ZIRCONIA- AND SILICA-BASED STATIONARY PHASES FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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LIST OF SYMBOLS AND ABBREVIATIONS

A	constant
A_{254}	absorption unit at wavelength 254 nm
α	selectivity factor
C_B	concentration of B in the mobile phase
C_s	concentration of eluting salt in the mobile phase
d_p	particle diameter
f	fraction of the molecules ionized
F	flow rate
ϕ_{mono}	phase ratio of the monomeric stationary phase
ϕ_{poly}	phase ratio of the polymeric stationary phase
h, hr	hour
φ	volume fraction of the organic eluent
k'	retention factor
k_p	retention factor of the parent molecule
k_w	retention factor in pure water
K	thermodynamic equilibrium constant
λ	wavelength
M	molecular weight of the solute
n	coefficient proportional to the size of solute molecule
θ_B	concentration of B in the stationary phase
S	slope of the linear function
t_g	gradient time

ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
B/L	breadth to length ratio
Con A	Concanavalin A
CRT	cathode-ray tube
CVD	carbon vapor deposition
DEAE	N,N-dimethylaminoethanol
DMF	N,N-dimethylformamide
Dns-AA	dansyl amino acid
d.p.	degree of polymerization
EDA	electron donor-acceptor
ESR	electron span resonance
Fig.	figure
FTIR	Fourier transform infrared spectroscopy
GDP	guanosine-5'-diphosphate
GMP	guanosine-5'-monophosphate
GTP	guanosine-5'-triphosphate
HPLC	high performance liquid chromatography
I.D.	inner diameter
IDA	iminodiacetic acid
IUPAC	International Union of Pure and Applied Chemistry
LCA	*lens culinaris* agglutinin
µg	microgram
µm	micrometer
µmol	micromole
MeCN	acetonitrile

N/D	not determined
N/M	not measured
NMR	nuclear magnetic resonance
N/R	not retained
ODA	octadecyl-bonded alumina
ODS	octadecyl-bonded silica
PAD	pulsed amperometric detector
PAH	polycyclic aromatic hydrocarbon
PA-XG	2-pyridylamino derivatives of xyloglucan oligosaccharides
PBD	polybutadiene
PEG	poly(ethylene glycol diglycidyl ether)
PEI	polyethyleneimine
pI	isoelectric point
pK_a	negative logarithmic acid dissociation constant
PSDVB	poly(styrene) divinyl benzene
psi	pound per square inch
PVA	poly(vinyl alcohol)
PVAN	poly(vinyl alcohol) with quaternary amine functions
SEM	scanning electron micrography
TEOS	tetraethyl orthosilicate
TFA	trifluoroacetic acid
TRAMQ	quaternarized triamine stationary phase
UDP	uridine-5'-diphosphate
UMP	uridine-5'-monophosphate
UTP	uridine-5'-triphosphate
v/v	volume to volume
WGA	wheat germ agglutinin

w/v	weight per volume

CHAPTER I

BACKGROUND AND RATIONALE

Introduction

Major advances in high performance liquid chromatography (HPLC) have been mostly the results of developments in instrumentation and column technology. Currently, a large variety of chemical and biochemical species of complex nature and different properties can be separated by HPLC due to the availability of various stationary phases that offer the analysts the freedom of choosing the proper separation mechanism to achieve the desired resolution and selectivity. The improvements in physical and chemical properties of column packing materials have significantly increased the column efficiency, performance and lifetime. Very fast and highly efficient separations can be accomplished in less than one minute with modern HPLC techniques, which would require several hours of elution time in earlier stage of liquid chromatography.

Column packing materials have evolved over the years of HPLC development and applications. First, silica-based packing materials provided columns with sufficient mechanical stability to withstand the high operating pressure, generally encountered in HPLC. In addition, the well-studied surface chemistry enabled the formation of a variety of stationary phases on silica surface. Thereafter, organic polymer packing materials were introduced to supplement the shortcomings of silica-based materials with superior chemical stability to be used under a variety of chromatographic conditions, e.g. high pH. In search

for rapid separations under minimal mass transfer resistance, and in turn, minimal band-broadening, nonporous and flow-through pore packing materials were introduced, especially for the separation of macromolecules. Recently, researchers have been evaluating inorganic metal oxides as column packings which can afford excellent mechanical strength and chemical stability under modern chromatographic conditions. In addition, surface modification proved to be feasible for the formation of a large variety of stationary phases. These inorganic sorbents including alumina, zirconia, and titania based stationary phases demonstrated unique selectivities which can not be obtained with conventional silica- or organic polymer-based packing materials.

As a contribution to the development of novel column packing materials for HPLC, our research has focused on the synthesis of monodispersed, microspherical zirconia particles as column support. Surface modifications were studied to form the stationary phases for reversed phase, hydrophilic interaction and ion exchange chromatography. The chromatographic properties of these stationary phases were evaluated and their applicability to a large variety of species of importance to the life sciences was examined. Comparative studies were also carried out with the conventional silica-based stationary phases. The results of these studies are reported in the following chapters of this dissertation.

In this introductory chapter, the physical and chemical properties of traditional column packing materials, silica and organic polymers, as well as the latest development in inorganic support based packing materials, such as alumina and zirconia, will be reviewed. The influences of support properties such as particle size, pore size and distribution, mechanical strength, surface chemistry, modification for stationary phase formation, and chemical stability, etc. on chromatographic performances will be discussed for each type of sorbents. Emphasis will be placed on zirconia as HPLC column support. The overview on the published studies of surface chemistry and chromatographic behavior of zirconia sorbents serves as background information for our research and development of microspherical zirconia particles and the subsequent modification of their surface to form a

variety of stationary phases, such as nonpolar, hydrophilic and ionic sorbents for practical application as HPLC packing materials in the separation of a large variety of species.

Important Physical and Chemical Properties of Chromatographic Supports

Column packing materials for HPLC have over the years received continuous improvements in terms of particle size, mechanical strength, and chemical stability, etc.. This is because the physical and chemical properties of chromatographic sorbents strongly influence column efficiency, selectivity and resolution, as well as column lifetime.

Among the important physical properties of column supports are the particle shape, mean particle size, specific surface area, mean pore diameter and specific pore volume. Microparticles are generally available in irregular and spherical shapes. Irregular microparticles are less expensive and still widely used, largely because, in practice, it has not been found that their chromatographic properties are significantly inferior. Spherical microparticles, however, have been preferred in modern column technology since they yield more homogeneous, stable and permeable column beds. Particle shape may become more important as the particle size is reduced, and spherical microparticles are considered superior for particle diameters less than 5 μm [1]. For analytical application, microparticles of 5 to 10 μm in diameter are the most widely used, and to a lesser extent, 3 μm microparticles. Larger diameter particles, generally between 15 to 40 μm, are used in preparative-scale liquid chromatography.

The specific surface area can be separated into two components: the surface area within the pores and the external surface area of the particle. The pore surface area is several orders of magnitude larger than the external surface area and, in general, the larger the surface area of the packing material the smaller will be the pore diameter. Micropores are particularly undesirable as they may give rise to size-exclusion effects or irreversible adsorption due to the high surface energy existing within the narrow pores. According to

IUPAC recommendation [2], micropores are considered to have a diameter < 20 Å, mesopores between 20 - 500 Å, and macropores > 500 Å.

The chemical properties of column sorbents strongly influences the modification and formation of stationary phases. In the covalent bonding process, functional groups are attached to the support surface usually through already existing sites such as the surface hydroxyl groups of inorganic supports (e.g., silica, alumina, zirconia) or through sites that were grafted to the surface of the support (e.g. hydroxylation of polystyrene divinyl benzene). The concentration and the nature of attachment sites can influence the extent of stationary phase formation. Steric limitations allow only part of the support attachment sites to be reacted with stationary phase ligands. Thus, the chemical properties (acidity, basicity, hydrophilicity, hydrophobicity, etc.) of the support proper can effect solute retention process. Solute can interact with column support through mechanisms such as acid-base, hydrophobic interaction, electrostatic or charge transfer. These secondary retention mechanisms usually result in low column efficiency and poor resolution. However, in some instances, the residual interactions with the support can modulate selectivity and may prove to be effective in separation of certain species that normally can not be resolved with totally inert sorbent packing materials. In general, the aim of surface modification is to minimize secondary mechanisms arising from the support.

Overview of Traditional Column Packings for HPLC

In the early stages of liquid chromatography developments, the column packing materials were relatively large particle sizes of totally porous texture. These were usually polysaccharides such as cellulose, agarose, and cross-linked dextrans [3-5]. These column packings had good sample capacity but only limited efficiency accompanied by long separation times, due to the large size and unfavorable size distribution of the particles through which sample molecules diffuse in and out very slowly. The development of

modern liquid chromatography has required that the support be mechanically strong to withstand higher operating pressure and of small particle size to yield higher separation efficiencies.

Silica Sorbents

The introduction of silica microparticles in the early 1970s significantly improved the speed and the efficiencies in chromatographic separations. Packing materials of relatively high mechanical stability allow the separation process to be performed at higher mobile phase flow velocity, which brings about shorter analysis time. The surface chemistry of silica gels has been well studied over the years. A large variety of surface modification procedures have been established to form the stationary phases of different selectivity which made silica gels the most widely used column support for HPLC.

Silica gels with mean pore diameters of 50-150 Å and surface areas of 150-600 m^2/g have been preferred for the separation of low molecular weight samples, while silica gels with pore diameters greater than 300 Å are suitable for the separation of biopolymers to avoid restricting the accessibility of the solutes to the stationary phase [6-9]. In the case of macromolecules such as large molecular weight proteins and DNA fragments, even macroporous packings still have drawbacks in terms of low performance, mass recovery and solute biological activity [10]. These phenomena are to some extent associated with the restricted mass transfer of biopolymers migrating through the pore network. One approach to solve the problem of slow diffusion is to eliminate the pores altogether and use particles of smaller size (d_p = 0.5-2.5 μm) to counterbalance the loss in internal surface area. The concept of using nonporous packings was introduced in 1984 by Unger's research group [11], and a series of studies were carried out on the application of nonporous column support in the reversed-phase [12], hydrophobic interaction [13], and affinity [11] chromatography. These studies on the nonporous particles of smaller size (1-3 μm)

indicated that high speed, high resolution and high separation efficiency of macromolecules can be easily attained. To achieve very rapid separation with nonporous sorbents on the time scale of seconds, higher column temperature [14,15], 50-80 °C, had to be used in order to reduce the higher column back pressure that arose from the mobile phase viscosity when the separation was carried out at higher flow velocity.

The silica surface consists of a network of silanol groups, some of which may be hydrogen bonded to water, and siloxane groups. A fully hydroxylated silica surface contains about 8 μmol of silanol groups per m^2, or 4.8 silanols per nm^2. Chemically bonded phases are prepared by reaction between the surface silanol groups of silica and reactive organosilanes to form siloxane bonds [16-18, 8, 19-21]. The major disadvantage of silica-based bonded phases is their limited hydrolytic stability which restricts their use to the pH range 2-8. At lower pH, their use is restricted by the hydrolysis of the siloxane bonded phases and at higher pH by the solubility of silica.

Efforts have been made to reduce the degradation of silica-based stationary phases and extend column lifetime. Pre-column packed with silica sorbents was placed between mobile phase pump and sample injector to let the mobile phase pass through before entering the analytical column. In this way, the mobile phase would be saturated with dissolved siliceous components which would prevent further dissolution of the packings in the analytical column. Mobile phase solvents saturated with siliceous components were commercialized for silica-based HPLC columns to minimize the deterioration of the stationary phases.

Recognizing the superior chemical stability of zirconia over a wide pH range, researchers [22-24] utilized zirconia to dope silica gel to form stabilized packing materials for ion exchange chromatography.

In order to improve the chemical stability of silica-based stationary phases, Kirkland described an approach using a monofunctional silanizing agent having steric-protecting groups to shield the hydrolytically labile bond between the silane and the silica

support [18]. This technique, which could extend the operating pH up to perhaps 10, was also introduced in HPLC '93 meeting in Hamburg, Germany [26, 27]. Silane compounds with large hydrophobic blocking groups, such as diisobutyl-*n*-octadecyl silane, were used to keep the silica support from contact with aqueous mobile phases. Other techniques including high coverage, and exhaustive end-capping were also applied to protect the silica support from attack by aqueous mobile phases of extreme pH conditions. It was reported in recent years [28-30] that, by use of mixed trifunctional silanes in the modification of silica, a monolayer film is formed on the silica surface, and the process was termed horizontal polymerization. Acid and base hydrolysis studies with FTIR have shown that this type of stationary phase formation is much more stable than the end-capped, conventional monomeric surface. Polymer bonded and polymer coated (i.e., polymer encapsulated) packings were among the new bonded phases that have been introduced in the last few years [31-34]. The silica gels are coated with a poly(siloxane) or poly(butadiene) prepolymer which is then immobilized by peroxide azo-*tert*-butane, or γ-radiation induced chemical crosslinking reactions. The availability of a large number of useful monomers and prepolymers has enabled a wide variety of reversed phase, polar, and ion exchange packings to be prepared using the same general reaction.

Polymer Sorbents

Another stage of support development began in the late 1970s with the introduction of rigid organic resins. The polymer supports are mechanically stable for HPLC at moderate flow rate, and chemically stable in the pH range of 2-12. Organic polymer based packings have found increasing use for those applications where silica-based packings are inadequate due to their chemical instability or surface heterogeneity [17, 31-33]. Current use of rigid polymer packings in the mode of ion-exchange chromatography, hydrophobic interaction chromatography, metal affinity chromatography, and other biospecific affinity

chromatography of biopolymers probably exceeds that of silica-based materials. Typically, commerical products can be prepared with particle size of 5-20 μm, pore diameters of about 20-6,000 Å, and surface areas of about 2-500 m^2/g [33,34].

Organic supports have been modified after polymerization to incorporate an octadecyl or other alkyl groups into the polymer structure. Direct reaction of the aromatic rings of poly(styrene) based packings with oleum or chlorosulfonic acid, or chloromethylation with chloromethyl ether under catalysis followed by amination, are widely exploited reactions for introducing ionic functional groups such as sulfonate and tetraalkylammonium groups to produce packings for ion exchange and ion chromatography [17,8,9,31,32,35,36].

For life science applications, ion exchangers have been prepared by covalently bonding a poly(ethyleneimine) polymer to nonporous poly(methacrylate) resin beads to obtain fast separations of proteins [37]. Nonporous cross-linked polystyrenes [38, 39] and synthetic hydrophilic resins [40,41] have been prepared and examined for the separation of biopolymers. The exact chemical composition of resin materials, commercialized by Tosoh, Tokyo (Japan), is not available, but it contains many primary hydroxyl groups. Diethylaminoethyl, sulphopropyl, octadecyl and butyl groups were chemically bonded on the surface of nonporous hydrophilic polymer spheres having 2.5 μm diameter. They are chemically very stable and can be operated over a wide pH range, 2-12. These packing materials are relatively rigid owing to the nonporous structure. The columns can be operated at flow rate up to 1.6 mL/min. Proteins [42] were separated with a higher resolution and in much shorter time on the nonporous sorbents which was one fifth to one sixth of the elution time on porous packings. The recovery of proteins from the nonporous columns was almost quantitative. On the other hand, the loading capacity was low owing to the small surface areas of the nonporous packings. This is the greatest disadvantage of nonporous packing materials.

A recent development in polymer-based packings is the introduction [43,44] of flow-through particles, in 10 and 20 μm sizes, produced from poly(styrene-divinylbenzene) with a thin film of cross-linked polymer. The support has two types of pore structure. One family of pores ranges from 6,000-8,000 Å in diameter, which form a network that transects the particle. Thus, mobile phase flows through particles and solutes are rapidly conducted by convection into the interior of sorbents, and the technique has been termed perfusion chromatography. The flow-through pores are thought to be of relatively low surface area and therefore make a small contribution to loading capacity. A second family of pores ranging from 800-1,500 Å, interconnecting the through pore network, are of much larger surface area and responsible for the bulk of the loading capacity in these sorbents. Solute transport in these smaller pores is more likely to occur by diffusion. The intraparticle solute transport with a combination of convective and diffusive transport modes is used to affect rapid solute transport into porous chromatographic sorbents. This type of packing was used for high speed and high resolution separations of proteins and peptides with high capacity in reversed phase, ion exchange, hydrophobic interaction, immobilized metal affinity and bioaffinity modes [43,44]. Stability studies showed that 10 μm particles packed into 250 x 4.6 mm columns are mechanically stable at pressures as high as 2,900 psi without bed compression. The packing is chemically stable with either high and low pH conditions, and can be used with most organic solvents. But it was noted that there may be residual double bonds in this support which can be oxidized if the matrix is exposed to strong oxidizing agents. This could change the surface characteristics of the support and may adversely affect separation. It is also recommended that the support not be used above 85°C, the temperature at which the sorbent is synthesized. There may be some temperature-induced structural change if the polymer packing is operated above this temperature.

Still, there are significant drawbacks in the chromatographic application of organic polymer packings. Many of the undesirable properties of polymer packings based on

poly(styrene) divinyl benzene (PSDVB) copolymers (the most popular type), have been attributed to the unavoidable incorporation of micropores into the polymeric structure [45-47]. The polymer packings possess both macroporosity, due to pores among the microspheres and their agglomerates, and microporosity, due to pores within the microspheres. For porous polymers, the column permeability depends on the physical properties of the solvent used as the mobile phase, and the efficiency depends strongly on the sample type, solvent and temperature. Efficiency generally decreases with increasing retention time. In good solvents, such as moderately polar organic solvents and aqueous mixtures containing acetonitrile or tetrahydrofuran, the micropores are swollen and column properties are most predictable. In poor solvents the microspheres are less swollen, the column efficiency is lower, and the retention time is greater. The poor efficiency is due to hindered diffusion within the micropores and increasing retention from the enhanced sorption effect due to the larger surface area contained within the micropores [46,47]. Polymer-based stationary phases are most often used for separations that require a chemically stable stationary phase, e.g., for the separation of basic compounds, where high pH mobile phase conditions are used to supress the ionization of solutes, but not many theoretical plates are necessary.

PSDVB has strong hydrophobicity which is another major disadvantage when used for the separation of biomacromolecules. The non-specific hydrophobic interactions between solutes and support matrix yield higher retention with low resolution and asymmetric peaks due to slow sorption kinetics which arises from the presence of the secondary mechanism. These undesirable effects were also observed with small molecules. For instance, aromatic hydrocarbons showed unusually higher retention on PSDVB-based reversed phase stationary phase [48,49], and consequently severe peak tailing was obtained. Problems are often encountered when low molecular weight solutes with an aromatic group are eluted under isocratic conditions. These results were often

attributed to the contribution of the backbone structure of the packing material, i.e., π–π charge-transfer interaction between solutes and polymer support.

Even though PSDVB can be modified through chloromethylation and sulfonation to form strong ion-exchange functional groups on the surface, these stationary phases are not suitable for the separation of proteins and other biopolymers. Their largely uncovered hydrophobic matrix can exert non-specific interactions with the solutes, which will yield low column efficiency. The hydrophobic nature of the uncovered support surface can cause protein denaturation and low recovery of biological activity of the biomacromolecule solutes. Efforts have been made to shield the organic backbone of PSDVB support surface by hydrophilic functionalities prior to the attachment of the interactive ligands [50,51].

One approach involves the formylation of PSDVB surface with α,α-dichloromethyl methyl ether catalyzed by aluminum chloride [51]. The carbonyl groups introduced on the aromatic rings are subsequently reduced using sodium borohydride in the presence of isopropanol. This procedure produces covalently attached hydroxyl groups on the PSDVB surface to provide very stable hydrophilic functions on the surface which can be utilized to attach ligands for chromatographic separations. Although the degree of surface coverage by the attached hydroxyl groups may be relatively high, it can be speculated that the aromatic and hydrophobic nature of PSDVB can not be totally shielded in this process.

In a recent study [50], hydrophilic coatings were formed on PSDVB in three different chemistries. Polyglycerol coating was realized first with the formation of a copolymer, through reaction between epibromohydrin or epichlorohydrin and glycidol in the presence of boron trifluoride. The copolymer is adsorbed onto the hydrophobic surface of PSDVB with the nonpolar halide containing functional groups oriented inward toward the PSDVB surface and hydroxyl containing functional groups outward. Finally, the polymer coating was cross-linked by ether bond formation in the presence of 3 M KOH. Since there are no hydrolyzable functional groups in the coating, this sorbent may be operated in either strong acid or base.

Polyglycerol-methacrylate coating was synthesized by achieving first the polymerization of glycidyl methacrylate to form the glyceryl methacrylate polymer in the presence of boron trifluoride. Then the polymer was deposited onto PSDVB. In an aqueous solution containing methacrylic acid and ammonium persulfate, cross-linking of methacrylate groups in the coating and polymerization of methacrylic acid in the solution was initiated simultaneously upon addition of the catalyst, tetramethylene diamine. During the course of reaction, methacrylate oligomers in the solution were grafted to the surface of the sorbent to form weak cation exchange functions. The coating was stable to both organic and aqueous mobile phases in the pH range of 2-10.

Diol phase was formed on PSDVB support that had been coated with the epibromohydrin-glycidol copolymer and incompletely cross-linked [50]. Sorbitol was coupled to the residual halide ($-CH_2Br$) functions through an ether linkage. Subsequent coupling of proteins to the sorbitol sorbent could be carried out by the cyanogen bromide activation and protein immobilization through an isourea linkage to amino groups.

Hydrophilic packings with non-aromatic backbone of lower hydrophobicity, such as those based on poly(vinyl alcohol), poly(alkyl methacrylate), poly(vinyl pyridine) and poly(hydroxyalkyl methacrylate) are commercially available [33,52,53] to improve the chromatographic properties of polymer packings. But, these hydrophilic gels can not be totally free of hydrophobic interactions with solutes [54]. Aromatic solutes still exhibited considerable retention in aqueous organic mobile phases in the reversed-phase mode.

Two types of highly hydroxylated gels (TSK) were produced by the Japanese firm Toyo Soda (Tokyo). The exact structures have not yet been described. Basically, one type is a hydroxylated polyether copolymer [55]. This type of gel is employed in the separation of synthetic organic polymers, but have also been applied to biopolymers such as proteins. The other type is a semirigid spherical gel, prepared on the basis of a hydrophilic vinyl polymer [56], and its use in the separation of proteins has been reported [57]. In general, hydrophilic polymer supports are less mechanically stable than their hydrophobic

counterparts which are essentially based on PSDVB network. In fact, PSDVB-based stationary phases are the most rigid organic polymer packings, and have found wide use in HPLC. However, as discussed above, a rather tedious procedure with a carefully chosen reaction conditions has to be followed in order to produce a surface with hydrophilic nature of very low hydrophobicity.

Novel Inorganic Sorbents

In recent years, there have been growing interests in inorganic packing materials for HPLC. Among these sorbents, alumina [58-60], zirconia [61-63], and, to a lesser extent, titania [59,63] based stationary phases have been evaluated in HPLC. These inorganic sorbents have the mechanical strength of silica, and chemical stability over a wide pH range like organic polymer packings. These properties provide more flexibility in the selection of separation conditions than silica- and polymer-based stationary phases. They possess different surface chemistry from that of the well-studied silica support. Although more studies are still needed to be carried out for better understanding of their properties, these inorganic sorbents have already shown some features that can be utilized to improve the chromatographic selectivity and resolution for complex sample separations.

Alumina Sorbents

The alumina support generally used in chromatography is the crystalline γ-alumina and is obtained by dehydration of the mineral Bayerite at elevated temperature, 200-600 °C [16,64]. It is available in several types with pore diameters from 60-150 Å, specific surface areas between 70-250 m^2/g, and pore volumes ranging from 0.2 to 0.3 mL/g. Its solubility at high pH is much lower than that of silica and can be used over the pH range of 2-12.

Alumina can be chemically modified in a manner similar to that of silica by use of an organochlorosilane reagent [65]. In fact, octadecyl-bonded alumina (ODA) has been reported [66] and the ODA was quite useful for the separations of low-molecular-weight organic compounds. Because alumina is quite stable at high pH, octadecyl-alumina packing proved useful for the separation of basic solutes with alkaline mobile phases which can suppress the ionization of these solutes.

To produce nonpolar alumina-based sordents, coating of alumina with polymeric layers was also practiced such as with polymeric butadiene via a free radical initiation of the cross-linking process [67]. Recent studies [68] indicated that the difference in solvent strength between methanol and acetonitrile is greater with polybutadiene-coated alumina than with C-18 silica, which suggests that coated alumina should prove advantageous for gradient elution of mixtures with a very wide range of polarities. A procedure for the preparation of polymeric cyano stationary phase was also developed [69].

The alumina surface, however, is more heterogeneous than silica, containing both hydroxyl groups and Lewis acid sites associated with partially coordinated aluminum ions. There are two alternative possibilities for interactions between alumina and solutes [70,71]. Many solutes with their electron density or basicity may adsorb on the acidic sites of alumina through nucleophilic interaction. The same sites may also form charge-transfer complexes with typical electron donors, such as aromatic solutes. Alternatively, acidic solutes may interact with basic sites on the alumina surface through transfer of a proton leading to chemisorption. In general, alumina interacts strongly with polar molecules, so much that its applicability is often limited by the adverse influence of chemisorption on the shape of the eluting peaks. On octadecyl-bonded alumina (ODA) stationary phase, there is a higher degree of hydrogen-bonding and solute-support interactions than on silica-based octadecyl (ODS) stationary phase, and multiple-ring aromatic compounds are more strongly adsorbed on ODA stationary phase than on ODS columns [72]. It has been reported [73] that peak broadening increases more rapidly with solute size on the polybutadiene-coated

alumina stationary phase with peptides, proteins and smaller molecules. This was attributed to the mass transfer resistance due to solute interaction with the uniquely-shaped alumina support.

Zirconia Sorbents

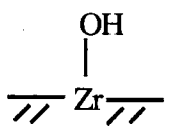
Preparation of zirconia particles. Microspherical zirconia particles are generally prepared by two methods, namely, the spray-pyrolysis method [74-77] and sol-gel processing method [78-81]. The spray-pyrolysis involves the atomization of the starting solution, e.g., zirconyl chloride dissolved in an aqueous solution, using an ultrasonic atomization technique. The mist is then introduced with an air carrier gas into a hot furnace, heated below 400° C to prevent crystallization. Each droplet acts as a separate "container", and reactions can be conducted within the droplets to yield spherical particles. Powders are collected with an electrofilter and calcinated in the air at 410° C for 1 hr and then at 700° C for 2 hrs. Sol-gel process involves the formation of highly concentrated zirconia sols by partial hydrolysis of zirconyl chloride in water. Prior to gelation, sols are emulsified in a water-immiscible liquid. Emulsion is stabilized by utilizing the hydrophilic-lipophilic balance system containing various nonionic surfactants. Small droplets are generated within the organic phase. Gelation reaction is initiated inside the droplets to form zirconium oxide (i.e., zirconia).

Zirconia prepared by precipitation from solution can exist in any of the following three metastable morphologies [82,83] depending on the thermal treatment. Below 375 °C zirconia remains amorphous, between 375 °C and 700 °C under vacuum the amorphous zirconia is transformed to the tetragonal phase. Above 700 °C under vacuum or 500 °C in the air, zirconia is converted to the monoclinic phase. The stable phase of zirconia is monoclinic up to 1170 °C, at which it transforms to the tetragonal phase. In a study reported by Unger's research group [81], neither native nor calcinated xerogels were crystalline as was revealed by X-Ray powder diffraction patterns. Crystallization is always

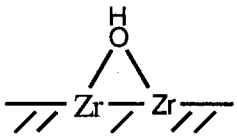
accompanied by a drastic decrease in specific surface area and porosity [25]. However, a considerable decrease of *ca.* 70% in surface area after thermal treatment at about 400°C was reported. Further increase in temperature did not have much effect on the specific surface area of the zirconia microparticles.

Surface chemistry of zirconia. Zirconia is very stable at extreme pH conditions. Studies [84] carried out under static conditions have shown that in 0.1 M hydrochloric acid (pH 1), and in 1.0 M carbon dioxide-free sodium hydroxide (pH 14) solutions, the dissolution of zirconia was not detected by inductively coupled plasma emission spectrometry with the detection limit of 0.03 μg/mL. By contrast, in a comparative study with alumina, detectable levels of aluminium were found at pH 1, 3, 12 and 14. Also, under dynamic conditions involving the perfusion of a zirconia column with a mobile phase of 1 M sodium hydroxide at 100 °C for 3.25 hrs at a flow rate of 1 mL/min, no zirconia was detected in the column effluent.

The surface of zirconia consists of a number of distinct classes of sites contributing to the surface chemistry. These include Brönsted acid and base sites, and Lewis acid sites [83-87]. Brönsted acid/base sites arise from the acidity of surface bound hydroxyls and tightly bound water molecules. Infrared studies of zirconia have confirmed the presence of at least two types of surface hydroxyl groups [87-89]: a terminal hydroxyl (A) similar to the free silanol of silica, and a bridging hydroxyl (B) in which a single oxygen is shared by two zirconium atoms.



(A)



(B)

These surface hydroxyl groups are partly responsible for the amphoteric nature of the surface. They may be protonated or deprotonated depending on the solution pH, giving the zirconia anion- or cation-exchange properties, respectively [90]

$$Zr\text{-}OH_2^+ \rightleftharpoons Zr\text{-}OH + H^+ \rightleftharpoons Zr\text{-}O^- + 2H^+$$

For example, zirconia acts as an anion exchanger at pH 5 in an acetate buffer and at pH 8 in a tris buffer; at pH 9.2 in a borate buffer, only cations are retained on the zirconia [84]. The transition from an anion exchanger to a cation exchanger occurs between pH 8 and pH 9.2. Coordinatively bound water molecules may also contribute to the ion-exchange properties of zirconia.

The presence of coordinatively unsaturated zirconium (IV) ion sites contribute to the Lewis acidic properties of the zirconia surface. These Lewis acid sites were detected by FTIR studies [83] involving ammonia or pyridine which were found to form coordinate bonds with the surface through their unshared pairs of electrons. The use of a nitroxy ion paramagnetic probe and electron spin resonance (ESR) [91] also confirmed the presence of Lewis acid zirconium ion sites. Since zirconium (IV) ion has an especially high charge to radius ratio, it can form very strong coordination complexes with hard Lewis bases [92,93]. It is well documented that zirconia interacts strongly with oxygen containing polyanions, such as borate, carbonate, phosphate, sulfate, chromate, arsonate, arsenite, molybdate and tungstate [94]. Although zirconia has been reported to have a very strong interaction with borate, the exposure of zirconia to borate did not cause any irreversible changes in the properties of the zirconia. Phosphate, however, has an essentially irreversible effect on the properties of zirconia [84]. At pH 8 in Tris buffer, treatment of zirconia packed in a column with 5 mM phosphate resulted in retention of only cations. Zirconium phosphate is very stable in acidic solutions. Washing the phosphate-treated zirconia with 0.1 M nitric acid has no apparent effect on the surface adsorption property.

Washing the zirconia with 0.1 M sodium hydroxide after phosphate treatment gave a product with certain recovery of the adsorption properties of the native zirconia, although not as much as the untreated zirconia [84].

Zirconia Lewis acid-base properties were thoroughly investigated by chromatographic studies in a series of publications [95-98]. A wide variety of derivatives of benzoic acid were used as probes, and it was found that the retention factors of the derivatives were closely correlated with their Brönsted acidities. The eluent pH was a critical factor in determining the magnitude of the retention factor, but it does not have much influence on chromatographic selectivity of the zirconia packing. Ligand-exchange interaction was the predominant mechanism for the retention of Lewis base solutes on zirconia. The addition of Lewis base to the eluent could attenuate the retention of these solutes. By comparison of the retention factors of *p*-benzoic acid derivatives, the relative elutropic strength of a number of Lewis bases was determined [96]. A wide variety of acidic, neutral and basic proteins was chromatographed on porous zirconia to evaluate their retention properties [97]. It was concluded that the proteins were retained by a complex balance of ion-exchange (i.e., on anionic sites) and ligand-exchange (i.e., on surface Lewis acid sites) mechanisms, since elution could be initiated by either increasing the ionic strength or by increasing the concentration of the Lewis base. The most critical parameter in achieving acceptable retention and peak shape, however, is the initial concentration of the Lewis base in the eluent. The retention of solutes on zirconia was attributed to four major types of interactions [98]: (i) cation exchange interactions at deprotonated surface hydroxyl groups and/or adsorbed negatively charged Lewis base components of the eluent; (ii) anion exchange interactions at the positively charged Lewis acid sites with non-coordinating anionic solutes; (iii) ligand exchange interactions at the Lewis acid surface sites with Lewis base solutes; and (iv) secondary interactions at the adsorbed Lewis base components of the eluent, which might involve hydrophobic interaction, hydrogen bonding, cation exchange or anion exchange interactions depending on the adsorbed Lewis base.

Zirconia as HPLC column packing materials. In searching for an inorganic material that has the mechanical stability of silica, yet chemically stable over a wider pH range, zirconia was investigated as a packing materials for HPLC in a variety of forms. Earlier studies were carried out in the normal mode [99,100], or dynamically modified (i.e., *in situ*) with reagents present in the mobile phase [61]. Zirconia was found to have different adsorption mechanism from that of silica gel, and was suggested for separation of compounds that could not be achieved on silica gel. Studies found that when the specific surface area and pore diameter of these alternative materials is equivalent to silica, there are certain differences in chromatographic properties, most of which could not be explained.

In recent years, a series of attempts have been made to chemically modify the surface of zirconia microparticles for use in HPLC. Organosilane compounds were used to form reversed-phase stationary phases on zirconia support in the same way as on silica gel [63,101]. It was reported that the retention and selectivity of octadecyl-bonded zirconia packing were identical to those of silica-based materials. The stationary phases exhibited a pronounced pH stability up to pH 12 in aqueous-organic mobile phases. The study on the coating of zirconia sorbent with polybutadiene (PBD) was also reported [102]. PBD coating was cross-linked with dicumyl peroxide or by irradiation with an accelerated electron beam. The potential limitation of polymer-coated chromatographic support is the loss in chromatographic efficiency that may result from restricted mass transfer of solutes in a polymer layer or in partially plugged pores by the polymer coating. Evaluation of the PBD stationary phase with alkylbenzenes and alkylarylketones revealed reversed phase retention mechanism. However, elution of benzoic acid, phthalic acid, cinnamic acid and phenyl phosphonate was not observed at all in methanol-water (40:60), even in the presence of 100 mM NaCl. Solute-zirconia interactions were not diminished on the support with a thicker (1.5 nm) film. There was a very strong interaction between these anionic solutes and the zirconia support which the PBD film did not completely inhibit,

regardless of its thickness. Static phosphate adsorption experiments indicated that zirconia-PBD support has substantial anion-exchange capacity. The presence of accessible anion-exchange sites leads to mixed-mode retention mechanism. One possible approach for inhibiting the interaction of these solutes with zirconia surface is to use phosphate-containing mobile phases so that inorganic phosphate competes for anion-exchange sites with solute molecules. Other modification of zirconia support to form reversed phase type of packings involved chemical vapor deposition (CVD) in which 1-butanol [103] or toluene [104], used as the carbon source, was passed over zirconia particles at elevated temperatures. Carbonaceous coatings were formed on zirconia surface. More than 90% of the available surface was covered with carbon. In some cases, carbon-coated particles were further treated with hydrogen at elevated temperatures in an attempt to hydrogenate the surface in the hope of removing high-energy adsorption sites, thereby creating a more energetically homogeneous surface. In another case, the zirconia particles coated by CVD process with toluene was also coated with polybutadiene [104], and then cross-linked by dicumyl peroxide.

Chromatographic studies of 1-butanol CVD coated zirconia revealed that an increase in solute dipolarity increases retention on the carbon-clad zirconia stationary phase. Aromatic isomers could be better resolved on the carbon-clad zirconia (i.e. 1-butanol CVD coated zirconia) than on silica-based ODS stationary phase, especially for those with polar functional groups such as nitro and hydroxyl groups. Reversed phase chromatographic properties were exhibited toward homologous series of alkylbenzenes and alkylphenones on CVD-toluene, hydrogen-treated toluene and polybutadiene-coated CVD toluene phases. Solute-adsorbent interaction was observed as the alkylphenone solutes, which are slightly polar, were much more retained than were the alkylbenzenes on the CVD toluene and on the hydrogen-treated CVD toluene stationary phases, a behavior that is different from that observed on silica-based ODS phase. Polybutadiene-coated CVD toluene behaved typically as a conventional bonded reversed phase on which alkylphenones were less retained than

alkylbenzenes. Hydrogen treatment modified some of the strong adsorption sites on the carbon surface as was manifested by the degree of solute-support interactions. The carbon-clad zirconia surface was extremely heterogeneous and was comprised of many different types of sites. A rather poor sample loading capacity was obtained on the carbon-clad zirconia relative to a conventional bonded phase column. It was observed that the carbon-clad zirconia surface had strong interactions with oxyanion functional groups, e.g., phosphate, carboxylic acids, sulfates, nitrates, etc..

The strong anion binding properties of zirconia were investigated by some researchers [105-109] in the aim of modifying zirconia surface to ion exchanger type of stationary phase. The dominant features of zirconia surface are Lewis acid sites and ionizable hydroxyl groups. Other groups such as the Brönsted base groups may be present in small numbers, but these Brönsted base sites may be responsible for the hydrogen bond accepting properties observed for strong hydrogen bond donor solutes at high pH. The heterogeneity of zirconia surface binding sites was thought to be responsible for the band broadening exhibited in solute elution.

Fluoride is a very hard Lewis base which could interact strongly with the hard Lewis acid sites on zirconia surface. Studies [105,107] indicated that the use of fluoride ion in the mobile phase could effectively block the surface Lewis acid sites. In the presence of fluoride, anionic solutes were not retained and were excluded from the zirconia packing to some extent. The strongly binding anions such as phosphonates and phosphates could be readily displaced by buffers containing higher concentration of fluoride. In the presence of fluoride, cations were retained most strongly. They were retained through interaction with the ionized anionic hydroxyl groups and /or coordinated fluoride ions. Solutes containing weak Lewis base groups were either very weakly retained or did not interact with fluoride-modified zirconia. Biopolymers, e.g., proteins, however, displayed mixed mode retention mechanism, involving contributions from the Lewis acid sites and the

protonated Brönsted base sites. In the absence of fluoride in the eluent, the proteins did not elute.

Zirconia modified with phosphate [106], the strong binding anions, exhibited primarily cation exchange properties, but the phosphate concentration in the mobile phase should be maintained above 0.15 M. The phosphate groups associated with the zirconia surface were most likely responsible for the cation-exchange sites. As the mobile phase pH was lowered below one of the pK_a values of the surface-bound phosphate, an oxygen of the phosphate becomes protonated and thus reduces the number of possible ion-exchange sites. No mixed mode retention mechanism was observed. For potassium, sodium and ammonium displacing cations, potassium ion showed the greatest displacing power of the three, followed by sodium. This is consistent with the order of elution power found for protein chromatography on strong cation-exchange columns [106]. It appears, though, that interactions between proteins and metallic sites on phosphate-modified zirconia surface were too strong to be chromatographically useful.

Ligand exchange chromatographic properties of phosphated zirconia packing was investigated [108] by dynamically loading copper (II) ions. The elution of amino acids followed a unique elution order in that many of the neutral amino acids eluted before the acidic ones while basic amino acids were very strongly retained, which is different from that on silica and resin based supports where acidic amino acids were weakly retained and always eluted before the neutral ones [109,110]. It was concluded that under these experimental conditions, ligand exchange might be less important to retention than was ion exchange. Surface Lewis acid sites, Zr (IV), played a very major role in retaining Lewis base solutes, even though many of these sites were covered by phosphate in the pre-treatment. It was reported [108] that in the absence of phosphate in the mobile phase, there were still many available Lewis acid sites on phosphated zirconia surface as suggested by NMR studies.

Zirconia surface modification for biospecific affinity chromatography and immobilized enzyme bioreactors was reported recently [111]. Zirconia was bonded with iminodiacetic acid (IDA) via γ-glycidoxypropyltrimethoxysilane. The zirconia thus treated was suspended in a solution of copper sulfate to form IDA-metal chelate functions on zirconia support surface. The metal chelate stationary phase was evaluated in metal interaction chromatography of proteins. In formation of zirconia-Concanavalin A (Con A) immobilized protein stationary phase, γ-isothiocyanatopropyltriethoxysilane was used to introduce reactive isothiocyanate groups onto the zirconia surface followed by the immobilization of Con A, primarily via ε-amino groups on accessible lysine residues. Adsorption isotherm studies with proteins indicated that immobilized Con A retained its biological activities, i.e., the binding of the horse radish peroxidase to the Con A sorbent was due to specific interactions between the carbohydrate binding sites of Con A and the polysaccharide chains of the glycosylated horse radish peroxidase, and was specifically eluted with methyl-α-D-mannopyranoside. Langmuir type adsorption isotherm were obtained with zirconia-Con A system as well as with the zirconia-IDA-Cu(II) system, indicating a single type of interaction.

Objectives of the Research and Overall Strategies

The objectives of this research are (i) to develop zirconia microparticles of controlled size, (ii) to introduce reproducible and well structured surface modification to form various stationary phases, and (iii) to evaluate the chromatographic properties of these novel packing materials and their practical applications in the separation of a wide variety of species.

In this regard, we evaluated the polymerization conditions for the synthesis of monodispersed, microspherical zirconia particles that are suitable for high performance column liquid chromatography as packing materials. Efforts were made to produce

microparticles with "nonporous" texture, having particle diameter in the range of 0.5-2.0 μm for high resolution and fast separation techniques.

These zirconia particles were modified through covalent bonding attachment of functional groups to the support surface to form a variety of stationary phases. Octadecylsilane compounds of mono- and tri-functional groups were used to form monomeric and polymeric reversed-phase stationary phases, respectively. Polar bonded stationary phases for hydrophilic interaction chromatography of carbohydrates were formed by either the covalent attachment of aminopropyl alkoxysilane compounds, or tetraethylenepentamine through γ-glycidoxypropyltrimethoxysilane on zirconia support surface. Ion exchange stationary phases of different degree of charge density were also synthesized and evaluated in the separation of proteins.

In all cases, the chromatographic properties of these stationary phases were evaluated with suitable probe compounds in order to better understand the retention behavior and the influence of zirconia surface chemistry. The potentials of these zirconia-based stationary phases for applications in the life sciences were investigated with standard solutes such as dansyl amino acids, bioactive peptides, proteins, nucleic acids, oligosaccharides, etc..

Finally, for comparative studies with the conventional silica-based stationary phases, monodispersed, nonporous, microspherical silica particles in the size range of 0.7-1.2 μm in diameter were synthesized by seeded growth sol-gel process. The silica microparticles were surface modified to form a variety of stationary phases for the reversed-phase, hydrophilic, and affinity chromatography. The rapid separation on these nonporous silica-based stationary phases was also investigated.

Overall, the various studies have demonstrated that microspherical zirconia support can be converted, via appropriately chosen and well controlled surface modification, into useful bonded stationary phases for various modalities of chromatography. As with other supports, e.g. silica, the residual adsorptivities of zirconia support can be minimized via

surface chemistry and/or mobile phase additives. In some instances, the residual adsorptivities (when moderate) may be beneficial for the separation.

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CHAPTER II

REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH MICROSPHERICAL OCTADECYL-ZIRCONIA BONDED STATIONARY PHASES*

Abstract

Microspherical zirconia particles were synthesized and surface modified with octadecylsilane compounds for reversed-phase high performance liquid chromatography (HPLC). Monomeric and "polymeric" octadecyl-zirconia bonded stationary phases were obtained by reacting the support with octadecyldimethylchlorosilane or octadecyltrichlorosilane, respectively. The surface coverage of the zirconia-based stationary phases with octadecyl functions was approximately the same as that of octadecyl-silica sorbents. These phases were evaluated in terms of reversed-phase chromatographic properties with non polar, slightly polar and ionic species over a wide range of mobile phase composition and pH. Monomeric octadecyl-zirconia with end-capping exhibited some metallic interactions with both basic and acidic solutes, but these interactions were greatly reduced in the presence of competing agents (e.g., tartarate ions) in the mobile phase. The polymeric octadecyl-zirconia sorbents exhibited higher retention than the monomeric ones with the various solutes investigated, and their residual adsoptivities

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* *J. Yu and Z. El Rassi, J. Chromatogr., 631 (1993) 91; Presented as a poster at the 16th International Symposium on Column Liquid Chromatography (HPLC '92), Baltimore, MD, June 14-19, 1992.*

toward acidic solutes were much lower. The retention of non polar and slightly polar aromatic compounds was quasi homoenergetic on both types of octadecyl-zirconia stationary phases. Stability studies conducted at extreme pH conditions (pH 2.0 and pH 12.0), have shown that polymeric octadecyl-zirconia are more stable than their monomeric counterparts. These stationary phases were quite useful in the separation of polycyclic aromatic hydrocarbons, alkylbenzene and phenylalkylalcohol homologous series, oligosaccharides, dansyl-amino acids, peptides and proteins.

Introduction

The enormous expansion of high performance liquid chromatography (HPLC) has been largely the result of the development of rigid microparticulate stationary phases. Very recently, the various aspects of support materials and their bonded stationary phases have been reviewed in a special issue of Journal of Chromatography [1].

Silica-based stationary phases are still the most widely used sorbents in all modalities of HPLC owing to their excellent mechanical strength and availability in a wide range of pore size and particle diameter. These attractive features of silica-based sorbents have apparently overshadowed their limited chemical stability at extreme pH and undesirable adsorptive properties toward basic species.

The search for rigid microparticulate stationary phases having the mechanical strength of silica gels and yet affording a wider pH range stability for use in HPLC has been a continuing theme of research since the introduction of the technique. Rigid polymer-based stationary phases, e.g., polystyrenedivinylbenzene and other resins based on polyacrylate, hydroxylated polyether copolymers or polyvinyl alcohol, are now available as column packing in various modes of HPLC; for recent review on polymeric packing see Ref 2. Although, polymeric supports afforded the preparation of chemically

stable stationary phases over a wide range of pH, they have been less mechanically stable than silica.

Recently, there has been an increasing interest in inorganic sorbents that combine the mechanical strength of silica to the chemical stability of polymeric-based stationary phases. In fact, stationary phases based on alumina [3-7], zirconia [8-12] and to a lesser extent titania [4, 10] have been evaluated in HPLC. However, an ideal column matrix that is free of all undesirable properties seems to be unrealistic. Thus, it can be anticipated that both organic- and inorganic-based sorbents will continue to coexist and complement each other. This would allow the separation of a wide range of species and satisfy the need of many users.

Although, alumina-based stationary phases with reversed-phase chromatographic properties such as polymer encapsulated- [5], octadecyl- [7] and polybutadiene alumina [3, 6] have showed an excellent chemical stability at high pH, the heterogeneous structure of their pores has limited their applications. Standard alumina has a bimodal pore size distribution with most of the surface hidden in small pores [13]. This property has yielded columns with much lower efficiencies than silica columns. In addition, alumina-based stationary phases show residual adsorptivities toward species with phosphate and carboxylate functional groups even if the stationary phase consisted of thick polymer coatings [9].

