyranoside in 10 m*M* phosphate containing 0.1 *M* NaCl, pH 7.0.

This short study had shown that PEG coated polystyrene supports can be activated with bifunctional ligands and the resulting surface can be utilized to immobilize affinity ligand and other functionalities for the separation of proteins by various modes of HPLC.

Conclusions

The procedures previously developed (Chapter V) for the hydrophilic coating and attachment of chelating ligands on both non-porous and macroporous poly(styrene-divinylbenzene) proved to be particularly useful in this study. The results have demonstrated that the chemically modified non-porous polystyrene supports are well suited for the rapid and efficient separation of proteins. Also, this study showed that macroporous polystyrene microparticles packed into short columns can be used for relatively rapid separation of proteins. However, non-porous microparticles exhibited

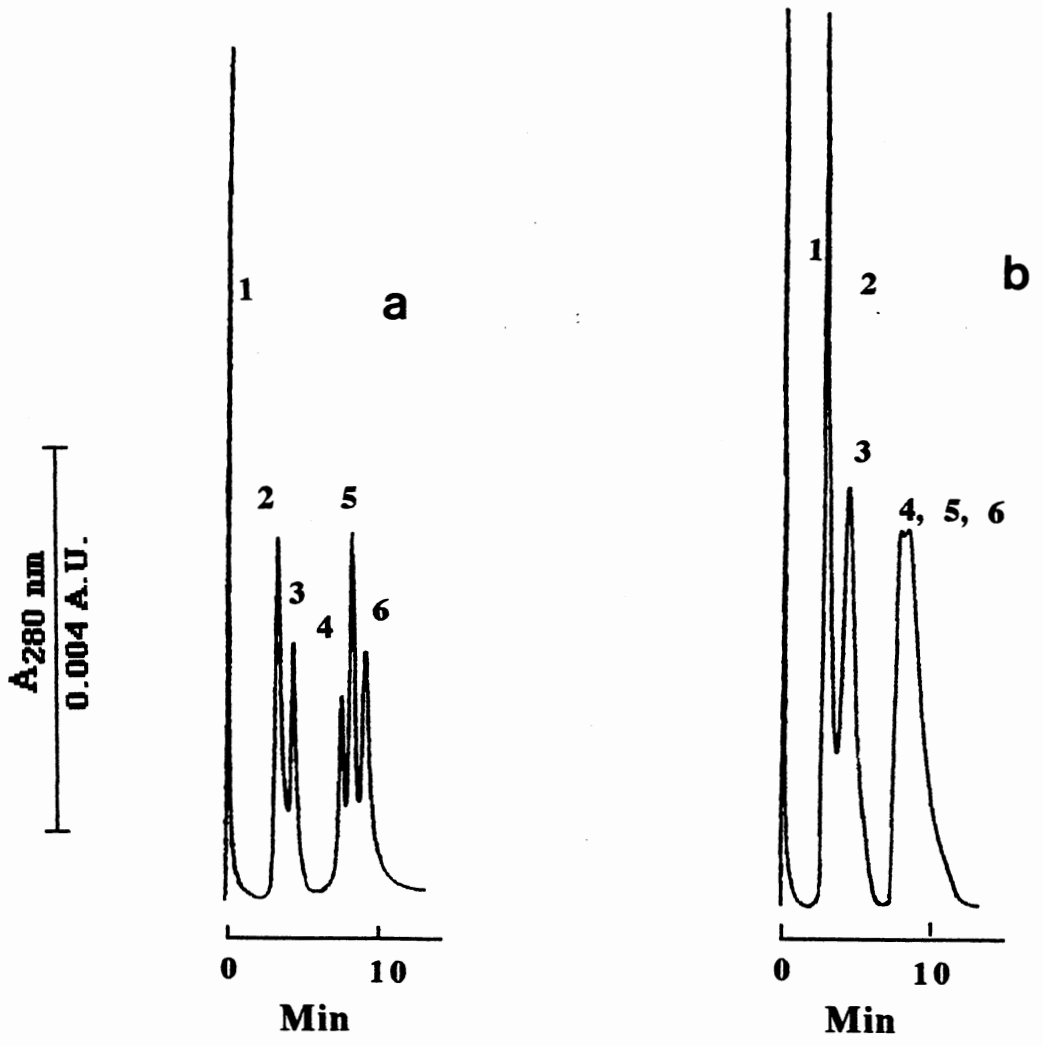


Figure 46. Rapid Separation of Six Model Proteins on (a) Zn(II)-IDA—PSDVB-2 and (b) Zn(II)-IDA—PLRP Columns.
Column, 30 x 4.6 mm I.D.; flow-rate, 2.0 mL/min. 6.0 min linear gradient from 0 to 1.0 *M* NaCl followed by 6.0 min linear gradient from 0 to 50 m*M* imidazole in 10 m*M* phosphate containing 1.0 *M* NaCl, pH 6.0. Proteins: 1, α-lactalbumin; 2, cytochrome c A; 3, lysozyme; and 4, iron-free transferrin; 5, carbonic anhydrase; 6, lactoferrin.