Octadecyl-titania stationary phases have been very briefly explored [10], and a detailed investigations of their chromatographic properties with various types of analytes are lacking. On the other hand, a few reports have recently appeared on hydrocarbonaceous zirconia stationary phases for reversed-phase HPLC. Gahemi and Wall [8] were the first to introduce dynamically modified zirconia with hydrophobic quaternary amine to HPLC [20]. Very recently, Carr and co-workers [9, 11, 12] and Trüdinger *et al* [10] introduced and evaluated microparticulate zirconia reversed-phase chromatographic sorbents. These studies have shown the excellent mechanical strength

and chemical stability of zirconia based stationary phases. However, polyoxy anions still exhibited strong interactions with the zirconia matrix even when the support was coated with a thick layer of cross linked polybutadiene [9], and these interactions could be minimized in the presence of phosphate ions in the eluent. The residual adsorptive properties could be further inhibited by coating the zirconia with carbon-clad, and the resulting sorbent did not exhibit peak tailing for amines or metallic interactions with phosphates and carboxylates [11, 12].

This report is concerned with the introduction of "nonporous", amorphous zirconia microparticles having a mean particle diameter in the range 1.5-2.8 μm and the evaluation of their bonded octadecyl derivatives in reversed-phase chromatography of small and large molecules. The surface modification of these zirconia microparticles with octadecyl silane reagents yielded sorbents having surface coverage in octadecyl functions similar to that obtained on non porous silica supports. In addition, the octadecyl-zirconia stationary phases were stable at acidic and alkaline pH for a long period of use, and allowed the rapid separation of proteins, peptides, dansyl-amino acids, oligosaccharides, polycyclic aromatic hydrocarbons and other polar and non polar aromatic compounds.

Experimental

Instrumentation

The liquid chromatograph was assembled from an LDC Analytical (Riviera Beach, FL, U.S.A.) ConstaMetric 3500 solvent delivery system with a gradient programmer, which was used to control a ConstaMetric Model III solvent delivery pump, a UV interference filter photometric detector Model UV-106 from Linear Instruments (Reno, NV, U.S.A.), and a sample injector Model 7125 from Rheodyne (Cotati, CA, U.S.A.).

Chromatograms were recorded with a computing integrator Model C- R6A from Shimadzu (Columbia, MD, U.S.A.).

Chemicals

HPLC grade acetonitrile, reagent grade as well as technical grade isopropanol and methanol, reagent grade isoamylacetate, benzene, carbon tetrachloride, sodium phosphate monobasic, dibasic, and tribasic were from Fisher (Fair Lawn, NJ, U.S.A.). Ethylbenzene, propylbenzene, butylbenzene, benzylamine, hexamethylenetetramine, zirconyl chloride octahydrate, and trimethylchlorosilane, were purchased from Aldrich (Milwaukee, NJ, U.S.A.). Toluene, anhydrous denatured ethanol, butanol, *n*-heptane, petroleum ether, sodium chloride, and sodium hydroxide were from EM Science (Gibbstown, NJ, U.S.A.). *p*-Xylene was from Estman Kodak (Rochester, NJ, U.S.A.). Octadecyldimethylchlorosilane and octadecyltrichlorosilane were obtained from Hüls America Inc. (Bristol, PA, U.S.A.). Polyoxyethylene 23-lauryl ether (Brij 35), polyoxyethylenesorbitan trioleate (Tween 85), sorbitan monooleate (Span 80), dansyl-L-amino acids, peptides, cytochrome c, lactoferrin, lysozyme, ribonuclease A and *p*-nitrophenyl maltooligosaccharides were from Sigma (St. Louis, MO, U.S.A.). Polyaromatic hydrocarbons were gifted by Dr. Eisenbraun from our Department.

Synthesis of Zirconia Microparticles and Zirconia Bonded Phases

Microspherical zirconia beads were synthesized according to the procedure of Trüdinger *et al* [10]. Typically, 160 g of zirconyl chloride was dissolved in 120 mL of water. The zirconium hydroxide sol thus formed was emulsified in 1400 mL of n-heptane, which was stabilized by the use of the following emulsifiers: 22.0 g Span 80, 6.70 g Tween 85 and 5.00 g Brij 30. The emulsion was homogenized using a Brinkmann dispersion unit (Westbury, NY, U.S.A.) at 8000 rpm for 2 min. Thereafter, 140 g

hexamethylenetetramine and 140 g urea were added to the emulsion to initiate the gelation reaction. The reaction mixture was stirred in a 2.0 L round-bottom flask at room temperature for at least 48 hrs.

After the reaction, the unreacted chemicals and organic solvents were removed in a multi-solvent cleaning process [10], using butanol-petroleum ether (1:1), methanol, and water. Zirconia microspheres were calcinated at 400°C to clean the organic residues adsorbed or deposited on the surface of the support [10].

To produce "nonporous" zirconia materials, the organic free zirconia microparticles were further calcinated at 800 °C for 6 hrs, a process during which the pore volume has been shown to approach zero [10]. The calcinated materials were then rehydroxylated using an established procedure [14]. The specific surface area, S_{BET}, of the support thus obtained was determined by nitrogen adsorption at Leeds and Northrup Co. (Petersburg, FL, U.S.A.) and was found to be 7.3 m^2/g. A scanning electron micrograph (SEM) of the same support taken at the electron microscope facilities of our University revealed that the starting product is polydisperse containing a sizable amount of 0.5-0.8 μm particles, which can explain the relatively moderate specific surface area obtained by nitrogen adsorption. These fine particles did not settle when the starting materials were suspended in isopropanol, and they were washed out by discarding the tops of repetitive suspensions. The classified particles thus obtained were in the size range 1.5-2.8 μm as can be seen in the SEM shown in Fig. 1. It has been shown that non porous supports in this particle size range would have on the average a specific surface area of ca. 1.4 m^2/g [15].

The above zirconia microspheres of narrower particle size range were modified with either monomeric or "polymeric" octadecyl functions according to the following procedures. The monomeric otadecyl-zirconia stationary phase was prepared by first heating a round bottom flask containing a suspension of 3 g of zirconia microspheres in toluene at 115 °C while stirring for at least 30 min. This step was to evaporate the water molecules that might have been adsorbed on the support surface from atmospheric moisture

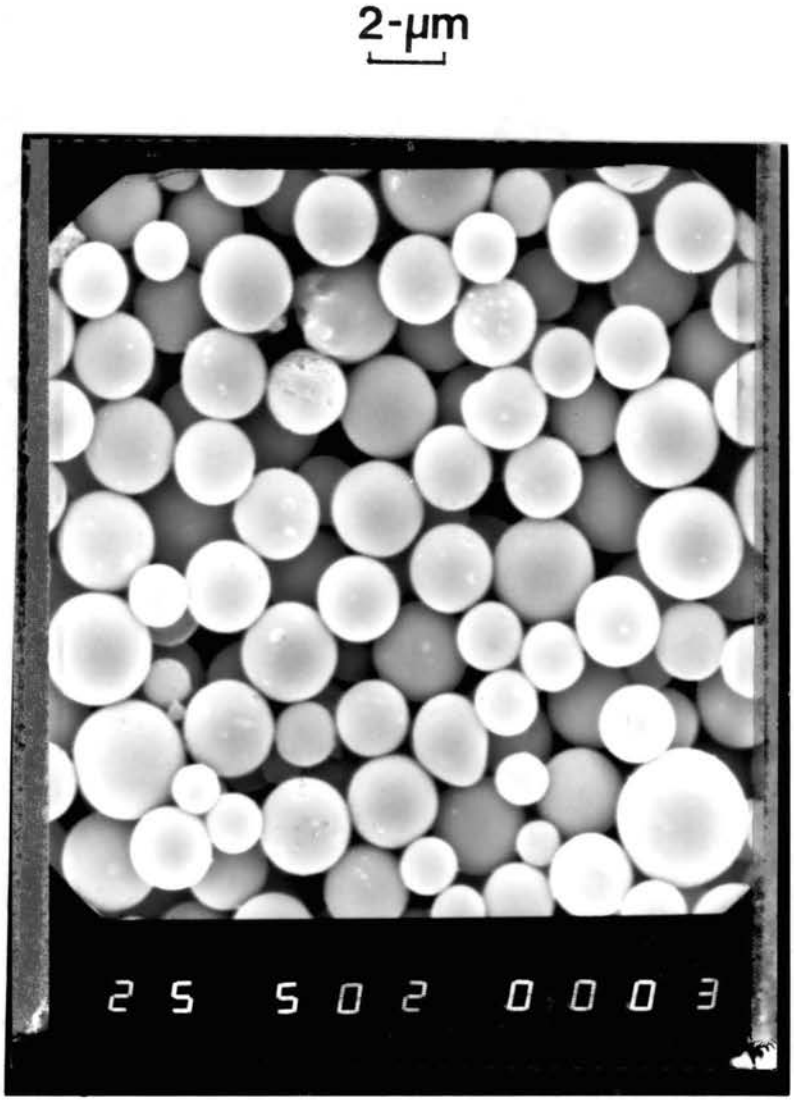


Figure 1. Scanning electron micrograph of the zirconia microspheres.

since toluene and water form a positive heteroazeotrope with a b.p. 84.1 °C [16]. Thereafter, 1g of octadecyldimethylchlorosilane was added to the zirconia suspension. The reaction solution was stirred for 12 hrs at 115 °C. After the reaction, the octadecyl-zirconia sorbent was separated from the solution by centrifugation. Technical grade methanol was used to wash the support thoroughly from unreacted silane and hydrogen chloride formed during the reaction. When the zirconia microsphere suspension was brought to neutral, the support was washed several times with toluene to completely remove methanol solvent. Then the octadecyl-zirconia thus obtained was suspended in toluene and heated to 80 °C for at least 30 min to evaporate the residual methanol. Following, the temperature was lowered to 50 °C, and 3 mL of trimethylchlorosilane were added to the suspension of modified zirconia in toluene. The reaction was stirred at 50 °C for 12 hrs. The modified zirconia particles were washed from the unreacted silane compound and hydrogen chloride with methanol, and let to dry in the air.

The "polymeric" octadecyl-zirconia stationary phase was prepared by following the same steps as in the above procedure using octadecyltrichlorosilane with the exception that the final product was not end-capped.

Column Packing

All columns used in this study were precision bore 316 stainless steel tubing from Alltech Associate (Deerfield, IL, U.S.A.), and having 30 x 4.6 mm I.D. as the dimensions. Column end fittings were also 316 stainless steel fitted with 0.5-μm stainless steel frits and distributor disks from Alltech Associates.

The modified zirconia microspheres were then packed from a carbon tetrachloride slurry at 8000 psi with isopropanol using a Shandon column packer instrument (Keystone Scientific, Bellefonte, PA, U.S.A.). Typically 1.5 g of surface modified zirconia microspheres were needed since zirconia is a relatively dense material (5.8 g/cm^3) [17].

Carbon tetrachloride, which is characterized by its high density, gave satisfactory results when a relatively high viscosity solvent such as isopropanol was used as the packing solvent.

Results and Discussion

Surface Modification of Zirconia

Infrared spectroscopic studies [18, 19] have shown that there are at least two types of hydroxyl functional groups at the zirconia surface. The free hydroxyl groups bound to single cations, Zr-OH, similar to those encountered on the silica surface, and bridging hydroxyl groups coordinated to more than a single cation, Zr-(OH)-Zr. The surface concentration of zirconia in hydroxyl groups has been found to be ca. 9.8 $\mu mol/m^2$ [20]. Although the relative chemical reactivity of both types of surface hydroxyl groups has not been yet established, these groups can be used for the covalent attachment of ligands. In addition, zirconia surface contains coordinatively unsaturated zirconium (IV) ions, i.e., hard Lewis acid sites [21]. The Lewis acid sites have been shown to undergo strong interactions with oxyanions such as phosphate containing compounds [20] and their presence can lead to undesirable chromatographic behavior such as peak tailing and irreversible binding of Lewis base solutes. Thus, the major concern in the preparation of zirconia bonded stationary phases is to shield the zirconium sites, and consequently minimize the solute-metal interactions.

Silane derivatives having one or more reactive functional groups can react with surface hydroxyl groups of zirconia to form monomeric or polymeric bonded phases, respectively.

When octadecyldimethylchlorosilane is used, a monomeric layer of octadecyl functions covalently bonded to the zirconia surface would result. Because of the steric

hindrance caused by the large size of the octadecyl hydrocarbon chains, there still would be some hydroxyl groups on the zirconia surface that remains unreacted. Smaller silane compounds such as trimethylchlorosilane was used as end-capping agent to scavenge the unreacted hydroxyl groups and to minimize their contribution to solute retention in the ensuing chromatographic separation. This approach is widely practiced with silica bonded stationary phases to minimize silanophilic interactions [22].

The use of octadecyltrichlorosilane would result in a "polymeric" octadecyl stationary phase bonded on the zirconia surface. Besides reacting with the hydroxyl groups of the zirconia surface, multi-functional silane reagents can react with each other to form a cross linked octadecyl polysiloxane layer. In this process, the zirconia support would have a higher surface coverage with octadecyl functions, which would provide a better sealing of the surface hydroxyl groups and zirconium sites.

Stability Studies

The chemical stability of the bonded octadecyl functions of the zirconia-based sorbents as well as the support itself was investigated under extreme pH conditions, i.e., pH 2.0 and pH 12.0. Monomeric and "polymeric" octadecyl-zirconia columns were perfused with 10 mM phosphate buffer solutions at a flow-rate of 1.0 mL/min. At 5-hour intervals, the column was first washed with water, and then equilibrated with the mobile phase. The retention of toluene, *p*-xylene, benzylamine and t-cinnamic acid test solutes was evaluated as a function of the number of column void volume of buffer solutions perfused through the column, and the results are shown in Figs. 2 and 3.

It is interesting to note that throughout the entire stability studies at low and high pH on both monomeric and "polymeric" bonded stationary phases, the column void volume remained unchanged, and no bed compaction was observed. This corroborates earlier

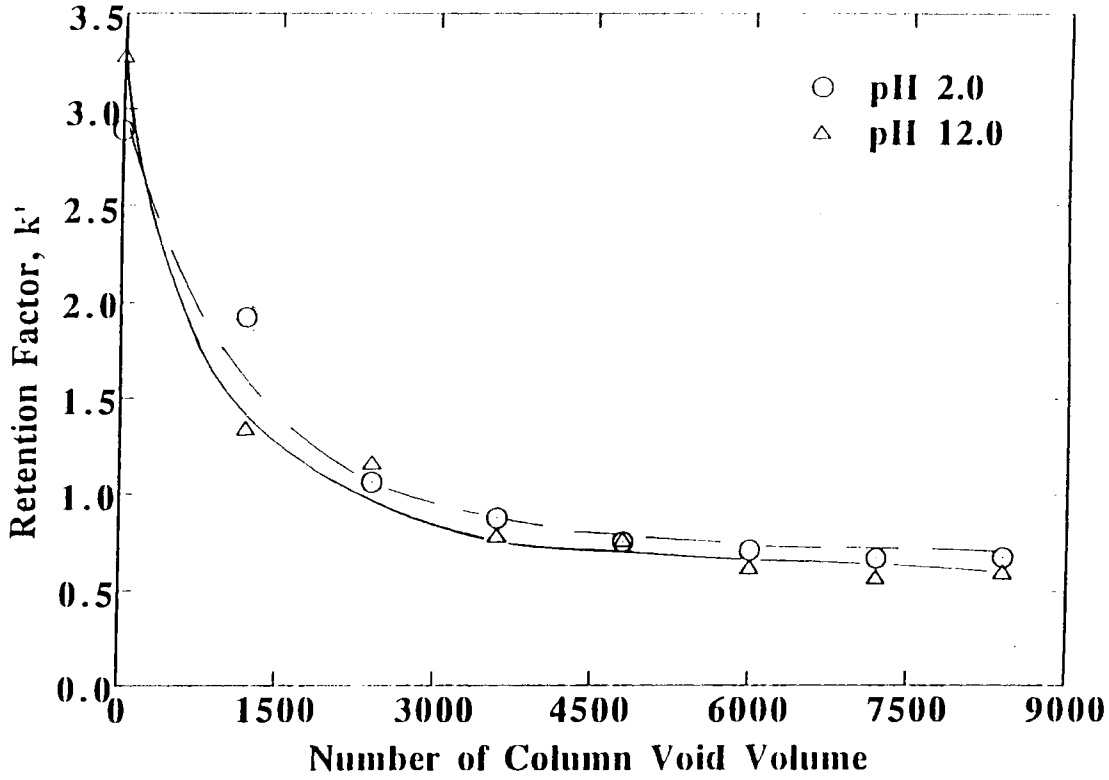


Figure 2. Plot of retention factor of *p*-xylene versus the number of column void volume of solutions perfused through the column. Solutions used in the stability test, 10 mM NaH_2PO_4, pH 2.0 and 10 mM Na_3PO_4, pH 12.0 ; flow-rate, 1.0 mL/min. Column, monomeric octadecyl-zirconia with end-capping, 3.0 x 0.46 cm I.D.; mobile phase used in the measurement of solute retention, water at 5% (v/v) acetonitrile; flow-rate, 1.0 mL/min.

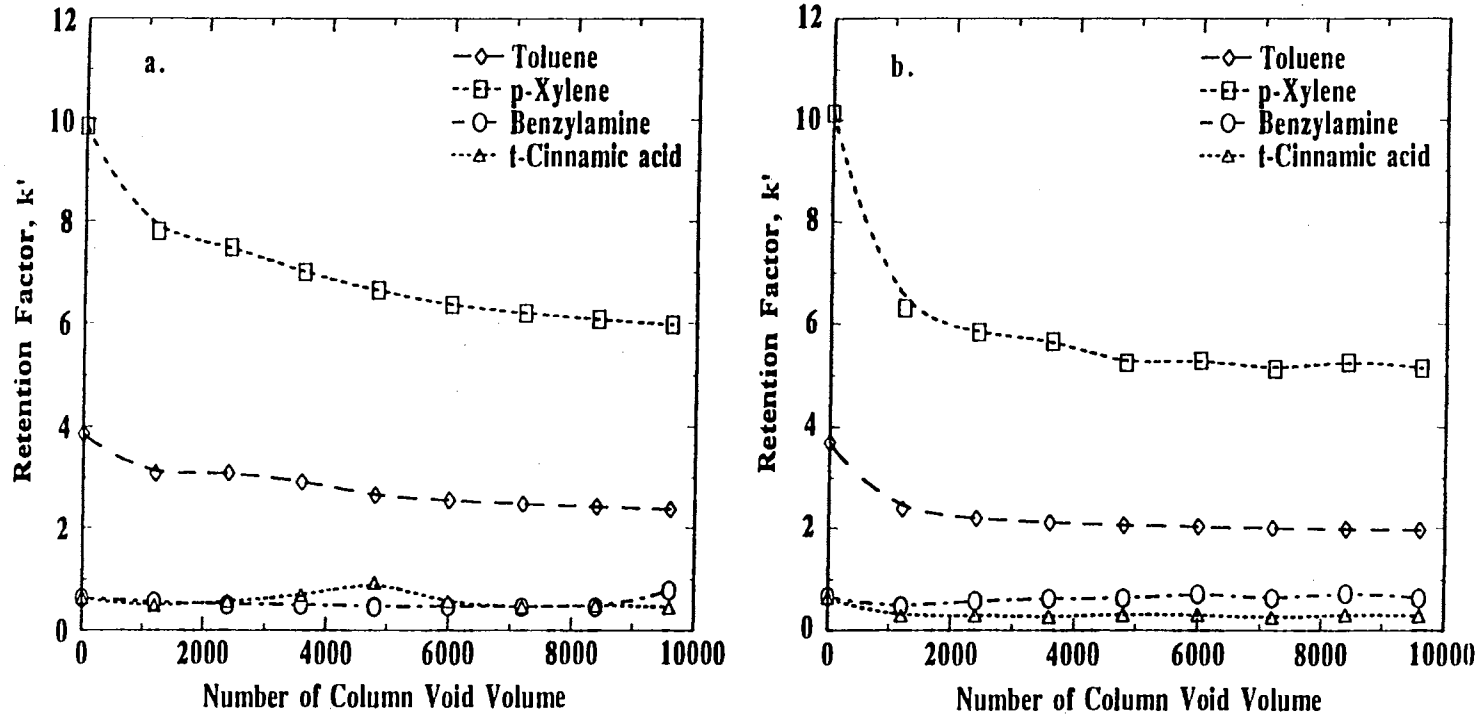


Figure 3. Plot of retention factor of the test solutes versus the number of column void volume of solutions perfused through the column. Solutions used in the stability test, 10 mM NaH_2PO_4, pH 2.0 and 10 mM Na_3PO_4, pH 12.0 ; flow-rate, 1.0 mL/min. Column, "polymeric" octadecyl-zirconia, 3.0 x 0.46 cm I.D.; mobile phase used in the measurement of solute retention, water at 20% (v/v) acetonitrile; flow-rate, 1.0 mL/min.

findings in that zirconium oxide was very stable with 0.1M hydrochloric acid, i.e., pH 1.0, and 1.0 M sodium hydroxide, i.e., pH 14.0 [20].

As shown in Figs 2 and 3, on both monomeric and polymeric octadecyl-zirconia stationary phases the retention of the test solutes reached a constant value after a certain number of column void volume. On the monomeric octadecyl column, the retention stabilized after 4000 column void volume, whereas on the polymeric column the constancy in retention was attained after 1000-2000 column void volume.

It has to be noted that a preconditioned polymeric octadecyl-zirconia column (i.e., after perfusion with acidic or basic solutions) kept its performance in terms of retention and separation efficiencies for a longer period of use than a monomeric octadecyl-zirconia column. The greater stability of the polymeric octadecyl-zirconia stationary phases may be attributed to the presence of a cross linked alkylpolysiloxane layer, i.e., silicon rubber, that establishes a greasy layer or strong hydrophobic shield and protects the Zr-O-Si as well as the Si-O-Si bonds from hydrolysis. In fact, the preconditioned polymeric octadecyl-zirconia column yielded the same retention for toluene and *p*-xylene after an additional 12,000 column void volume with a variety of mobile phases including pH 2.0 and pH 12.0, and the column is still in use. These results are in agreement with those reported by Trüdinger *et al* [10] on the stability of octadecyl-zirconia stationary phases. Their results have shown that polymeric octadecyl-zirconia sorbents are stable up to pH 12.0 even after a period of 500 hours of use.

Chromatography of Non-Polar and Slightly Polar Solutes

The reversed-phase chromatographic properties of the octadecyl-zirconia stationary phases were evaluated with non-polar and slightly polar aromatic compounds. First, monomeric octadecyl-zirconia microspheres without end-capping packed into a 3.0 x 0.46 cm column were evaluated with benzene, toluene, *p*-xylene and naphthalene at various

acetonitrile concentrations in the mobile phase. Under these circumstances, plots of the logarithmic retention factors of the aromatic solutes versus the percent acetonitrile in the mobile phase were not linear. This behavior may indicate the presence of interactions between the π-electrons on the aromatic rings and the exposed zirconium sites on the surface of the support.

Based on the above findings, the monomeric octadecyl-zirconia stationary phases were then reacted with trimethylchlorosilane, i.e., end-capping, to minimize solute-zirconia associations.

Figure 4a and b illustrates plots of logarithmic retention factor versus the volume percent of acetonitrile in the mobile phase for benzene, toluene, *p*-xylene and naphthalene obtained on monomeric and "polymeric" octadecyl-zirconia, respectively. These plots are linear with a correlation coefficient varying between 0.996 and 1.000 over a wide range of acetonitrile concentration in the mobile phase. As can be seen in Fig. 4a and b, solute that has larger hydrophobic surface area showed greater response in terms of retention to changes in the organic content of the mobile phase. That is the slope of the line increased in the order of benzene < toluene < *p*-xylene < naphthalene. As expected, the "polymeric" stationary phase having higher surface coverage, i.e., higher phase ratio, exhibited higher retention toward non-polar species.

On both types of bonded stationary phases a switch in the elution order between *p*-xylene and naphthalene was observed, see Fig. 4a and b. This change in the elution order occurred at lower acetonitrile concentration on the monomeric stationary phase.

To further characterize these phases, alkylbenzene homologous series were chromatographed under reversed-phase conditions. The results are shown in Fig. 5a and b in terms of logarithmic retention factor of the solutes versus the number of carbon atoms in their alkyl chains. In all cases, log k' increased linearly with increasing number of methylene groups in the homologous series, which confirmed the reversed-phase

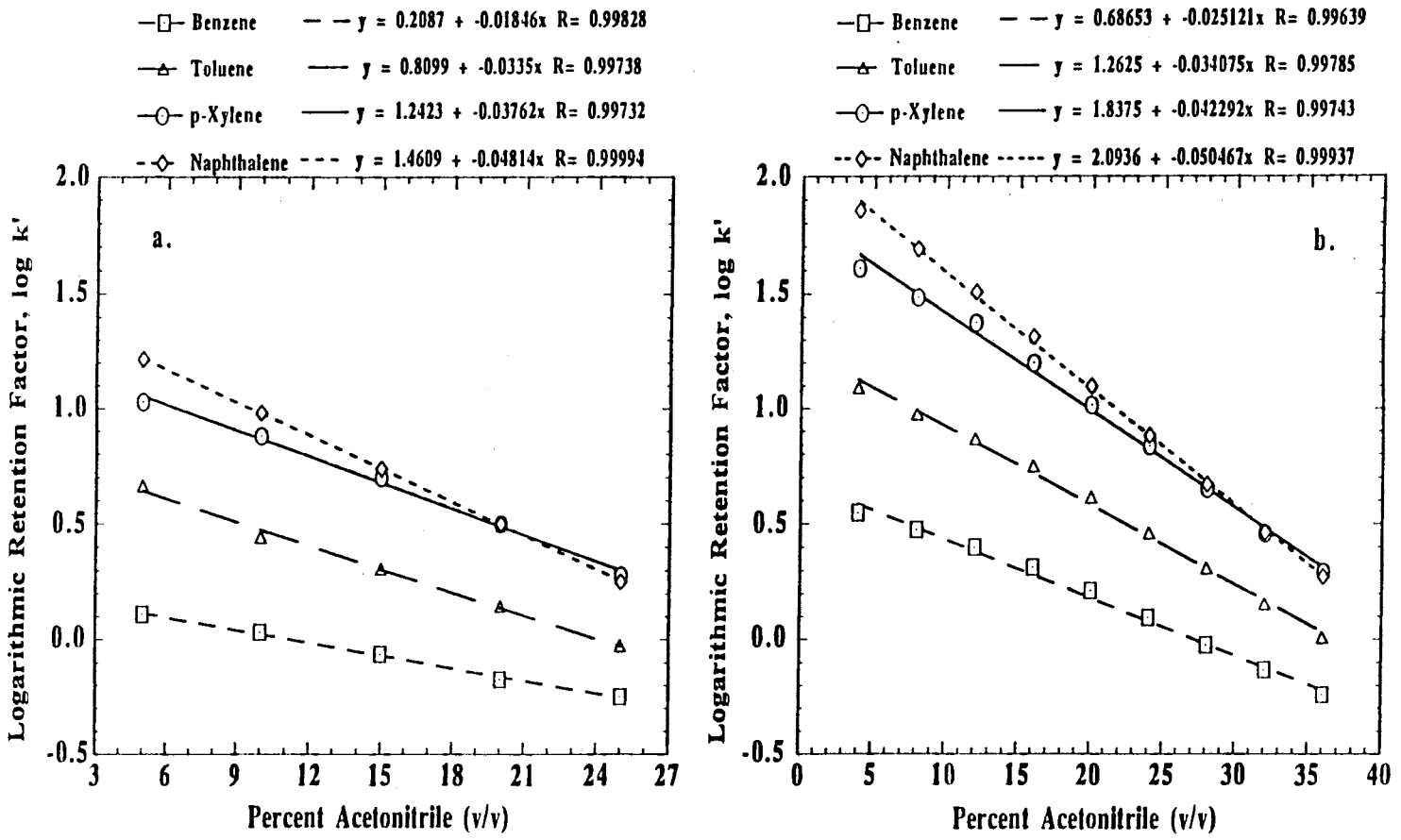


Figure 4. Plots of logarithmic retention factor versus the volume percent acetonitrile in mobile phase for both monomeric (a) and "polymeric" (b) octadecyl-zirconia stationary phases. Columns, 3.0 x 0.46 cm I.D.; mobile phase, water at various volume percent acetonitrile; flow-rate, 2.0 mL/min.

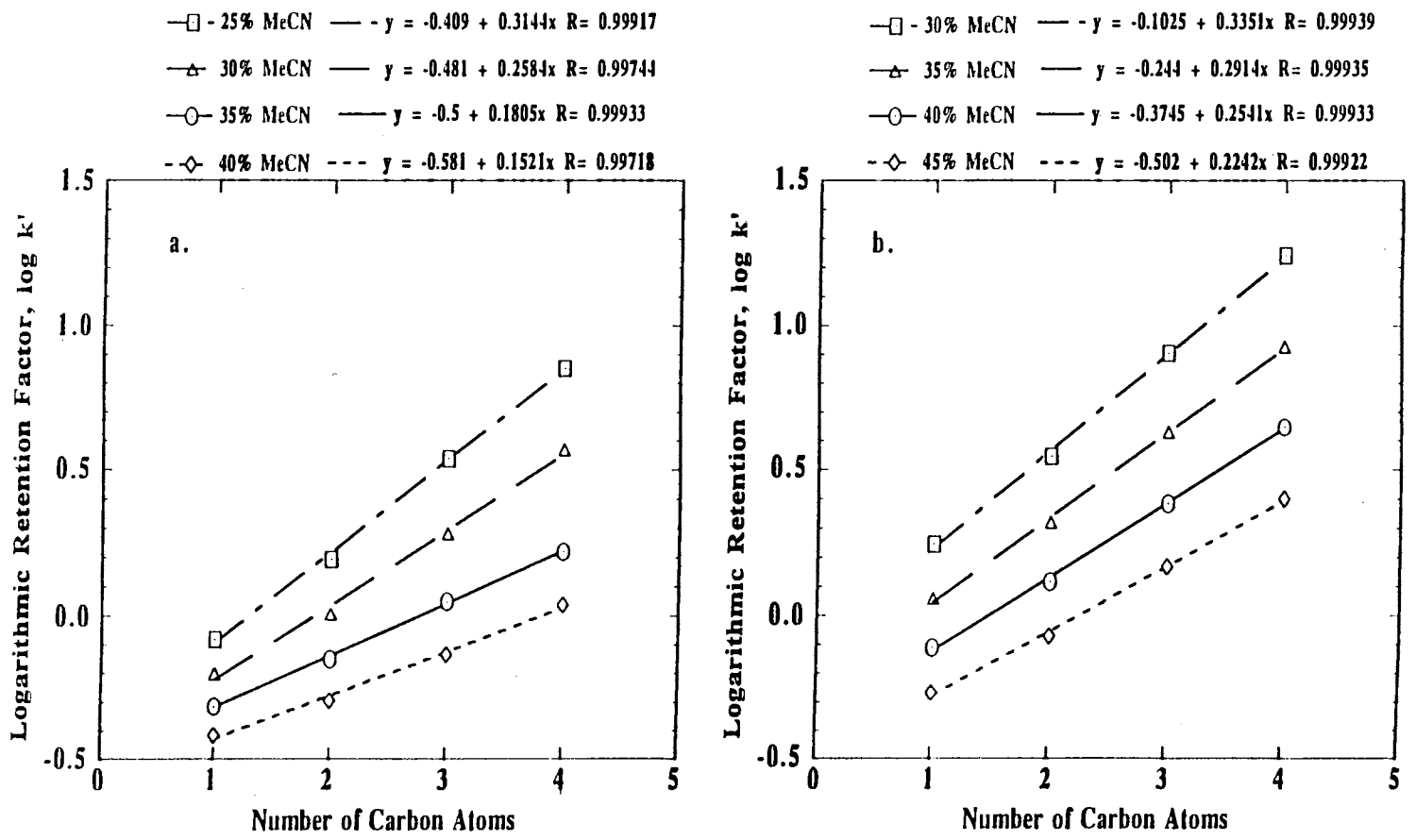


Figure 5. Plots of logarithmic retention factor versus the number of carbon atoms in the alkyl chain of alkylbenzene homologous series for both monomeric (a) and "polymeric" (b) octadecyl-zirconia stationary phases. Columns, 3.0 x 0.46 cm I.D.; mobile phases, water at various volume percent acetonitrile (MeCN); flow-rate 2.0, mL/min. Solutes: toluene, ethylbenzene, propylbenzene and butylbenzene.

chromatographic property of the octadecyl-zirconia bonded stationary phases. The slope of the lines, which is the methylene group retention increment, showed that the hydrocarbonaceous phases had higher selectivity toward the homologous series when organic-lean eluents were used, a behavior typical of reversed-phase chromatography.

The selectivity of monomeric and "polymeric" bonded stationary phases toward alkylbenzene homologous series was compared using the methylene group retention increment. The acetonitrile concentration in the mobile phase was adjusted for both types of stationary phases so that the retention of toluene (the smallest solute in the homologous series) on both monomeric and "polymeric" stationary phases would be nearly the same; compare curves obtained at 25% and 40% (v/v) acetonitrile in Fig. 5a and b, respectively. The slope of these two lines shows clearly that both monomeric and polymeric stationary phases yield nearly the same selectivity toward alkylbenzene homologous series, with the difference that it would take lower organic concentration in the mobile phase to bring about the elution and separation of the homologous series with the monomeric bonded phase.

Phenylalkylalcohols homologous series were used to study the retention behavior of slightly polar solutes on the octadecyl-zirconia bonded stationary phases. The results are shown in Fig. 6a and b in terms of logarithmic retention factor versus the number of carbon atoms in the homologous series. Straight lines were obtained in pure water as well as in the presence of acetonitrile in the mobile phase. Again, octadecyl-zirconia based stationary phases exhibited reversed-phase properties toward Phenylalkylalcohols. As reflected by the slope of the lines, the methylene group retention increments for the phenylalkylalcohols homologous series decreased with increasing acetonitrile concentration in the eluent on both monomeric and "polymeric" bonded octadecyl-zirconia stationary phases.

It has been shown [23, 24] that plots of log k' obtained on one stationary phase versus those obtained on another with the same mobile phase can be utilized to compare the

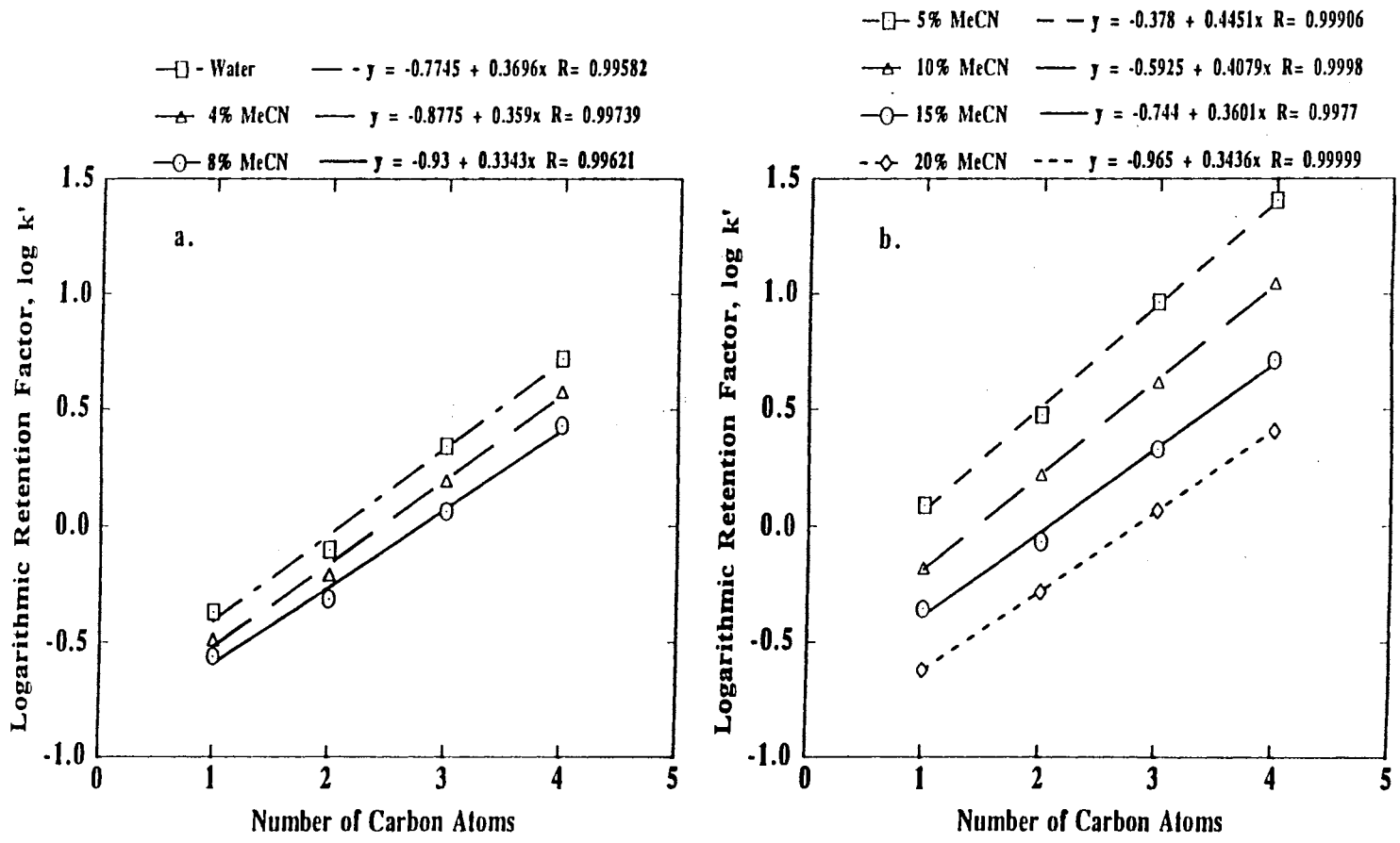


Figure 6. Plots of logarithmic retention factor versus the number of carbon atoms in the alkyl chains of phenylalkylalcohol homologous series for both monomeric (a) and "polymeric" (b) octadecyl-zirconia stationary phases. Columns, 3.0 x 0.46 cm I.D.; mobile phase, water at various volume percent acetonitrile (MeCN); flow-rate, 1.0 mL/min (a) and 2.0 mL/min (b). Solutes: benzyl alcohol, phenethyl alcohol, 3-phenyl-1-propanol, and 4-phenyl-1-butanol.

energetics of solute retention on different columns. If the Gibbs free energies for a given solute are identical in both columns, i.e., the retention is homoenergetic, then plots of log k'-log k' obtained on the two stationary phases yield a straight line with unit slope and the intercept is the logarithmic quotient of the two columns phase ratios [22]. If the corresponding Gibbs free energies in the two chromatographic systems are not identical but proportional to each other, linear plots are still obtained with a slope different from unity and such retention behavior is termed homeoenergetic [23].

The log k'-log k' plot illustrated in Fig. 7 was graphed from retention data obtained with alkylbenzene homologous series on the monomeric and polymeric bonded stationary phases. As can be seen in Fig. 7, the log k' -log k' plot is linear with a slope slightly larger than unity indicating that the retention of alkylbenzenes is quasi-homoenergetic on both types of columns, i.e., the retention mechanism is based essentially on hydrophobic interaction between the solute and the hydrocarbonaceous chains of the stationary phase. The antilog of the intercept of the line, which is the quotient of the two columns phase ratios, ϕ_{poly}/ϕ_{mono}, was equal to 3.33 indicating that the polymeric octadecyl-zirconia column has a phase ratio (ϕ_{poly}) of ca. 3 times higher than the monomeric one (ϕ_{mono}).

Both types of octadecyl-zirconia bonded stationary phases showed quasi homoenergetic retention with the slightly polar phenylalkylalcohols, as illustrated by Fig. 7. In fact, the slope of log k'-log k' plot is ca. 1.27. The quotient of phase ratios ϕ_{poly}/ϕ_{mono}, defined as the phase ratio of a column relative to that of the reference column, was evaluated as the antilog of the intercept of the log k'-log k' plot and its value was ca. 4.22. Based on these results and those obtained with alkylbenzene homologous series the phase ratio of polymeric octadecyl-zirconia is higher than that of monomeric by a factor of ca. 3.0-4.0. The retention of these slightly polar species on both monomeric and "polymeric" bonded stationary phases was primarily through hydrophobic interaction between the solutes and the bonded stationary phases.

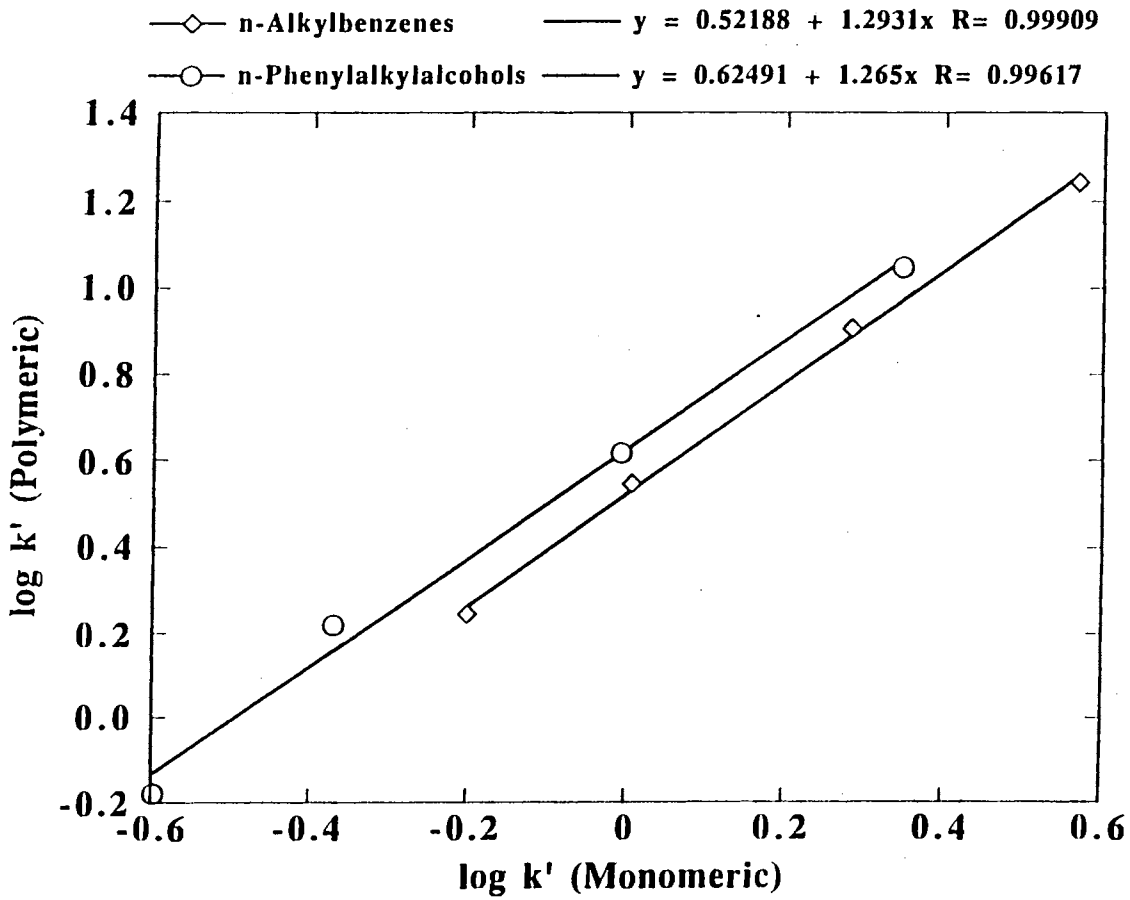


Figure 7. Plots of logarithmic retention factor of phenyalkylalcohol and alkylbenzene homologous series on the "polymeric" phase versus that on the monomeric phase. Columns, 3.0 x 0.46 cm I.D.; mobile phase, water at 30% acetonitrile (v/v) for alkylbenzenes and 10% acetonitrile (v/v) for phenyalkylalcohols; flow-rate 2.0 mL/min. Alkylbenzenes: toluene, ethylbenzene, propylbenzene and butylbenzene; phenylalkylalcohols: benzyl alcohol, phenethyl alcohol, 3-phenyl-1-propanol, and 4-phenyl-1-butanol.

Furthermore, the retention of non polar alkylbenzenes was much higher than that of the slightly polar phenylalkylalcohols, suggesting that there was no interaction between the hydroxyl group of the latter homologous series and the zirconia support matrix.

Comparison with Octadecyl-Silica Stationary Phases

To further evaluate the reversed-phase chromatographic property of the octadecyl-zirconia bonded stationary phases, the retention behavior of *p*-xylene and naphthalene obtained on the zirconia sorbents was compared to that observed on non porous octadecyl-silica stationary phases. The specific surface areas of both sorbents are low, and the comparison of their energetics of retention for the non polar solutes would therefore be meaningful. The non porous microspherical silica support of mean particle diameter of 0.8 μm was synthesized in our laboratory by a seeded growth technique according to well established procedures [25], and was bonded with octadecyl functions by following the same procedure described for the zirconia support, see experimental.

The values of the logarithmic retention factor obtained at different concentrations of acetonitrile in the mobile phase on zirconia-based stationary phases were plotted against those obtained on octadecyl-silica columns, see Fig. 8. Referring to this figure, log k' - log k' plots were linear, meaning that the retention energetics of the aromatic compounds on the end-capped octadecyl-zirconia stationary phases was the same as that obtained on the octadecyl-silica stationary phases. The antilog of the intercepts of *p*-xylene and naphthalene curves are 1.67 and 1.31, respectively, indicating that the phase ratio of octadecyl-zirconia is slightly higher than that of octadecyl-silica. This may be explained by the higher surface area per unit volume for zirconia than for silica. The packing density of non porous silica has been estimated to be 1.5 g/mL [15]. The packing density of the zirconia particles used in this study was ca. 5.6 g/mL. Based on literature data [15], a non porous silica of 0.8 μm particle diameter as that used in this study would have a specific

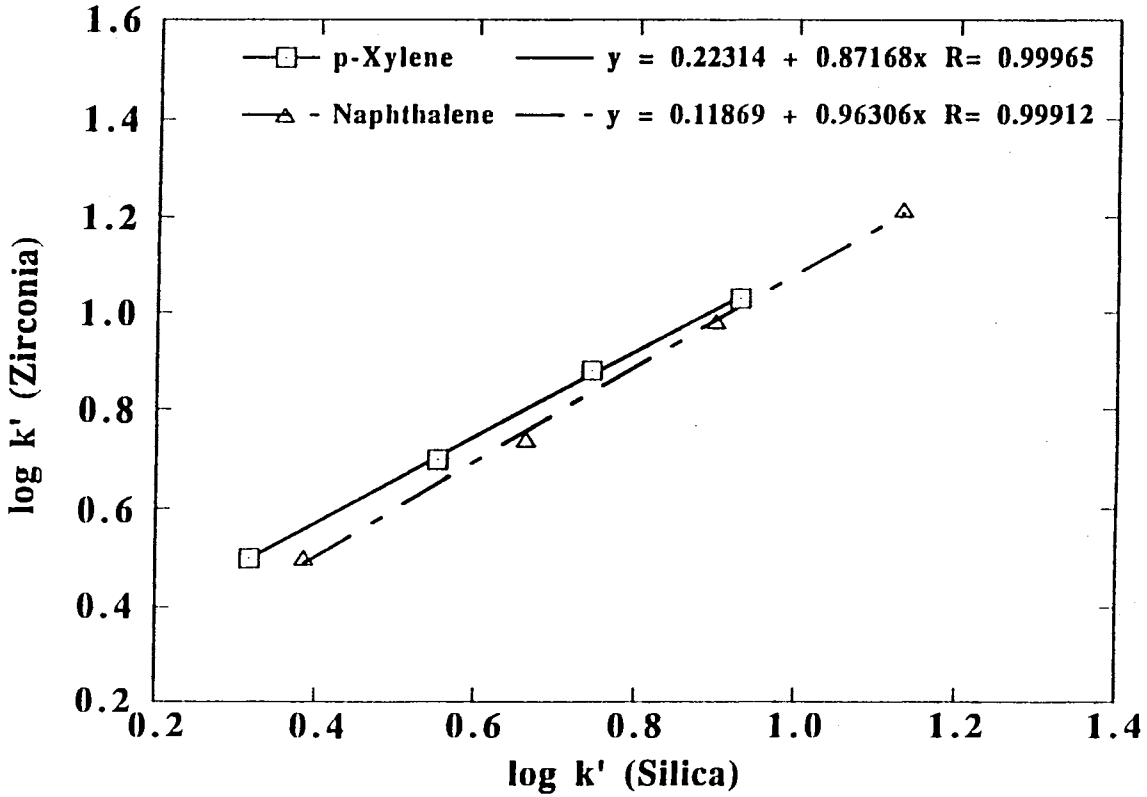


Figure 8. Plots of logarithmic retention factor of *p*-xylene and naphthalene on octadecyl-zirconia versus that on octadecyl-silica. Columns, monomeric with end-capping, 3.0 x 0.46 cm I.D.; mobile phase water at various volume percent of acetonitrile, 1%, 2%, 5%, 10%, 15%, and 20% (v/v).

surface area of 3.4 m^2/g, whereas the specific surface area of the nonporous zirconia of 1.5-2.8 μm would be on the average 1.4 m^2/g. From these data the surface area per unit volume for silica is 5.1 m^2/mL versus 7.8 m^2/mL for that of zirconia.

As expected, "polymeric" bonded octadecyl-zirconia stationary phases compared favorably with the silica-based stationary phases, Fig. 9a and b. The zirconia surface was well covered with octadecyl functions in this modification process. The antilog of the intercepts of log k'-log k' plots for *p*-xylene and naphthalene were 1.82 and 1.86, respectively. These results and those obtained with monomeric zirconia suggest that the extent of surface modification of silica and zirconia with octadecyl functions are approximately the same.

Chromatography of Charged Species

The chromatographic properties of the octadecyl-zirconia stationary phases under investigation were further evaluated with ionizable species at different pH. Benzylamine and t-cinnamic acid were chosen as solute probes, and they were chromatographed on both monomeric and polymeric octadecyl columns in the presence or absence of 50 mM tartrate in the eluents. Tartrate was added to the mobile phases as a competing agent for the active sites on the zirconia surface, and to minimize solute-support interactions. Other competing agents were very recently investigated in the elution and separation of benzoic acid derivatives and proteins on bare zirconia [26-28].

Bare zirconia has been shown to have both anion and cation exchange properties for charged species as well as ligand exchange behavior towards Lewis bases [20, 26-28]. The isoelectric point of the ampholytic surface can range from below 3 to above 10 depending on the source and the type of zirconia support [29]. Whereas the ligand exchange property of zirconia is the result of the presence of coordinatively unsaturated

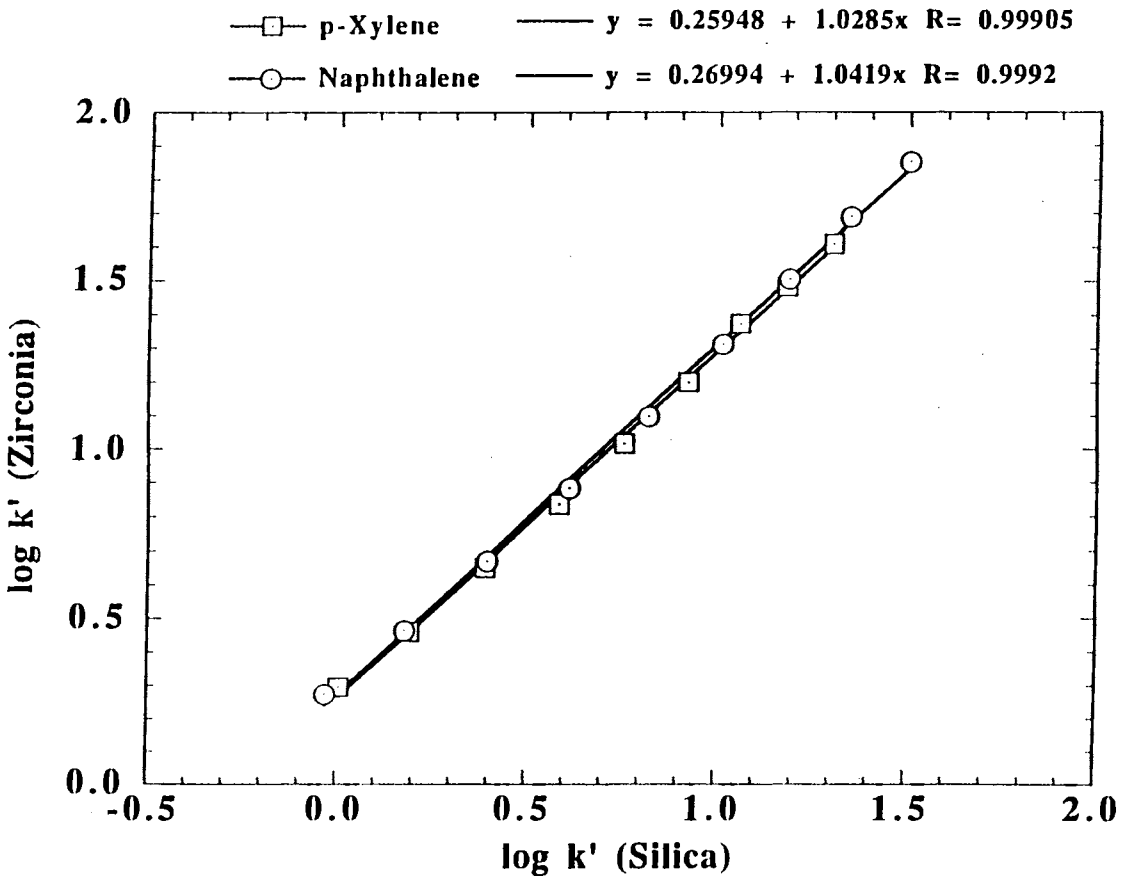
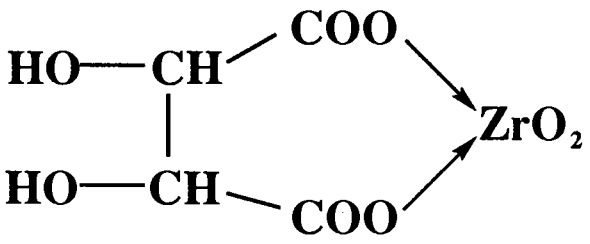


Figure 9. Plots of logarithmic retention factor *p*-xylene and naphthalene on octadecyl-zirconia versus that on octadecyl-silica. Columns, "polymeric" octadecyl-zirconia and octadecyl-silica, 3.0 x 0.46 cm I.D.; mobile phase water at various volume percent of acetonitrile, from 4% to 36% (v/v) with an increment of 4% .

zirconium sites, the anion and cation exchange behavior is thought to arise from the protonation and deprotonation of surface hydroxyl groups [30], respectively.

When tartarate is added to the mobile phase, this hard Lewis base ligand would form metal chelates with the exposed zirconium sites of the surface of the stationary phase according to the following scheme:



Under these conditions, the empty valence orbitals of the zirconium sites will be filled with the electron pairs donated from the mobile phase additive, and consequently there would be little interaction between the solute and the support matrix. In addition, the doubly charged tartarate ions may reduce the residual ion-exchange of the zirconia matrix.

In the absence of tartarate, benzylamine which was completely ionized at pH below 4.0 (pK_a = 9.33), exhibited very weak hydrophobic interaction with the monomeric and end-capped octadecyl-zirconia stationary phases. When the pH of the eluent was increased to 6.0, the fully ionized benzylamine showed strong interaction with the zirconia matrix of the end-capped monomeric octadecyl-zirconia column, see curve 1 in Fig. 10. As the pH of the mobile phase was increased to above the pK_a value of the solute, benzylamine was totally deprotonated and therefore exhibited stronger interaction with both the zirconia matrix and the bonded octadecyl functions of the stationary phase. Thus, solute retention was the highest at pH > 9.0.

Since zirconium sites are rather hard Lewis acids, the residual adsorptivity of the monomeric octadecyl-zirconia toward benzylamine, an intermediate soft Lewis base, may be largely due to the cation exchange property of the support matrix. In fact, in the pH range 6.0-9.0 whereby benzylamine exhibited strong interaction with the monomeric

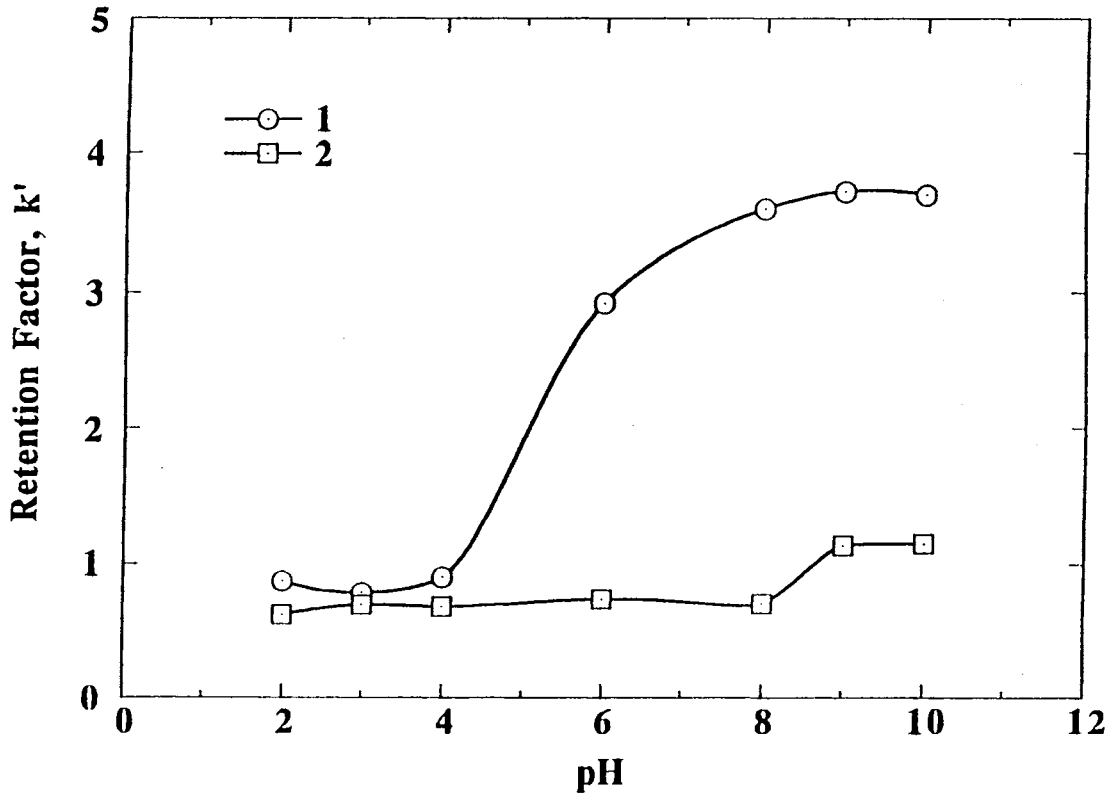


Figure 10. Plots of the retention factor of benzylamine versus the pH of the mobile phase. Column, monomeric bonded octadecyl-zirconia with end-capping, 3.0 x 0.46 cm I.D.; mobile phases: curve 1, 1% (v/v) acetonitrile in all the buffer solutions; curve 2, same mobile phases as in curve 1 in the presence of 50 mM tartarate. Buffers: (all containing 50 mM NaCl) pH 2.0 and 3.0, 5 mM NaH_2PO_4; pH 4.0, 5 mM sodium acetate; pH 6.0 and 8.0, 5 mM Na_2HPO_4; pH 9.0 and 10.0, 5 mM sodium borate; flow-rate, 1.0 mL/min.

octadecyl-zirconia, the protonation of the amino group of the analyte would exclude ligand exchange type of retention. However, at pH above 9.0, benzylamine becomes less protonated, and ligand exchange interaction may predominate.

In the presence of tartarate ions in the mobile phase, and at pH below 8.0, the residual interaction of the surface proper of the zirconia support with the fully protonated benzylamine was greatly reduced, and its retention *via* hydrophobic interaction with the hydrocarbonaceous chains of the stationary phase was also low, see curve 2 in Fig. 10. At higher pH where benzylamine became deprotonated, its retention increased since its hydrophobic interaction with the bonded stationary phase increased. Thus, upon adding tartarate to the mobile phase, the chromatographic retention was mainly due to hydrophobic interaction.

In the absence of tartarate, benzylamine was eluted as a sharp peak at pH 2.0 and 3.0. At pH 4.0, the solute peak started to show tailing and broadening. This behavior became more pronounced at pH 6.0 and 8.0 due to dual retention mechanism, i.e. ion-exchange and reversed-phase. Benzylamine has been shown to undergo some interactions with bare zirconia [20]. When the pH reached 9.0 and 10.0, benzylamine peak became less broader indicating that residual adsorptivity from the support matrix was still operating. When tartarate was added to the mobile phase, slight peak tailing appeared only at pH 4.0 and 6.0.

Figure 11 shows the behavior of benzylamine on polymeric octadecyl-zirconia in the absence and presence of tartarate in the mobile phase at different acetonitrile concentrations. As can be seen in Fig. 11, in the absence of tartarate in the mobile phase the retention factor of benzylamine remained almost unchanged over the entire pH range studied, and decreased with increasing the acetonitrile content of the eluent, see curves 1, 2, 3 and 4 in Fig. 11. The retention factor of benzylamine decreased slightly upon adding tartarate to the eluents, and again showed no dependence on pH, see curves 3' and 4' in Fig 11. The addition of small amount of tartarate would enhance the ionic atmosphere

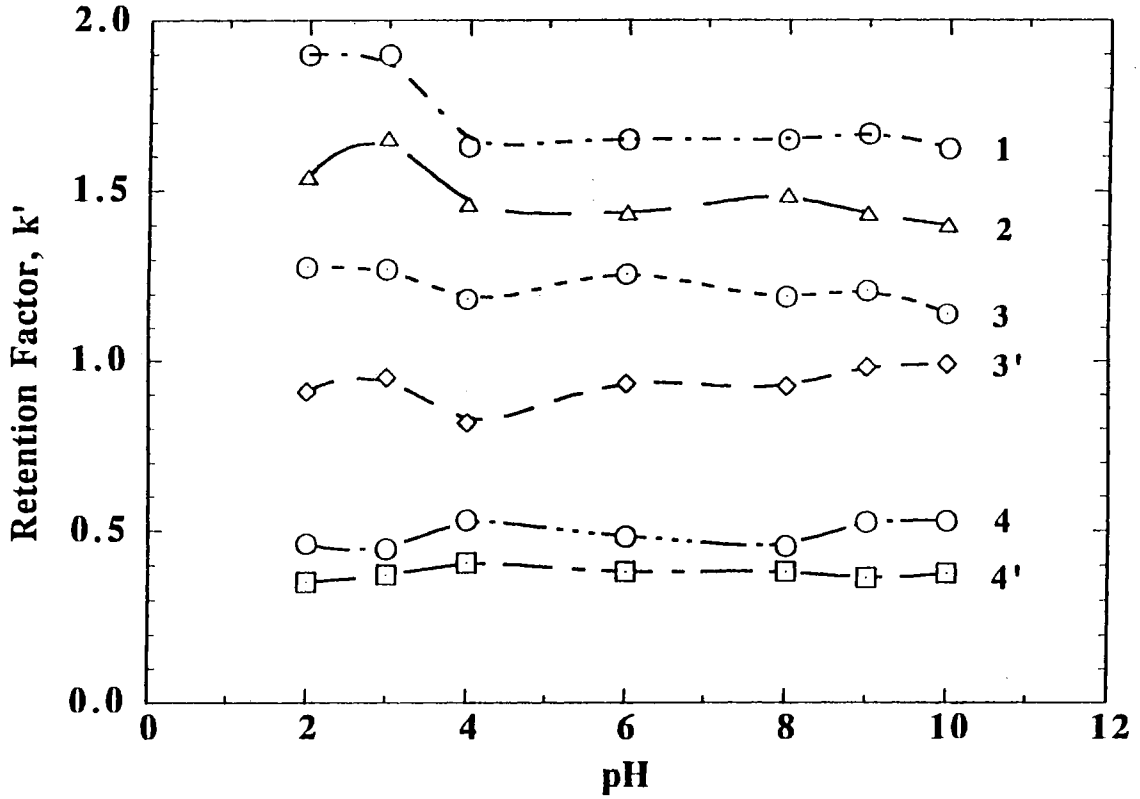


Figure 11. Plots of the retention factor of benzylamine versus the pH of the mobile phase. Column, polymeric bonded octadecyl-zirconia , 3.0 x 0.46 cm I.D.; mobile phases: curve 1, aqueous buffer solutions; curves 2, 3 and 4, 1%, 2% and 14% (v/v) acetonitrile, respectively, in the same buffers as in curve 1; curves 3'and 4', 50 mM tartarate in the same buffers as in curves 3 and 4. Buffers and flow-rate are the same as in Fig. 12.

about the solute molecules, which would cause solute interaction with the hydrophobic phase to decrease [22]. It should be noted that no peak tailing was observed for benzylamine on polymeric octadecyl-zirconia column in the presence or absence of tartarate ions in the eluent.

t-Cinnamic acid exhibited the same chromatographic behavior regardless of the bonding chemistry of the stationary phases, see curves 1 in Fig. 12a and b. In the absence of tartarate, t-cinnamic acid had the highest retention at pH 4.0 on both monomeric and polymeric octadecyl-zirconia columns, which may be explained by a dual retention mechanism of the solute. t-Cinnamic acid was also reported to undergo solute-support interaction even with zirconia having thick and cross linked polybutadiene coatings [9].

As can be seen in Fig. 12a and b, the extent of solute interaction with the support proper by ligand exchange and/or ion exchange was higher on the monomeric than on polymeric octadecyl zirconia column. This is manifested by the fact that at pH 4.0 the retention modulus of cinnamic acid, which is defined as the ratio of its retention factor k' observed in the absence to that obtained in the presence of tartarate, was ca. 2.0 on the monomeric versus 1.5 on the polymeric octadecyl columns. At pH above its pK_a value of 4.44, t-cinnamic acid became fully ionized, a fact that explains the decrease in its retention through hydrophobic interaction with the bonded octadecyl stationary phase.

In the presence of tartarate the solute-support interaction was minimized, and t-cinnamic acid was retained primarily by hydrophobic interactions, see curves 2 in Fig. 12a and b. At pH higher than 6.0, t-cinnamic acid was completely ionized and showed no interaction with the support matrix, and very little or no retention in the hydrophobic stationary phase. The surface of zirconia may posses cation exchange property at high pH and cinnamic acid would undergo coulombic repulsion from the surface. This may explain the fact that at high pH the retention of the solute was almost the same in the presence or absence of tartarate in the mobile phase.

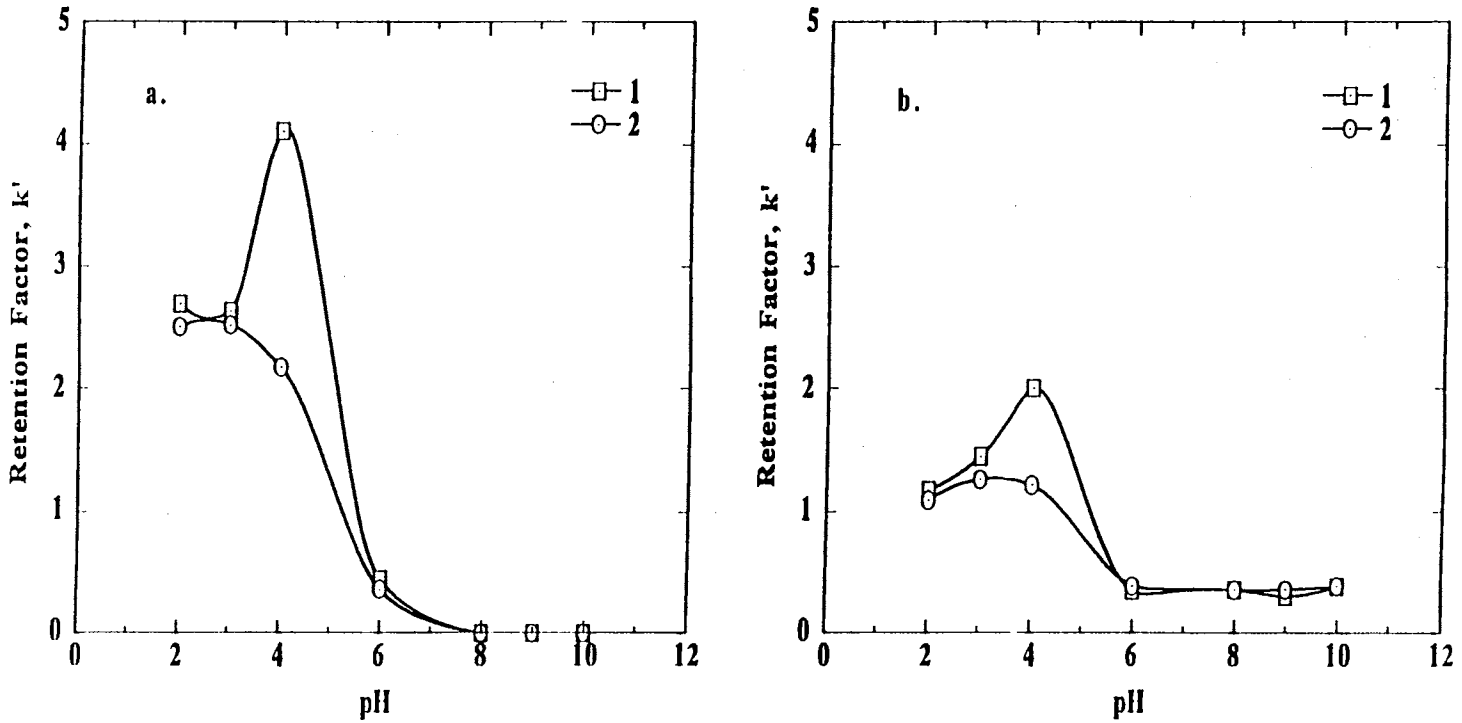


Figure 12. Plots of retention factor of t-cinnamic acid versus the pH of the mobile phase. Column, monomeric octadecyl-zirconia with end-capping in (a) and polymeric octadecyl-zirconia in (b), 3.0 x 0.46 cm I.D.. Mobile phases: in (a) 1% acetonitrile and 50 mM NaCl in all the buffer solutions in the absence (1) and presence of 50 mM tartarate (2); in (b) 15% (v/v) acetonitrile and 50 mM NaCl in all buffer solutions in the absence (1) and presence of 50 mM tartarate (2). Buffers are the same as in Fig 12; flow rate, 1.0 mL/min.

On both bonded stationary phases, t-cinnamic acid showed increased band broadening in the pH range 4.0 to 6.0. When tartarate was added to the mobile phase, the solute-support interaction was minimized and t-cinnamic acid eluted as a sharp peak.

Selected Applications

Polycyclic aromatic hydrocarbons. Reversed-phase liquid chromatography with non polar stationary phases has been the most widely used HPLC technique for the separation of polycyclic aromatic hydrocarbon (PAH), for recent reviews see Refs 31 and 32. It is well documented [31] that octadecyl stationary phases with high carbon loading are required for the high resolution of PAH isomers. Fig. 13, illustrates the separation of a series of PAHs and their isomers on "polymeric" bonded octadecyl-zirconia stationary phase. The retention of the polyaromatic hydrocarbons increased in the order of increasing hydrophobic area of the molecules, or in another word with increasing number of aromatic rings. Within the same groups of isomers, i.e., PAH having the same number of aromatic rings, the breadth to length ratio (B/L) determined the relative retention of the isomers. For pyrene and benz[a]pyrene, their corresponding B/L are 1.27 and 1.58, whereas for benzo[e]pyrene and benzo[a]pyrene their respective B/L are 1.12 an 1.50 [31]. As can be seen in Fig. 13 the isomer of higher B/L was more retarded. This is identical to that reported [33] on silica-based reversed-phase column. No evidence of solute-support interaction was revealed with this polymeric octadecyl column. On the other hand, with monomeric and end-capped octadecyl-zirconia stationary phase, the PAHs revealed some interaction with the zirconia surface proper and tailing peaks were observed.

Maltooligosaccharides. Fig. 14a illustrates the retention behavior of p-nitrophenyl maltooligosaccharides on polymeric octadecyl-zirconia stationary phases. It can be seen in this figure that the plots of log k' versus the number of glucose units in the homologous

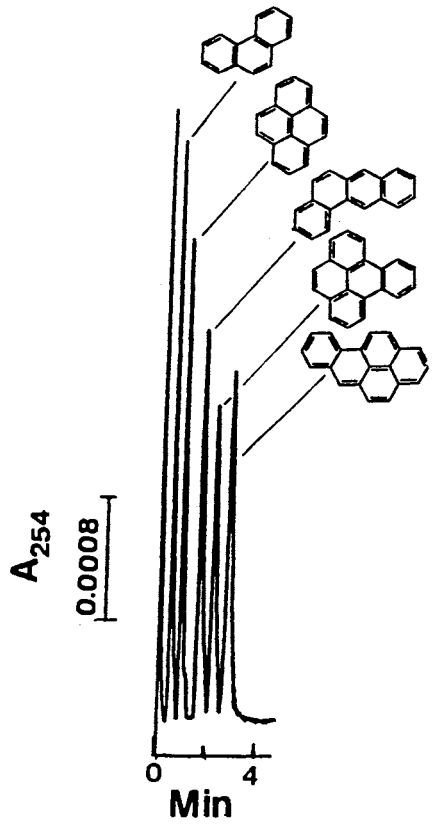


Figure 13. Separation of polycyclic aromatic hydrocarbons. Column, "polymeric" octadecyl-zirconia, 3.0 x 0.46 cm I.D.. Linear gradient in 6 min from 40% to 70% (v/v) acetonitrile in water; flow-rate, 2.0 mL/min. Solutes from left to right: phenanthrene, pyrene, benz[a]anthracene, benzo[e]pyrene and benzo[a]pyrene.

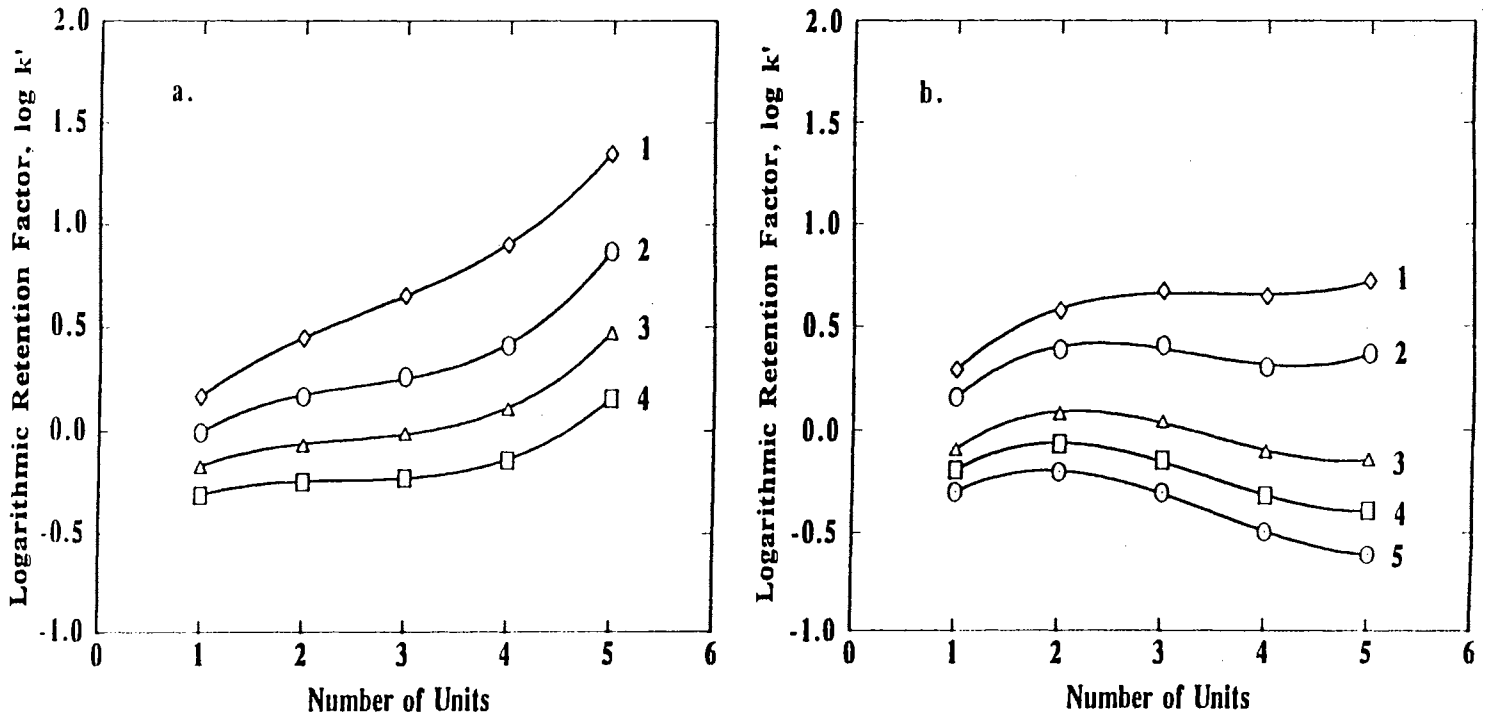


Figure 14. Plots of logarithmic retention factor of p-nitrophenyl derivatives of maltooligosaccharides versus the number of glucose units in the homologous series. Column, "polymeric" octadecyl-zirconia in (a) and polymeric octadecyl-silica in (b), 3 x 0.46 cm I.D.. Mobile phases, 0.05% trifluoroacetic acid in water at various volume percent of acetonitrile; in (a): 1, pure water; 2, 2.0%; 3, 4.0%; and 4, 6.0%; in (b): 1, pure water; 2, 1.0%; 3, 2.0%; 4, 3.0%, and 5, 4.0%. Flow-rate, 1.0 mL/min. Solutes: p-nitrophenyl derivatives of glucopyranoside, maltoside, maltotrioside, maltotetraoside, and maltopentaoside.

series are not linear, and the deviation from linearity increased as the acetonitrile content of the mobile phase increased.

For comparison, Fig. 14b shows the retention behavior of p-nitrophenyl maltooligosaccharides on nonporous polymeric octadecyl-silica stationary phases. With these sorbents, increasing the organic content of the mobile phase resulted in a switch of the elution order of the homologous series, and the five homologues could not be resolved even with plain aqueous mobile phase. This irregular behavior has been previously observed by us with xyloglucan oligosaccharides on porous octadecyl-silica stationary phases [34].

The different elution patterns of the maltooligosaccharides observed on octadecyl-silica and octadecyl-zirconia sorbents may be attributed to the difference in solute-support interactions between the two types of stationary phases.

Figure 15a and b portrays the rapid separation of p-nitrophenyl maltooligosaccharides obtained on a short polymeric octadecyl-zirconia column. These homologues could be separated in less than 40 sec when the mobile phase flow-rate was increased to 4.0 mL/min

Dansyl-amino acids. As shown above, polymeric octadecyl-zirconia stationary phases did not exhibit significant interaction with benzylamine and the residual adsorptivity of the sorbent toward t-cinnamic acid was greatly attenuated at high pH and/or in the presence of competing agent such as tartarate. Based on these results, amino acids, which are ampholytic compounds having amino and carboxylic groups in their structure should then be better chromatographed at high pH. Some preliminary studies conducted in our laboratories have already confirmed this prediction. An in-depth study on the chromatographic behavior of dansyl amino acids on octadecyl-zirconia stationary phases is underway and the results will be published in an upcoming article.

Fig. 16 shows a typical chromatogram for the separation of dansyl amino acids obtained on polymeric octadecyl-zirconia stationary phases. Seven amino acids can be

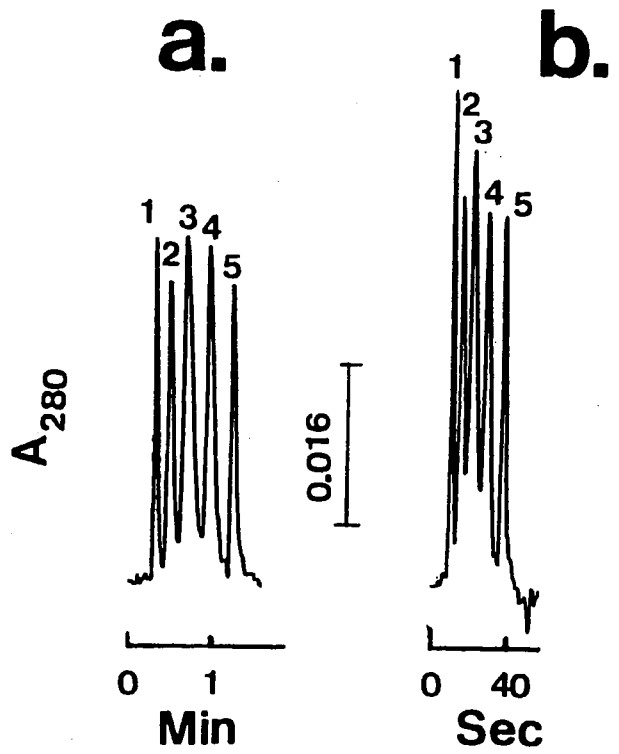


Figure 15. Chromatograms of p-nitrophenyl maltooligosaccharides. Column, "polymeric" octadecyl-zirconia, 3.0 x 0.46 cm I.D.. Linear gradient in 1 min in (a) and 0.5 min in (b) from 0 to 20 % (v/v) acetonitrile in water at 0.05% (v/v) trifluoroacetic acid; Flow-rates, 1.0 mL/min in (a) and 4.0 mL/min in (b). Solutes: p-nitrophenyl derivatives of 1, glucopyranoside; 2, maltoside; 3, maltotrioside; 4, maltotetraoside; and 5, maltopentaoside.

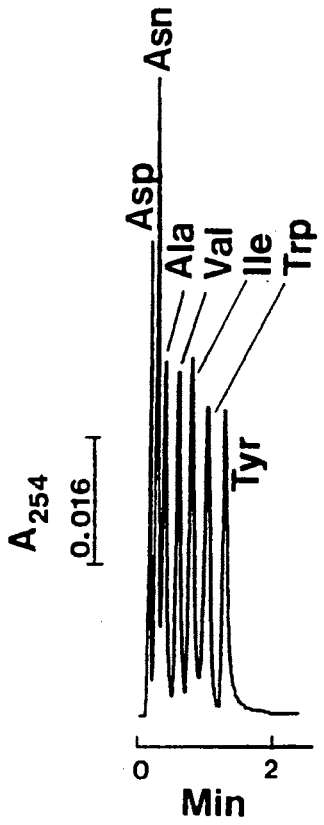


Figure 16. Chromatogram of dansyl-amino acids. Column, "polymeric" octadecyl-zirconia, 3.0 x 0.46 cm I.D.. Consecutive linear gradients, 0.8 min from 0 to 19.5%, and 0.1 min from 19.5 to 30% followed by isocratic elution for 0.2 min with 30% (v/v) acetonitrile in 5 mM Na_3PO_4, pH 12.0; flow-rate, 2.0 mL/min. Solutes: Asp, aspartic acid; Asn, asparagine; Ala, alanine; Val, valine; Ile, isoleucine; Trp, tryptophan; Tyr, tyrosine.

separated in less than 2 min with a rapid gradient of acetonitrile at pH 11.0. The retention of amino acids followed more or less the reversed-phase mechanism. The species with charged side chain, e.g., aspartic acid, was the least retained, followed by the amino acid having uncharged polar side chain, e.g., asparagine. The dansyl-amino acids with non-polar moieties were more retained and eluted in the order of increasing hydrophobicity, i.e., in the order alanine, valine, isoleucine, tryptophan and tyrosine.

Peptides. Since "polymeric" octadecyl-zirconia stationary phases exhibited lower metallic interaction than their monomeric counterparts toward charged solutes, these phases were employed in the separation of closely related peptides.

Fig. 17a illustrates the baseline resolution of Phe-Leu-Glu-Ile and Phe-Leu-Glu-Val on the "polymeric" bonded octadecyl-zirconia stationary phase using phosphate buffer, pH 2.0. These two peptides did not separate at pH 6.0, despite the fact that they differ in the amino acid residues at the C-terminal , i.e., isoleucine and valine, with the former being more hydrophobic. On the other hand, at pH 11.5, both peptides did not have much retention.

To further investigate the potential of octadecyl-zirconia, relatively hydrophilic peptides were chromatographed. As expected, Val-Gly-Ser-Glu and Val-Gly-Asp-Glu, having relatively low hydrophobicity, and differing only in one amino acid residue, serine and aspartic acid, showed little retention on the octadecyl stationary phase. To bring about their retention and separation, decyltrimethylammonium bromide ion-pairing agent was used in the mobile phase. Under this condition the peptides were resolved, and, as expected, the peptide with aspartic acid residue was more retained, see Fig. 17b.

Angiotensin I (Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu), angiotensin II (Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe) and angiotensin III (Arg-Val-Tyr-Ile-His-Pro-Phe) were not well resolved on polymeric octadecyl-zirconia bonded stationary phase at low pH. But at pH 11.0, these three peptides were very well separated, see Fig. 17c. As mentioned earlier, at this pH, solute-support interaction was minimized. Although angiotensin II has

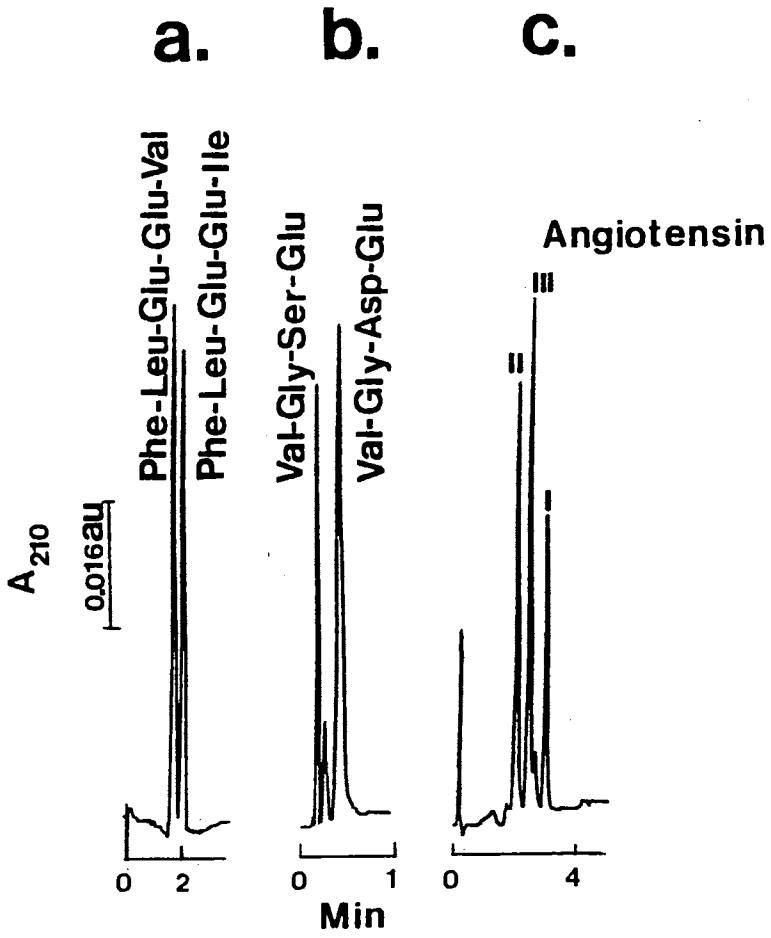


Figure 17. Chromatograms of peptides. Column, "polymeric" octadecyl-zirconia, 3.0 x 0.46 cm I.D.. Linear gradients: in (a) 2 min from 0 to 20% (v/v) acetonitrile in 5 mM phosphate, pH 2.0; in (b), 2 min from 0 to 20% (v/v) acetonitrile in 5 mM sodium phosphate containing 10 mM decyltrimethylammonium bromide, pH 6.0; in (c) 4 min from 0 to 40% (v/) acetonitrile in 5 mM phosphate, pH 11.0; flow-rates, 2.0 mL/min in (a) and (b) and 1.0 mL/min in (c).

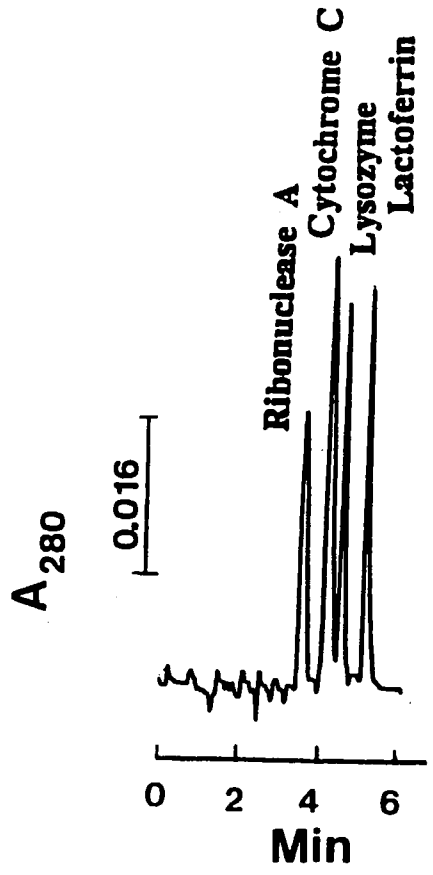


Figure 18. Chromatogram of proteins. Column, monomeric octadecyl-zirconia, 3.0 x 0.46 cm I.D.. Linear gradient in 7.0 min from 0 to 70% (v/v) acetonitrile in water at 0.05% (v/v) trifluoroacetic acid; flow-rate, 1.0 mL/min.

four more amino acid residues than angiotensin III, (i.e., Ala-Pro-Gly-Asp) the presence of an amino acid residue with ionizable side chain (Asp) in this tetrapeptide fragment may have caused angiotensin II to be the least retained, and separated from the other two peptides. Angiotensin I was the most retained of the three peptides on the octadecyl-zirconia bonded stationary phases. The two extra amino acid residues, histidine and leucine on the C-terminal of angiotensin I may have increased the hydrophobicity of this peptide, and consequently has brought about its higher retention on the reversed-phase column.

Proteins. Although monomeric octadecyl zirconia columns exhibited residual adsorptivities toward small and charged species, large molecular weight proteins chromatographed nicely on these phases, and a typical chromatogram is shown in Fig. 18. The four proteins eluted and separated in less than 6 min at a flow-rate of 1.0 mL/min with a linear gradient at moderate acetonitrile concentration in the eluent.

The absence of any significant solute-support interaction may be due to steric hindrance imposed on the protein analyte by the long octadecyl chains of the stationary phase, thus preventing the large protein molecule from getting into close proximity to the metallic sites as well as the unreacted hydroxyl groups on the surface of the stationary phase.

Acknowledgements

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CHAPTER III

REVERSED-PHASE LIQUID CHROMATOGRAPHY OF DANSYL AMINO ACIDS WITH MICROSPHERICAL OCTADECYL-SILICA AND OCTADECYL-ZIRCONIA BONDED STATIONARY PHASES*

Abstract

A series of non-porous, microspherical octadecyl-silica and octadecyl-zirconia bonded stationary phases were introduced and evaluated in the HPLC of dansyl-amino acids over a wide range of elution conditions. The microspherical silica and zirconia particles were coated with either polymeric or monomeric octadecylsilyl layers. Polymeric octadecyl-silica columns afforded virtually no solute-support interaction, whereas polymeric octadecyl-zirconia bonded stationary phases exhibited metallic interaction with some dansyl amino acids, and their residual adsorptivities toward the separated analytes were comparable to those observed on monomeric octadecyl-silica columns without end-capping. These metallic interactions, which are of the electron donor-electron acceptor (EDA) type, predominate in the acidic pH region. However, the presence of small amounts of tartrate or phosphate ions in the eluent greatly reduced EDA interaction, and consequently allowed the high resolution separation of dansyl amino acids (Dns-AA). Under optimal gradient elution conditions, eleven or fourteen different Dns-AA could be

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separated in less than 6.0 min on short polymeric octadecyl-zirconia or octadecyl-silica columns, respectively.

Introduction

Reversed phase packing materials based on inorganic supports other than silica are being increasingly employed in HPLC separations. These phases include polymer encapsulated alumina (1-3), alkyl bonded alumina (4), octadecyl-titania (5), polybutadiene coated-zirconia (6) zirconia sorbents having vapor-deposited carbons (7), polybutadiene-carbon composite zirconia sorbents (8) and octadecyl-zirconia (5,9). The rationale for the introduction of these non traditional stationary phases has been to provide sorbents with greater hydrolytic stability than the commonly used alkyl-silica bonded stationary phases. Indeed, hydrocarbonaceous zirconia, alumina and titania have been shown to combine the mechanical strength of alkyl-silica sorbents to the chemical stability of organic copolymer-based stationary phases such as rigid polystyrene-divinylbenzene, a feature that might soon increase the popularity of these inorganic phases. For recent reviews on the reversed-phase chromatographic properties and applications of polybutadiene alumina and zirconia see Refs. 10 and 11.

We have recently been investigating the potentials of non-porous octadecyl-zirconia bonded stationary phases in the rapid and high-resolution reversed-phase chromatographic separations of large and small molecules. Our initial studies (9) have shown that the non-porous octadecyl-zirconia sorbents are complementary to non-porous octadecyl-silica columns in the sense that their chemical stability spans a wider pH range and their composite chromatographic properties provide a unique selectivity in the rapid separation of proteins, peptides, dansyl-amino acids and oligosaccharides. In the meantime, work from other laboratories (5-8) involved porous, hydrocarbonaceous zirconia stationary phases,

which described their preparation, surface modification, chemical and mechanical properties and reversed-phase chromatographic behaviors.

In this article, which is an extension to our recent studies (9), we aimed at providing (i) another contribution to the understanding of the reversed-phase chromatographic properties of octadecyl-zirconia phases, (ii) a more comprehensive comparison with the more established octadecyl-silica bonded stationary phases and (iii) rapid analytical separations of practical importance with non-porous octadecyl-zirconia bonded stationary phases. In this regard, we have also prepared non-porous, microspherical silica particles of 1.1 μm, and determined the appropriate reaction conditions for their production in the desired size. Both supports were coated with monomeric and polymeric octadecyl functions in order to compare and evaluate the contribution of these inorganic matrices to the retention of charged solutes. Dansyl-amino acids having widely differing structural characteristics in terms of hydrophobicity, acidity and basicity were ideal solutes to probe the various types of possible interactions on the surface of the different stationary phases.