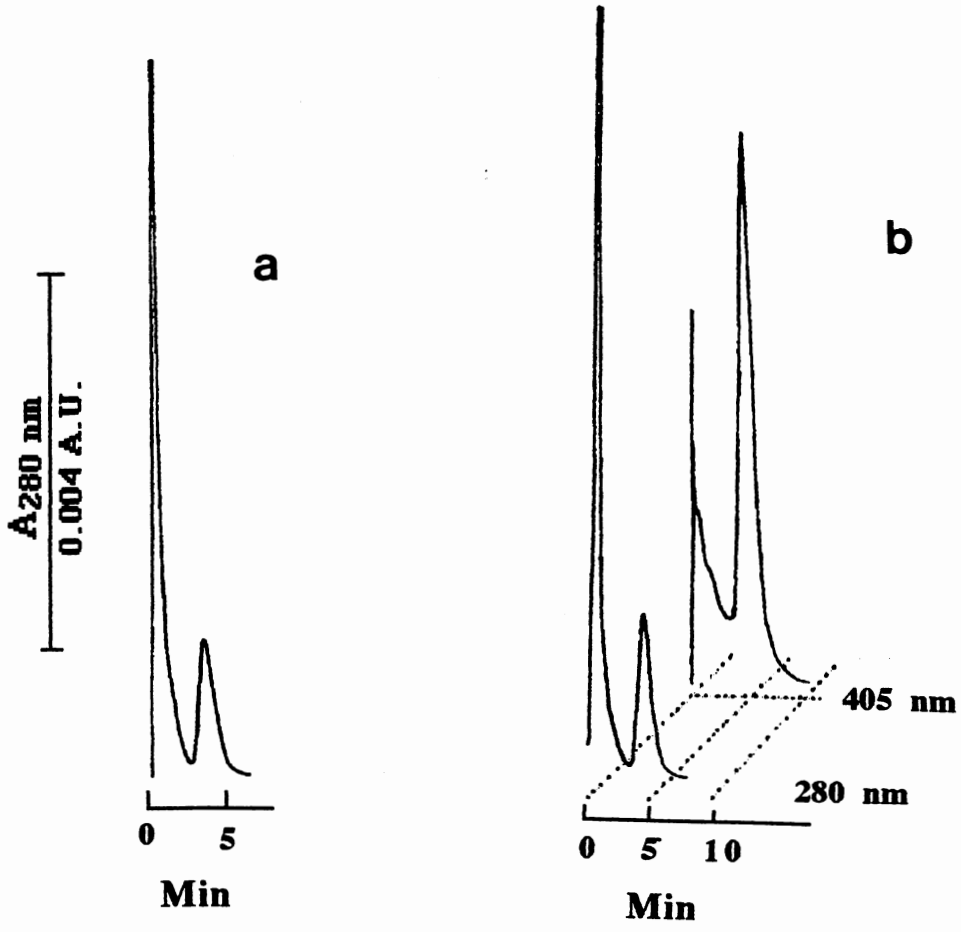


Figure 47. Chromatograms of (a) Ovalbumin and (b) Peroxidase from Con A-PLRP-S Column.
7.0 min linear gradient from 0 to 50 m*M* methyl-α-*D*-mannopyranoside in 10 m*M* phosphate containing 0.1 *M* NaCl at pH 7.0. Column, 30 x 4.6 mm I.D.; flow-rate, 1.0 mL/min.

better performance than wide pore microparticles when utilized for rapid separation of proteins especially for those having low relative retention factors.

References

1. Porath, J.; Olin, B. *Biochemistry* **1983**, 22, *1621.*

2. Fatiadi, A. J. Critical Reviews in Analytical Chemistry; CRC Press:Boca Raton, FL, **1987**, 1.

3. Corradini, D.; El Rassi, Z.; Horvath, C.; Guerra, G.; Horne, W. *J. Chromatogr.* **1988**, *458*, 1.

4. Hutchens, T. W.; Henry, J. F.; Yip, T. -T. *Clin. Chem.* **1989**, *35*, 1928.

5. Dayhoff, M. O. *Atlas of Proteins Sequence and Structure*; National Biomedical and Research Foundation: U.S.A., **1979**, 105.

6. Righetti, P. G.; Tudor, G. *J. Chromatogr.* **1981**, *220*, 115.

7. El Rassi, Z.; Horvath, C. in *HPLC of Biological Macromolecules*, Gooding, K. M.; Regnier, F. E., Eds., Marcel Dekker, Inc.: New York, **1990**, Chapter 2.

8. Sulkowski, E. *Makromol. Chem. Macromol. Symp.* **1988**, *17*, 335.

9. Porath, J. *Biotechnol. Prog.* **1987**, *3*, 14.

10. Sulkowski, E. in *Protein Purification: Macro to Micro, UCLA Symposium on Molecular and Cellular Biology*, Burgess, R., Ed.,Vol. 68, Allan R. Liss: New York, **1987**, 149.

11. Figueroa, A.; Corradini, C.; Feibush, B.; Karger, B. *J. Chromatogr.* **1986**, *371*, 335.

12. Voet, D.; Voet, J. *Biochemistry*; John Wiley and Sons: New York, **1990**, 181.

13. El Rassi, Z.; Horvath, C. *J. Chromatogr.* **1986**, *359*, 241.

14. Pearson, R. G. *J. Am. Chem. Soc.* **1963**, *85*, 3535.

15. Pearson, R. G. *Science* **1966**, *151*, 172.

16. Borchert, A.; Larsson, P; Mosbach, K. *J. Chromatogr.* **1982**, *244*, 49.

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