Experimental

Instrumentation

The liquid chromatograph consisted of the following components: a multiple solvent delivery system Model CM 4000 and a variable wavelength detector SpectroMonitor Model 3100 from LDC Analytical (Riviera Beach, FL, U.S.A.), a sample injector Model 7125 from Rheodyne (Cotati, CA, U.S.A.) and an integrator Model C-R5A from Shimadzu (Columbia, MD, U.S.A.).

Chemicals

HPLC grade acetonitrile was purchased from Baxter Diagnostic Inc. (McGraw Park, IL, U.S.A.). Reagent and technical grade isopropanol and methanol, and reagent grade sodium phosphate monobasic, dibasic and tribasic were from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Tetra-ethyl orthosilicate (TEOS) and trimethylchlorosilane were purchased from Aldrich (Milwaukee, WI, U.S.A.). Toluene, anhydrous denatured ethanol, butanol, sodium chloride and sodium hydroxide were from EM Science (Gibbstown, NJ, U.S.A.). Octadecyldimethylchlorosilane and octadecyltrichlorosilane were obtained from Hüls America Inc. (Bristol, PA, U.S.A.). Dansyl-L-amino acid were purchased from Sigma (St. Louis, MO, U.S.A.).

Synthesis of Inorganic Supports and Octadecyl Bonded Stationary Phases

Microspherical silica particles were synthesized by the hydrolysis of tetra-ethyl orthosilicate (TEOS) in aqueous ethanol solutions containing ammonia (12-15). Typically, 1000 mL of ethanol were put in a three-necked round bottom flask which was cooled in an ice bath. Anhydrous ammonia gas (99.99%) from a cylinder was bubbled into the ethanol through a glass capillary until saturation was reached. At this point, the flow of ammonia gas was stopped and the solution was allowed to warm up to room temperature. The final volume of the ethanol-ammonia solution was ca. 1200 mL. To maintain ammonia near saturated level throughout the reaction, 254 mL of concentrated ammonia solution were added to the alcohol solution. Thereafter, 16 mL of tetra-ethyl orthosilicate (TEOS) were added to the saturated ammoniacal ethanol solution. Total water and ammonia contents were computed by adding up the fractional amounts introduced by the components of the reaction mixture. At this initial point the solution had a volume of ca. 1470 mL, and contained 4.9 M NH_3, 7.0 M water and 0.05 M TEOS. Under these conditions, the polymerization reaction would normally start in about 5 min as was indicated by an increasing opalescence of the mixture. The reaction mixture was stirred with a paddle

stirrer and left at room temperature throughout the polymerization process. After an initial period of 12 hrs, 12.0 mL of TEOS (0.054 mol) and 2.8 mL of H_2O (0.16 mol) were added to the reaction solution, i.e., in a molar ratio of water:TEOS of 2.96 . The addition of TEOS and water was repeated every 12 hrs time interval for a total of five additions. When the reaction was completed, the silica microparticles were separated from the solution by centrifugation. Ethanol and water were used to wash away the unreacted chemicals until the wash became neutral. Silica microspheres were let dry on the air. As determined with scanning electron microscopy, the silica thus obtained has a mean particle diameter of ca. 1.1 μm.

Surface modification was carried out with octadecylsilane compounds using either mono- or trifunctional silane derivatives (9). Typically, 5.0 g of silica microparticles were suspended in 50 mL of toluene to which 1.5 g of octadecyldimethylchlorosilane or octadecyltrichlorosilane were added. The reaction was stirred for 12 hrs at 115 °C. After the reaction, the octadecyl-silica sorbents were separated by centrifugation from the reaction solution, and washed thoroughly first with toluene and then with methanol to clean the unreacted silane compound and hydrogen chloride formed during the reaction. Silica microspheres thus treated were let dry on the air.

The silica microspheres that were treated with octadecyldimethylchlorosilane were further reacted with trimethylchlorosilane. This end-capping process was to minimize the amount of unreacted surface silanols and to yield a higher surface coverage of homogeneous, nonpolar bonded stationary phase. Typically, 1.5 g of the octadecyl-silica were suspended in 30 mL of toluene and then heated at 50 °C. Thereafter, 3.0 mL of trimethylchlorosilane were added to the suspension together with 0.50 mL of pyridine which was introduced to neutralize the HCl formed during the reaction. The reaction solution was stirred at 50 °C for 12 hrs. After the reaction, the modified silica gel was washed with methanol and let dry on the air.

The synthesis of zirconia microspherical particles was carried out using well established procedures (5), which were described in a recent contribution from our laboratory (9). The surface modification of the zirconia microparticles were performed using the same procedures outlined above for silica microparticles.

Column Packing

Unless otherwise stated, all columns used in this study were precision-bore 316 stainless steel tubing from Alltech Associates (Deerfield, IL, USA) having 3.0 x 0.46 cm I. D. as the dimensions. Column end fittings were also 316 stainless steel fitted with 0.5-μm frits and distributor disks from Alltech Associates.

The octadecyl-silica and octadecyl-zirconia stationary phases were packed using slurry column packing technique at 7000 p.s.i. with a Shandon column packer instrument from Keystone Scientific (Bellefonte, PA, USA). Isopropanol or carbon tetrachloride were used to prepare the octadecyl-silica or the octadecyl-zirconia suspension, respectively. In both cases the packing solvent was isopropanol.

Results and Discussion

Synthesis of Silica Microspherical Particles

It is well established (12) that alkoxides (e.g., tetra-ethyl orthosilicate), the esters of the weak silicic acid $Si(OH)_4$, undergo hydrolysis in the presence of water, thus forming a siloxane network by a condensation reaction which is often referred to as a precipitation or more adequately polymerization reaction. This reaction can proceed at neutral pH but it is much faster in acidic or basic media. While acidic media lead to the formation of porous gel network, basic media favor the formation of "non-porous" microparticles or sol (16).

In the present study, we were interested in the base catalyzed precipitation of TEOS to produce microspherical silica particles.

Although silica microparticles of relatively large sizes could be obtained by a single step precipitation of TEOS in ammoniacal alcohol media (15), these attempts have been met with a limited success in our laboratory and elsewhere (13). It has been shown that narrow size distributions can be reproducibly synthesized over a narrow size range of 20-800 nm diameter, and near the maximum achievable size for any TEOS precipitation reaction, broad or multimodal particle size distributions are often obtained (13). In addition, in one step growth process, the solids content in the resulting suspension achieves a maximum values of 3 % at a TEOS concentration of 0.5 M, and attempts to increase the solid weight fraction above this value by simply increasing the initial TEOS concentrations resulted in heterodisperse particle size distributions (13).

To achieve large silica microparticles with narrow particle size distributions and high mass fractions, we have adopted a seeded growth technique developed by Bogush *et al* (13). This technique involves the preparation of a seed suspension in the first place, and then mixtures of TEOS and water at 1:2 molar ratios are added at predetermined time intervals to affect additional hydrolysis of TEOS and in turn increase the size of the seed. In this process, the seed should be produced under controlled conditions since the size of the seed would largely affect the desired final size and mass fraction. As seen in Experimental, the seed suspension was obtained from an initial reaction mixture containing 4.9 M NH_3, 7.0 M water and 0.05 M TEOS. At room temperature, this suspension yielded a seed with an average size of ca. 0.50 μm as determined by electron microscopy (see Fig. 1a). In order to obtain a final product for which the particle diameter is slightly above 1.0 μm, TEOS and water were added to the seed suspension in 1:2.96 mole ratio after the seed suspension has stopped reacting. It has been reported that during ensuing hydrolysis and condensation the number of colloidally stable particles remains constant but their size increases (13).

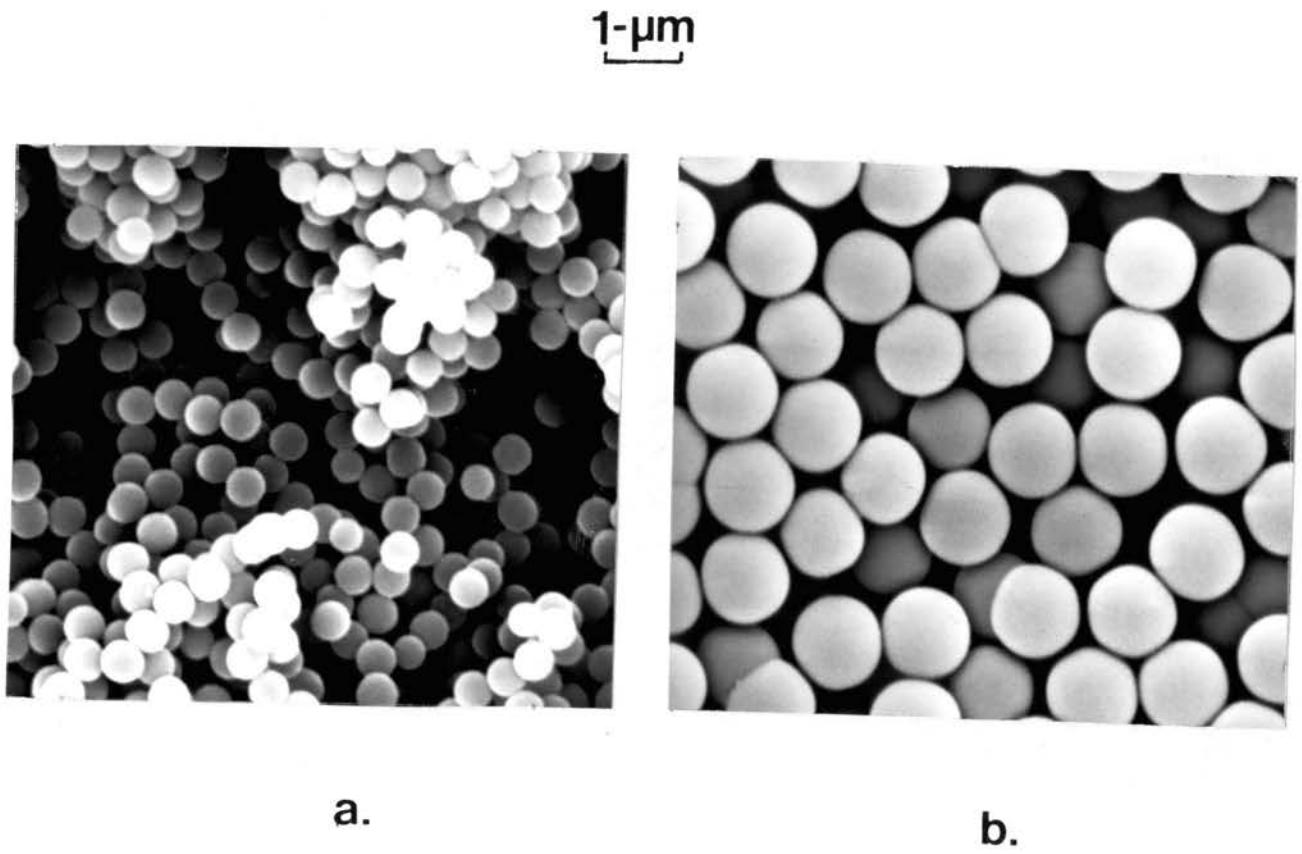

Figure 1. Scanning electron micrographs of the silica seed in (a) and the silica microspheres obtained by the seeded growth technique in (b).

In the seed suspension step, the conditions were selected while bearing in mind that the size and size distribution of the microspherical seed particles are largely influenced by the reaction conditions such as the relative concentration of TEOS and water used to initiate the polymerization reaction, ammonia concentration in the reaction solution, and temperature (12-15). Depending on the reaction conditions (14), the hydrolysis of silicon alkoxides could lead to either the growth of the existing microsol formed during the initial reaction, whereby the condensation of the monomer on the surface of small particles would occur, or nucleation by reaction between few monomers in which smaller particles of second generation are formed. Thus, the control of the hydrolysis rate is of primary importance as far as the size and dispersity of the product are concerned.

To produce monodispersed seed, we have selected to work at room temperature. As shown in Fig. 1a, this condition yielded monodispersed silica particles of 0.5 μm as determined by scanning electron microscopy. It has been shown that decreasing the reaction temperature of the seed suspension to subambient temperature would produce relatively large seed particles, but as the particle size increases the silica gel becomes less monodisperse (15). This is because a slower hydrolysis reaction rate is obtained at lower temperature, and consequently particle growth predominates. When the reaction rate is markedly reduced, a much smaller number of nuclei will be required to relieve the high supersaturation of silicic acid, and consequently the average particle size must increase with decreasing temperature (15).

To ensure monodispersity of the seed particles, we have maintained the concentration of ammonia at near saturation level. Although the reaction started at 4.9 M NH_3, loss of this reagent by evaporation during the prolonged reaction cannot be precluded even though the reaction vessel was tightly closed. The inevitable loss of ammonia may not be very critical since it was demonstrated that at concentration above 2 to 3 M ammonia (near saturation) the particle size did not show further increase under otherwise constant experimental conditions, i.e., temperature, TEOS concentration, water concentration (15).

At or near saturation, fast kinetics was observed (14), but the effect of ammonia was considered to promote the polymerization rate to a higher degree. It has been reported that at low concentration of ammonia (i.e., below 1.0 M), the silica flocculated in irregularly shaped particles (12). Ammonia apparently influences the morphology and creates spherical particles. This is because at high pH (i.e., high concentration of ammonia), the silica microparticles are highly ionized and are, therefore, stabilized against aggregation.

To keep the rate of hydrolysis at moderate level, we have utilized 0.05 M TEOS for the seed suspension. This is also essential for the monodispersity of the microparticles, since higher concentration of TEOS could lead to a faster hydrolysis rate, and consequently broader size distribution may be obtained (14). This is because at higher monomer concentration the nucleation rate would increase, thus leading to the formation of smaller particles. Simultaneously, however, the condensation rate would also increase, which may result in a faster growth rate. Aggregation might also occur which would yield silica particles with irregular shapes. However, and as is the case of our reaction, whereby saturated ammoniacal alcoholic solutions having relatively large water content (i.e., 7.0 M) were utilized, silicon alkoxide concentration can be varied between 0.02 and 0.5 M without affecting significantly the particle size (13).

The condensation rate depends strongly upon the water content of the system (14). Moderate water concentration favors the production of larger particles but excess water has the opposite effect. It was reported earlier (13) that with saturated ammoniacal solution, maximum particle size can be attained when 6.0 M water was present, and beyond a water concentration of 8.0 M a sharp decrease in the particle size was observed. In our experimental set up the concentration of water in the reaction solution was about 7.0 M at the onset of the reaction.

Under optimal reaction conditions, single-step sol-gel process seemed to have a limited potential for achieving large particle size. The maximum particle size in a single

growth process was reported to be 0.8 μm (13). As shown in Fig. 1a, under the conditions specified above monodispersed silica particles of 0.5 μm were obtained.

The 0.5 μm seed particles thus obtained were further grown using the seeded growth technique (13) in which mixtures of TEOS and water were added to the seed suspension at predetermined time intervals. As shown in Fig. 1b, this approach yielded particles with an average diameter of 1.1 μm, which was larger than what can be obtained with a one step growth process. Under seeded growth conditions, reported kinetic studies (13) have shown that smaller particles grew faster than larger ones. Bigger particles stopped interacting and grew primarily through aggregation with smaller particles and freshly formed nuclei. As a result, the size distribution became narrower. As can be seen in Fig. 2a, after the first addition of TEOS and water at the mole ratio 1:2.96 to the seed suspension, a sharp particle growth was observed, and the size grew at a much slower rate in the following additions. The particle size of the product was twice the size of the seed after five consecutive additions.

By increasing the amount of TEOS and water to 0.10 M and 7.1 M, respectively, in the seed suspension while keeping the other conditions (i.e., ammonia concentration and temperature) the same as in the preceding experiment, the initial seed was about 0.4 μm, which is slightly smaller than that obtained in the previous reaction. This can be explained by the increase in the rate of hydrolysis due to increasing the amount of water and TEOS in the seed suspension. Under these conditions, and by affecting successive additions at the same mole ratios of TEOS and water as in the preceding experiment, i.e., 12.0 mL TEOS and 2.8 mL water, the particle size increased steadily and leveled off after ca. 10 additions, see Fig. 2b. The final product was smaller in size than the product shown in Fig. 1b, and more additions were required to increase the size of the seed particles. This is because of the presence of more particles per unit volume which resulted from doubling the TEOS concentration and increasing the water content. This also explains the shallower increase in particle size upon the successive additions (compare Fig 2a and 2b). Above 10 additions

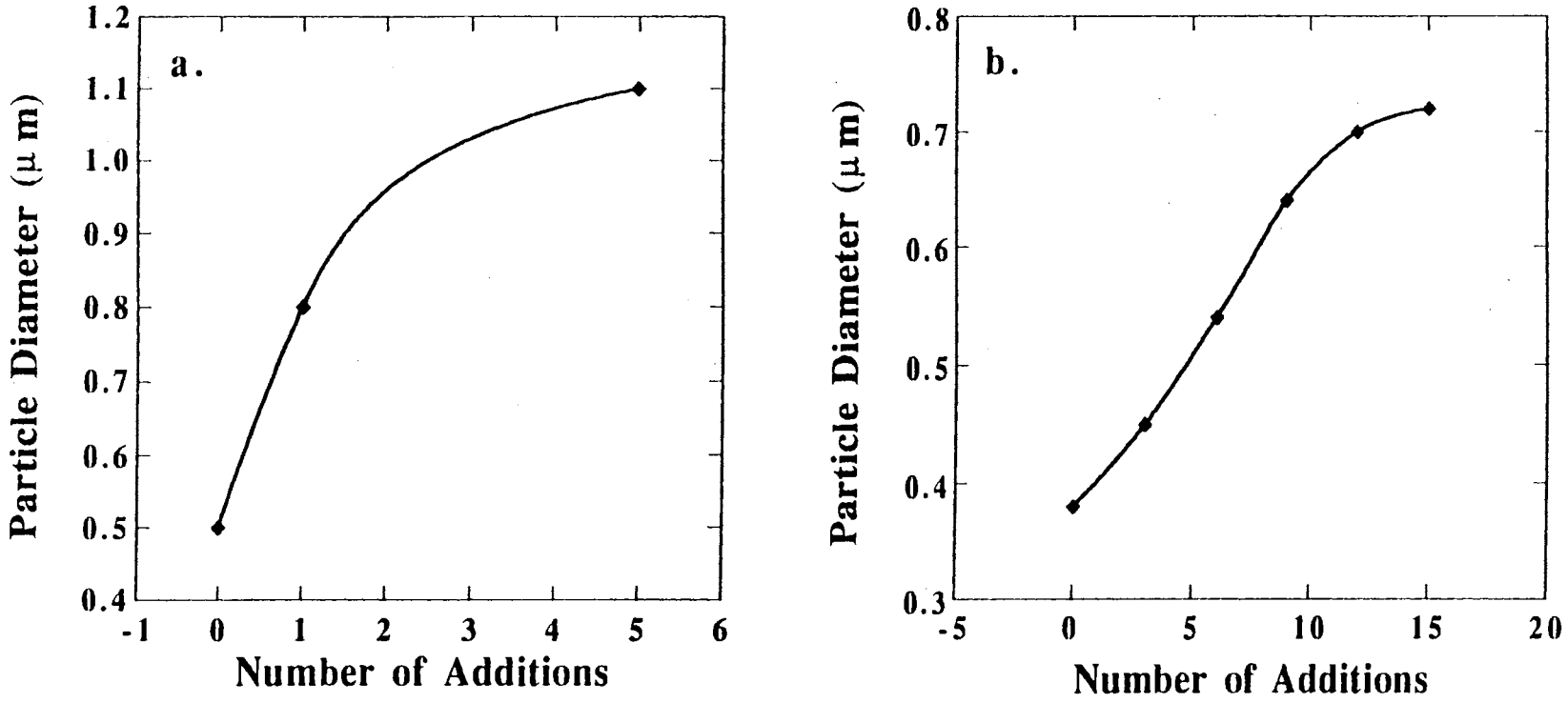

Figure 2. Plots of particle diameter as determined from scanning electron microscopy of the silica microspheres *versus* the number of additions of TEOS and water to the seed suspension. For experimental details see text.

no further gain in particle size was observed and the product became more polydisperse with the formation of aggregated particles. This indicates the importance of keeping the TEOS at concentration below 0.10 M in the seed suspension so that slightly larger seed particles are produced, and thus less successive additions are needed.

Chromatographic Behavior of Dansylated Amino Acids on Octadecyl-Silica and Octadecyl-Zirconia Bonded Stationary Phases

Surface treatment. Non-porous stationary phases have the advantage of eliminating intraparticulate diffusional mass transfer resistance in the stagnant mobile phase, thus allowing high speed separations without sacrificing separation efficiencies. However, due to their relatively low specific surface area (< 5 m^2/g), nonporous supports yield stationary phases of low phase ratio (i.e., ratio of the volume of the stationary phase to that of the mobile phase) and low linear sample capacity (i.e., amount of solute injected per gram of sorbent) when compared to porous sorbents. In other words, the concentration of surface-bound ligands per unit column volume is low, and consequently the column is more quickly overloaded with the injected samples. While the low sample capacity limits the utility of nonporous sorbents to microscale and analytical purposes, the low phase ratio can be regarded as an advantage in terms of bringing chromatographic retention to practical range, especially in the chromatography of biomacromolecules, e.g., proteins and DNA fragments. Under these circumstances, biopolymers may be separated under mild elution conditions which would preserve their biological activity and allow their high mass recovery. On the other hand, the low phase ratio of non-porous sorbents may not provide sufficient retention and consequently resolution for relatively small, polar substances. Thus, while advantageous for the separation of large molecules, the inherent low phase ratio of nonporous sorbents represents a major drawback as far as their utility in the

separation of small molecules is concerned and may explain why these stationary phases have been exclusively used in the area of large biomacromolecules (17-20).

To overcome, at least in part, the above impediments, the silica and zirconia microparticles used in this study were produced with particle diameters of 1.1 μm and 1.5-2.8 μm, respectively, which correspond to a calculated specific surface area of 2.6 m^2/g for silica and an average calculated specific surface area of 1.4 m^2/g for zirconia (21). In addition, the microspherical silica and zirconia particles were coated with polymeric octadecyl functions by reacting the non-porous supports with octadecyltrichlorosilane. Besides increasing the phase ratio, and concomitantly the retention of Dns-AA, the polymeric coating has also reduced residual adsorptivities between the analytes and the support proper. This type of treatment would also achieve the same results with porous supports. But in the case of porous supports, the polymeric layer may result in pore constriction which would give rise to reduction in the intraparticulate diffusion rate of the solute and poor column efficiency (22). This may explain the limited use of porous stationary phases with polymeric octadecyl coatings. With nonporous supports, this situation does not exist, and polymeric coatings should provide increased retention and reduced solute-support interactions.

As expected, when the microspherical, non-porous silica support was modified with octadecyldimethylchlorosilane, the monomeric stationary phases thus obtained did not provide a satisfactory separation for the solutes under investigation, and the peaks of the Dns-AA were relatively broad. The band broadening may be explained by the poor sorption kinetics of these solutes on the partially alkylated silica surface, a phenomenon that has been also observed with monomeric, porous octadecyl-silica bonded stationary phases (22). As with porous octadecyl-silica sorbents, end-capping the nonporous monomeric octadecyl-silica with a small silane compound, e.g., trimethylchlorosilane, yielded slightly higher retention and improved peak symmetry. This is because the end-capping would render the surface more hydrocarbonaceous and uniform (22).

The Dns-AA were better resolved on the polymeric octadecyl-silica columns than on the monomeric octadecyl-silica sorbents under otherwise identical elution conditions. It should be mentioned that in both cases, i.e., monomeric and polymeric octadecyl-silica, the addition of small amounts of amino compounds such as triethylamine to the mobile phase was beneficial for the separation of Dns-AA, see below. The triethylamine may have further reduced the magnitude of silanophilic interactions, and formed ion-pairs with the Dns-AA. This is in agreement with studies reported by other researchers (23,24) on the improvement of retention behavior of polar compounds in the presence of small amounts of triethylamine in the mobile phase.

For the zirconia-based stationary phases, the situation was further complicated by the metallic nature of the support. Because of the excessive metallic interaction, the monomeric octadecyl-zirconia did not yield satisfactory results as far as the separation efficiencies is concerned even after end-capping, see below. As we reported earlier (9), the magnitude of this interaction was minimized by coating the surface with polymeric octadecyl layer.

Table 1 summarizes the above observations in terms of adjusted retention volume (i.e., solute retention volume minus dead volume of the column) obtained at pH 6.0 with gradient elution at increasing acetonitrile concentration in the eluents. Dns-AA were eluted essentially following the order of increasing hydrophobicity on both monomeric and polymeric octadecyl-silica or octadecyl-zirconia stationary phases. In the case of silica-based stationary phases, the monomeric octadecyl-silica without end-capping exhibited strong solute-support interaction of hydrophilic nature, i.e., silanophilic interactions. For example, Dns-arginine and Dns-lysine, which have amino groups in their side chains, showed relatively higher retention than some other Dns-AA of higher hydrophobicity. In fact, arginine was more retained than glycine, threonine and alanine while lysine was more retarded than proline, valine and methionine. Also, proline showed higher retention than valine on the monomeric stationary phase possibly for the same reason. End-capping of

Table 1. Adjusted retention volume of Dns-AA obtained on the various octadecyl-silica and octadecyl-zirconia stationary phases using linear gradient elution from 0 to 50.0% (v/v) acetonitrile in 10.0 mM sodium phosphate (I) or in10 mM sodium phosphate containing 0.50 M NaCl (II) or in 10.0 mM sodium phosphate containing 0.20M NaCl (III) or in 50.0 mM ammonium phosphate (IV and V); all buffers were pH 6.0. Columns, 30.0 x 4.6 mm; flow-rates: 1.0 mL/min in I and V, and 2.0 mL/min in II, III and IV. NM = not measured.

	Adjusted Retention Volume (mL)				
	Octadecyl-silica			Octadecyl-zirconia	
Dns-AA	Monomeric I	Monomeric (End-capped) II	Polymeric III	Monomeric (End-capped) IV	Polymeric V
Cysteic acid	0.36	1.54	2.93	2.06	1.26
Aspartic acid	0.34	1.55	3.02	2.07	1.44
Glutamic acid	0.45	1.92	3.17	NM	1.50
Asparagine	0.85	2.52	3.39	1.76	1.94
Serine	0.76	2.84	3.66	1.98	2.10
Glutamine	1.30	2.95	3.70	1.89	2.18
Arginine	2.80	3.25	3.73	2.80	3.22
Glycine	1.28	3.02	3.78	2.12	2.36
Threonine	1.14	3.04	3.90	2.01	2.28
Alanine	1.45	3.01	4.00	2.26	2.30
Lysine	3.05	3.63	4.24	NM	4.44
Proline	2.90	3.78	4.40	2.52	3.00
Valine	2.52	3.88	4.56	2.68	3.06
Methionine	3.00	4.00	4.84	2.91	3.42
Isoleucine	3.25	4.36	5.20	3.00	3.74
Leucine	3.25	4.37	5.22	3.00	3.80
Phenylalanine	3.52	4.64	5.50	3.32	4.18
Tryptophan	3.48	4.56	5.54	3.42	4.24
Tyrosine	5.00	5.24	6.05	NM	7.36

the monomeric octadecyl-silica yielded a stationary phase with chromatographic behavior that paralleled the behavior of the polymeric type. Elution order of Dns-AA was almost the same as that on the polymeric stationary phase. Although 0.5 M NaCl was added to the eluents, arginine still showed silanophilic interaction. As can be seen in Table 1, even though the ionic strength of the mobile phase was decreased from 0.5 to 0.2 M NaCl, the polymeric octadecyl-silica stationary phase showed higher retention and the order of elution followed the normal reversed-phase behavior.

When compared to silica-based stationary phases, octadecyl-zirconia showed significant differences as far as the elution pattern of Dns-AA is concerned. End-capped monomeric octadecyl-zirconia stationary phases showed the presence of solute-support interaction involving electron donor-electron acceptor (EDA) type of interaction, which may be responsible for the higher retention of Dns-AA with nucleophilic properties such as cysteic acid and aspartic acid, see Table 1, column IV. These findings corroborate earlier observations reported by other researchers (6,8,25-27) in that the metallic properties of zirconia led to strong interactions with ionic species having phosphoric or carboxylic groups. Also, we have shown in our previous study (9) that while end-capped monomeric octadecyl-zirconia stationary phases exhibited reversed-phase chromatographic property toward non-polar and slightly polar species and compared favorably to monomeric octadecyl-silica stationary phases, this process (i.e., end-capping) still could not effectively mask the metal interaction of zirconia support with highly nucleophilic solutes such as *trans*-cinnamic acid. As can be seen in Table 1 (column IV), arginine with an amino side chain group exhibited pronounced interaction with the sorbent, perhaps *via* the unreacted (or residual), deprotonated surface hydroxyl groups as well as the surface exposed zirconium sites.

As can be seen in Table 1, polymeric octadecyl coating had effectively shielded the surface active zirconium sites. For example, the retention of cysteic acid and aspartic acid decreased on the polymeric octadecyl-zirconia when compared to the end-capped

monomeric octadecyl column. Arginine and lysine, however, still exhibited higher retention, a phenomenon that was only observed on monomeric octadecyl-silica sorbent without end-capping. Similarities of elution pattern on polymeric octadecyl-zirconia and on monomeric octadecyl-silica without end-capping could also be found with solutes such as glycine and threonine, which may indicate that the latter solute is interacting with the zirconia support. The addition of triethylamine to the mobile phases did not show much effect on the performance of the polymeric octadecyl-zirconia column toward the elution of Dns-AA. The secondary retention mechanism is thought to arise from both the active zirconium sites and the residual hydroxyl groups on the support surface, although metal interaction (i.e., EDA type of interaction) might play a major role.

pH of the eluent. The retention behavior of a group of selected Dns-AA with the general chemical formula $(CH_3)_2NC_{10}H_6SO_2NH$-AA (where NH-AA denotes the amino acid moiety of the derivative) was studied at different eluent pH on both octadecyl-zirconia and octadecyl-silica stationary phases of the polymeric type. The results are depicted in Figs 3-8 in terms of retention factor k' versus the pH of the eluents. In all cases, the pH of the eluent largely influenced the retention of the solutes under investigation. According to recent studies on the ionization of Dns-AA (28,29), the pK_a value of the dimethylamino group of Dns-AA , i.e., for the protonated form $(CH_3)_2N^+HC_{10}H_6SO_2NH$-AA, is between 3.0 and 4.0, and this value is largely independent of the ionic properties of the side chain of the amino acid. The amino group adjacent to the sulfonyl group of the dansyl moiety has a pK_a of 11.7, i.e., for the deprotonated form $(CH_3)_2NC_{10}H_6SO_2N^-$-AA, and would dissociate only at extreme alkaline pH. Earlier study on the separation of amino acids on a porous polystyrene-divinylbenzene copolymer (30) reported that the retention behavior of the dansyl derivatives was typical of ampholytes without zwitterion properties. A retention maximum occured at about pH 3.5.

As can be seen in Fig. 3a and b, all the Dns-AA studied showed an increase in retention in the pH ranging from 2.5 to 4.0, which correspond to the deprotonation of the dimethylamino group of the dansyl moiety, see above. The retention factor of Dns-AA having an acidic side chains, e.g., aspartic acid and glutamic acid, decreased when the mobile phase pH was raised above pH 4.0 and leveled off above pH 6.0. This can be explained by the complete ionization of the carboxyl group of the side chain at pH above 6.0. The retention factor of Dns-AA having a side chain amino group such as lysine and arginine increased slightly at pH above 4.0 and leveled off in the pH range 6.0-7.0. This may be due to the fact that after pH 4.0 whereby the dimethylamino group of the derivative has become deprotonated, the net charge of the derivative stayed the same due to complete ionization of both the side chain amino group and the carboxyl group of the derivatives. Unexpectedly, the retention factor of glutamine was affected almost in the same way when compared to that of arginine and lysine. As expected, the retention factor of Dns-AA without ionizable side chain groups such as methionine and valine passed through a maximum at pH 4.0 as with those having carboxyl groups in the side chain. This again is due to the interplay of the successive deprotonation of the dimethylamino group and carboxyl group of the Dns-AA.

The retention-pH dependency of Dns-AA on octadecyl-zirconia columns of the polymeric type was investigated in the pH range 2.0-12.0. Mobile phase additive such as d-tartrate was also used to study the effect of the presence of a competing agent on attenuating the electron donor-electron acceptor (EDA) type of interactions between the Dns-AA and the accessible metallic sites of the zirconia support. In this process, the Dns-AA solutes function as electron donors while the surface exposed zirconium sites play the role of electron acceptors. Typical results are presented in Figs 4-8 in terms of retention factor, k', *versus* pH. As shown in these figures, the retention behaviors of Dns-AA were similar to those obtained on the silica-based stationary phases in the sense that (except for lysine) all the Dns-AA studied showed the highest retention at pH 4.0. Although this

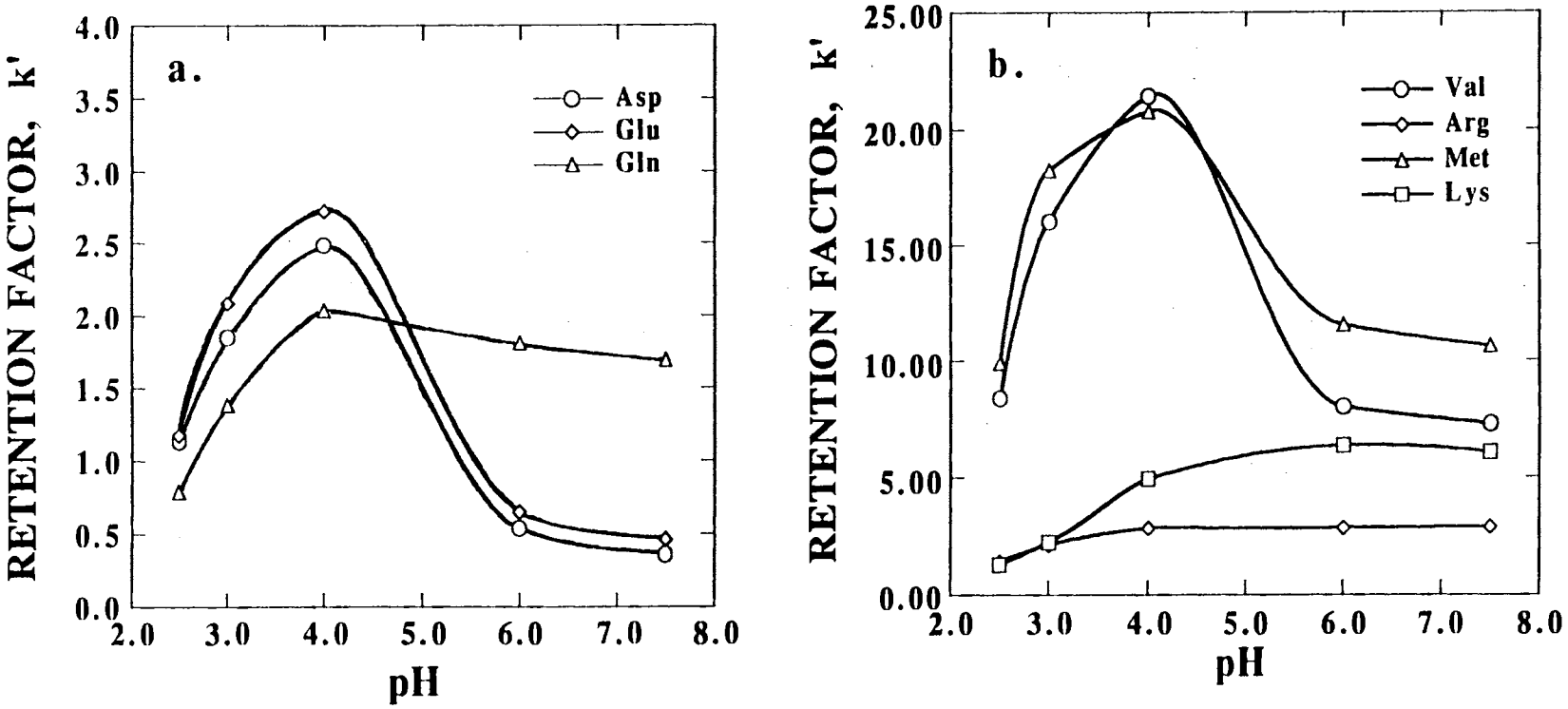

Figure 3. Plots of retention factor of Dns-AA *versus* pH of the eluent. Column, 30.0 x 4.6 mm, polymeric octadecyl-silica; mobile phases: 10.0 mM phosphate, pH 2.5, 3.0, 6.0, and 7.5; 10.0 mM sodium acetate, pH 4.0; all mobile phases contained 10.0% (v/v) acetonitrile; isocratic elution at 1.0 mL/min.

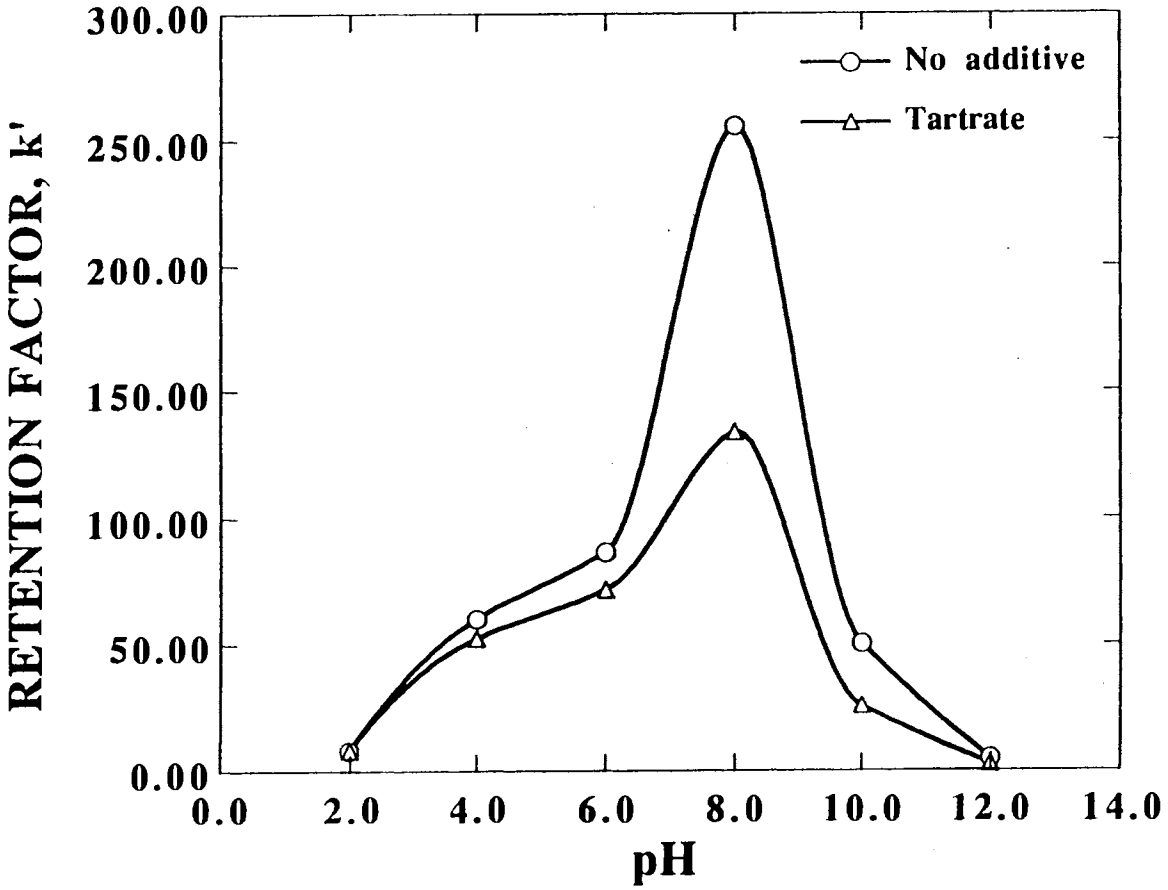

Figure 4. Plots of retention factor of Dns-lysine *versus* the pH of the eluent in the presence and absence of tartrate. Column, 30.0 x 4.6 mm, polymeric octadecyl-zirconia; mobile phases: 10.0 mM sodium phosphate, pH 2.0, 6.0, 8.0, 10.0 and 12.0; 10.0 mM each sodium phosphate and sodium acetate, pH 4.0; all mobile phases contained 5.0% (v/v) acetonitrile in the absence or presence of 50.0 mM sodium tartrate; isocratic elution at 1.0 mL/min.

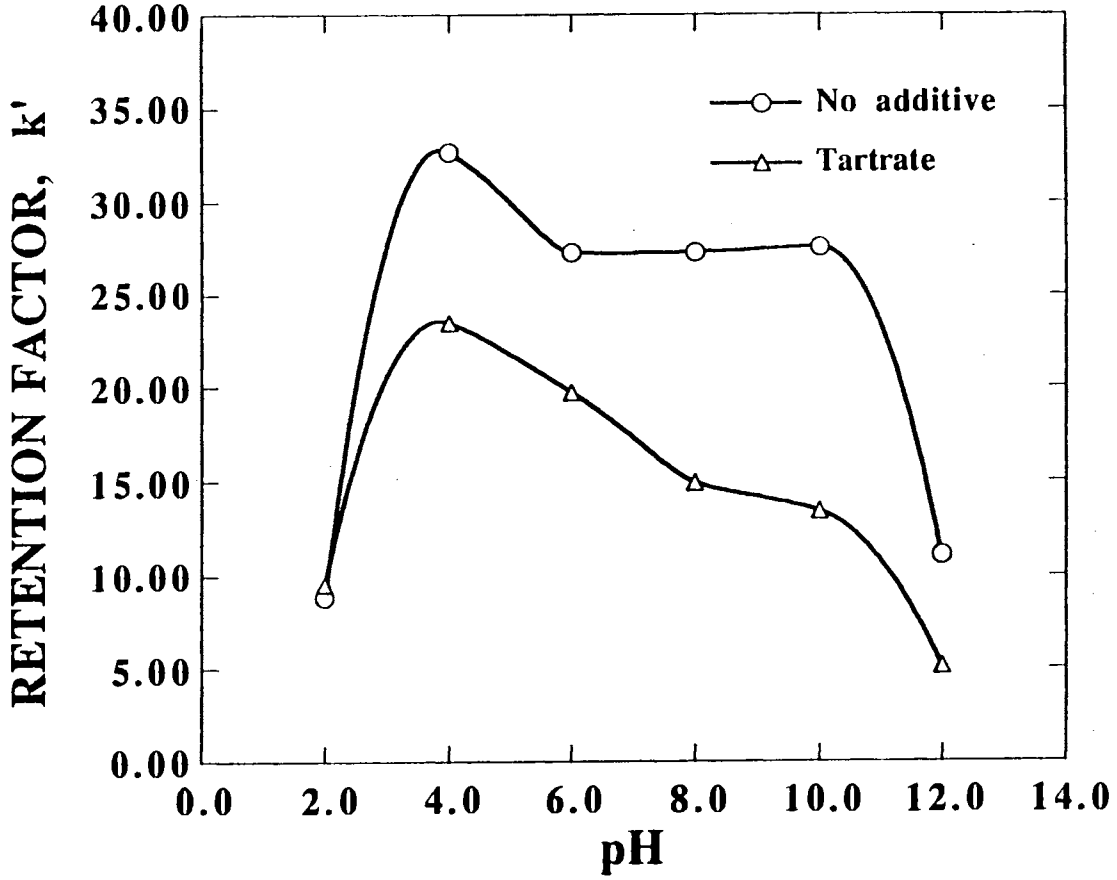

Figure 5. Plots of retention factor of Dns-arginine *versus* pH of the eluent in the presence or absence of tartrate. Conditions are as in Fig. 4.

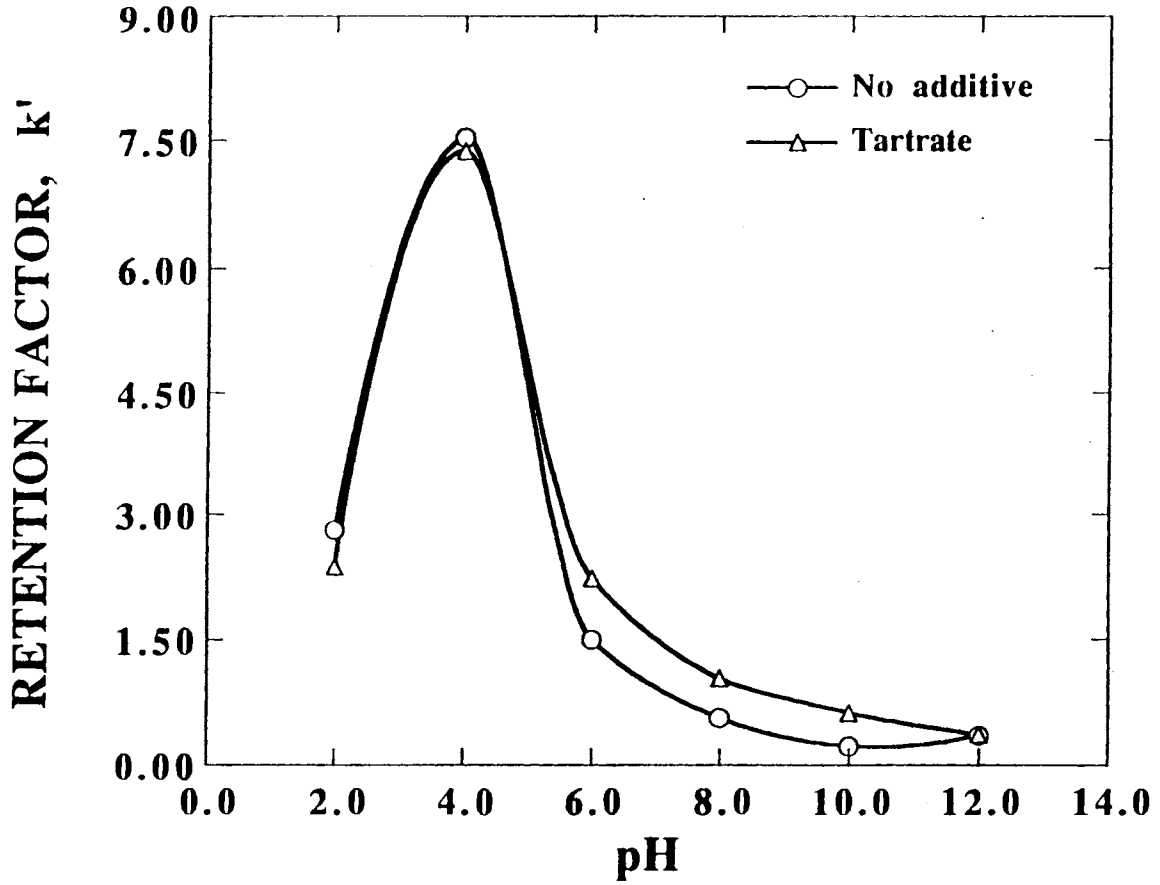

Figure 6. Plots of retention factor of Dns-glutamate *versus* pH of the eluent in the presence or absence of tartrate. Conditions are as in Fig. 4.

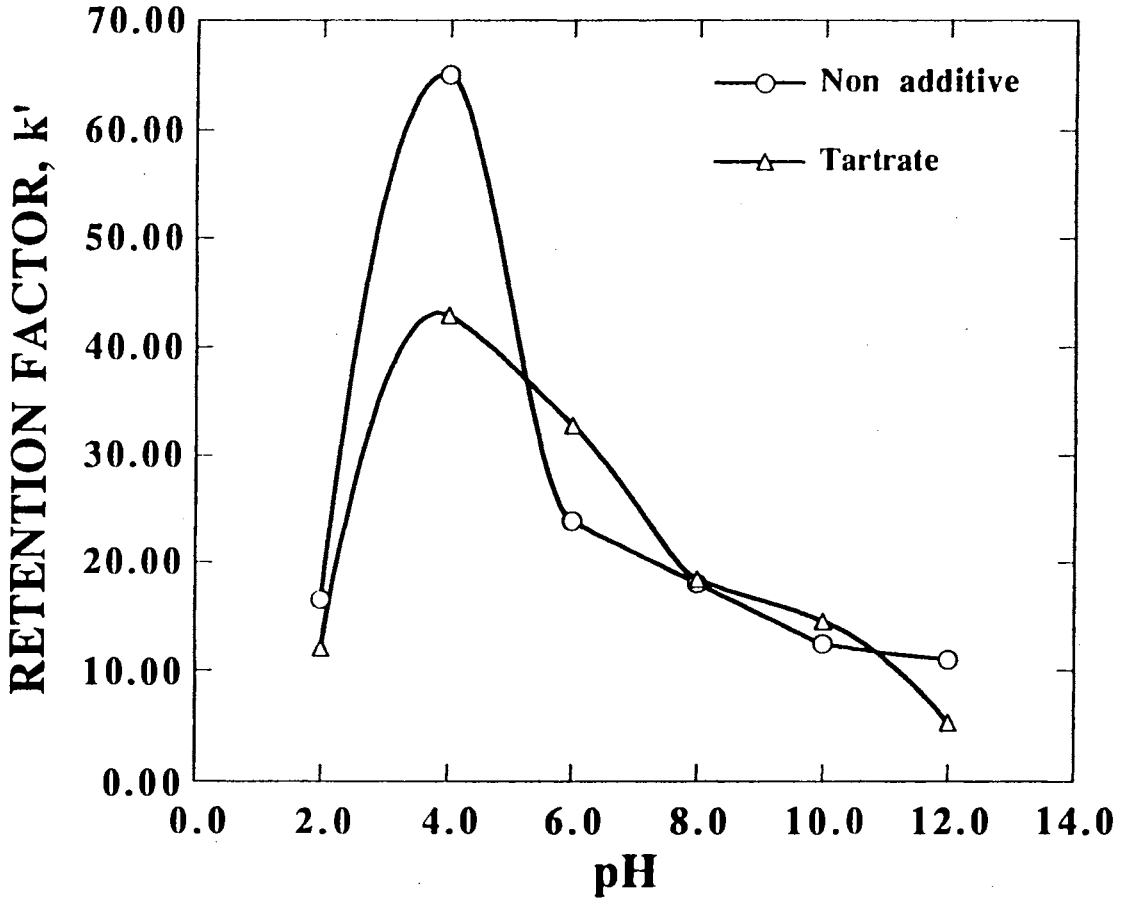

Figure 7. Plots of retention factor of Dns-methionine *versus* pH of the eluent in the presence or absence of tartrate. Conditions are as in Fig. 4.

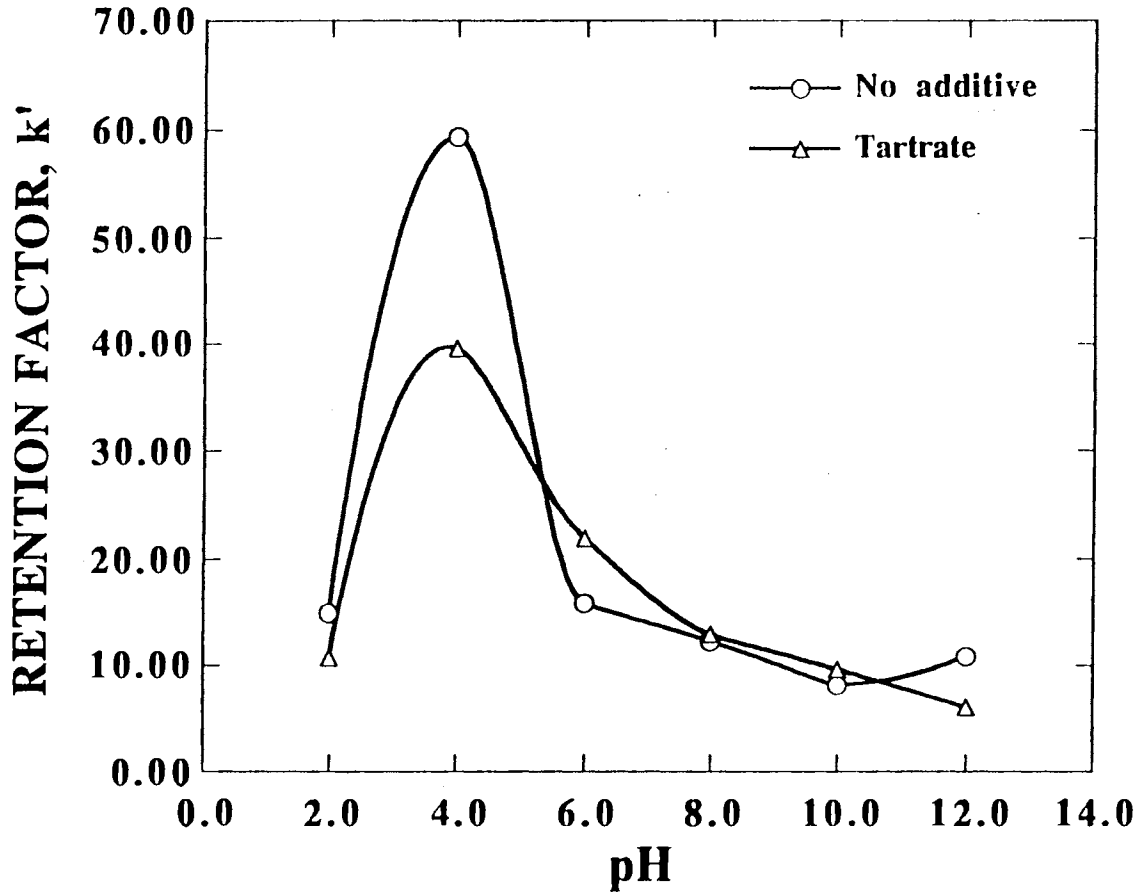

Figure 8. Plots of retention factor of Dns-valine *versus* pH of the eluent in the presence or absence of tartrate. Conditions are as in Fig. 4.

behavior arises mainly from the ionization of the Dns-AA solutes, in some cases, however, the magnitude of retention maxima obtained at pH 4.0 was much higher on the zirconia-based stationary phases than on the silica columns. For instance, the ratio of k' of methionine obtained at pH 4.0 to that observed at pH 6.0 on silica column was ca. 1.7, whereas this same ratio was 2.7 on the zirconia column. The ratio of k' obtained at pH 4.0 to that at pH 6.0 for valine was 3.8 on octadecyl-zirconia and 2.9 on octadecyl-silica. This may indicate the presence of EDA retention mechanism between the solutes and the metallic sites of the zirconia support. Possible electron donor atoms in valine are those of the carboxyl group whereas for methionine these electron donor atoms are the sulfur in side chain and the oxygen of the carboxyl group. It should be mentioned that at pH 4.0 some of the Dns-AA could not be eluted from the octadecyl-zirconia column when 10.0 mM sodium acetate buffer was used as the running mobile phase, and their elution necessitated the inclusion of 10.0 mM sodium phosphate in the mobile phase. This is another evidence of the presence of EDA interactions.

As shown in Fig. 4, lysine exhibited a retention maximum at pH 8.0. Unlike on octadecyl-silica column, arginine showed a retention maximum at pH 4.0, and the magnitude of its retention decreased slightly but stayed constant in the pH range 6-10, see Fig. 5. For these two amino acids, both the surface exposed zirconium sites and the unreacted hydroxyl groups of the zirconia may be contributing to their retention.

As discussed above the polymeric octadecyl-zirconia behaved normally toward glutamic acid reason for which its retention was almost the same in the presence or absence of tartrate in the eluent, see Fig. 6. On the other hand, and as can be seen in Figs 4, 5, 7 and 8, the addition of tartrate to the eluent generally alleviated solute-support interactions for the other Dns-AA investigated, namely lysine, arginine, methionine and valine. The competing agent, tartrate, had the strongest effect at pH 4.0 for methionine and valine, at pH 8.0 for lysine and almost across the pH range studied for arginine.

For valine, methionine and glutamate, the addition of tartrate generally enhanced their retention at pH above 6.0 due to increasing ionic strength, which may have lowered the ionic repulsion of these acidic solutes from the negatively charged surface and increased hydrophobic interaction with the octadecyl ligands. On the other hand, in the case of lysine and arginine, the addition of tartrate to the mobile phase attenuated solute-support interaction, whether the solute interactions were of the EDA type with the zirconium sites or electrostatic with the unreacted hydroxyl groups of the support. As we have shown earlier (9), tartrate seems to function as hard Lewis base ligand, and would form "metal chelates" with the exposed zirconium sites of the surface of the stationary phase. It is thought that the empty valence orbital of the zirconium sites will be filled with the electrons pairs donated by the mobile phase additive, and consequently there would be little interaction between the solute and the support matrix. In addition, the doubly charged tartrate ions increases the ionic strength of the eluent and may reduce the residual ion-exchange property of the zirconia matrix.

In general, the metallic property of the support was most effective in the acidic region, and especially below pH 6.0. When the mobile phase pH was increased to 6.0, Dns-AA retention by EDA mechanism decreased, and that was reflected by an improved peak symmetry. When the mobile phase pH was raised to pH > 11.0, the surface exposed zirconium sites were effectively shielded by the high concentration of hydroxide ions in the mobile phases. Also, the support acquires a net negative charge at such high pH conditions, which may further contribute to the decrease in retention of Dns-AA *via* electrostatic repulsion between the support and the negatively charged solutes.

Illustrative separations. Monomeric octadecyl-silica and octadecyl-zirconia stationary phases were not sufficiently retentive toward Dns-AA and/or yielded excessive band broadening. Thus, they were not used further in this study. To achieve high peak capacity on the polymeric octadecyl-zirconia or silica stationary phases, we have examined

various eluents. First, the ionic strength of the mobile phase seemed to influence the retention of Dns-AA. Second, the nature of the buffer and mobile phase additives were also important for achieving high resolution separation of the Dns-AA.

Figure 9a illustrates the separation of fourteen Dns-AA in about 5.0 min at a mobile phase flow-rate of 2.0 mL/min using a 7.0 min gradient consisting of three consecutive linear segments at increasing acetonitrile concentration in 10.0 mM phosphate containing 0.50 M NaCl and 0.008% (v/v) triethylamine, pH 6.0. The inclusion of sodium chloride for up to 0.50 M in the eluent increased the retention of Dns-AA having ionizable polar side chain such as cysteic acid and glutamic acid. This is due to the shielding effect of the salt, which decreased the magnitude of Coulombic repulsive forces between the negatively charged analytes and the sorbent, and consequently enhanced hydrophobic interaction. For Dns-AA with uncharged polar and nonpolar side chains, retention increased in the region of low salt concentration in the mobile phase, i.e., for up to about 40.0 mM NaCl. Further increase in the ionic strength of the mobile phase did not significantly affect the retention, but did improve peak symmetry, and consequently the quality of the overall separation.

A 7.0 min linear gradient at increasing acetonitrile concentration in 50.0 mM ammonium phosphate, pH 6.0, allowed the separation of fourteen Dns-AA in about 6.0 min, as shown in Fig. 9b, without the need of high ionic strength as in the preceding experiment. This is because ammonium phosphate has effectively minimized the interaction between the solutes and the active residual silanol groups (although not high) on the stationary phase surface. Under these conditions, complete resolution of solutes of close hydrophobicity like lysine and proline could be obtained, and amino acid isomers, e. g., leucine and isoleucine, could be slightly resolved. Further increase in ammonium phosphate concentration in the mobile phase did not show much effect on retention and resolution of the solutes. When compared with Fig. 9a, better resolution was achieved for the solutes with ionizable polar side chain, such as cysteic acid and glutamic acid, in the ammonium phosphate mobile phase system. With a steeper gradient of acetonitrile in

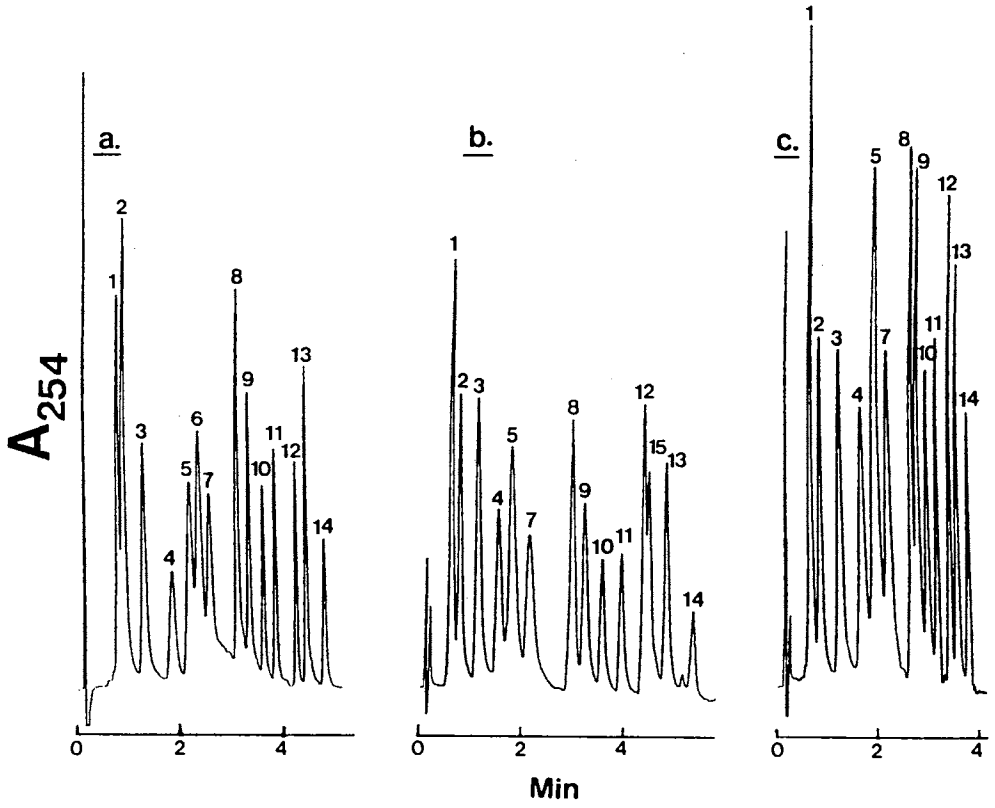

Figure 9. Chromatograms of Dns-AA obtained on polymeric octadecyl-silica stationary phase. Column, 30.0 x 4.6 mm; (a): consecutive linear gradients, 0.70 min from 5.0 to 6.5% (v/v), 0.10 min from 6.5 to 10.0% and 6.2 min from 10.0 to 40.0% (v/v) acetonitrile in 10.0 mM sodium phosphate containing 0.50 M NaCl and 0.008% (v/v) triethylamine, pH 6.0; (b): linear gradient in 7.0 min from 5.0 to 40.0% (v/v) acetonitrile in 50 mM ammonium phosphate, pH 6.0; (c) : consecutive linear gradients, 0.10 min from 5.0 to 6.5% and 3.0 min from 6.5 to 30.0% (v/v) acetonitrile in 50.0 mM ammonium phosphate, pH 6.0; in all cases the mobile phase flow-rate was 2.0 mL/min. Dns-AA: 1, cysteic acid; 2, glutamic acid; 3, asparagine; 4, serine; 5, glycine; 6, threonine; 7, alanine; 8, lysine; 9, proline; 10, valine; 11, methionine; 12, leucine; 13, tryptophan; 14, tyrosine; 15, isoleucine.

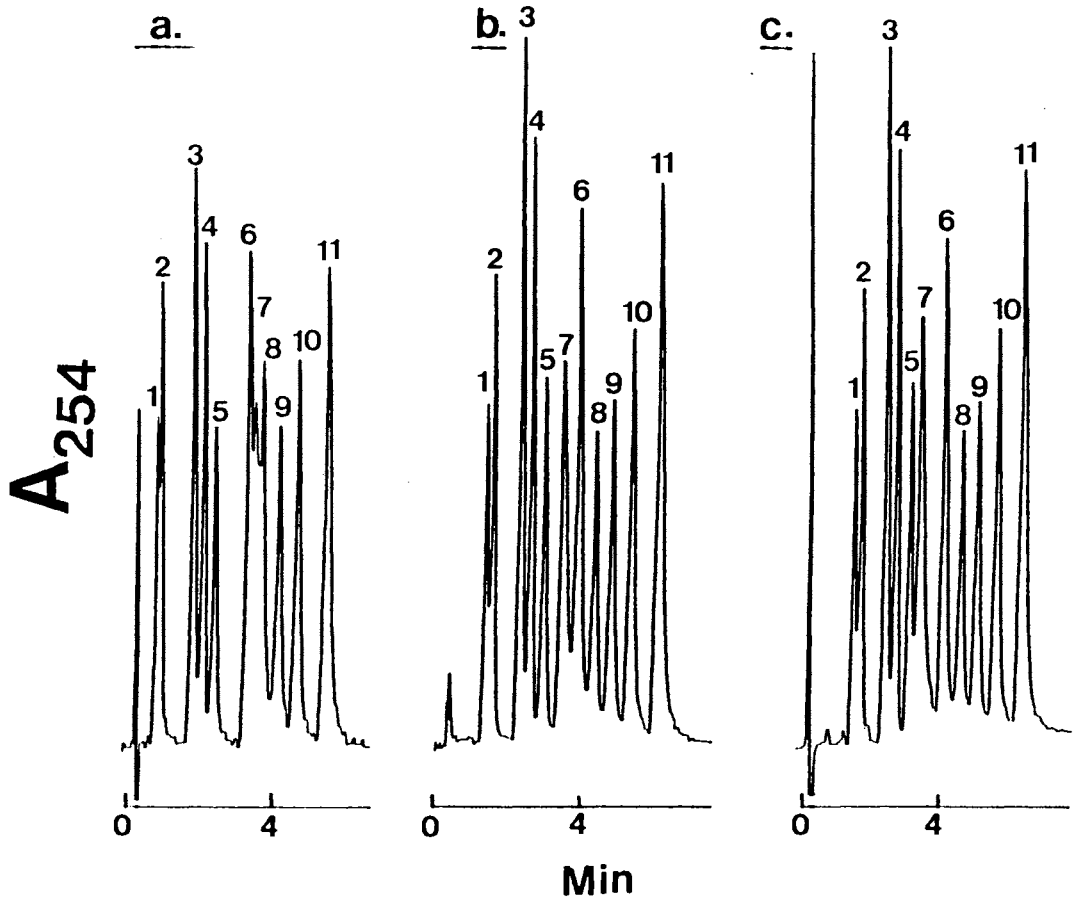

Figure 10. Chromatograms of Dns-AA obtained on polymeric octadecyl-zirconia stationary phase. Column, 50.0 x 4.6 mm; consecutive linear gradient, 2.0 min from 6.0 to 14.0%, 9.0 min from 14.0 to 36.0% (v/v) acetonitrile in 20.0 mM ammonium phosphate containing 0 in (a) 50.0 in (b) and 100.0 mM tartrate in (c), pH 8.0; flow-rate, 1.0 mL/min. Dns-AA: 1, aspartate; 2, glutamate; 3, asparagine; 4, glutamine; 5, threonine; 6, proline; 7, arginine; 8, valine; 9, methionine; 10, isoleucine; 11, tryptophan.

50.0 mM ammonium phosphate, thirteen dansyl amino acids could be separated in less than four minutes at 2.0 mL/min.

Figure 10 a, b and c shows the separation of eleven Dns-AA on polymeric octadecyl-zirconia column using an 11.0 min gradient consisting of two consecutive linear segments at increasing acetonitrile concentration in 20 mM phosphate containing 0, 50.0 or 100.0 mM tartrate, pH 8.0, respectively. As can be seen in Fig. 10, the inclusion of tartrate in the eluent improved the quality of the overall separation. At this pH, whereby the ligand exchange property of the zirconia matrix is diminished, the addition of tartrate enhanced the retention of the Dns-AA and reduced the interaction of arginine with the support. In fact, without tartrate this solute interacted with the zirconia matrix and eluted between proline and valine. Upon adding 50.0 mM tartrate to the eluent, the retention of arginine decreased slightly, and it eluted before proline. Also the addition of tartrate provided better resolution for aspartate and glutamate. Going to pH 11.0 or higher, the retention of Dns-AA decreased rapidly with concomitant decrease in the peak capacity of the chromatographic system. Under these conditions, and as shown in our previous report (9), the octadecyl-zirconia column is adequate for the separation of hydrophobic Dns-AA.

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CHAPTER IV

CHROMATOGRAPHIC PROPERTIES OF ZIRCONIA-BASED STATIONARY PHASES HAVING SURFACE BOUND CATIONIC FUNCTIONS*

Abstract

A series of non-porous, microspherical zirconia-based stationary phases with surface bound cationic functions has been introduced and evaluated in ion exchange chromatography of proteins and small acidic solutes. Different surface modification procedures were evaluated in the covalent attachment of weak, strong or hybrid anion exchange moieties on the surface of non-porous zirconia microparticles. *N,N*-Diethylaminoethanol (DEAE) was used as the weak anion exchange ligand while glycidyltrimethylammonium chloride, which was covalently attached to poly (vinyl alcohol) layer (PVAN) on the zirconia surface, constituted the strong anion exchange moiety. Partially quaternarized poly(ethyleneimine) hydroxyethylated (PEI) was used as the hybrid type of anion exchange coating. DEAE-zirconia microparticles acted as purely cation exchange stationary phases toward basic proteins indicating the predominance of electron donor-electron acceptor interaction (EDA) with surface exposed zirconium sites as well as cation exchange mechanism *via* electrostatic interaction with unreacted and unshielded hydroxyl groups. PVAN-zirconia stationary phase exhibited anion exchange chromatographic properties toward acidic proteins, but EDA interaction has stayed as an

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important contributor to solute retention despite the presence of a relatively thick layer of poly(vinyl alcohol) on the surface of the zirconia particles. The modification of zirconia surface with partially quaternarized PEI proved to be the most effective approach to minimize Lewis acidic metallic properties of the support. In fact, PEI-zirconia stationary phase operated as an anion exchanger toward acidic proteins and other small acidic solutes.

Introduction

Very recently, there has been an increasing interest in zirconia-based stationary phases mainly because of the chemical stability of the zirconia support over a relatively wide pH range. Thus far, the most widely used zirconia-based stationary phases have been the non-polar types for reversed-phase chromatography. Octadecyl-zirconia [1-4], polybutadiene coated zirconia [5], zirconia microparticles coated with vapor-deposited carbons [6] and polybutadiene-carbon composite zirconia sorbents [7] are typical examples of reversed-phase packing materials for HPLC. Other modifications of zirconia microparticles for use in HPLC included the impregnation of the support with fluoride ions [8] or phosphate ions [9]. Both fluoride and phosphate impregnated zirconia microparticles behaved as cation exchange sorbents. Phosphated zirconia microparticles were further modified by dynamically loading the sorbent with copper (II) ions for use in ligand exchange chromatography [10]. The chemical stability of zirconia has very recently been exploited in biospecific affinity chromatography and metal chelate interaction chromatography of proteins by introducing zirconia sorbents with surface bound concanavalin A or iminodiacetic acid [11], respectively.

Thus far, no attempt has been made yet to modify the surface of zirconia with charged ligands for ion-exchange chromatography despite the strong need for chemically stable ion-exchange stationary phases. This paper is concerned with the investigation of different chemical modification procedures for the covalent attachment of weak, strong or

hybrid anion exchange moieties on the surface of non-porous zirconia microparticles. *N,N*-Diethylaminoethanol (DEAE) was used as the weak anion exchange ligand while glycidyltrimethylammonium chloride, which was covalently attached to poly(vinyl alcohol), constituted the strong anion exchange moiety. Partially quaternarized poly(ethyleneimine) hydroxyethylated (PEI) was used as the hybrid type of anion exchange coating. The various coatings were characterized over a wide range of elution conditions including eluent pH, salt concentration and nature of salt using small acidic probe solutes as well as high molecular weight proteins of various isoelectric points.

Experimental

Instrumentation

The liquid chromatograph was assembled from an LDC Analytical (Riviera Beach, FL, U.S.A.) ConstaMetric 3500 solvent delivery system with a gradient programmer which was used to control a ConstaMetric Model III solvent delivery pump, a sample injector Model 7125 from Rheodyne (Cotati, CA, U.S.A.) and a UV interference filter photometric detector Model UV-106 from Linear Instruments (Reno, NV, U.S.A.). Chromatograms were recorded with a computing integrator Model C- R6A equipped with a floppy disk drive and a cathode-ray tube (CRT) monitor from Shimadzu (Columbia, MD, U.S.A.).

Chemicals

Reagent grade ammonium sulfate, sodium phosphate, sodium chloride, and iodomethane, and reagent grade as well as technical grade isopropanol, methanol and *N,N*-dimethylformamide (DMF) were from Fisher (Pittsburgh, PA, U.S.A.). Triethylamine, glycidyltrimethylammonium chloride, *N,N*-diethylaminoethanol (DEAE) and boron

trifluoride etherate were purchased from Aldrich (Milwaukee, WI, U.S.A.). γ-Glycidoxypropyltrimethoxysilane was obtained from Hüls America Inc. (Bristol, PA, U.S.A.). Trizma base, uridine-5'-monophosphate (UMP), uridine-5'-triphosphate (UTP), myoglobin from horse skeletal muscle, ribonuclease A as well as ribonuclease B, α-chymotrypsinogen A and carboxypeptidase A from bovine pancreas, cytochrome c from horse heart, lysozyme from chicken egg, β-lactoglobulin A and α-lactalbumin from bovine milk, transferrin (approx. 90% substantially iron-free, and holo approx. 98% iron saturated) from human, carbonic anhydrase from bovine erythrocytes, α_1-acid glycoprotein from bovine serum, ovalbumin, albumin from human and lipoxidase as well as trypsin inhibitor, type I-S and type II-S from soybean were purchased from Sigma (St. Louis, MO, U.S.A.). Benzoic acid, terephthalic acid (1,4-benzenedicarboxylic acid), isophthalic acid (1,3-benzenedicarboxylic acid), trimesic acid (1,3,5-benzenetricarboxylic acid), and pyromellitic acid (1,2,4,5-benzenetetracarboxylic acid) were from Chem Service (West Chester, PA, U.S.A.). Triglycidoxyglycerol, poly(vinyl alcohol) (PVA) 87-89% hydrolyzed (M.W. 124,000-186,000), poly(ethyleneimine) (PEI) hydroxyethylated (M.W. 60,000-80,000) 31% in water, and poly(ethylene glycol 200 diglycidyl ether) (diepoxy PEG 200) were obtained from Polysciences, Inc. (Warrington, PA, U.S.A.).

Stationary Phases

Synthesis of DEAE stationary phase. The zirconia microparticles used in this study were in the size range 1.5-2.8 μm, and were prepared as previously described [2,3]. Typically, 3.0 g of zirconia were suspended in 30.0 mL of 5.0 mM Na_2HPO_4 buffer solution, pH 7.0, in a round bottom flask and the mixture was heated at 98.0 °C. To this suspension, 5.0 mL of γ-glycidoxypropyltrimethoxysilane were added, and the reaction mixture was stirred for 2 hrs. Thereafter, the epoxy activated zirconia thus obtained was first washed successively with water and dry DMF and then resuspended in 30.0 mL of

DMF:DEAE solution of 1 : 1 volume ratio to which 3.0 mL of triglycidoxyglycerol were added. The reaction was stirred at 70.0 °C for 5 hrs. After the modification, the support was washed with DMF and methanol, and was let dry in the air.

Synthesis of PVAN stationary phase. Zirconia with surface-bound PVA to which quaternary amine functions (PVAN) were covalently attached was synthesized in a multistep process as follows. Typically, 5.0 g of zirconia were suspended in 50.0 mL of DMF and heated at 120.0 °C in a round bottom flask. To this suspension, 3.0 mL of γ-glycidoxypropyltrimethoxysilane were added, and the reaction mixture was stirred at 120.0 °C for 12 hrs. After the reaction, zirconia support was separated from the solution and re-suspended in 20.0 mL of dry DMF and heated to 125.0 °C. Meanwhile, in a separate vessel, 2.0 g of PVA were added to 50.0 mL of boiling DMF. The upper portion of the viscous PVA solution (about 30 mL) was poured into the zirconia suspension. Triethylamine (2.0 mL) was used as the catalyst for the reaction. After the reaction, zirconia thus treated was suspended again in 50.0 mL of DMF and heated at 120.0 °C. Then, 3.0 mL of diepoxy PEG 200 and 2.0 mL of triethylamine were added to the suspension, and the resulting reaction mixture was stirred for 5 hrs. Then, the support was separated and suspended again in another 50.0 mL DMF to which 2.0 mL of triglycidoxyglycerol and 1.0 mL of triethylamine were added. The reaction mixture was stirred for 3 hrs at 60.0 °C .

After the PVA coating, the support was suspended in 50.0 mL dry DMF to which 3.0 g of glycidyltrimethylammonium chloride and 1.0 mL of triethylamine were added. The reaction was stirred at 60.0 °C for 12 hrs. Zirconia support thus treated was washed with DMF and methanol, and let dry in the air.

Synthesis of PEI stationary phase. Zirconia (5.0 g) was first reacted with γ-glycidoxypropyltrimethoxysilane as described above for the synthesis of PVAN stationary phase. The epoxy-activated zirconia was then suspended in 30.0 mL of DMF containing 1% partially quaternarized PEI hydroxyethylated, and the reaction mixture was stirred for

12 hrs at 120.0 °C in a round bottom flask. Thereafter, the support was first separated and washed with DMF, and then suspended in another portion of 50.0 mL DMF. To cross-link the quaternarized PEI hydroxyethylated coating on the zirconia surface, 3.0 mL of diepoxy PEG 200 and 1.0 mL of triethylamine were added, and the reaction was stirred at 80.0 °C for 12 hrs. After the reaction, the zirconia was washed successively with DMF, water and methanol, and was let dry in the air.

The quaternarized PEI hydroxyethylated was prepared according to previously described procedure [12]. Typically, 40 g of PEI hydroxyethylated (M.W. 60,000-80,000) were dissolved in 20.0 mL of DMF. To this solution, 20.0 mL of iodomethane were added, and the reaction mixture was stirred for a total of 120 hrs at room temperature. Three consecutive additions of iodomethane, 10.0 mL each, were made during the reaction. After 120 hrs, the quaternarized PEI hydroxyethylated was precipitated out with methanol, and the iodine formed during the reaction was washed away with methanol.

Column Packing

All columns used in this study were precision bore 316 stainless steel tubing from Alltech Associates (Deerfield, IL, U.S.A.), and having 30 x 4.6 mm I.D. as the dimensions. Column end fittings were also 316 stainless steel fitted with 0.5-μm stainless steel frits and distributor disks from Alltech Associates. The modified zirconia microspheres were packed from a 0.10 M NaCl slurry at 7000 psi with 0.10 M NaCl as packing solvent using a Shandon column packer instrument (Keystone Scientific, Bellefonte, PA, U.S.A.).

Results and Discussion

DEAE Stationary Phase

As shown in Figure 1, the DEAE-zirconia stationary phase behaved as a cation-exchange rather than an anion-exchange column permitting the separation of basic proteins such as ribonuclease B, α-chymotrypsinogen A and cytochrome c. As the pH was lowered from 8.0 to 6.0, myoglobin, which is a neutral protein, became slightly retained since its net charge changed to positive at pH 6.0. Unexpectedly, in the same pH range, acidic proteins, e.g., ovalbumin, α_1-acid glycoprotein and α-lactalbumin, were not retained by the DEAE-zirconia stationary phase, and eluted in the dead volume of the column. This may be an indication that the surface properties of the zirconia support are playing major roles in solute retention. Zirconia is known to contain zirconium ions on the particle surface which function as Lewis acid sites through which electron donor-electron acceptor (EDA) interaction can take place. Other possible sites for interaction (i.e., cation exchange sites) on the zirconia surface are the unreacted (or unmasked) hydroxyl groups of the zirconia support. While the zirconium Lewis acid sites and the unmasked hydroxyl groups are possible sites for the retention of basic proteins, the weaker affinity of acidic proteins for the DEAE-zirconia may be caused by the presence of a large number of ionized, unmasked hydroxyl groups which render the net charge of the modified surface to be negative.

In conclusion, the surface modification of zirconia with DEAE did not yield the desired stationary phase (i.e., a weak anion exchange column), and the contribution of the support proper to retention is still significant, for further discussion see section 3.4.

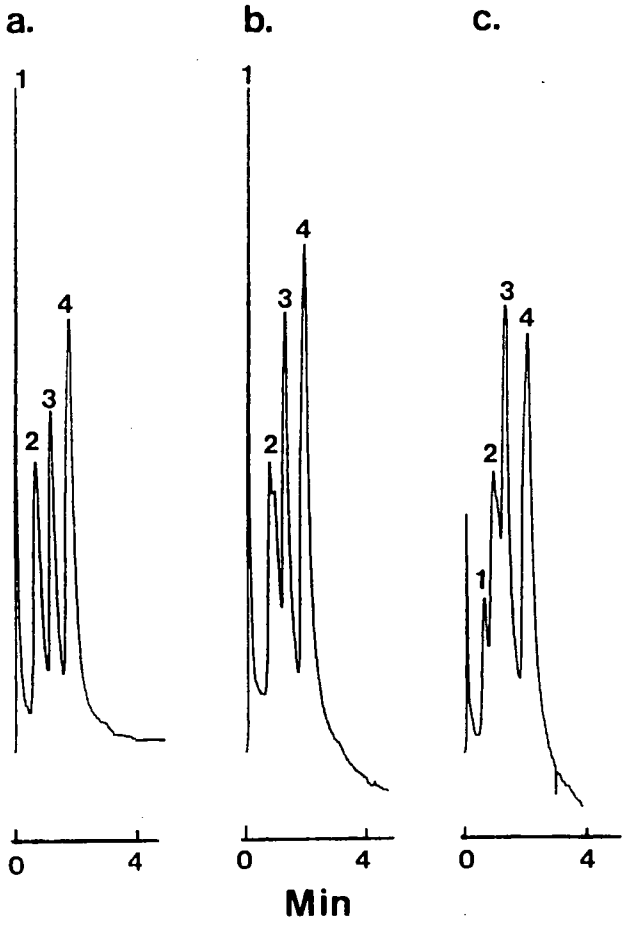

Figure 1. Chromatograms of proteins obtained on DEAE-zirconia stationary phase. Column, 30.0 x 4.6 mm; 5.0 min linear gradient from 10.0 mM to 500.0 mM sodium phosphate, pH 8.0 in (A), pH 7.0 in (B) and pH 6.0 in (C) ; flow rate, 1.0 mL/min. Samples: 1, myoglobin (pI = 6.8-7.3); 2, ribonuclease B (pI = 9.4); 3, α-chymotrypsinogen A (pI = 9.5); 4, cytochrome c (pI = 10.7). Detection, UV at 280 nm.

PVAN Stationary Phase

As described in Experimental, the PVAN stationary phase is characterized by a thicker hydrophilic coating which is expected to diminish the extent to which the metallic properties and the acidic character of the hydroxyl groups of the zirconia surface will affect the anion exchange behavior of the bonded stationary phase. Unlike the DEAE stationary phase, which acted as a purely cation exchange sorbent, the PVAN stationary phase retained some acidic proteins. This is shown in Figure 2, where 4 acidic proteins were separated on PVAN stationary phase, at pH 6.0. The column was able to distinguish the two forms of human transferrin, i.e., iron-saturated and iron-free. Iron-free transferrin is slightly more acidic than holo transferrin [13], and consequently was slightly more retained on the PVAN column.

Besides its anion exchange property, the PVAN column exhibited a mixed-mode type of behavior and allowed the separation of both acidic and basic proteins having pI values ranging from 5.1 to 11.0 as shown in Figure 3. The separation was performed at pH 8.0 using a linear gradient of increasing phosphate concentration in the eluent. As can be seen in Figure 3, the higher retention of basic proteins, i.e., α-chymotrypsinogen A, lysozyme and cytochrome c, indicates that the stationary phase still exhibit mixed-mode type of behavior (i.e., ion exchange and electron donor-electron acceptor properties) despite the use of polymeric hydrophilic coating. This behavior is also manifested by the fact that the elution pattern did not follow any order of pI values of the proteins. Nevertheless, the PVAN stationary phase was useful for the separation of acidic proteins having very close pI values, i.e., α-lactalbumin, ovalbumin and human albumin, as shown in Figure 4.

The rather complex retention mechanism exhibited by the PVAN stationary phase is portrayed in Figure 5 which shows plots of the adjusted retention volumes for a group of acidic, basic and neutral proteins versus pH of the eluent. The retention data of Figure 5

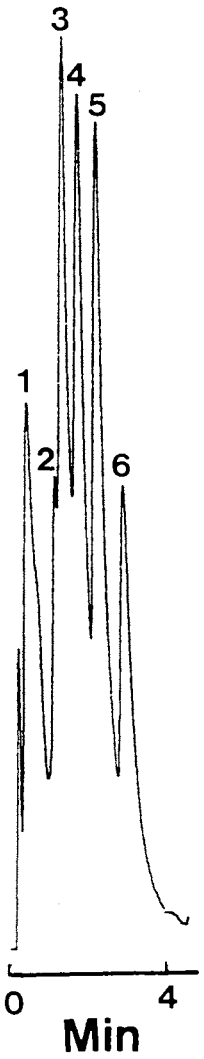

Figure 2. Chromatogram of proteins obtained on PVAN-zirconia stationary phase. Column, 30.0 x 4.6 mm; 5.0 min linear gradient from 5.0 mM to 500.0 mM sodium phosphate, pH 6.0; flow rate, 1.0 mL/min. Samples: 1, α-lactalbumin (pI = 4.8); 2, iron-saturated transferrin; 3, iron-free transferrin (pI = 5.2-6.1); 4 and 5, carboxypeptidase A (pI = 3.5-5.2); 6, carbonic anhydrase (pI = 6.2). Detection, UV at 280 nm.

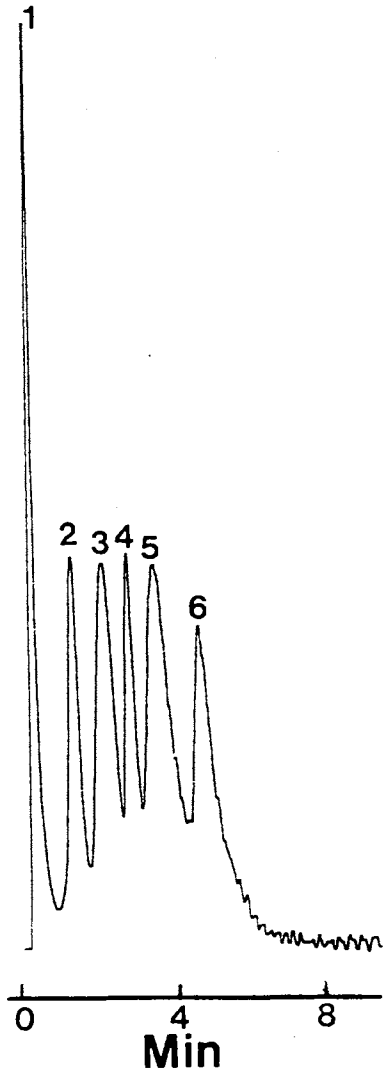

Figure 3. Chromatogram of proteins obtained on PVAN-zirconia stationary phase. Column, 30.0 x 4.6 mm; 10.0 min linear gradient from 19.8 mM to 401.0 mM sodium phosphate, pH 8.0; flow rate, 1.0 mL/min. Samples: 1, myoglobin; 2, α_1-acid glycoprotein; 3, β-lactoglobulin A (pI = 5.1); 4, α-chymotrypsinogen A; 5, lysozyme (pI =11.0); 6, cytochrome c. Detection, UV at 280 nm.

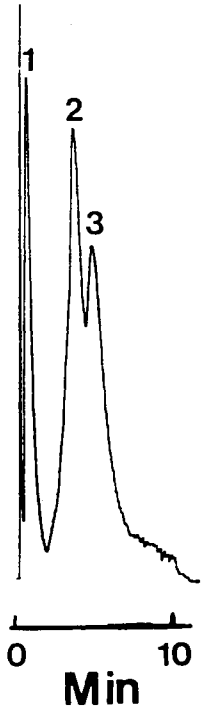

Figure 4. Chromatogram of proteins obtained on PVAN-zirconia stationary phase. Column, 30.0 x 4.6 mm; 10 min linear gradient from 5.0 mM to 104.0 mM sodium phosphate, pH 8.0; flow rate, 1.0 mL/min. Samples: 1, α-lactalbumin; 2, ovalbumin (pI = 4.7); 3, human serum albumin (pI = 4.7). Detection, UV at 280 nm.

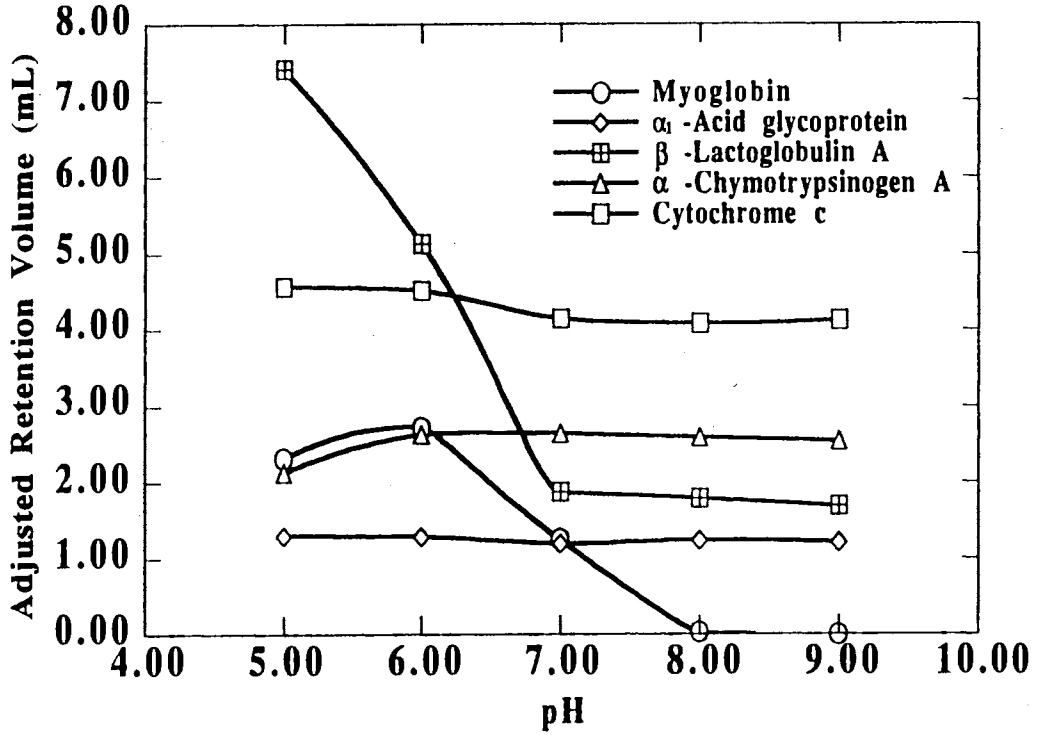

Figure 5. Plots of adjusted retention volume of proteins *versus* mobile phase pH. Column, 30.0 x 4.6 mm, PVAN-zirconia; 5.0 min linear gradient from 0 to 0.50 M NaCl in 5.0 mM sodium phosphate at various pH; flow rate: 1.0 mL/min. Samples: 1, cytochrome c; 2, α-chymotrypsinogen A; 3, β-lactoglobulin A; 4, α_1-acid glycoprotein; 5, myoglobin.

were obtained by gradient elution at increasing sodium chloride concentration in the eluent. This pH-retention map shows that the PVAN stationary phase exhibits retention toward acidic, neutral and basic proteins over a relatively wide range of pH. While the retention of strongly basic proteins (e.g., cytochrome c and α-chymotrypsinogen A) and acidic proteins (e.g., α_1-acid glycoprotein) stayed the same, that of neutral and weakly acidic protein such as myoglobin and β-lactoglobulin A, respectively, decreased with increasing pH. In a recent study from our laboratory on the retention behaviors of dansyl amino acids on octadecyl-zirconia stationary phases, most amino acids showed extensive EDA interaction with the zirconia support [3]. While neutral and acidic amino acids exhibited the highest EDA interaction with the zirconia support at pH 4.0, basic amino acids such as arginine and lysine showed EDA interaction over a wider range of pH. In the case of proteins, only the amino acid residues situated at the protein surface will undergo EDA interaction. The exposure of amino acid residues of a given protein to the solvent is pH and medium dependent, a condition that complicates the interpretation of pH-retention map of proteins even in the case where the retention is predominantly of one kind, e.g., ion-exchange.

To further gain insight into protein retention on zirconia-based stationary phases, the retention behaviors of α-chymotrypsinogen A, lysozyme and myoglobin were examined at various salt concentrations using isocratic elution. Sodium phosphate, sodium chloride and ammonium sulfate were selected as the eluting salts for these studies. The results are shown in Figure 6a, b and c in terms of logarithm of retention factor versus logarithm of salt molarity. In all cases the plots are linear, and the retention of the proteins decreased with increasing concentration of the eluting salts in the mobile phase. This behavior is similar to that observed with pure ion-exchange sorbents. Thus, the interactions of proteins with zirconia support can be attenuated by increasing the salt concentration in the eluent.

In ion exchange chromatography, the dependence of solute retention factor on the salt concentration in the eluent can be expressed as [14]

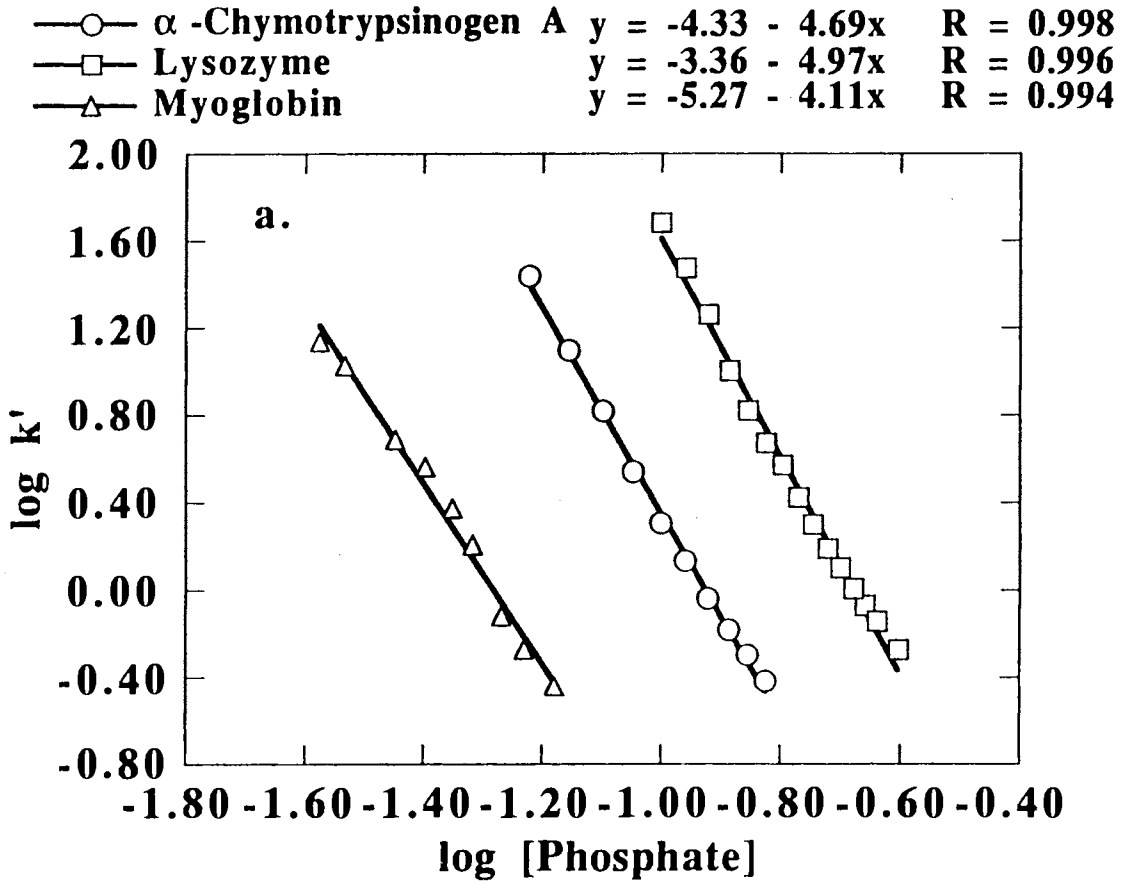

Figure 6. Plots of logarithmic retention factor of proteins *versus* logarithmic molar concentration of salt in the eluent. Column, 30.0 x 4.6 mm, PVAN-zirconia; mobile phases: (a), Na_2HPO_4 at various concentration, pH 6.0; (b), 5.0 mM sodium phosphate, pH 6.0, at various concentrations of NaCl; (c), 5.0 mM sodium phosphate, pH 6.0, at various concentrations of ammonium sulfate; isocratic elution at flow rates of 1.0 mL/min.

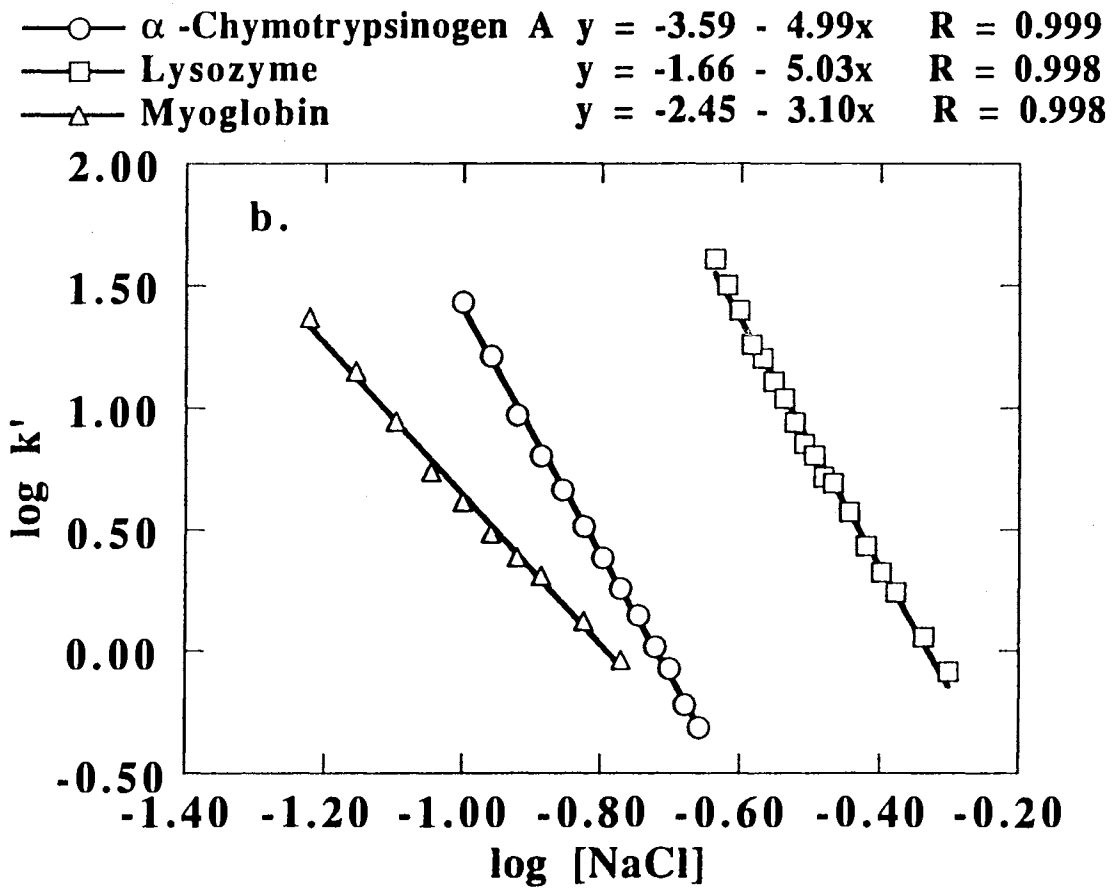

Figure 6, continued.

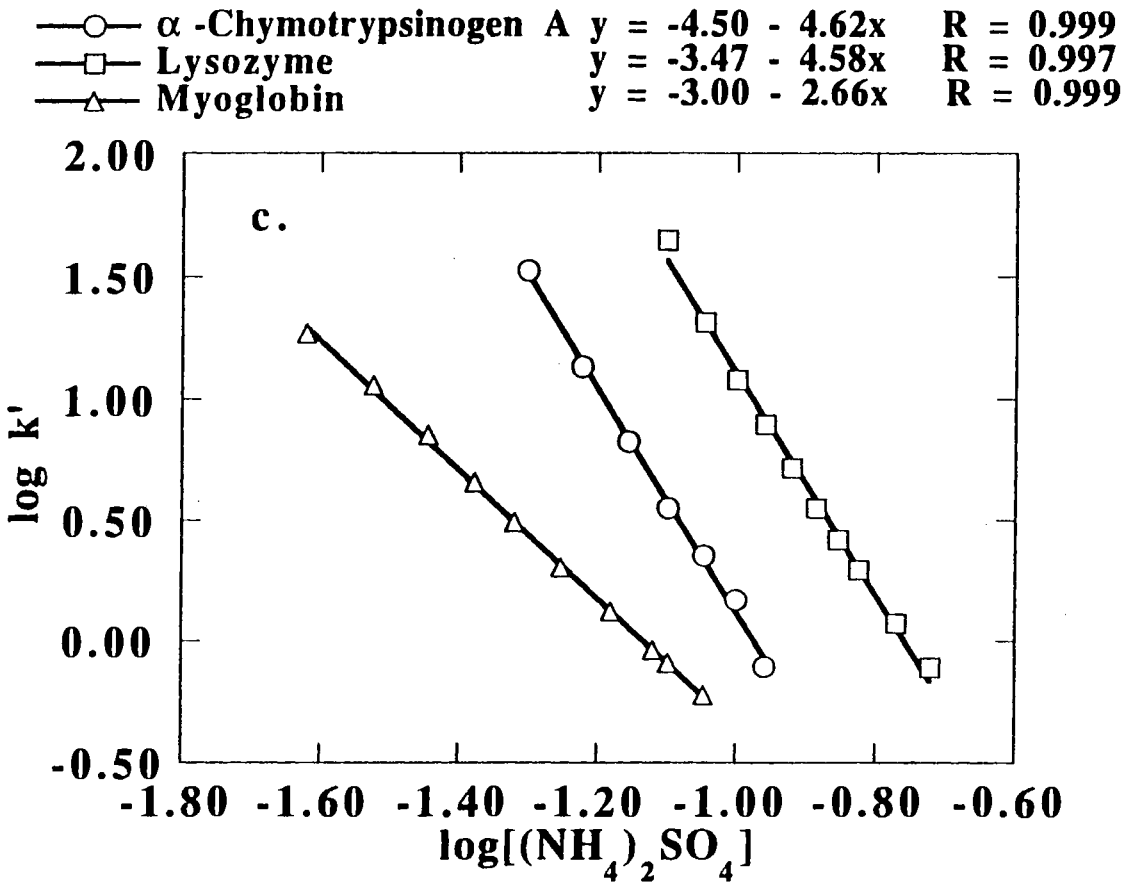

Figure 6, continued.

$$k' = (constant) f C_s^{-n} \qquad (1)$$

where f is the fraction of the molecules ionized, C_s is the concentration of eluting salt in the mobile phase and n is the ratio of the charge of the solute to that of the eluent ion. In the case of polyionic solutes with three-dimensional structures and asymmetrical charge distribution such as proteins, the value of n would be proportional to the number of accessible surface charges that interact with the sorbent. Equation 1 can be rewritten as

$$\log k' = A - n \log C_s \qquad (2)$$

where A is a constant. According to eqn 2 a straight line should be obtained when plotting *logk' vs. log* C_s. The eluting strength of the three salts studied was about the same for lysozyme. For α-chymotrypsinogen A, NaCl was the strongest eluting salt while for myoglobin, sodium phosphate ranked the strongest eluent. Since phosphate is the stronger eluent for myoglobin, this solute is likely to be retained principally by EDA mechanism, whereas the other two solutes are retained by both EDA and cation exchange *via* interaction with the zirconium sites and the hydroxyl groups, respectively.

PEI Stationary Phase

Retention behavior of proteins. The chromatographic properties of the PEI stationary phase were evaluated with various model proteins. As shown in Figure 7a and b, the PEI stationary phase revealed some of the microheterogeneity of ovalbumin and α_1-acid glycoprotein. Besides its various glycoforms, about 75% of ovalbumin possesses two phosphate groups per molecule while the remaining of the protein consists largely of one component having one phosphate group per molecule and a small amount of phosphate-free component [15,16]. Also, α_1-acid glycoprotein is known for its heterogeneity caused by

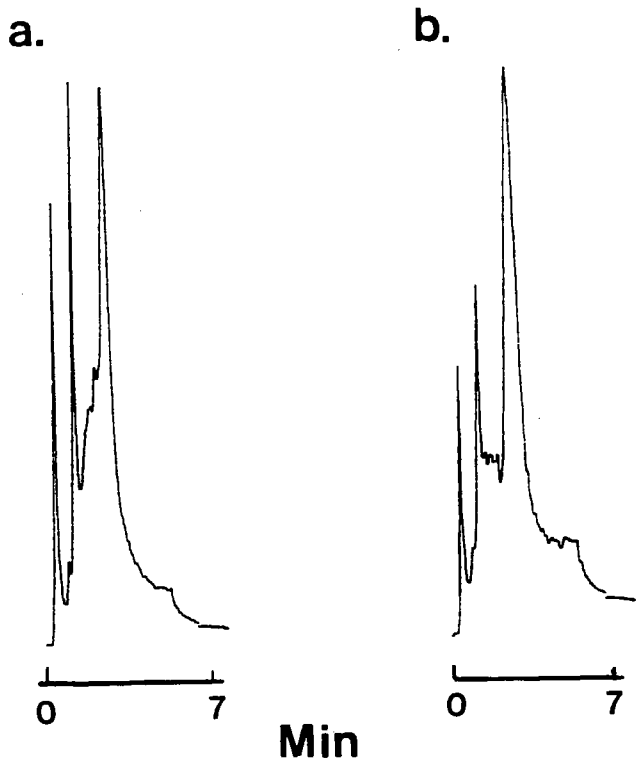

Figure 7. Chromatograms of proteins obtained on PEI-zirconia stationary phase. Column, 30.0 x 4.6 mm; 5.0 min linear gradient from 0.00 to 0.20 M Na_2SO_4 in 10.0 mM Tris, pH 8.0; flow rate, 1.0 mL/min. Samples: (a), ovalbumin; (b) α_1-acid glycoprotein. Detection, UV at 280 nm.

the oligosaccharide chains attached [17]. The variation in the terminal sialic acid of the oligosaccharide chains of α_1-acid glycoprotein is the major source of heterogeneity causing charge difference among protein molecules having the same polypeptide backbone.

PEI-zirconia stationary phases was evaluated with crude soybean trypsin inhibitor type II-S and soybean lipoxidase type I-S obtained from Sigma. As shown in Figure 8a and b, mobile phase pH had certain influence on the resolution of the various components of these two protein preparations. Some components in lipoxidase were better resolved at pH 6.0 than at pH 8.0. The same observation can be made for the resolution of trypsin inhibitor, type II-S, see Figure 8c and d. Trypsin inhibitor of the purified form (type I-S), however, was eluted as a single peak at both pH 6.0 and 8.0 on PEI-zirconia stationary phase (results are not shown). The higher resolution obtained at pH 6.0, may be attributed to the fact that the ionization of the amine functions of the partially quaternarized PEI is higher at low than at high pH.

Retention behavior of small solute probes. To gain further insight into the chromatographic properties of PEI stationary phases, the retention behavior of small acidic molecules such as UTP and some benzene carboxylic acids were examined at various pH and salt concentrations. Unlike proteins, these small solutes can get close to the surface of the support and interact with some sites that are not accessible for large molecules. Figure 9 shows plots of log k' of UTP versus logarithm of sodium chloride concentration at various pH. The linearity of these plots is typical of the behavior normally encountered in ion exchange chromatography. The pK_a values of the primary and secondary phosphate groups of UTP are 1.02 and 5.88, respectively. Thus, at pH 3.0 and 5.0 the UTP carries one charge whereas at pH 6.0 and above the UTP carries four negative charge (one primary phosphate and 3 secondary phosphate groups). As can be seen in Figure 9, the slope of log k' *vs* log [NaCl], which in principle should be equal to the ratio of the charge of the solute to the charge of the eluent ion [14,18], increased from -1.00 to -1.23 when going from pH 3.0 to pH 8.0. While at pH 3.0 and 5.0 the slopes are in agreement with the

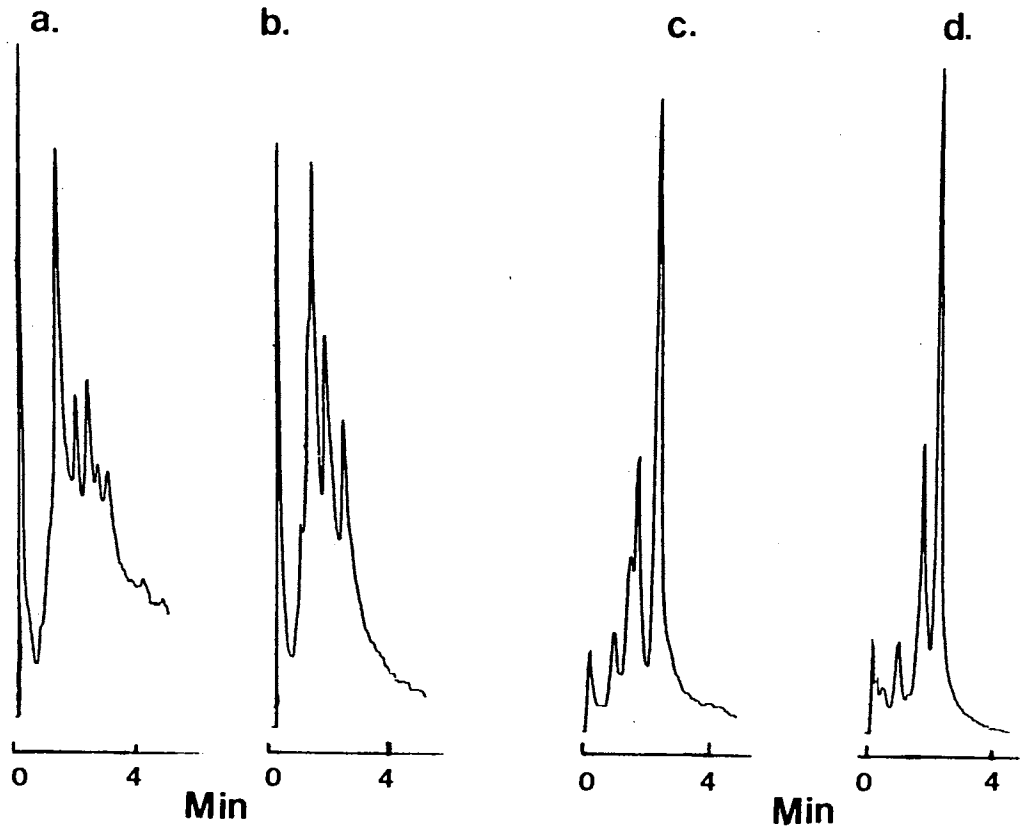

Figure 8. Chromatograms of lipoxidase in (a) and (b), and trypsin inhibitor type II-S in (c) and (d) obtained on PEI-zirconia stationary phase. Column, 30.0 x 4.6 mm; 5.0 min linear gradient from 5.0 to 500.0 mM sodium phosphate, pH 6.0 in (a) and (c) pH 8.0 in (b) and (d); flow rate, 1.0 mL/min. Detection, UV at 280 nm.

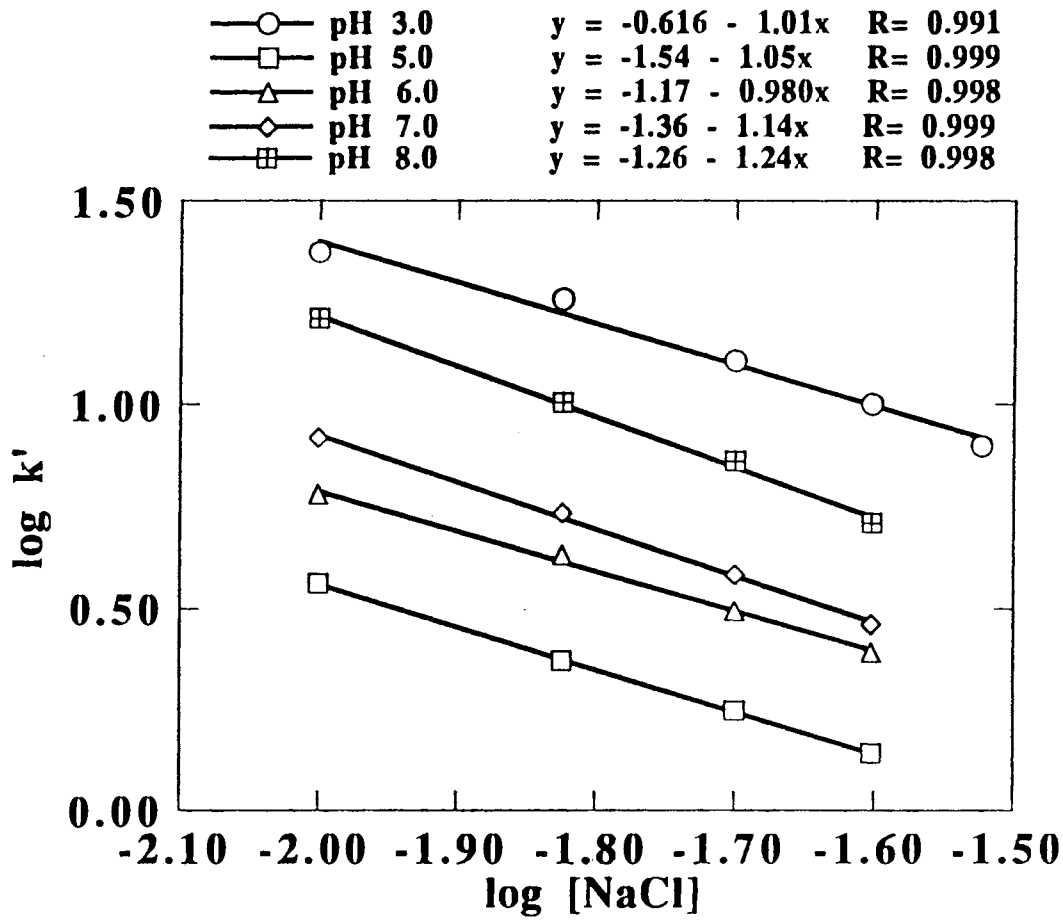

Figure 9. Plots of logarithmic retention factor of uridine-5'-phosphate *versus* logarithmic molar concentration of sodium chloride in the mobile phase. Column, 30.0 x 4.6 mm, PEI-zirconia; isocratic elution with 5.0 mM sodium phosphate at various pH and concentration of NaCl; flow rate: 1.0 mL/min.

values predicted from the theory, at pH equal or greater than 6.0 the values of the slopes are smaller than the predicted value, i.e., -4. The most likely cause for the deviation is the contribution of the zirconia surface to the overall retentive properties of the stationary phase. This would suggest that 5.0 mM phosphate in the mobile phase are not enough to completely eliminate the interaction of phosphate containing species such as UTP with the zirconia surface.

As with UTP, the retention behavior of benzenecarboxylic acids followed similar trends yielding linear plots for log k' *vs* log salt molarity at various pH. The results are summarized in Table I. Considering the ionization properties of benzenecarboxylic acids, at pH 3.0 only trimesic acid ($pK_1 = 2.12$) and pyromellitic acid ($pK_1 = 1.92$, $pK_2 = 2.87$) are singly and doubly negatively charged, respectively, and therefore are worth measuring their retention. As can be seen in this Table 1, at pH 3.0 the slopes of the lines are -1.29 for the singly charged trimesic acid and -1.80 for the doubly charged pyromellitic acid. These values more or less correspond to the ratio of charge of the solute to that of the eluent ion. At pH 5.0, the second carboxylic group of trimesic acid ($pK_2 = 4.10$) is almost fully ionized while half of the trimesic acid molecules have their third carboxylic group ionized ($pK_3 = 5.18$). For pyromellitic acid the third carboxylic group is almost fully ionized ($pK_3 = 4.49$) while the fourth carboxylic group is just slightly ionized ($pK_4 = 5.63$). At pH 5.0 the slope of the lines are -1.81 and -2.09 for trimesic and pyromellitic acid, respectively. For both acids, the values of the slopes of the lines are less than what can be predicted from the theory by almost one unit. At pH 6.0, benzoic acid ($pK = 4.2$) terephthalic ($pK_1 = 3.54$ and $pK_2 = 4.46$) and isophthalic acid ($pK_1 = 3.62$ and $pK_2 = 4.60$) are fully ionized as singly (benzoic acid) and doubly charged (isophthalic and terephthalic) species. On the other hand, trimesic and pyromellitic are triply and quadruply negatively charged, respectively. While the lines are quite linear (R values between 0.977 and 0.999), the values of the slopes are much less than predicted from the theory. This may be explained by some residual effects from the zirconia surface as well as from the fact that the PEI

Table I. Slope (s), y-intercept (y-int) and R values of plots of log k' of benzene carboxylic acids as a function of logarithmic NaCl concentration in the eluent. Column, 30.0 x 4.6 mm, PEI-zirconia; mobile phases, 5.0 mM sodium phosphate at various concentration of NaCl and pH; isocratic elution at a flow rate of 1.0 mL/min.

	pH 3.0			pH 5.0			pH 6.0			pH 7.0			pH 8.0		
	y-int	s	R	y-int	s	R	y-int	s	R	y-int	s	R	y-int	s	R
Benzoic acid	-	-	-	-	-	-	-0.89	-0.39	0.999	-0.45	-0.09	0.577	-0.24	0.09	0.834
Isophthalic acid	-	-	-	-	-	-	-0.67	-0.48	0.977	-0.31	-0.25	0.876	-0.33	-0.28	0.988
Terephthalic acid	-	-	-	-	-	-	-0.76	-0.47	0.993	-0.38	-0.21	0.954	-0.59	-0.35	0.899
Trimesic acid	-1.08	-1.29	0.997	-2.13	-1.81	0.999	-0.40	-0.79	0.994	0.04	-0.50	0.875	-0.09	-0.62	0.987
Pyromellitic acid	-1.09	-1.80	0.999	-2.43	-2.09	0.997	-0.58	-1.10	0.988	-0.39	-0.97	0.973	-0.19	-0.96	0.987

coating was only partially quaternarized, and therefore, the mobile phase pH had an effect on the protonation of the amine functional groups on the surface of the stationary phase.

As shown in Fig. 10a and b, UMP and UTP exhibited the highest retention at pH 3.0 where all types of amino groups at the surface of the PEI stationary phase are fully protonated. Both solutes showed decreased retention when mobile phase pH was increased to pH of around 6.0, which may be indicative of the deprotonation of primary, secondary and tertiary amino groups and ionization of the zirconia hydroxyl groups. In the pH range 7.0 to 8.0, retention increased again as the secondary phosphate groups become more and more ionized and the surface quaternary ammonium functions became the major sites for interaction. The increase in retention at pH above 7.0 may have arisen also from EDA secondary retention mechanism.

Comparison of DEAE, PVAN and PEI Stationary Phases

There are several major differences between PEI-, PVAN- and DEAE-zirconia stationary phases which originate primarily form the nature of the ligand and its covalent attachment to the zirconia surface. As described in the Experimental section, DEAE-zirconia stationary phase was formed through covalent bonding of γ-glycidoxypropyltrimethoxysilane to the zirconia surface, followed by reacting the epoxylated surface with diethylaminoethanol (DEAE). The DEAE coating contains tertiary amine functions which serve as weak anion exchangers. The PVAN- and PEI-zirconia stationary phases were obtained through coating epoxy activated zirconia particles with high molecular weight compounds. In PVAN the quaternary amine functions were incorporated into the coating by reacting the hydroxyl groups of the PVA layer with glycidyltrimethylammonium chloride, while in PEI the quaternary amine functions were produced by reacting PEI with iodomethane before coating the particles. From this brief description of the covalent bonding of the various zirconia sorbents, it is obvious that the

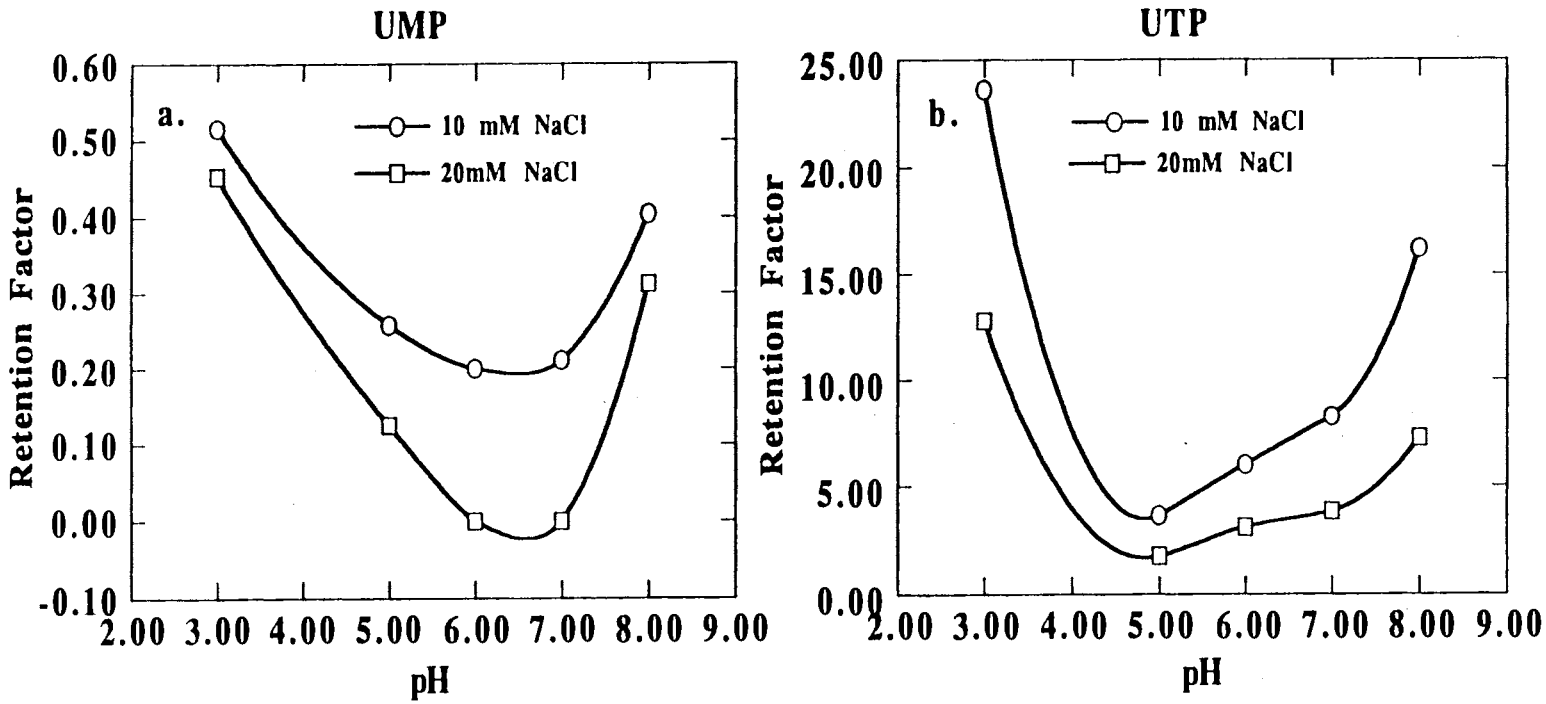

Figure 10. Plots of retention factor of UMP in (a) and UTP in (b) *versus* pH of the mobile phase. Column, 30.0 x 4.6 mm, PEI-zirconia; isocratic elution with 5.0 mM sodium phosphate, at various pH and concentration of NaCl; flow rate, 1.0 mL/min.

DEAE stationary phase would provide the lowest shielding of the zirconia surface toward the incoming solutes. But, as discussed above and will be seen below, the three types of stationary phases exhibited some degrees of solute-support interactions *via* unshielded zirconium sites or residual hydroxyl groups, and consequently, they possess different selectivity toward certain type of compounds.

Under isocratic elution conditions with a mobile phase containing 5.0 mM sodium phosphate and 5.0 mM NaCl, pH 7.0, UTP which was not retained on DEAE- and PVAN-zirconia columns yielded a k' of ca. 80 on the PEI-zirconia column. Because of the presence of phosphate ions in the eluent, the phosphate groups in UTP interacted mainly with the PEI stationary phase through ion exchange mechanism. The strong anion exchange interaction of UTP with PEI column is an indication of the presence of a larger population of anion exchange sites than on DEAE and PVAN. Using the same elution conditions as those with UTP, pyromellitic acid was not retained on PVAN-zirconia column but was slightly retained on DEAE-zirconia column (k' = 0.4), and could not be eluted from PEI-zirconia stationary phase. The slight retention of pyromellitic acid observed on DEAE may be attributed to chelating interaction with surface exposed zirconium sites. The PVAN stationary phase seems to have less exposed zirconium sites than DEAE may be due to the presence of the bulky PVA layer on the zirconia surface, which weakened the EDA interaction between solute and zirconia proper. However, the density of quaternary amine functions on PVAN seem to be relatively low for solute to be retained through ion exchange mechanism under the elution conditions used in the experiment.

To assess the extent of shielding of the Lewis acid sites (i.e., zirconium sites) and hydroxyl groups on the PVAN and PEI stationary phases toward large polyionic solutes, a group of fifteen proteins were chromatographed on both stationary phases using a 5.0 min linear gradient from 5.0 to 500.0 mM sodium phosphate at pH 6.0 and pH 8.0. The adjusted retention volumes are listed in Table II.

Table II. Adjusted retention volumes of proteins obtained on PVAN- and PEI-zirconia statioanry phases. Column, 30.0 x 4.6 mm; 5.0 min linear gradient from 5.0 to 500.0 mM sodium phosphate; flow rate, 1.0 mL/min.

Proteins	pI	PVAN		PEI	
		pH 6.0	pH 8.0	pH 6.0	pH 8.0
α_1-Acid glycoprotein		1.127	1.057	1.392	1.350
Trypsin inhibitor (type I-S)	4.5	ND	1.390	2.322	2.270
Ovalbumin	4.7	1.045	1.108	1.443	1.508
α-Lactalbumin	4.8	0.283	0.400	1.067	1.167
Transferrin (Iron sat'd)	5.2-6.1	0.895	0.908	1.145	1.048
Transferrin (Iron free)	5.2-6.1	1.077	0.908	1.145	1.153
β-Lactoglobulin A	5.1	2.967*	1.502	2.537	2.383
β-Lactoglobulin B	5.1	2.872*	1.457	2.208	2.150
Carbonic anhydrase	6.2	2.788	NR	1.932	NR
Myoglobin	6.8-7.3	1.177	NR	1.025	NR
α-Chymotrypsin	8.8	2.873	2.072	ND	1.842
Ribonuclease B	9.4	1.790	1.392	ND	1.115
α-Chymotrypsinogen A	9.5	2.288	1.958	ND	1.730
Cytochrome c	10.7	3.333	2.767	ND	2.232
Lysozyme	11.0	3.025	2.420	ND	1.768

* Broad elution peaks. ND, not determined. NR, not retained

Acidic proteins such as α_1-acid glycoprotein, trypsin inhibitor (type I-S), ovalbumin, α-lactalbumin, and transferrin exhibited higher retention on PEI than on PVAN at both pH 6.0 and pH 8.0. At pH 6.0, β-Lactoglobulin A and B had higher retention on PVAN than on PEI, but elution peaks were broad, probably due to the presence of dual retention mechanism. In general, proteins that have pI values higher than 6.0 showed higher retention on PVAN than on PEI stationary phase at both pH 6.0 and pH 8.0. These results may indicate a higher surface concentration in quaternary amine functions for the PEI stationary phase than for PVAN-zirconia column. The higher density of amine groups on the surface of the PEI stationary phase, would provide a better shielding of the zirconia surface toward the protein solute. Basic proteins which were positively charged at the pH

of the study, all exhibited higher retention on PVAN-zirconia stationary phase, a behavior that may indicate that the retention of these proteins was through EDA interaction with unshielded zirconium ions as well as by cation exchange *via* interaction with unreacted deprotonated hydroxyl groups.

Conclusions

The modification of zirconia surface with PEI proved to be the most effective approach to minimize the Lewis acidic metallic properties of the support. PEI-zirconia stationary phase operated as an anion exchanger toward proteins and other small acidic solutes. However, the support proper could still play a certain role in solute retention through EDA mechanism, which increased the selectivity and resolving power of the stationary phase. This is especially true when compared with DEAE- and PVAN-zirconia stationary phases. In fact, heterogeneous and crude proteins could be resolved on PEI stationary phase but not on either DEAE or PVAN stationary phases. The retention on DEAE was thought to be mainly due to EDA interaction and cation exchange between solute and zirconia support. On the other hand, both ion-exchange and EDA interaction seem to be important contributors to solute retention on PVAN stationary phase. In this latter case, the retention process is ascribed as through dual mechanism. The solute can exhibit higher retention time, but the elution peak is normally broad or asymmetrical. Mixed-mode chromatography best functions when one mechanism contributes predominantly to the retention while the presence of a secondary mechanism is to increase the selectivity and resolution of the stationary phase. This is the case of PEI-zirconia stationary phase.

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CHAPTER V

PREPARATION OF AMINO-ZIRCONIA BONDED PHASES AND THEIR EVALUATION IN HYDROPHILIC INTERACTION CHROMATOGRAPHY OF CARBOHYDRATES WITH PULSED AMPEROMETRIC DETECTION*

Abstract

A series of non-porous, microspherical zirconia-based stationary phases with surface bound amine functionality have been introduced and evaluated in hydrophilic interaction chromatography (HILIC) of underivatized, neutral carbohydrates and anion exchange chromatography of nucleotides using pulsed amperometric detection and ultraviolet detection, respectively. Three aminopropyl alkoxysilane compounds were used in the surface modification of the non-porous zirconia support, namely 3-aminopropyltrimethoxysilane (monoamine), N-(2-aminoethyl)-3-aminopropyltrimethoxysilane (diamine) and trimethoxysilylpropyldiethylenetriamine (triamine). Due to the relatively low specific surface area of the non-porous zirconia support used in this study (ca. 7.3 m^2/g), zirconia with surface coating of the triamine type yielded the best results as far as the separations of chitooligosaccharides and maltooligosaccharides are concerned. Since a non-porous zirconia could be readily modified with amine functionality via Zr-O-Si bonds, it is expected that all the three aminopropyl alkoxysilane compounds would yield satisfactory results with porous zirconia

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microparticles because of their much higher specific surface areas. Although, the non-porous zirconia exhibited some limitations, the present study has demonstrated that microspherical zirconia particles are suitable support for the production of polar sorbents for HILIC of carbohydrates. Another surface modification, which involved the activation of the zirconia surface with aldehyde groups followed by reductive amination with tetraethylenepentamine, was also evaluated. Although this chemistry would in principle yield sorbents with higher concentration in amine groups, the conversion of the majority of the primary amine groups of the tetraethylenepetamine molecules to secondary amine functions in the course of the reductive amination reaction have provided a stationary phase that did not afford satisfactory resolution for carbohydrates. However, this same stationary phase behaved as a weak anion exchanger and allowed the high resolution separation of nucleoside-5'-mono-, -di- and triphosphates. Overall, the results obtained with zirconia-based hydrophilic sorbents paralleled those obtained on amino-silica bonded phases.

Introduction

Amino-bonded stationary phases are polar sorbents usually employed with less polar mobile phases (e.g., acetonitrile rich eluents) in the separations of polar species including carbohydrates [1, 2]. Historically, this mode of chromatography is referred to as normal phase chromatography. To emphasize the polar nature of intermolecular forces governing retention with such stationary phases, the name hydrophilic interaction chromatography has been proposed recently [3]. Since the abbreviation HIC is now used exclusively for hydrophobic interaction chromatography, the acronym HILIC has been suggested by Alpert [3] to designate hydrophilic interaction chromatography, and it is increasingly used in recent literature [4-8]. Besides amino-bonded phases [9-12], HILIC can be carried out with a wide spectrum of polar sorbents including bare silica [13], diol [14,15] and polyol bonded phases [16,17], cyclodextrin bonded phases [18,19] and more

recently polymer coated silica with a layer of polysuccinimide in which ethanolamine functions were incorporated [3].

In HILIC, the intermolecular forces governing retention encompass hydrogen bonding and dipole-dipole interactions. With polar bonded phases, e.g., amino-bonded silica packings, the chromatographic separation is based mainly on differential partitioning of the solutes between the mobile phase and the water-enriched solvent layer adsorbed onto the surface of the column packing material [1].

Thus far, HILIC of carbohydrates has been primarily carried out on silica-based hydrophilic (polar) sorbents, although polymer based packings for HILIC, e.g., amino-bonded vinyl alcohol copolymer gel and macroporous vinylpyridinium polymer gels in the phosphate or sulfate forms, have been introduced [20-23].

While silica-based polar stationary phases are mechanically strong for use in HPLC, polymer-based hydrophilic sorbents, which are more chemically stable than siliceous stationary phases under elution conditions normally used in HILIC, are inherently characterized by relatively low mechanical strength. To overcome these drawbacks encountered with silica- and polymer-based sorbents, zirconia-based stationary phases, which combines the mechanical strength of silica to the chemical stability of polymer supports, have been recently introduced for use in HPLC; for recent review, see Ref. 24.

Thus far, the most widely used zirconia-based stationary phases in HPLC have been of the non-polar types for reversed phase chromatography [25-28], and to a lesser extent of the impregnated types with inorganic ions such as fluoride [29] or phosphate ions [30] for use in protein HPLC. Very recently, other zirconia sorbents have been described including (i) surface modified zirconia with immobilized concanavalin A or iminodiacetic acid [31] for lectin affinity and metal interaction chromatography, respectively, (ii) polyphosphate-modified zirconia for HPLC of proteins and nucleic acids [32] and (iii) zirconia-based weak, strong and hybrid ion-exchangers for HPLC of proteins [33].

This study, is a continuation to our previous efforts [26,27,33] aimed at introducing novel zirconia-based stationary phases for HPLC of a wide variety of chemical and biochemical species. To the best of our knowledge, there has been no attempt to introduce well structured polar zirconia sorbent for HPLC of underivatized carbohydrates. This report describes the preparation of non-porous, microspherical zirconia particles the surface of which have been modified with various aminopropyl alkoxysilane compounds and polyfunctional amine compounds to form amino-zirconia bonded stationary phases. The synthetic procedures of zirconia-based amine stationary phases as well as the evaluation of their chromatographic properties in the separations of oligosaccharides with pulsed amperometric detection are discussed.

Experimental

Instrumentation

The liquid chromatograph consisted of the following components: (i) an LDC Analytical (Riviera Beach, FL, U.S.A.) ConstaMetric 3500 solvent delivery system with a gradient programmer, which was used to control a ConstaMetric Model III solvent delivery pump, (ii) a sample injector Model 7125 from Rheodyne (Cotati, CA, U.S.A.), (iii) a UV interference filter photometric detector Model UV-106 from Linear Instruments (Reno, NV, USA), and (iv) a pulsed amperometric detector (PAD) with gold working electrode from Dionex (Sunnyvale, CA, U.S.A.). The PAD settings for pulse potentials and duration were E_1 = 0.10 V, t_1 = 540 msec; E_2 = 1.00 V, t_2 = 120 msec; and E_3 = - 0.80 V, t_3 = 300 msec. Chromatograms were recorded with a computing integrator Model C- R6A equipped with a floppy disk drive and a cathode-ray tube (CRT) monitor from Shimadzu (Columbia, MD, U.S.A.).

For the PAD of sugar solutes, 0.50 M NaOH (pH 13.7) was added to the column effluent at a flow rate of 0.5-0.6 mL/min. This post-column addition of sodium hydroxide

was accomplished through a T-connector that was connected at one end to the column outlet and at another end to a vessel containing sodium hydroxide under nitrogen gas pressure. The mixing between NaOH and column effluent occurred in a polyethylene tubing reactor (150 cm x 0.45 mm I.D.) filled with glass beads, which was placed between the T-connector at the column outlet and the PAD detector sample cell.

Chemicals

HPLC grade acetonitrile was obtained from Baxter Diagnostics Inc. (McGraw Park, IL, U.S.A.). Reagent grade ammonium acetate, hydrochloric acid, acetic acid, sodium phosphate dibasic, sodium iodate, sodium cyanoborohydride and sodium borohydride, and reagent grade as well as technical grade isopropanol, methanol, and *N*, *N*-dimethylformamide (DMF) were from Fisher (Pittsburgh, PA, U.S.A.). D-(+)-Glucose, boron trifluoride etherate and tetraethylenepentamine were purchased from Aldrich (Milwaukee, WI, U.S.A.). *N*-(2-Aminoethyl)-3-aminopropyl-trimethoxysilane, γ-glycidoxypropyltrimethoxysilane, 3-aminopropyltrimethoxysilane and trimethoxysilylpropyldiethylenetriamine were obtained from Hüls America Inc. (Bristol, PA, U.S.A.). *N*-Acetyl-D-glucosamine, adenosine-5'-mono-, -di- and -tri-phosphate (AMP, ADP and ATP, respectively) guanosine-5'-mono-, -di- and -tri-phosphate (GMP, GDP and GTP, respectively), cytidine-5'-mono-, -di- and -tri-phosphate (CMP, CDP and CTP, respectively) and uridine-5'-mono-, -di-, and -tri-phosphate (UMP, UDP and UTP, respectively), inosine-5'-monophosphate (IMP) and maltooligosaccharides were from Sigma (St. Louis, MO, U.S.A.). Chitooligosaccharides were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Zorbax silica having 150Å mean pore size and 4.6 μm mean particle diameter was from DuPont de Neurours (Willmington, DE, U.S.A.). Triglycidoxyglycerol and poly(ethylene glycol 200 diglycidyl ether) were obtained from Polysciences, Inc. (Warrington, PA, U.S.A.).

Surface Modification and Column Packing

Modification with aminopropyl alkoxysilane compounds. Non-porous microspherical zirconia and silica particles were synthesized following the same procedures described earlier [26,27]. Surface modification with aminopropyl alkoxysilane compounds was carried out in aqueous solution. Typically, 5.0 g of zirconia were suspended in the aqueous medium containing 0.10 M sodium acetate, pH 4.0, and the suspension was heated to 98.0 °C. To this suspension, 3.0 mL of 3-aminopropyltrimethoxysilane, *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane or trimethoxysilylpropyldiethylenetriamine were added, and three separated surface modifications were carried out. In the case of silica, 5.0 g were treated with 3-aminopropyltrimethoxysilane using the same conditions outlined for zirconia particles. In all cases, the reaction solution was stirred at 98.0 °C for 12 hrs. The amino-bonded phases thus obtained were separated from the solution by centrifugation. Technical grade methanol was used to wash the product from the unreacted reagent. The modified zirconia or silica microspheres were let dry in the air.

Modification with tetraethylenepentamine. Typically, 5.0 g of zirconia were added to 50.0 mL of DMF, and the suspension was heated to 120.0 °C. γ-Glycidoxypropyltrimethoxysilane and distilled water in the amount of 3.0 mL and 1.0 mL, respectively, were added to the suspension. The reaction was stirred at 120 °C for 12 hrs. After the reaction, the zirconia was separated by centrifugation and washed with DMF. Thereafter, the support was suspended in 50.0 mL DMF to which 3.0 mL of poly(ethylene glycol 200 diglycidyl ether) were added. Boron trifluoride etherate in the amount of 500 μL was used as the catalyst. After the reaction was stirred at room temperature for 4 hrs, 2.0 mL of triglycidoxyglycerol were added to the reaction mixture, and the stirring continued for an additional 2 hrs at room temperature. The zirconia thus treated was

washed successively with DMF and water, and then was suspended in 50.0 mL of 0.01 M aqueous HCl solution. The suspension was stirred at 90 °C for 1 hr. Thereafter, the support was washed with water to neutral, and treated with 0.5 g of sodium periodate in a volume ratio of 9:1 of acetic acid:water solution. The oxidation was carried out for 1 hr at room temperature. Thereafter, the aldehyde activated zirconia was suspended in 50.0 mL of 0.10 M phosphate, pH 6.8, containing 0.20 g sodium cyanoborohydride and 3.0 mL of tetraethylenepentamine, and the reaction was stirred at 70 °C for 12 hrs. Finally, the support thus modified was washed and suspended in isopropanol containing 0.20 g of sodium borohydride to reduce the aldehyde functional groups that remained. After this modification process, the zirconia support was washed with water and methanol, and then was let dry in the air.

Column packing. All columns used in this study were precision bore 316 stainless steel tubing from Alltech Associate (Deerfield, IL, U.S.A.), having 50.0 x 4.6 mm I.D. as the dimensions. Column end fittings were also 316 stainless steel fitted with 0.5-μm stainless steel frits and distributor disks from Alltech Associates.

Columns were packed using the slurry method with a Shandon column packer instrument (Keystone Scientific, Bellefonte, PA, U.S.A.). For the various amino-bonded stationary phases, a solvent mixture of isopropanol and water of 1:4 (v/v), was found to be suitable for making a stable well-dispersed suspension. Since zirconia particles have a density of 5.8 g/cm^3 [34] which is almost three times higher than that of silica, 1.5 g of surface modified zirconia microspheres were needed while 0.5 g of amino-bonded silica packings were sufficient to pack a column of 50.0 x 4.6 mm.

Results and Discussion

Evaluation of Stationary Phases Formed by Aminopropyl Alkoxysilane Compounds

As described in Experimental, three different types of aminopropyl alkoxysilane compounds were covalently attached to the zirconia surface. This surface modification was best achieved when the reaction was carried out in aqueous solution using 0.10 M sodium acetate at pH 4.0. When the reaction was performed in anhydrous DMF, the resulting stationary phases did not show the desired retention properties for carbohydrates, e.g., maltooligosaccharides. This behavior may have been caused by the surface property of zirconia support, which is known to contain Lewis acid, i.e., zirconium sites. When an aminopropyl alkoxysilane compound is added to the reaction solution, the amino group of the silanizing agent may play the role of Lewis base. The result of the Lewis interaction is the inverted orientation of the silane compounds with the amino groups toward the support surface, which does not favor the formation of amino-zirconia bonded phases with high surface coverage. On the other hand, the presence of sodium acetate at pH 4.0 would diminish the extent of adsorption of aminopropyl alkoxysilane compounds *via* their amino groups since carboxylic acid containing compounds (competing Lewis bases) have been shown to decrease metallic interactions of basic and acidic solutes with zirconium sites (Lewis acids) [26, 27, 35].

To assess the extent to which the zirconium sites and other surface functionality of the zirconia support would contribute to carbohydrate retention, bare zirconia, which was calcinated at 800 °C for 3 hrs, without any surface modification was packed into a 3.0 cm column. Maltooligosaccharides and chitooligosaccharides were each injected into the column individually using water as the mobile phase. No retention was observed and symmetrical peaks were obtained for up to maltoheptaose and *N*-acetyl-D-chitohexaose.

This may indicate that there is no interaction between the solutes and the column support matrix.

Based on the above observations, amino-zirconia bonded stationary phases were all prepared by reacting aminopropyl alkoxysilanes with zirconia using aqueous solutions containing 0.10 M sodium acetate, pH 4.0, as the reaction medium. Zirconia bonded stationary phases of the monoamine type obtained by reacting the support with 3-aminopropyltrimethoxysilane did not show enough retention for the sugar probes tested. The non-porous zirconia support having relatively low specific surface area (ca. 7.3 m^2/g) [26] did not allow the formation of stationary phases with adequate phase ratios. In other words, since the specific surface area of the non-porous support is low, the surface concentration of amino functional groups will also be low. Therefore, the monofunctional amino-zirconia phases were not considered further. For comparison, non-porous silica of mean particle diameter of 1.1 μm, which was prepared as described previously [27], was also coated with 3-aminopropyltrimethoxysilane. Similar to non-porous zirconia bonded stationary phase of the monoamine type, the non-porous amino-bonded silica packings did not exhibit enough retention toward homooligosaccharides, e.g., maltooligosaccharides.

To produce amino-zirconia bonded phases with higher phase ratios in amino functionality, zirconia modified with ethylenediamine functional groups was prepared as described in Experimental. These bonded phases exhibited some retentivity toward the *N*-acetyl-D-chitooligosaccharides and maltooligosaccharides, although the resolution was still not high enough for the complete resolution of the oligomers having one unit difference, see Figure 1a and b. Generally, maltooligosaccharides showed better separation than chitooligosaccharides. This may be due to the presence of *N*-acetyl groups in the chitooligosaccharides (i.e., less hydroxyl groups per molecule) which may have lowered the hydrophilic interaction between the solute and stationary phase.

With ethylenediamino-zirconia bonded phases and maltooligomer solutes, the addition of 20 mM ammonium acetate to the water-acetonitrile solvent system increased the

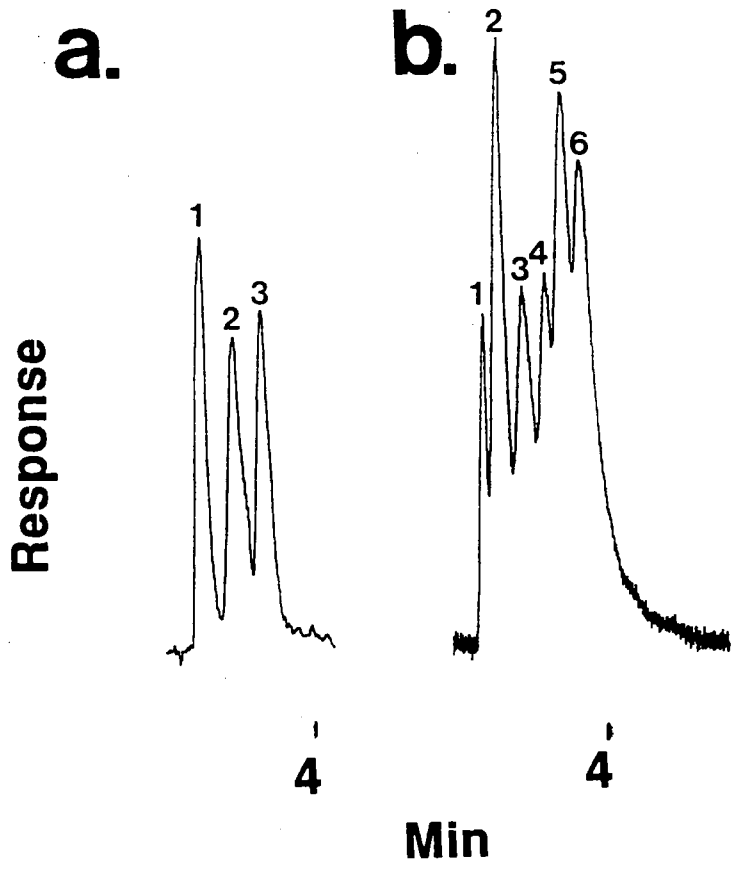

Figure 1. Chromatograms of *N*-acetyl-D-chitooligosaccharides in (a) and maltooligosaccharides in (b) obtained on diamine-zirconia stationary phase. Column, 50.0 x 4.6 mm; linear gradients in 5.0 min from 13.0% to 70.0% (v/v) H_2O (a) and from 16.0% to 70.0% (v/v) H_2O (b) in acetonitrile; flow rate, 1.0 mL/min. Samples, in (a): 1 = *N*-acetyl-D-chitobiose, 2 = *N*-acetyl-D-chitotetraose, 3 = *N*-acetyl-D-chitohexaose; in (b): 1 = glucose, 2 = maltose, 3 = maltotriose, 4 = maltotetraose, 5 = maltopentaose, 6 = maltohexaose.

solute-stationary phase hydrophilic interaction as was manifested by an increased retention of the sugar solutes, a condition that favored an increase in resolution.

Stationary phases formed by reacting zirconia microspheres with trimethoxy-silylpropyldiethylenetriamine having one more amino functional group yielded increased retention and resolution for both homooligosaccharides as compared to those formed by *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane. Better resolution was observed for oligomers of smaller number of repeating units (i.e., up to a degree of polymerization, d.p. = 5). When d.p. reached six, the resolution started to decrease, see chromatograms in Figure 2a and b. This is an indication that the effective interaction between the solute and the stationary phase decreased as the size of the oligomer increased, which is the limitation of non-porous supports.

The hydrophilic properties of zirconia-based stationary phases with diethylenetriamine functions were evaluated using maltooligosaccharide homologous series. Due to the low retention of glucose, maltose and maltotriose only maltotetraose, maltopentaose, maltohexaose and maltoheptaose were injected into the column using mobile phases at different proportions of water:acetonitrile. As can be seen in Figure 3, the logarithmic retention factor is linearly related to the number of repeating units (i.e., d.p.) in the homooligomers as follows

$$logk' = (log\alpha)n + logk_p'$$

where α is the selectivity factor of two neighboring homologous solutes, $log\ \alpha$ is the slope (hydrophilic selectivity) which characterizes nonspecific interactions, n is the number of repeating units (number of glucose residues in the present case), k_p' is the retention factor of the parent molecule (hydroxyl group in the present case) and $log\,k_p'$ is the intercept reflecting the specific interactions between the parent molecule and the mobile and stationary phase. The above equation implies a constant contribution to the free energy of

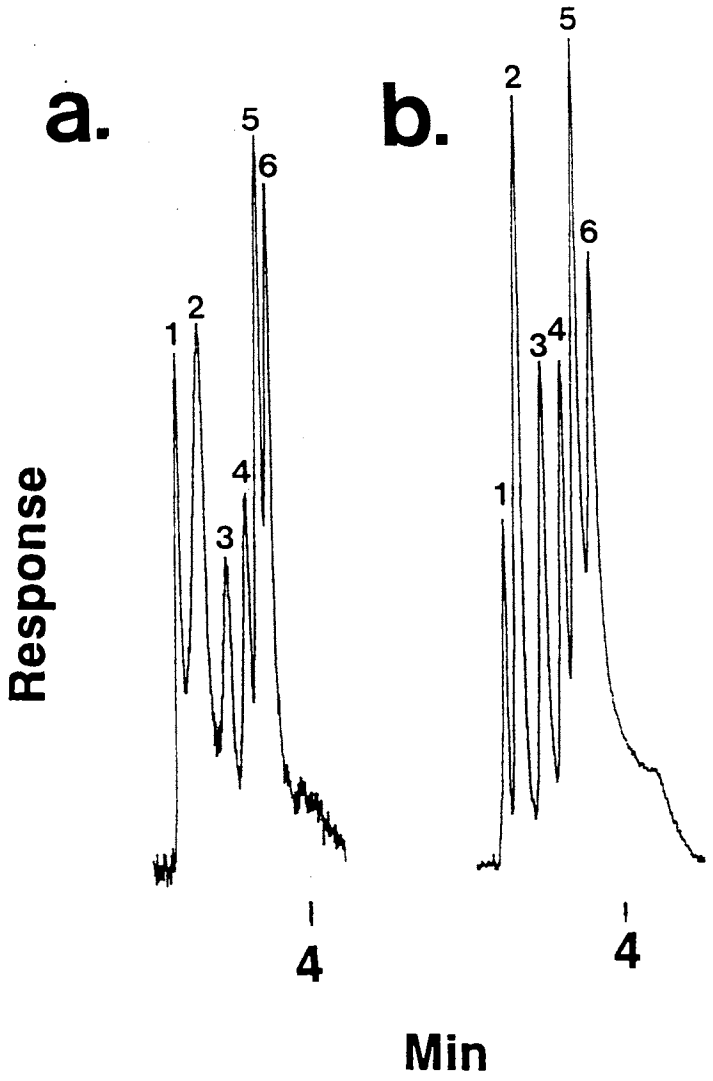

Figure 2. Chromatograms of *N*-acetyl-D-chitooligosaccharides in (a) and maltooligosaccharides in (b) obtained on triamine-zirconia stationary phase. Column, 50.0 x 4.6 mm; linear gradients in 5.0 min from 10.0% to 60.0% (v/v) H_2O in acetonitrile in (a) and from 15.0% to 60.0% (v/v) H_2O in acetonitrile; flow rate, 1.0 mL/min. Samples, in (a): 1 = *N*-acetyl-D-glucosamine, 2 = *N*-acetyl-D-chitobiose, 3 = *N*-acetyl-D-chitotriose, 4 = *N*-acetyl-D-chitotetraose, 5 = *N*-acetyl-D-chitopentaose, 6 = *N*-acetyl-D-chitohexaose; in (b): 1 = glucose, 2 = maltose, 3 = maltotriose, 4 = maltotetraose, 5 = maltopentaose, 6 = maltohexaose.

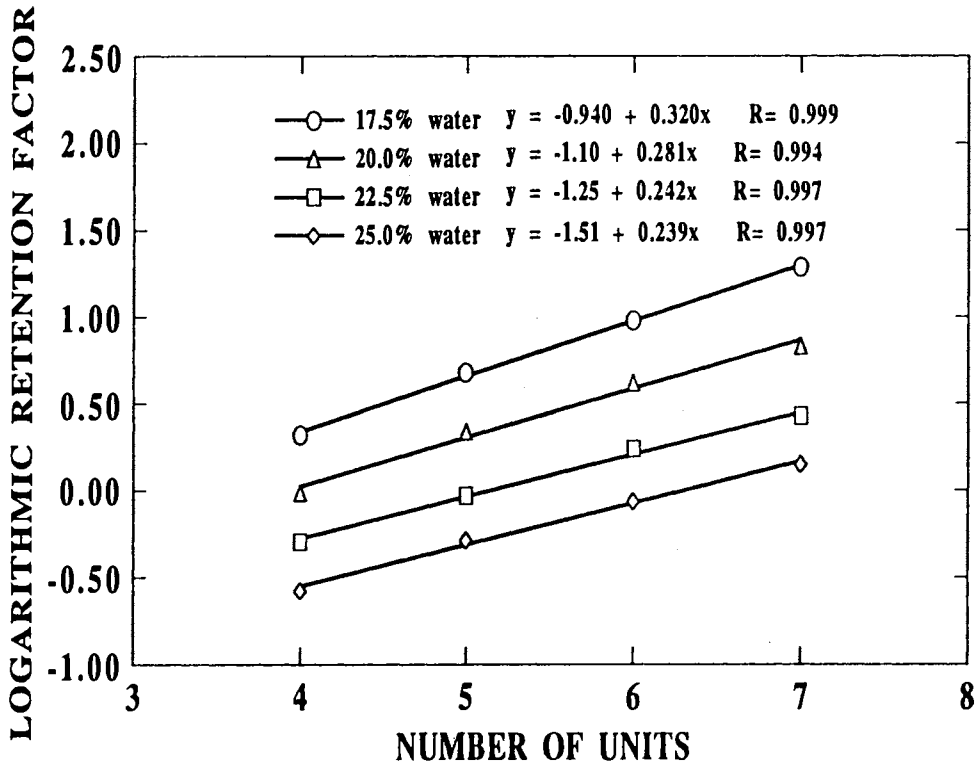

Figure 3. Plots of logarithmic retention factor of maltooligosaccharide homologues *versus* the number of repeating glucose units at various volume percent water concentration in acetonitrile. Column, 50.0 x 4.6 mm, triamine-zirconia; flow rate, 1.0 mL/min; isocratic elution.

solute transfer between mobile phase and stationary phase with each repeating unit (e.g., glucose residue) in the homologues [36].

Returning to Figure 3, the linear relationship between logarithmic retention factor of the homologous series and the number of repeating units may indicate that the chromatographic process is based on partitioning via hydrophilic interactions between the mobile phase and the water-rich diethylenetriamine stationary phase. In fact, as the water content of the mobile phase was increased, the values of the slopes of the lines (see Figure 3) decreased. The linear relationship between *log k' and n* (or d.p.) was also observed on amino-bonded silica column [37,38].

To further characterize the retention properties of diethylenetriamine stationary phase, a group of four maltooligosaccharides was chromatographed isocratically with mobile phases containing various percentage of water. Quasi-linear relationship was obtained between the logarithmic retention factor and % water (v/v) in the mobile phase for all the four solutes studied, see Figure 4. As expected, the slope of the lines increased with increasing the size of the molecule (i.e., increasing hydrophilicity) when going from maltotetraose to maltoheptaose by a factor of 1.23. This is another indication of the hydrophilic interaction properties of zirconia-based diethylenetriamine stationary phases. The same behavior was also observed on amino-bonded porous silica packings (see Experimental) of 150Å mean pore diameter and 4.6 μm particle diameter. As can be seen in Figure 5, plots of logarithmic retention factor decreased quasi-linearly with increasing % water (v/v) in the eluent. The slope increased by a factor of 1.62 and 1.47 when going from *N*-acetylglucosamine to chitobiose and from glucose to maltose, respectively. Straight lines were also obtained for galactose and xylose when *log k'* was plotted versus % water. The slope for galactose was 0.0300 and that for xylose was 0.0220, with an R value of 0.996 and 1.00, respectively. The higher slope obtained with galactose can be explained by the fact that galactose has one more hydroxyl group than xylose. Again, this observation may indicate that retention on amino-bonded phases is based on hydrophilic interaction

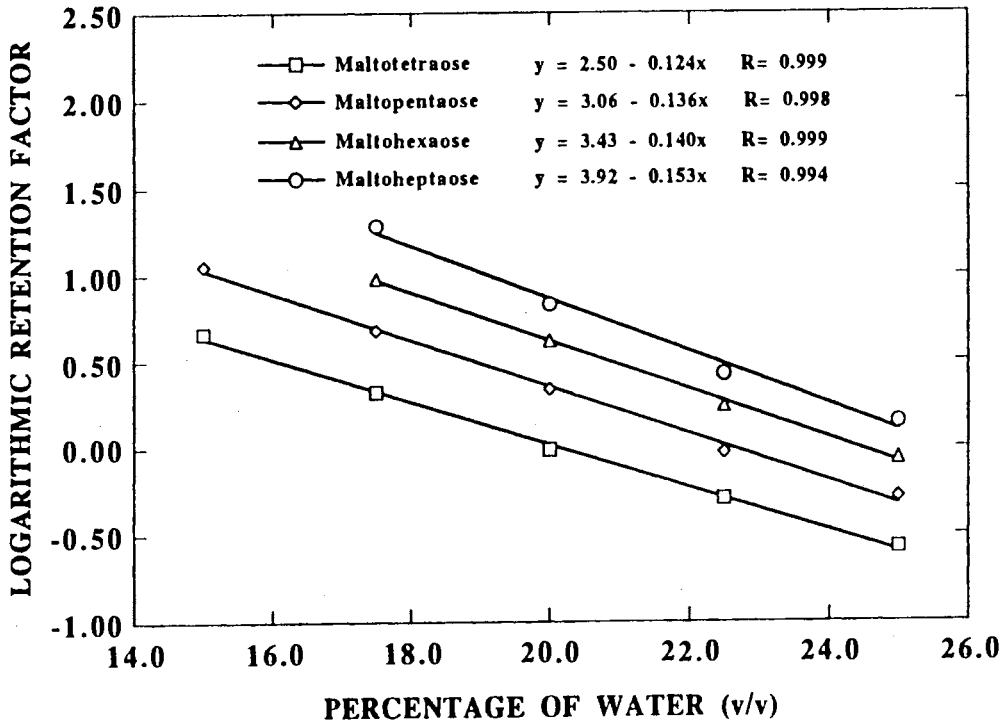

Figure 4. Plots of logarithmic retention factor of maltooligosaccharide homologues *versus* volume percent of water concentration in acetonitrile. Column, 50.0 x 4.6 mm, triamine-zirconia; flow rate: 1.0 mL/min; isocratic elution.

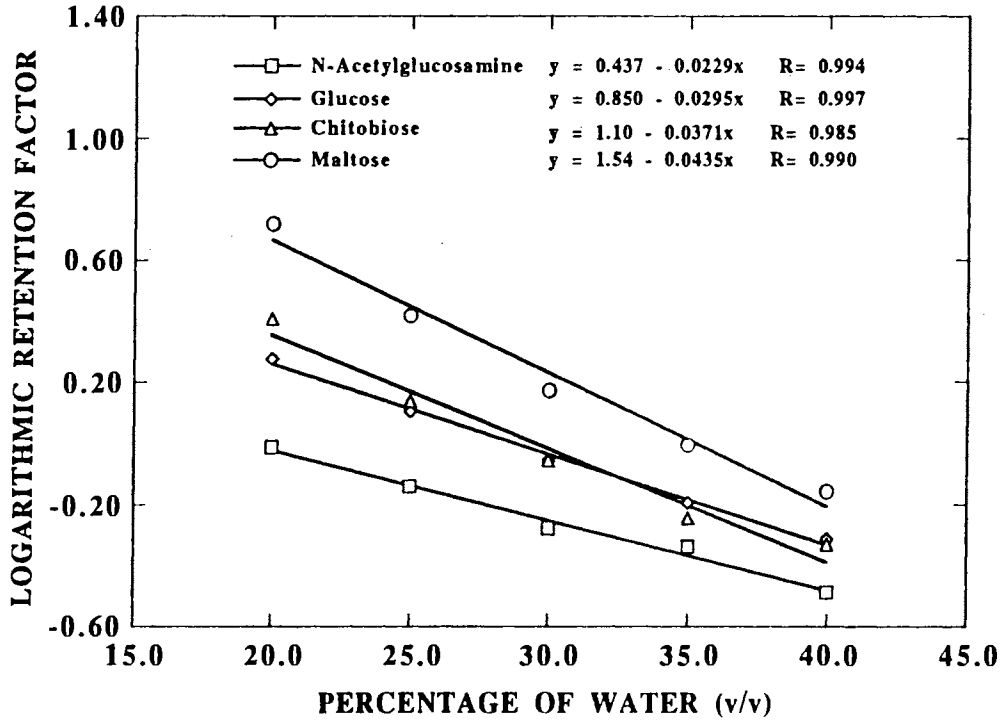

Figure 5. Plots of logarithmic retention factor of sugars *versus* volume percent of water concentration in acetonitrile. Column, 50.0 x 4.6 mm, monoamine-Zorbax, average particle diameter, 4.6 μm; average pore diameter, 150 Å; flow rate, 1.0 mL/min; isocratic elution.

mechanism. The linear dependence of *log k'* on % water or acetonitrile in the eluent was recently reported [37] on aminopropyl silica columns. As manifested by the R values, it should be noted (refer to Figures 4 and 5) that the plots of the *log k' vs* % water in the eluent were more linear on non-porous zirconia sorbents than on porous silica-based stationary phases may be due to the fact that in the latter case the % water in the mobile phase was higher than that in the former case. When a higher water content is used a mixed-mode hydrophilic interaction mechanism involving adsorption and partitioning may prevail.

Evaluation of Tetraethylenepentamine Stationary Phase

When compared with diethylenetriamine bonded phases, columns packed with zirconia-tetraethylenepentamine did not show better results as far as the separation of maltooligosaccharides is concerned (results are not shown). This may be attributed to the chemistry used in the formation of the pentamine stationary phase. In the process of covalent attachment of the pentamine functionality to the aldehyde activated zirconia surface (see Experimental), the reaction of tetraethylenepentamine with the aldehyde groups could occur at both ends (i.e., primary amine groups) of the molecule in a multi-attachment fashion. Therefore, even though the number of surface amino groups of the tetraethylenepentamine stationary phase might be relatively higher than that obtained by other modification processes described above, there will be less effective interaction sites, especially primary amino groups on the surface of tetraethylenepentamine-zirconia sorbent. Pentamine stationary phase, however, was useful in the separation of mono-, di- and triphosphate nucleotides, see chromatogram shown in Figure 6. The overall elution order followed the increasing number of phosphate groups in the nucleotide molecule indicating an anion-exchange mechanism. Nucleotides with monophosphate groups had lower retention and could be resolved with 5 mM $NH_4H_2PO_4$ under isocratic elution condition,

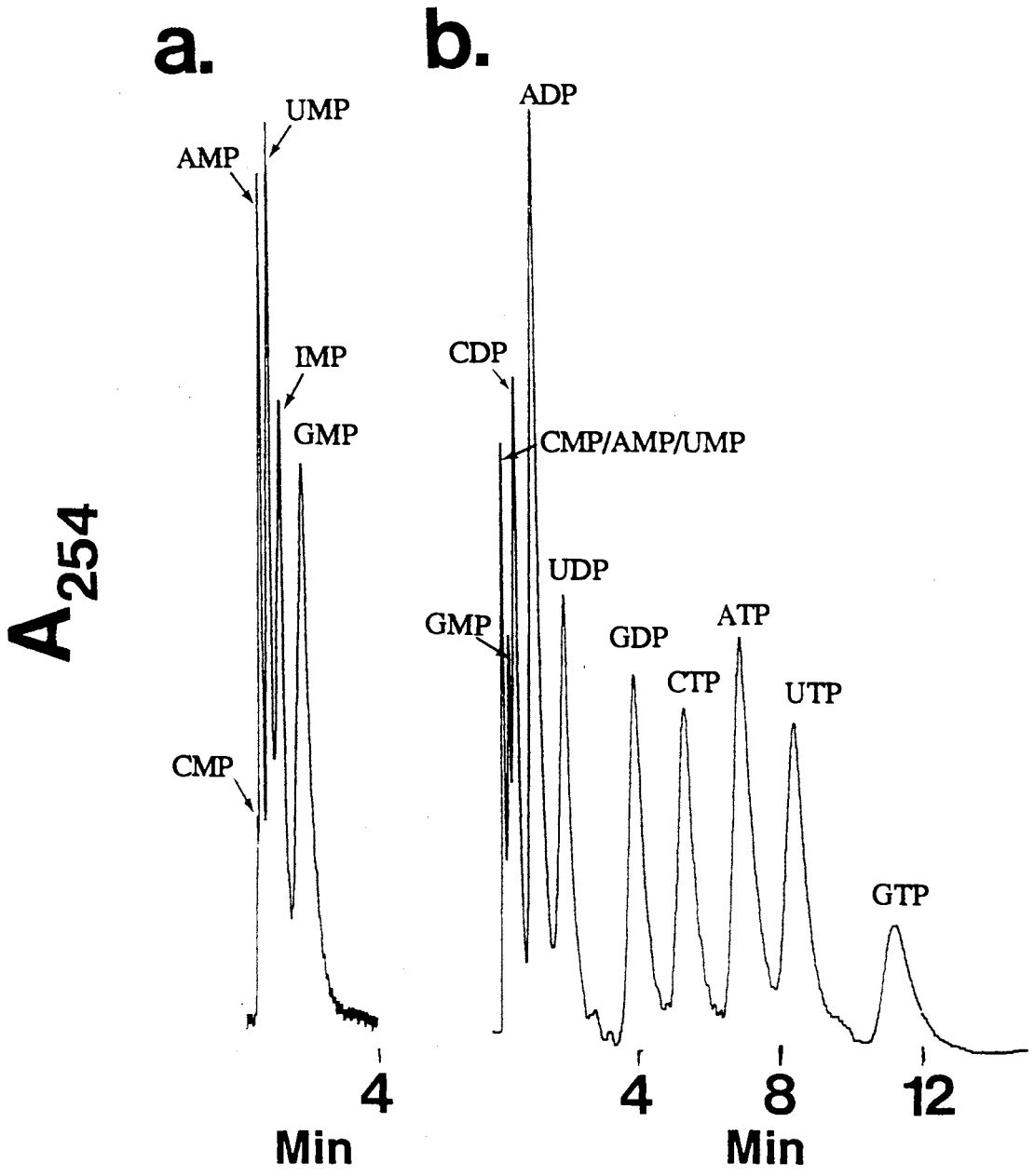

Figure 6. Chromatograms of nucleotides obtained on pentamine-zirconia stationary phase. Column, 50.0 x 4.6 mm; (a), isocratic elution with 5.0 mM $NH_4H_2PO_4$, pH 3.0; (b), linear gradient in 10.0 min from 0.0 to 0.50 M NaCl in 50.0 mM $NH_4H_2PO_4$, pH 3.0; and isocratic at 0.50 M NaCl in 50.0 mM $NH_4H_2PO_4$ for 5 min; flow rate: 1.0 mL/min. For symbols, see Experimental.

see Figure 6a. Nucleotides with di- and triphosphate groups were eluted with a linear gradient of increasing NaCl concentration in 50 mM $NH_4H_2PO_4$, see Figure 6b. Overall, the elution process is a weak anion exchange mechanism.

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CHAPTER VI

RAPID HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING NONPOROUS SILICA SUPPORT

Abstract

Nonporous, monodispersed, microsperical silica particles having mean particle diameters of 0.8 and 1.1 μm were surface-modified with octadecyl or triphenyl functions as well as with lectin proteins for rapid high performance liquid chromatography (HPLC). "Monomeric" or "polymeric" octadecyl-silica (ODS-silica) bonded phases were formed, depending on the silane compounds used. End-capped monomeric ODS-silica stationary phases yielded high resolution separation with various classes of species including aromatic compounds, 2-pyridylamino derivatives of xyloglucan oligosaccharides and proteins. The effect of the particle size of the support on the resolution of the stationary phase was also examined. Polymeric ODS-silica stationary phases yielded higher phase ratios and better support surface coverage which are important for the separation of hydrophilic species such as derivatized chitooligosaccharides of low degree of polymerization (d.p.). The number of theoretical plates and the slopes of the linear dependence of the logarithmic retention factor on the volume percent of organic modifier were determined for the monomeric and polymeric ODS-silica stationary phases with low molecular weight aromatic compounds. For comparative study, a separation of protein mixture on a triphenyl-silica stationary phase was also conducted. Lectin affinity stationary phases with *lens culinaris* agglutinin (LCA) and wheat germ agglutinin (WGA) covalently bound to nonporous silica support were examined for the application of nonporous stationary phases in rapid lectin affinity

chromatography of glycoproteins. In all cases, rapid separations on the time scales of seconds and minutes could be obtained because of the absence of mass transfer resistance in the nonporous stationary phase matrices.

Introduction

The development of rapid separation schemes by HPLC has been one of the themes of extensive studies in recent years. Fast chromatographic separations ensure short analysis time, reduced solvent consumption, rapid development of analytical methods, and high mass recovery with preserved bioactivity for biological species. Mass transfer resistance and solute adsorption-desorption kinetics are the two main factors that influence band broadening in chromatography. To reduce the mass-transfer resistance arising from slow diffusion commonly encountered in traditional porous column packing materials, one appraoch is to use macroporous particles with flow-through pores [1]. Another approach is to eliminate the pore structure using external particle surface. Nonporous silica packings of small particle diameter (0.5 - 2.0 μm) offer about the same specific area per unit column volume as a 400 nm pore size silica of 5 to 10 μm particle diameter and a packing density of 0.5 g/mL [2]. Columns packed with these particles have been used for the rapid separation of biomacromolecules, such as peptides, proteins, oligonucleotides and nucleic acid restriction fragments, by reversed phase [3-5], ion exchange [6,7] and hydrophobic interaction chromatography [8]. In one published study, the application of nonporous silica packing material (d_p = 5 μm) for the reversed phase separation of low-molecular-weight compounds was investigated [9].

This paper reports the use of nonporous, monodispersed, spherical silica particles having mean particle diameter of 0.8 and 1.1 μm. These particles were modified with octadecyl or triphenyl functions to form nonpolar stationary phase, and with lectins (e.g., LCA and WGA) to form lectin affinity stationary phases. The chromatographic properties

of the various modifications on these nonporous supports were evaluated with small and large molecular weight compounds.

Experimental

Instrumentation

The liquid chromatograph was assembled from (i) an LDC Analytical (Riviera Beach, FL, U.S.A.) ConstaMetric 3500 solvent delivery system with a gradient programmer, which was used to control a ConstaMetric Model III solvent delivery pump, (ii) a sample injector Model 7125 from Rheodyne (Cotati, CA, U.S.A.), and (iii) a UV interference filter photometric detector Model UV-106 from Linear Instruments (Reno, NV, USA). Chromatograms were recorded with a computing integrator Model C- R6A equipped with a floppy disk drive and a cathode-ray tube (CRT) monitor from Shimadzu (Columbia, MD, U.S.A.).

Chemicals

HPLC grade acetonitrile and methanol, reagent grade sodium phosphate mono- and dibasic, sodium hydroxide, sodium chloride, sucrose, glacial acetic acid, anhydrous ammonia, saturated ammonium hydroxide, trifluoroacetic acid (TFA), benzene, toluene, *p*-xylene, and naphthalene, and reagent grade as well as technical grade isopropanol, methanol, 200 proof, denatured ethanol, and *N, N*-dimethylformamide (DMF) were from Fisher (Pittsburgh, PA, U.S.A.). Tetraethyl orthosilicate (TEOS), trimethylchlorosilane, sodium cyanoborohydride and sodium periodate were purchased from Aldrich (Milwaukee, WI, U.S.A.). γ-glycidoxypropyltrimethoxysilane, triphenylchlorosilane, octadecyldimethylchlorosilane and octadecyltrichlorosilane were obtained from Hüls America Inc. (Bristol, PA, U.S.A.). Ribonuclease A from bovine pancreas, cytochrome c

from horse heart, lysozyme from chicken egg, glucose oxidase from *Aspergillus niger*, ovalbumin, fetuin from fetal calf serum, *p*-nitrophenyl-*N*-acetyl-α-D- and β-D-glucosaminide, *p*-nitrophenyl-*N*-acetyl-β-D-*N,N'*-diacetylchitobioside were purchased from Sigma (St. Louis, MO, USA). *N*-acetyl-D-glucosamine, *N*-acetyl-D-chitobiose, *N*-acetyl-D-chitotriose, and *N*-acetyl-D-chitotetraose were obtained from Seikagaku America, Inc. (Rockville, MD, USA) and were labelled with 2-aminopyridine using a well established procedure [10]. *Lens culinaris* agglutinin (LCA) and wheat germ agglutinin (WGA) were from Vector Laboratories, Inc. (Burlingame, CA, USA).

Column Support

Microspherical monodispersed nonporous silica particles were prepared by seed-growth process of base-catalyzed hydrolysis of TEOS following the procedures described earlier [11]. Typically, 500 mL of ethanol placed in a 1.0 L three-neck round bottom flask was first saturated with anhydrous ammonia by bubbling it through the solution. Thereafter, 127 mL of saturated ammonium hydroxide and 8.0 mL TEOS were added while stirring. After 10 hrs, addition of 6.0 mL of TEOS and 1.4 mL water were repeated every 12 hrs interval for a total of six additions. Silica particles thus prepared have a nonporous texture, monodispersed spherical shape (see Chapter III) with a mean diameter of *ca.* 0.8 μm. Nonporous monodispersed silica particles in the size of 1.1 μm were prepared as the above procedure except that 16 mL TEOS and 1.4 mL water were added for a total of five additions.

Commercially available Nucleosil silica of 7 μm mean particle diameter and 300 Å mean pore diameter was obtained from Machery-Nagel (Düren, Germany), and used as supplied.

Stationary Phases

ODS-silica stationary phases. Monomeric and polymeric ODS stationary phases were synthesized according to the procedures described in an earlier study [11], see Chapter III. The silica microspheres were treated with octadecyldimethylchlorosilane to form "monomeric" stationary phase. Further treatment with chlorotrimethylsilane was also performed with some monomeric octadecyl-silica to end-cap the residual silanol groups that might remain unreacted. Octadecyl-silica of the "polymeric" type was formed by reacting the silica with octadecyltrichlorosilane. Both modifications were carried out in toluene, refluxed at 120°C for 12 hrs. End-capping with chlorotrimethylsilane was carried out in toluene at 60°C for 12 hrs.

Triphenyl-silica stationary phase. Typically, 3.0 g of silica support was suspended in 30.0 mL of toluene, and heated to 120°C. Then 8.0 mL of triphenylchlorosilane was added to the solution. The reaction was stirred for 12 hrs at 120°C. After the reaction, the silica thus treated was separated from the solution, and then washed successively with toluene and methanol, and let dry in the air.

Lectin stationary phases. Microspherical silica particles were first activated by reacting them with γ-glycidoxypropyltrimethoxysilane in 1.0 mM Na_2HPO_4, pH 6.0. Reaction was stirred at 95°C for 4 hrs. Thereafter, the epoxy ring was opened with 0.01 M HCl at 90°C, and, then converted to aldehyde by allowing the diol-silica phase to react with sodium periodate in glacial acetic acid-water (9:1, v/v) at room temperature. The aldehyde-activated silica microparticles were reacted with WGA or LCA. In all cases, 50 mM phosphate buffer, pH 6.0, containing soldium cyanoborohydride was used as the reaction medium.

Column Packing

All columns, 3.0 x 0.46 cm I.D. No. 316 stainless steel tubes (Alltech Associates Inc., Deerfield, IL, USA), were packed at 7000 *psi* using slurry packing technique with Shandon column packer instrument (keystone Scientific, Bellefonte, PA, USA). ODS-silica stationary phases were packed from an isopropoanol slurry with isoproponal as packing solvent. Lectin stationary phases were packed from an aqueous sucrose-NaCl slurry containing 50% (w/v) sucrose, with 1.0 M NaCl as the packing solvent.

Results and Discussion

Rapid Reversed Phase Chromatography

Nonprous silica microparticles were modified to form two types of stationary phases, monomeric and polymeric. Compared to porous supports, nonporous packings have limited surface area, and, as a result, the phase ratio for this type of stationary phase is relatively low. On the other hand, the nonporous texture of the support has the advantage of eliminating intraparticulate diffusional mass transfer resistance in the stagnant mobile phase, thus allowing high speed separations without sacrificing separation efficiencies.

In reversed-phase HPLC, the dependence of solute retention factor (k') on the volume fraction (φ) of the organic solvent in the mobile phase can be expressed by [12]

$$\log k' = \log k_w - S\varphi$$

where S is the slope of the linear function, which is characteristic of the solute molecular weight for a given stationary phase. The value of S for a given sample has important consequences in the selection of optimum gradient conditions [13,14]. To a first approximation, S is given by [15]

$$S = 0.48M^{0.44}$$

where M is the molecular weight of the solute. Earlier studies [3] by Unger's research group have investigated and evaluated the reversed phase chromatographic properties of proteins with molecular weight of 12,000-162,000 on nonporous monodispersed 1.5 μm silica support. They reported S values in the range of 4-7 for proteins as opposed to S values of typically 30 - 95 for the reversed phase separation of the same protein samples using porous column packings.

In this study, small molecules, benzene, toluene, *p*-xylene and naphthalene were used to evaluate the chromatographic properties of ODS-modified nonporous silica of smaller diameter, 0.8 μm. Fig. 1A and 1B are the plots of *log k' vs.* φ for monomeric and polymeric ODS stationary phases, respectively. The S values of these four solutes obtained on the two types of stationary phases are listed in Table I.

Table I. Slopes of the plots of logarithmic retention factor versus the volume fraction of acetonitrile in the mobile phase. Column, 30.0 x 4.6 mm, end-capped monomeric and polymeric octadecyl-silica stationary phases; mobile phase, water at various volume fraction of acetonitrile; flow rate, 2.0 mL/min.

		S-value		
	M.W.	Calculated	Monomeric	Polymeric
Benzene	78.11	3.27	1.83	2.07
Toluene	92.14	3.51	3.19	3.18
p-Xylene	106.17	3.74	4.04	4.10
Napthalene	128.17	4.06	4.94	4.83

As can be seen in Table I the S value for the solutes obtained on monomeric and polymeric ODS stationary phases increased with increasing molecular weight of the solute.

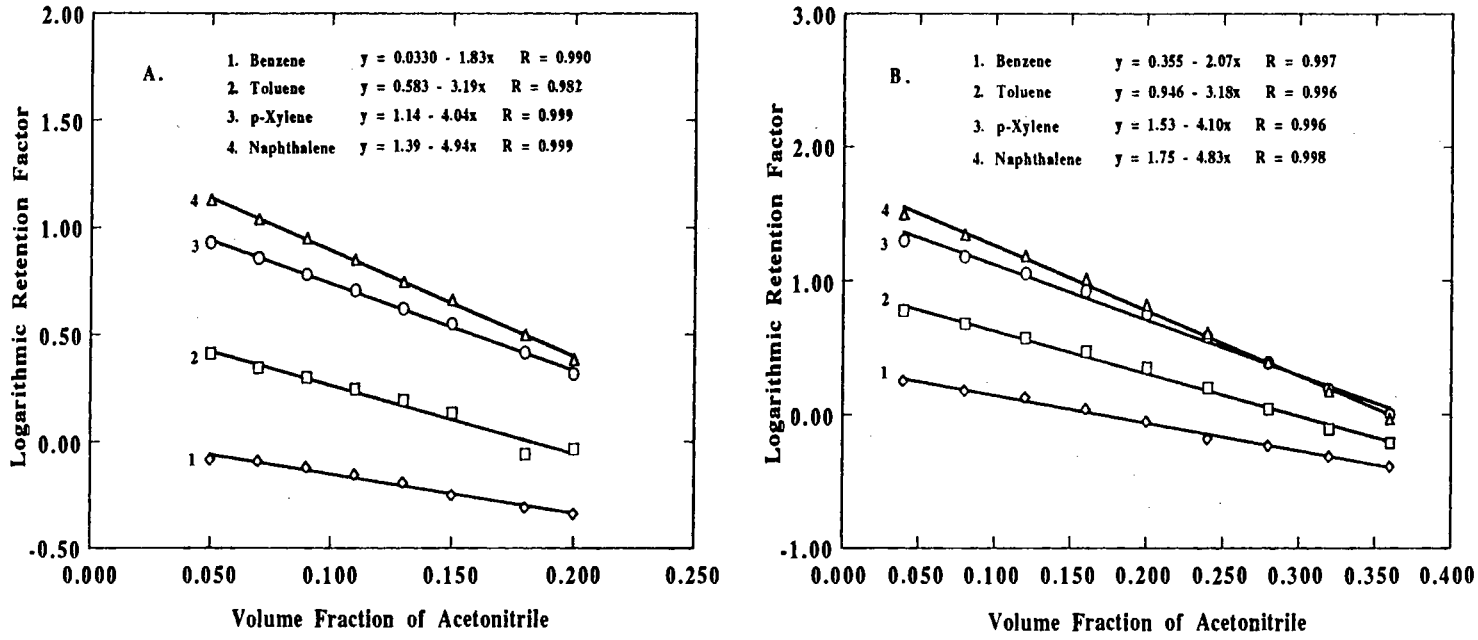

Figure 1. (A) Plots of logarithmic retention factor versus the volume percent acetonitrile in mobile phase for end-capped monomeric octadecyl-silica stationary phase. Column, 30.0 x 4.6 mm; mobile phase, water at various volume percent acetonitrile; flow rate, 2.0 mL/min. (B) Plots of logarithmic retention factor versus the volume percent acetonitrile in mobile phase for polymeric octadecyl-silica stationary phase. Column, 30.0 x 4.6 mm; mobile phase, water at various volume percent acetonitrile; flow rate, 2.0 mL/min.

Compared to the calculated *S* values, which were derived from porous packings, *S* values obtained on nonporous silica support are in the same range, except for that of benzene. These results differ from those reported by Unger *et al.*, who showed that the *S* values of proteins were 10-fold smaller on nonporous than on porous support. The rapid mass transfer of large molecules at the external surface area of the packing affects the rate constants of adsorption and desorption in such a way that lower equilibrium constants and distribution coefficients will result. For small molecules the difference in kinetics of the diffusion process will be much less significant, when comparing solute diffusion into and out of a pore system to that at exclusively external surfaces. This may explain why the *S* values for small molecules obtained on nonporous ODS-silica packings were similar to the calculated values.

In our previous studies [11,16] (see Chapters II and III), it was found that polymeric ODS stationary phases exhibit higher phase ratios and better support surface coverage than the monomeric ones. This was especially important in the separation of small molecules of closely related species. For the separation of polar and basic compounds, such as dansyl amino acids, better support surface coverage can eliminate the interactions between solute and support, *e.g.*, interactions with residual silanol groups, and improve the column efficiency and resolving power. Monomeric ODS stationary phase, however, can have higher resolving power than polymeric type, when the support is well covered to have sufficient functional groups in the modification process, and provides enough solute retention. Fig. 2 shows the isocratic elution of aromatic compounds on end-capped monomeric and polymeric ODS stationary phases. Better resolution (R_S = 1.94) between *p*-xylene and naphthalene was achieved on the monomeric phase (see Fig. 2A) than on the polymeric phase (R_S = 1.46, see Fig. 2B). Even when the mobile phase flow rate was raised to 4.0 mL/min, a resolution of 1.09 was obtained on the monomeric ODS stationary phase (chromatogram not shown). Polymeric formation of ODS stationary phase yielded a cross-linked and thick layer of octadecyl functions on the support surface.

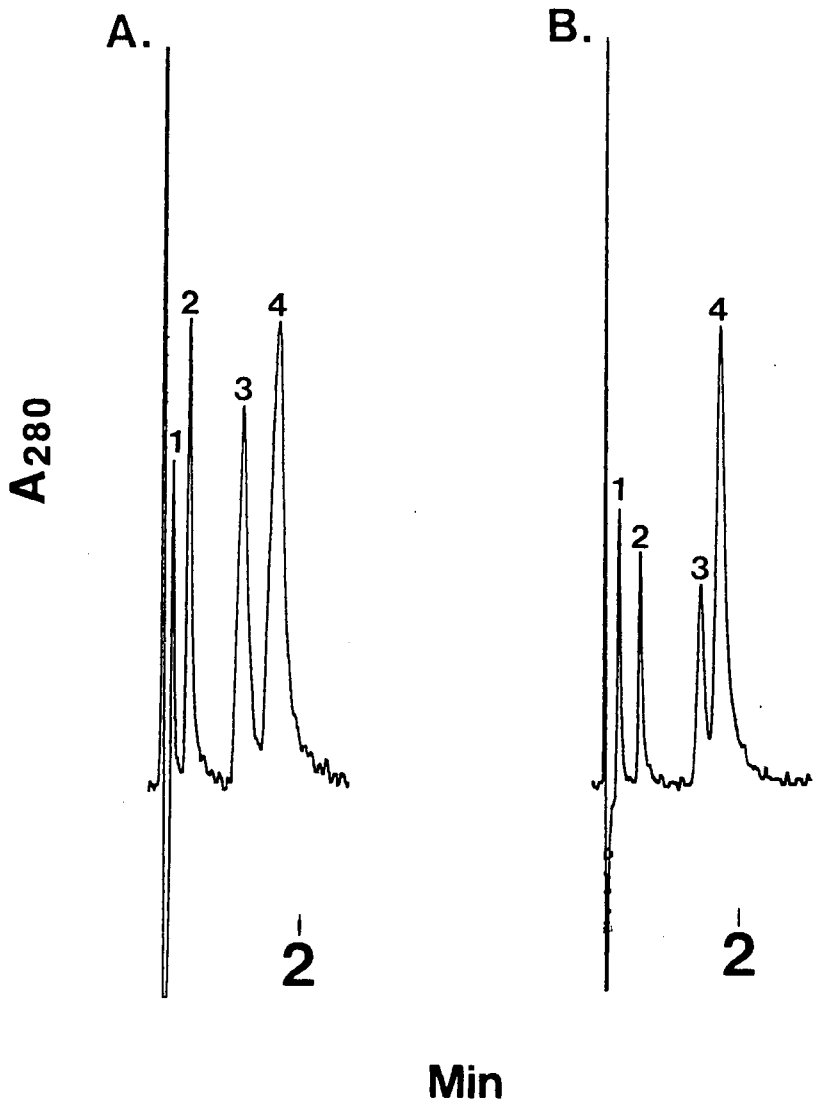

Figure 2. Chromatograms of aromatic compounds obtained on monomeric octadecyl-silica with end-capping in A and polymeric octadecyl-silica in B. Column, 30.0 x 4.6 mm; isocratic elution with aqueous mobile phase containing 10.0% (v/v) acetonitrile in A and 18.0% (v/v) acetonitrile in B; flow rate, 2.0 mL/min; samples: 1, benzene; 2, toluene; 3, *p*-xylene; 4, naphthalene; detection, UV, $\lambda = 280$ nm.

The density of octadecyl functions was higher compared to that of the monomeric type. The number of theoretical plates of the two types of ODS stationary phases with each aromatic compound were calculated and are listed in Table II. In all cases, polymeric stationary phase exhibited larger number of theoretical plates.

Table II. The number of theoretical plates calculated. Column, 30.0 x 4.6 mm; isocratic elution with aqueous mobile phase containing volume percent of acetonitrile of 10.0% for the end-capped monomeric and 18.0% for the polymeric octadecyl-silica; flow rate, 2.0 mL/min; detection, UV, λ = 280 nm.

	Number of Theoretical Plates		
	Monomeric (N_m)	Polymeric (N_p)	N_p/N_m
Benzene	98	340	3.5
Toluene	289	1079	3.7
p-Xylene	900	1314	1.5
Naphthalene	434	1174	2.7

Solute injected into the column would have larger hydrophobic interaction area on the polymeric stationary phase. As a consequence, higher retention would be observed, and stronger eluent of higher organic content would be required. To bring the retention of aromatic compounds to the same level on both monomeric and polymeric ODS stationary phase, aqueous mobile phase with 10% acetonitrile was needed for the monomeric phase as opposed to 18% acetonitrile for the polymeric phase. Since higher organic eluent was necessary to displace the solutes from the polymeric octadecyl stationary phase, solutes with small difference in hydrophobicity could not be well resolved in the strong partition process between mobile phase and stationary phase.

Because of its surface density in octadecyl function, the polymeric ODS-silica was useful for the separation of hydrophilic species such as the 2-pyridylamino derivatives of chitooligosaccharides of low d.p., see Fig. 3. As can be seen in Fig. 3, all the four solutes

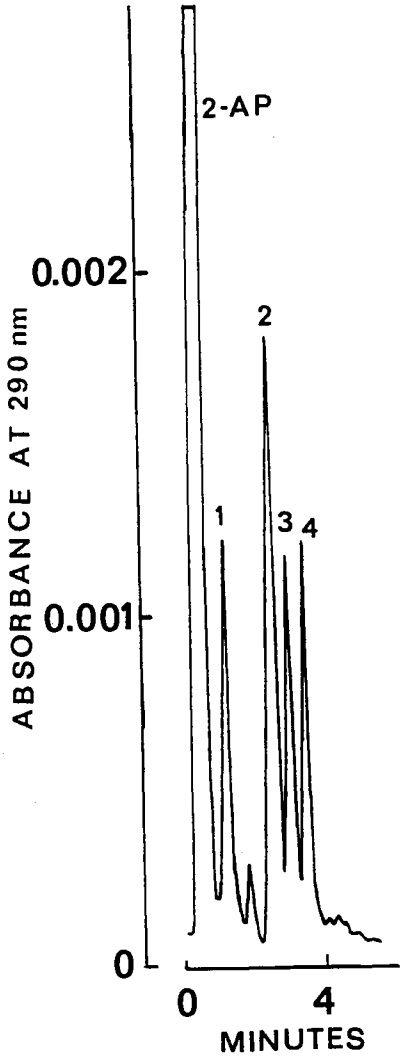

Figure 3. Chromatograms of 2-pyridylamino derivatives of chitooligosaccharides obtained on polymeric octadecyl-silica stationary phase. Column, 30.0 x 4.6 mm; linear gradient in 5.0 min from 0.0 to 10.0% (v/v) acetonitrile in 10.0 mM NaH_2PO_4, pH 3.0; flow rate, 2.0 mL/min; samples: 2-pyridylamino derivatives of 1, *N*-acetyl-D-glucosamine; 2, chitobiose; 3, chitotriose; 4, chitotetraose; detection, UV, λ = 290 nm.

could be eluted in less than four minutes with gradient elution of up to 10% (v/v) acetonitrile in the mobile phase at a flow rate of 2.0 mL/min.

In a recent report from our laboratory [17], we have examined the chromatographic behavior of 2-pyridylamino derivatives of xyloglucan oligosaccharides in reversed phase chromatography on wide-pore octadecyl-silica based stationary phases over a wide range of elution conditions, and reported the oligosaccharide maps of the cellulase digest of xyloglucan from cotton cell walls and tamarind seed. As a continuation of our method developments for the separation of oligosaccharides by HPLC, the chromatographic behavior of xyloglucan oligosaccharides was examined on nonporous octadecyl-silicas.

Fig. 4 illustrates the various maps of 2-pyridylamino derivatives of xyloglucan oligosaccharides (PA-XG) at different mobile phase flow rates and gradient times, performed on a short column (30.0 x 4.6 mm) packed with 0.8 μm nonporous octadecyl-silica monomeric stationary phase. As can be seen in the chromatograms, upon increasing the flow rate from 1.0 mL/min to 3.0 mL/min and gradient steepness from 0.40 to 0.85% (v/v) acetonitrile/min, respectively, the analytical information about the content of the mixture remained almost unchanged as far as the larger PA-XG fragments (i.e., retarded peaks) are concerned. In addition, the peaks were sharper at the mobile phase flow rate of 3.0 mL/min than at 1.0 mL/min, and, consequently, for the same signal the amount injected was 2 to 3 times lower at higher flow velocity. This clearly demonstrates that rapid reversed phase chromatography with appropriately designed nonporous octadecyl-silica stationary phase yields excellent separation for closely related oligosaccharides with high sensitivity and high separation effeciencies.

When the same oligosaccharide mixture was chromatographed on the same size silica gel (0.8 μm) but with polymeric octadecyl bonded phase, the amount of organic solvent needed to bring about the same time of analysis as with the monomeric octadecyl-silica packings is slightly higher, see Fig. 5A and B. This is expected as the phase ratio increased. However, the resolution did not improve when going from monomeric to

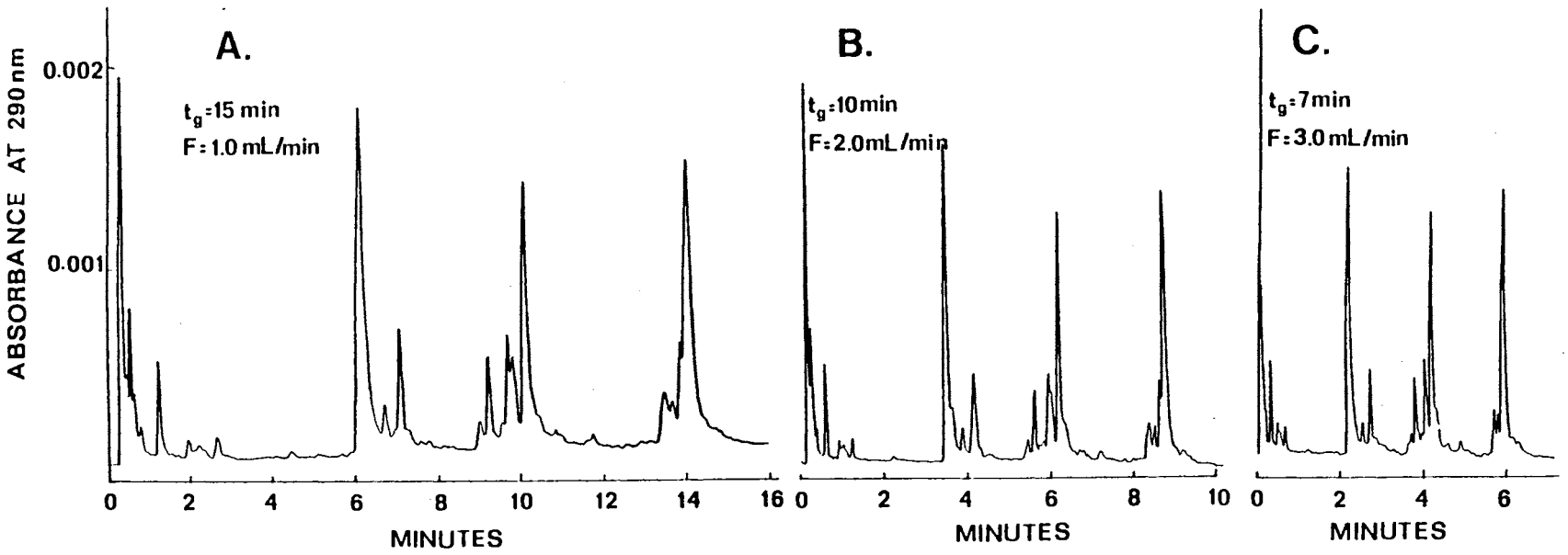

Figure 4. Chromatograms of 2-pyridylamino derivatives of xyloglucan oligosaccharides obtained on end-capped monomeric octadecyl-silica (d_p = 0.8 μm) stationary phase. Column, 30.0 x 4.6 mm; linear gradient from 0.0 to 6.0% (v/v) acetonitrile in 10.0 mM NaH_2PO_4, pH 3.0; the gradient time (t_g) and flow rate (F) are indicated in each panel; detection, UV, λ = 290 nm.

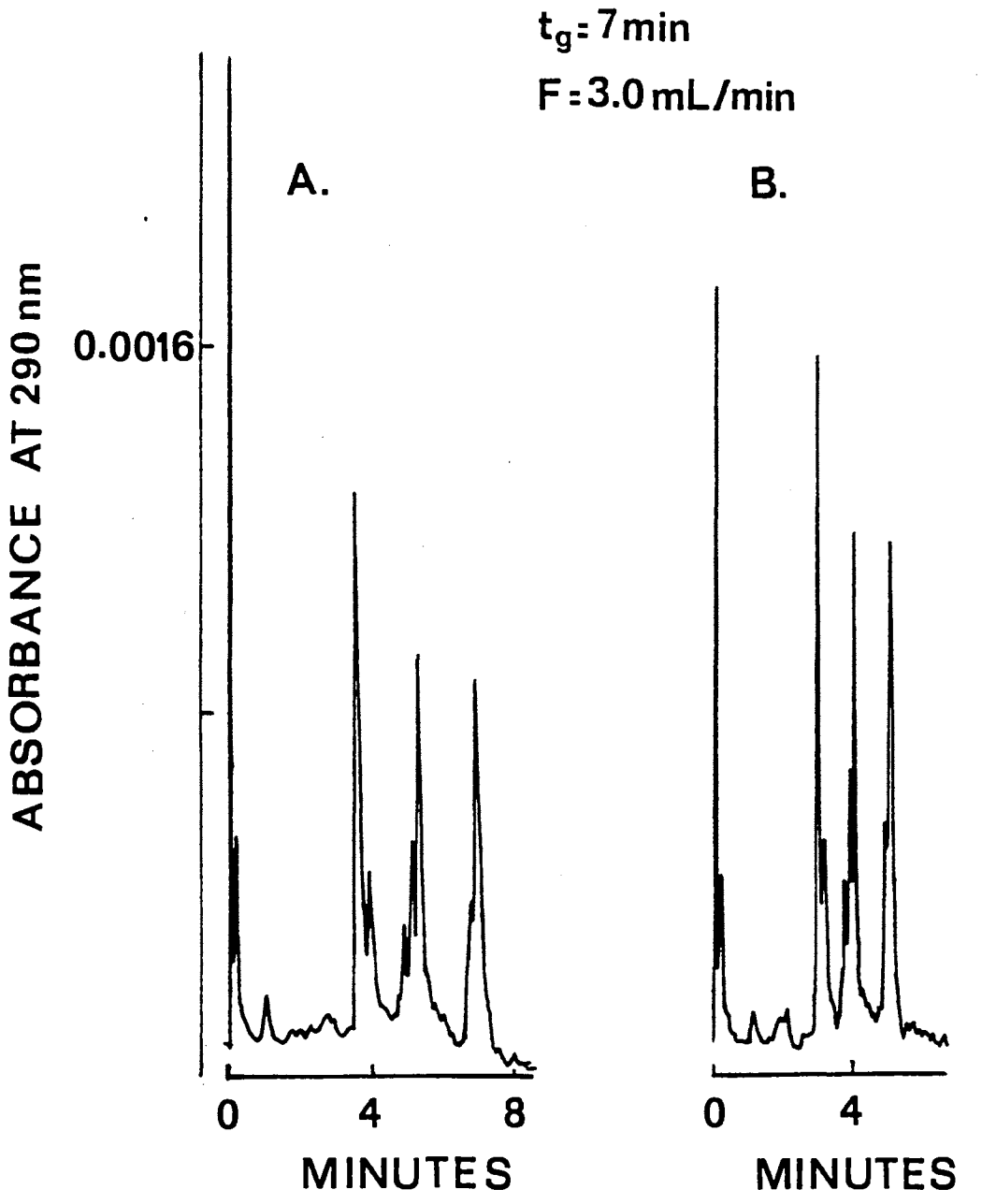

Figure 5. Chromatograms of 2-pyridylamino derivatives of xyloglucan oligosaccharides obtained on polymeric octadecyl-silica (d_p = 0.8 μm) stationary phase. Column, 30.0 x 4.6 mm; linear gradients: A, from 0.0 to 6.0% (v/v), B, from 0.0 to 10.0% (v/v) acetonitrile in 10.0 mM NaH_2PO_4, pH 3.0; the gradient time (t_g) and flow rate (F) are indicated in each panel; detection, UV, λ = 290 nm.

polymeric octadecyl bonded phase, and part of the analytical information about the mixture was lost, even when using the same gradient profile as with the monomreic octadecyl sorbent, compare Figs. 5A and 4C. This corroborates with our previous findings with porous octadecyl silica stationary phases in that higher phase ratio sorbents, which necessitates increased percent of organic solvent in the mobile phase, did not bring about good resolution for this mixture. This is probably due to organic solvent-induced conformational changes. At higher organic solvent mobile phase, the difference in molecular hydrophobic surface areas of the various sugar chains was diminished during the oligomer elution process from the stationary phase, and as a result low selectivity was yielded.

To determine the effect of particle size on the resolution of the oligomers, the PA-XG oligosaccharides were chromatographed on a 1.1 μm nonporous silica gel having monomeric octadecyl bonded phase. The results are shown in Fig. 6. As expected, increasing the particle size by a factor of 1.4 resulted in decreased retentivity of the column as a result of the decreased specific surface area of the silica particles. The percent organic solvent in the mobile phase needed to bring about the same time of analysis as with 0.8 μm decreased by a factor of 1.5. Also the resolution for the early eluting peaks decreased significantly, and part of the analytical information was lost. It seems that there is an optimum phase ratio that is best attained by decreasing the size of the support and using monomeric octadecyl bonded stationary phase for the rapid and high efficiency separation of these branched oligosaccharides.

Although the low phase ratio of monomeric ODS stationary phase on 1.1 μm silica support limits the resolution of hydrophilic species, this feature, on the other hand, can be regarded as an advantage in terms of bringing chromatographic retention to practical range, especially in the chromatography of biomacromolecules, *e.g.*, proteins. Under these circumstances, biopolymers may be separated under milder elution conditions which would preserve their biological activity and allow their high mass recovery. Fig. 7 is an example

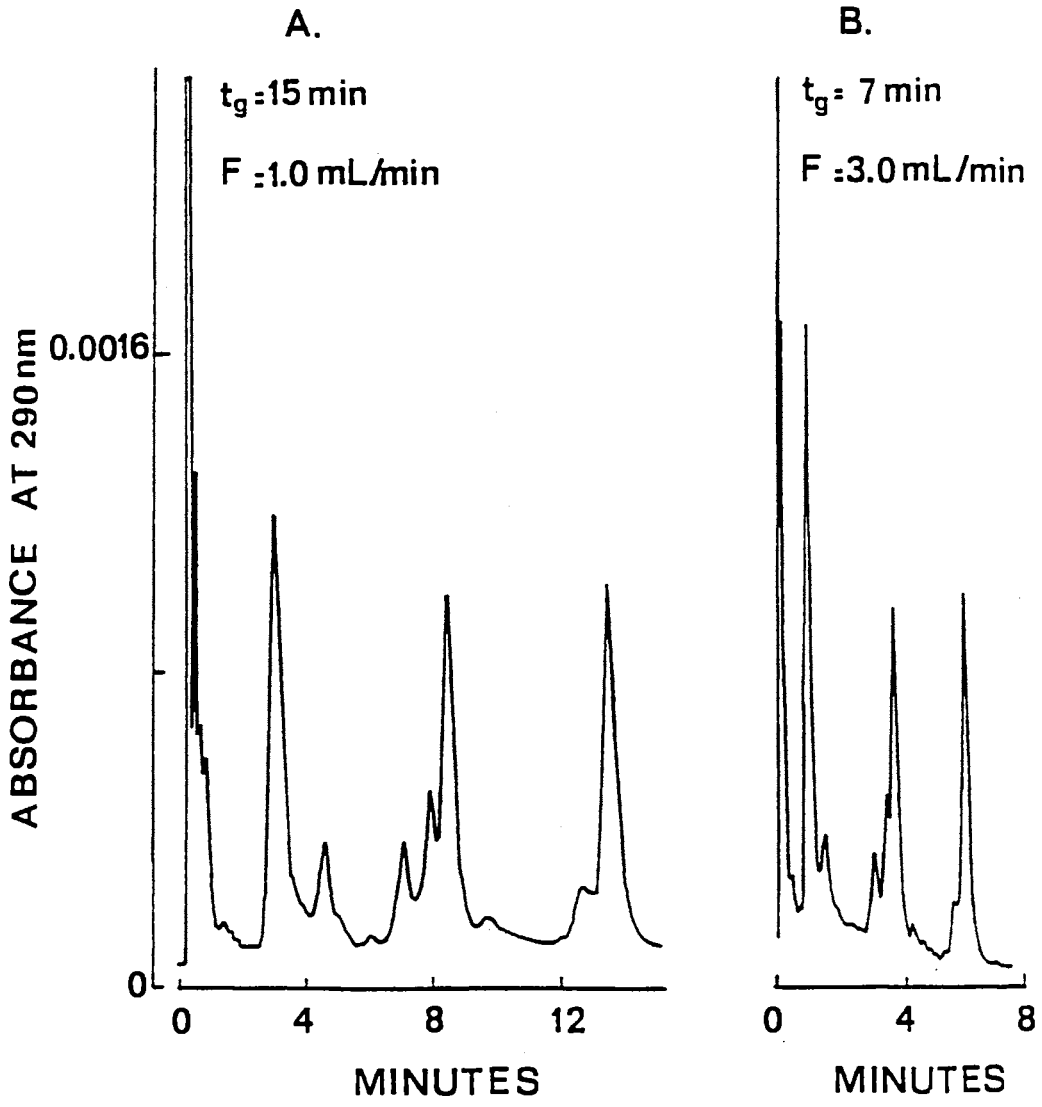

Figure 6. Chromatograms of 2-pyridylamino derivatives of xyloglucan oligosaccharides obtained on end-capped monomeric octadecyl-silica (d_p = 1.1 μm) stationary phase. Column, 30.0 x 4.6 mm; linear gradient from 0.0 to 6.0% (v/v) acetonitrile in 10.0 mM NaH_2PO_4, pH 3.0; the gradient time (t_g) and flow rate (F) are indicated in the figure; detection, UV, λ = 290 nm.

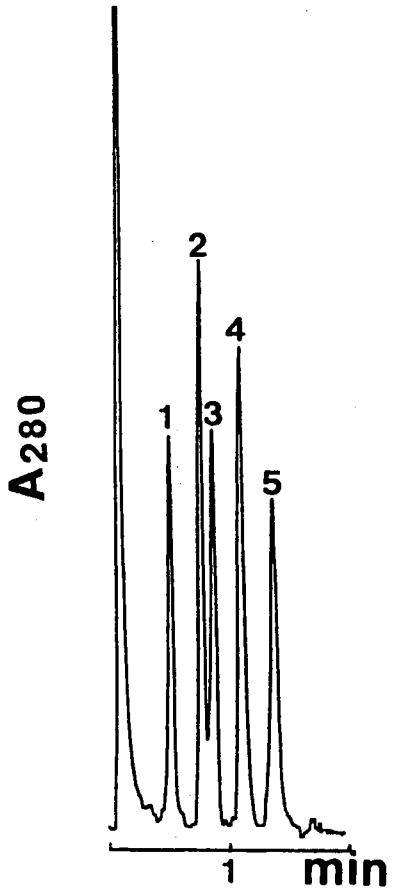

Figure 7. Chromatogram of proteins obtained on monomeric octadecyl-silica stationary phase without end-capping. Column, 30.0 x 4.6 mm; linear gradient in 2.5 min from 20.0 to 70.0% (v/v) acetonitrile in aqueous mobile phase with 0.05% (v/v) TFA; flow rate, 4.0 mL/min; samples: 1, ribonuclease A; 2, cytochrome c; 3, lysozyme; 4, albumin (bovine); 5, ovalbumin; detection, UV, $\lambda = 280$ nm.

of high speed separation of a group of five proteins on monomeric ODS stationary phase without end-capping having a mean particle diameter of 1.1 μm. The separation was accomplished in less than 1.5 min at mobile phase flow rate of 4.0 mL/min using a gradient elution from 20 to 70% (v/v) acetonitrile in the mobile phase. Even though the phase ratio was quite low for this stationary phase, its application for the separation of macromolecules could still produce enough resolving power.

Silica microparticles (1.1 μm) were also modified with triphenyl functional groups. The same group of proteins chromatographed on the ODS phase was separated on this stationary phase, see Fig. 8. The elution pattern was the same, except that 20.0 mM NaCl had to be added to the mobile phase since otherwise these proteins could not be eluted from the triphenyl stationary phase. This may indicate the presence of unreacted surface silanols which act as cation exchange sites for polyionic species such as proteins. When the same amount of organic eluent was used for both ODS and triphenyl stationary phases, slightly lower retention for all the proteins was observed on triphenyl phase, possibily because of the difference in hydrophobicity between triphenyl and octadecyl functions. Better resolution between cytochrome c and lysozyme was obtained on ODS than on triphenyl stationary phase, compare Fig. 7 and 8.

Rapid Lectin Affinity Chromatography of Glycoproteins and Other Glycoconjugates

Glycoproteins are heterogeneous species due to the variations in the oligosaccharide chains attached [18]. This heterogeneity is amplified when glycoprotein is multiply glycosylated with the variation in the number and location of the oligosaccharides as well as in the nature of the sugar chains. These microheterogeneities lead to multiple forms for a given glycoprotein. Lectins are sugar-binding proteins [19]. They bear at least two sugar-binding sites, and can precipitate polysaccharides, glycoproteins, and glycolipids. As these interactions are often reversed by monosaccharides or glycosides, lectins are powerful tools

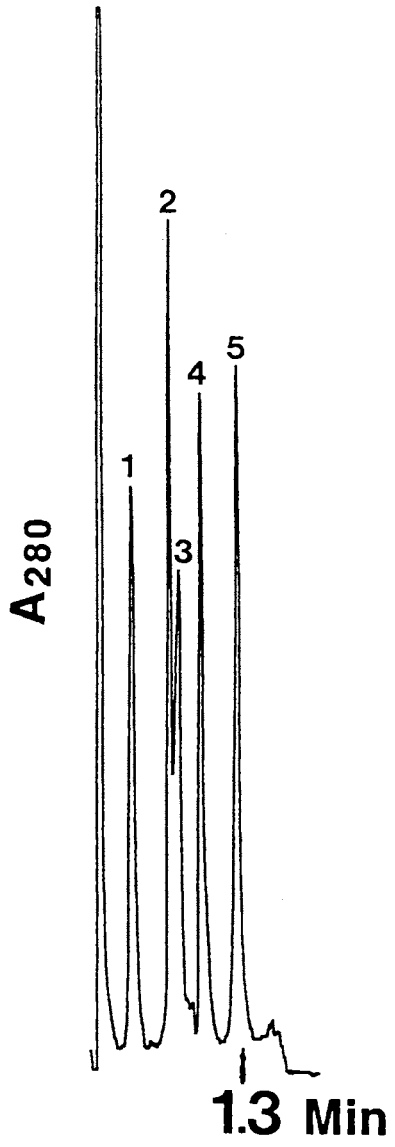

Figure 8. Chromatogram of proteins obtained on triphenyl-silica stationary phase. Column, 30.0 x 4.6 mm; linear gradient in 1.5 min from 20.0 to 70.0% (v/v) acetonitrile in aqueous mobile phase with 20.0 mM NaCl and 0.05% (v/v) TFA; flow rate, 4.0 mL/min; samples: 1, ribonuclease A; 2, cytochrome c; 3, lysozyme; 4, albumin (bovine); 5, ovalbumin; detection, UV, λ = 280 nm.

for studying the heterogeneity of glycoproteins and isolating sugar components [20,21]. In lectin affinity chromatography, the microheterogeneity of glycoproteins, imparted by their oligosaccharide chains, is often manifested by the presence of three different peaks: (i) non-reactive population of the molecules, i.e., molecules missing the oligosaccharide moieties that can be recognized by the lectin, which are eluted in the void volume of the column with the binding buffer; (ii) weakly reactive population of the molecules, i.e., molecules containing weakly reactive oligosaccharide chains; and (iii) strongly reactive molecules which are specifically desorbed and eluted with the debinding buffer containing the appropriate haptenic sugar [18].

Two lectins, LCA and WGA, were immobilized on nonporous silica microparticles for rapid lectin affinity chromatography of glycoconjugates. Fig. 9A and B show the rapid chromatography of glucose oxidase performed on a short column (30.0 x 4.6 mm) packed with 0.8 μm nonporous silica-bound LCA. As can be seen in Fig. 9, the lentil lectin column yielded two peaks for the protein. One peak eluted with the binding buffer, which is believed to correspond to the molecules missing the saccharide determinant, i.e., weakly reactive components. The other peak was specifically eluted with the debinding buffer containing the haptenic sugar, methyl-α-D-mannopyranoside, which corresponds to the molecules containing the oligosaccharide chains with the sequences that are necessary for lectin recognition, i.e., strongly reactive components. Note that glucose oxidase is a flavoprotein, the prosthetic group being flavine-adevine dinucleotide which is well detected in the visible region at 460 nm (see Fig. 9A); the chromatogram recorded at 280 nm corresponds to the specific UV absorbance of the peptide bonds of the protein. On the other hand, on porous silica (300 Å) with surface bound LCA, the weakly binding fraction of glucose oxidase seemed to elute with the lectin reactive fraction, and one single peak was recorded, see Fig. 9C. This can be explained by the much higher phase ratio obtained on the porous support. The strong binding interactions from the highly populated lectin stationary phase rendered the two fractions of glucose oxidase to have the same retention,

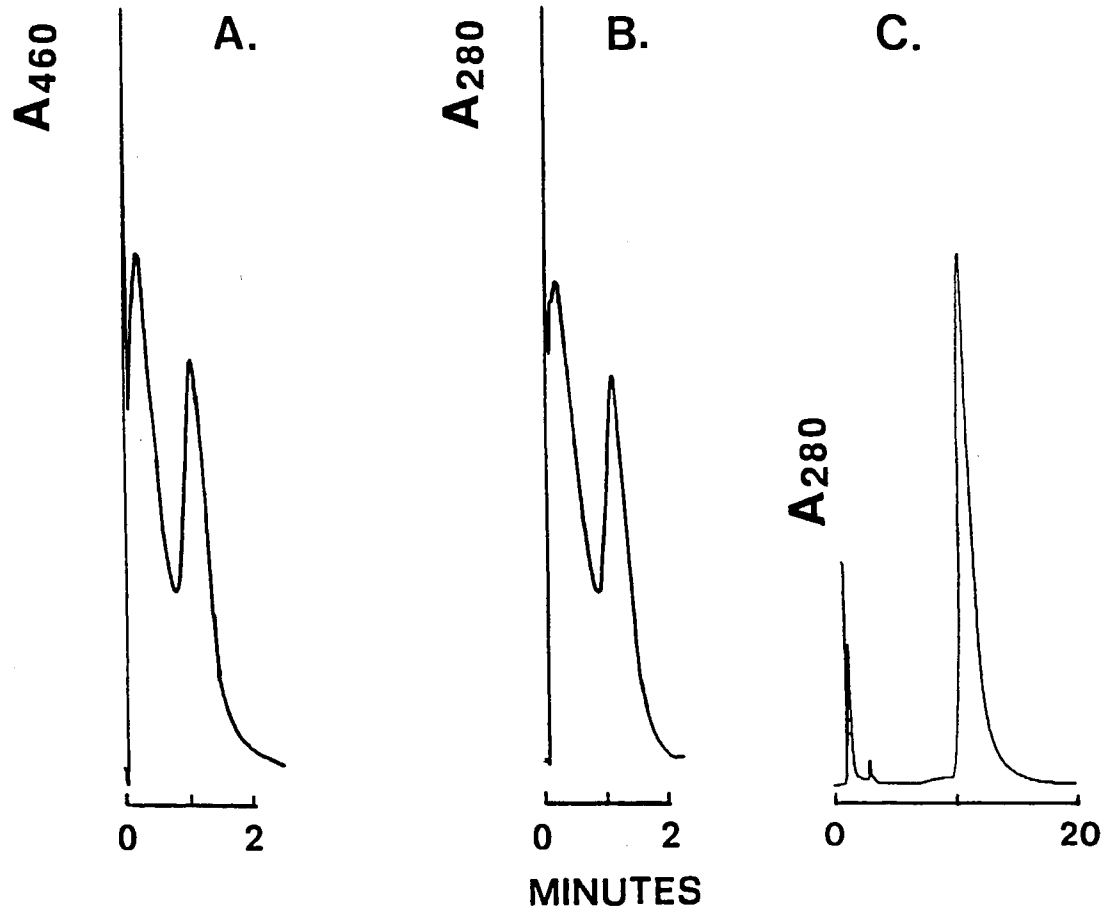

Figure 9. Chromatograms of glucose oxidase obtained on (A) and (B) nonporous (0.8 μm) silica-bound LCA, and (C) porous (7 μm, 300 Å) silica-bound LCA, stationary phases. (A) and (B): column, 30.0 x 4.6 mm; gradient from 0.0 to 25.0 mM methyl-α-D-mannopyranoside in 25.0 mM Na_2HPO_4, pH 6.0, and 200.0 mM NaCl, in 0.20 min, and isocratic elution for 2.0 min; flow rate, 3.0 mL/min; (C): column, 100.0 x 4.6 mm; linear gradient from 0.0 to 20.0 mM methyl-α-D-mannopyranoside in 20.0 mM $NaOOCCH_3$, pH 6.0, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 200.0 mM NaCl, in 1.0 min, and isocratic elution for 10.0 min; flow rate, 1.0 mL/min. Detection, UV, (A) λ = 460 nm, (B) and (C) λ = 280 nm.

when relatively higher concentration of debinding sugar was required to elute the protein from the porous silica-bound LCA stationary phase. With non-porous packing material, the elution condition was relatively mild under lower concentration of debinding sugar because of the lower phase ratio. Under this condition, lectin stationary phase could better differentiate the degree of affinity of glycoprotein heterogeneous fractions.

To determine the amount of lectin that can be immobilized on non-porous silica, 2.00 g of non-porous silica of mean particle diameter of 1.1 μm having aldehyde-activated surface was reacted with a solution containing 5.92 mg WGA. Reaction was carried out for 32 hrs. A calibration curve (see Fig. 10) was established using a series of WGA standard solutions. The quantitative measurement was conducted using isocratic elution of WGA on an ODS column (30.0 x 4.6 mm) with an aqueous mobile phase containing 0.05% (v/v) TFA and 17.0% (v/v) acetonitrile. The peak height was used in the measurement of WGA concentration. The WGA reaction solution, after the silica modification was completed, was determined to have 1.58 mg WGA left. Thus, the amount of WGA covalently attached to the nonporous silica support was determined to be 2.17 mg WGA per gram of silica support.

Fig. 11 illustrates the rapid affinity chromatography of bovine and human α_1-acid glycoproteins obtained on 1.1 μm nonporous silica-bound WGA. The selectivity of the lectin column permitted the illustration of the microheterogeneity of the glycoproteins. α_1-Acid glycoprotein from bovine was separated into two fractions (Fig. 11A) one unretained corresponding to the non-reactive components, and the other strongly reactive components highly retained and eluted with the haptenic sugar. α_1-Acid glycoprotein from human yielded three peaks (Fig. 11B): one non-reactive, another slightly retained containing the weakly reactive components, and the third one retarded containing strongly reactive components. Fetuin from calf serum was also fractionated on WGA stationary phase, see Fig. 12A. Two peaks were obtained: one containing the non-reactive molecules and the other containing the molecules with the sugar moieties that have binding affinity for the

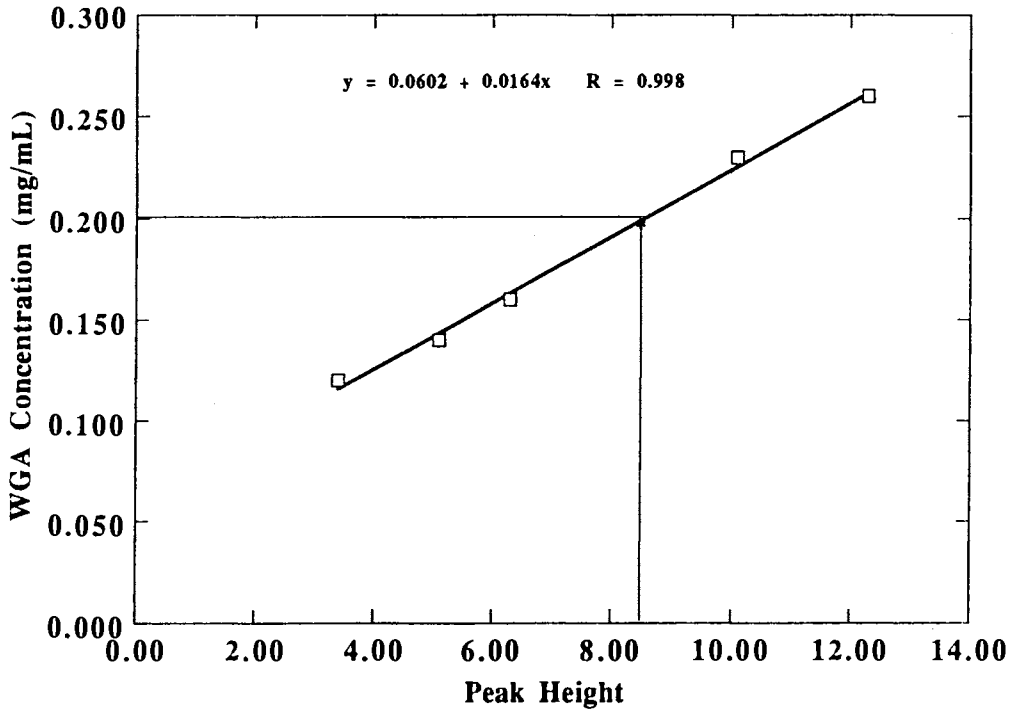

Figure 10. Calibration curve for the determination of WGA concentration. Column, 30.0 x 4.6 mm, monomeric octadecyl-silica (1.1 μm); mobile phase, 0.05% (v/v) TFA and 17.0% (v/v) acetonitrile in H_2O, flow rate, 2.0 mL/min; detection, UV, $\lambda = 280$ nm.

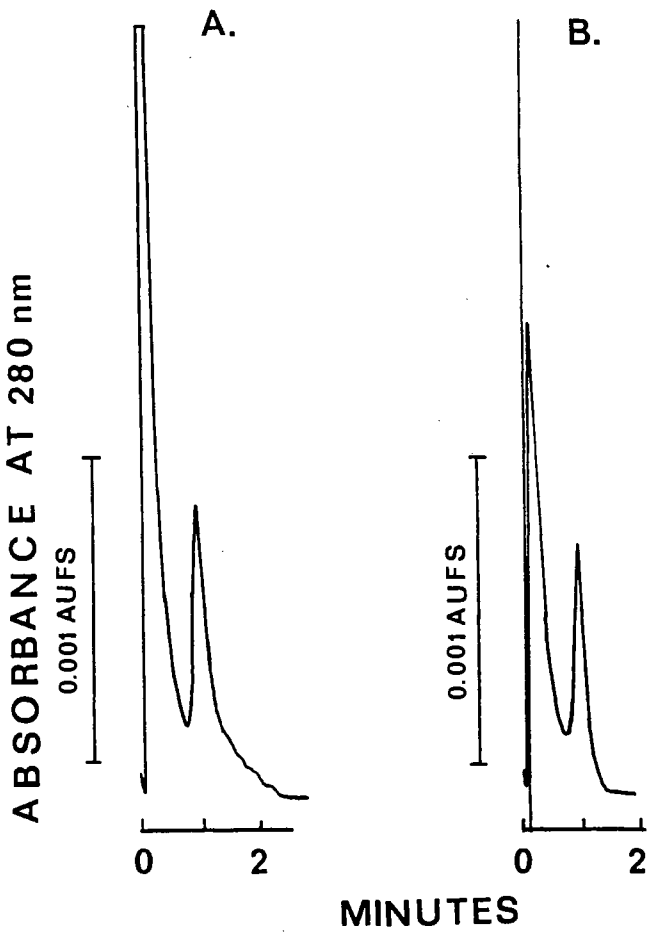

Figure 11. Chromatograms of α_1-acid glycoprotein from bovine in A and human in B obtained on nonporous silica-bound WGA stationary phase. Column, 30.0 x 4.6 mm; gradient from 0.0 to 25.0 mM *N*-acetylglucosamine in 25.0 mM Na_2HPO_4, pH 6.0, and 200.0 mM NaCl, in 0.20 min, and isocratic elution for 2.0 min; flow rate, 3.0 mL/min.

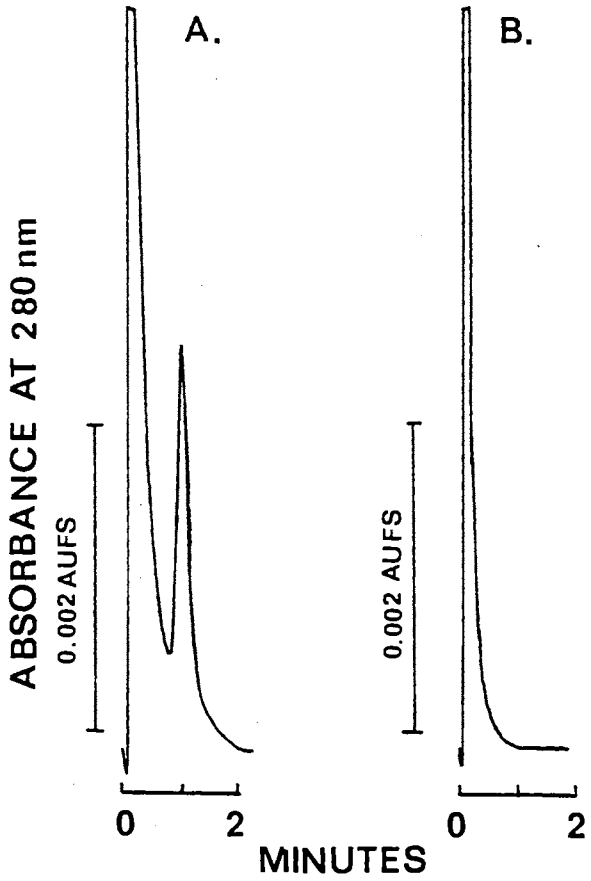

Figure 12. Chromatograms of fetuin in A and glucose oxidase in B obtained on nonporous silica-bound WGA stationary phase. Column, 30.0 x 4.6 mm; gradient from 0.0 to 25.0 mM *N*-acetylglucosamine in 25.0 mM Na_2HPO_4, pH 6.0, and 200.0 mM NaCl, in 0.20 min, and isocratic elution for 2.0 min; flow rate, 3.0 mL/min.

lectin stationary phase. silica-bound WGA, however, did not exhibit any reactivity toward glucose oxidase, Fig. 12B.

In order to bring about the retention of glycoproteins of different lectin affinity, tandem column technique was utilized. Two columns packed with nonporous silica-bound LCA and WGA stationary phases, respectively, were connected in a series, LCA→WGA. Separation of glucose oxidase and fetuin was carried out in a triple mobile phase elution mode, and the reactive components of the two glycoproteins were separated in less than five minutes, see Fig. 13. The non-reactive and slightly reactive components of fetuin and glucose oxidase could not be separated due to the low phase ratio of such column configuration. However, the tandem configuration has the advantage of doubling the peak capacity of lectin chromatography.

Lectin affinity chromatography is characterized by its inherent slow sorption kinetics which often leads to bandspreading. With porous particles, axial dispersion at high flow velocities would be influenced by two independent processes, kinetic resistance and intraparticular diffusion resistance. With nonporous affinity stationary phases, however, since there is no solute mass transfer in and out of the pores, the major contribution to bandspreading comes only from the sorption kinetics. Modification of such support having totally exposed surface of nonporous texture is expected to yield a more energetically uniform surface than the porous sorbents. As a consequence, nonporous stationary phases may exhibit the sorption kinetics that is significantly faster than that of the porous stationary phases. The high efficiency of nonporous silica-bound WGA stationary phase was demonstrated with the rapid separation of *p*-nitrophenyl derivatives of mono- and disaccharides, see Fig. 14. The high resolution was achieved even when steep gradient of strong eluent was used, and the separation was accomplished in less than 1 min.

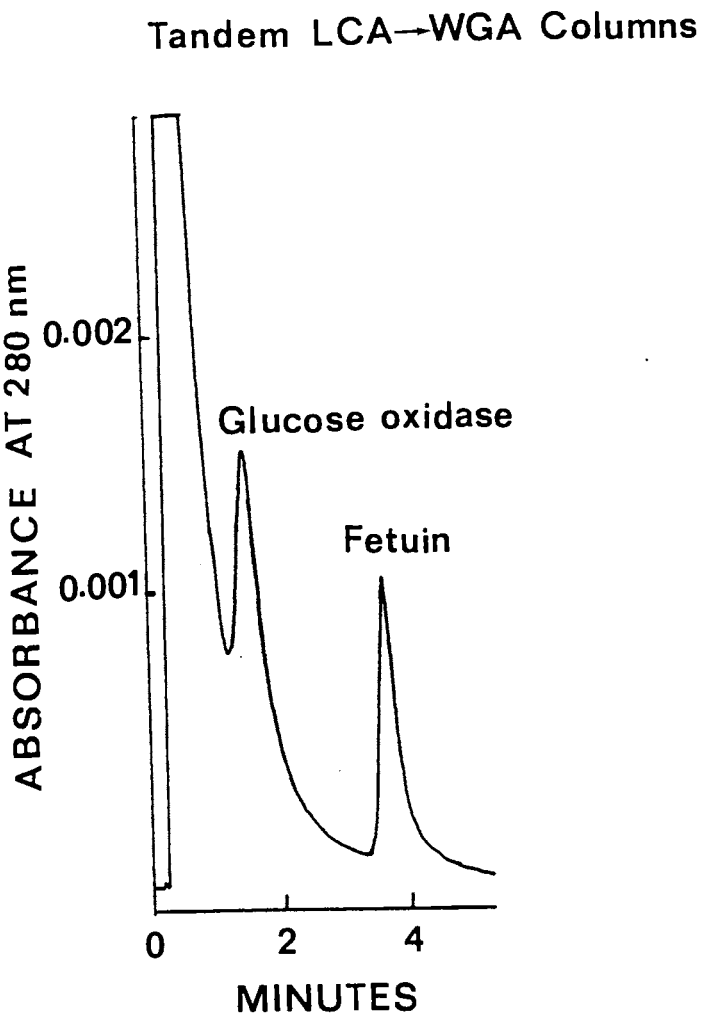

Figure 13. Chromatogram of proteins obtained on nonporous silica-bound LCA and WGA stationary phases. Columns, 30.0 x 4.6 mm; mobile phases: (A) 25.0 mM Na_2HPO_4, pH 6.0, 200.0 mM NaCl; (B) 25.0 mM methyl-α-D-mannopyranoside in mobile phase A; (C) 25.0 mM *N*-acetylglucosamine in mobile phase A; gradient from A to B in 0.20 min, isocratic elution with B for 2.0 min, then from B to C in 0.10 min, isocratic elution with C for 2.0 min.; flow rate, 3.0 mL/min.

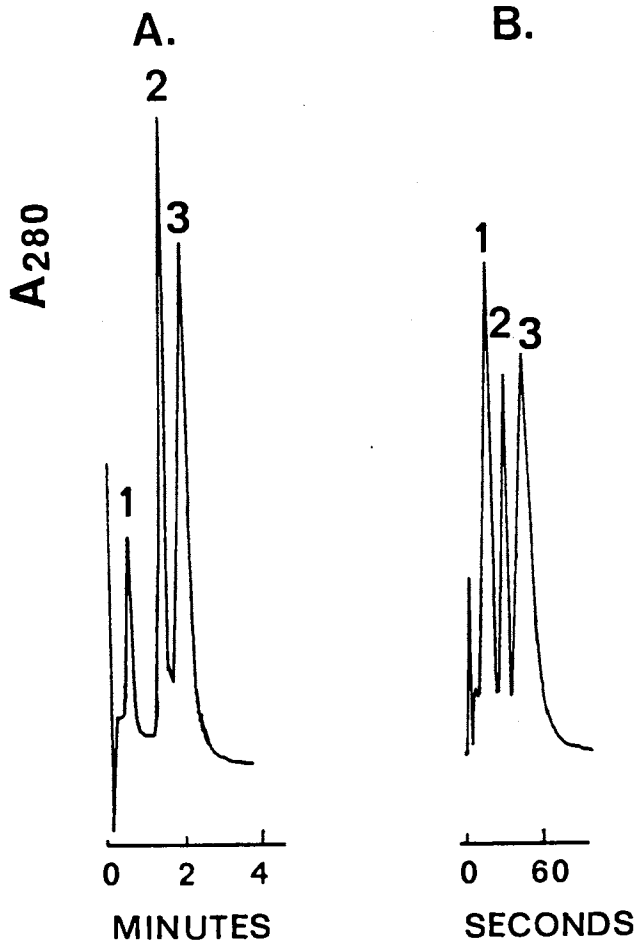

Figure 14. Chromatograms of *p*-nitrophenyl derivatives of saccharides obtained on nonporous silica-bound WGA stationary phase. Column, 30.0 x 4.6 mm; in A linear gradient from 0.0 to 50.0 mM *N*-acetylglucosamine in 100.0 mM Na_2HPO_4, pH 6.0, in 1.0 min, and isocratic elution for 2.0 min; in B linear gradient from 0.0 to 100.0 mM *N*-acetylglucosamine in 100.0 mM Na_2HPO_4, pH 6.0, in 0.50 min, and isocratic elution for 2.0 min; flow rate, 3.0 mL/min in both cases; samples, *p*-nitrophenyl derivatives of 1, *N*-acetyl-α-D-glucosaminide; 2, *N*-acetyl-β-D-glucosaminide; 3, β-D-*N*,*N*′-diacetylchitobioside; detection, UV, λ = 280 nm.

Conclusion

The chromatographic applications of monodispersed, nonporous, microspherical silica particles were examined for rapid HPLC. Surface modification with octadecyl functions for the formation of reversed phase have shown that the polymeric ODS-silica stationary phase exhibits higher phase ratio which is important for the separation of solutes of lower hydrophobicity; the monomeric ODS-silica, when the modification was properly carried out, can yield higher resolving power than the polymeric type, which is especially true when used for high-speed separation of samples of relatively higher hydrophobicity. Uniformly covered supports of lower ligand density, such as monomeric ODS without end-capping, and triphenyl functions, are suitable for the high-speed separation of biomacromolecules like proteins. The formations of lectin affinity stationary phases on the nonporous silica supports have proved to be useful for rapid affinity chromatography of heterogeneous glycoproteins.

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