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Reversed-phase liquid chromatography with microspherical octadecyl-zirconia bonded stationary phases

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ABSTRACT

Microspherical zirconia particles were synthesized and surface modified with octadecylsilane compounds for reversed-phase high-performance liquid chromatography. 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.*, tartrate 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 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 sorbents are more stable than their monomeric counterparts. These stationary phases were quite useful in the separation of polycyclic aromatic hydrocarbons, alkylbenzene and phenyl alkylalcohol 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.*, polystyrene–divinylbenzene 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 packings 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

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strength of silica with 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 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 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. 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].

Thus far, only porous zirconia packing materials have been evaluated in HPLC. Over the last few years, non-porous silica [15,16] and resin-based [17,18] stationary phases have been developed for the rapid and high-resolution HPLC of large biomolecules. Due to the absence of intraparticle mass transfer resistances, these non-porous sorbents permitted rapid separations of biomacromolecules at relatively high flow-rates without sacrificing column efficiency. This report is concerned with the introduction of "non-porous", microspherical zirconia particles 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, USA) 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, USA), and a sample injector Model 7125 from Rheodyne (Cotati, CA, USA). Chromatograms were recorded with a computing integrator Model C-R6A from Shimadzu (Columbia, MD, USA).

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, USA).

Ethylbenzene, propylbenzene, butylbenzene, benzylamine, hexamethylenetetramine, zirconyl chloride octahydrate and trimethylchlorosilane were purchased from Aldrich (Milwaukee, NJ, USA). Toluene, anhydrous denatured ethanol, butanol, *n*-heptane, light petroleum (b.p. 35–60°C), sodium chloride, and sodium hydroxide were from EM Science (Gibbstown, NJ, USA). *p*-Xylene was from Eastman Kodak (Rochester, NJ, USA). Octadecyldimethylchlorosilane and octadecyltrichlorosilane were obtained from Hüls America (Bristol, PA, USA). 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, USA). 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 were 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 35. The emulsion was homogenized using a Brinkmann dispersion unit (Westbury, NY, USA) 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 h.

After the reaction, the unreacted chemicals and organic solvents were removed in a multi-solvent cleaning process [10], using butanol light petroleum (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 "non-porous" zirconia materials, the organic free zirconia microparticles were further calcinated at 800°C for 6 h, a process during which the pore volume has been shown to approach zero [10]. The calcinated materials were then rehydroxylated using an established procedure [19]. The specific surface area, S_{BET}, of the support thus obtained was determined by nitrogen adsorption at Leeds & Northrup (Petersburg, FL, USA) and was found to be 7.3 m^2/g. Scanning electron micrography (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 [20].

The above zirconia microspheres of narrower

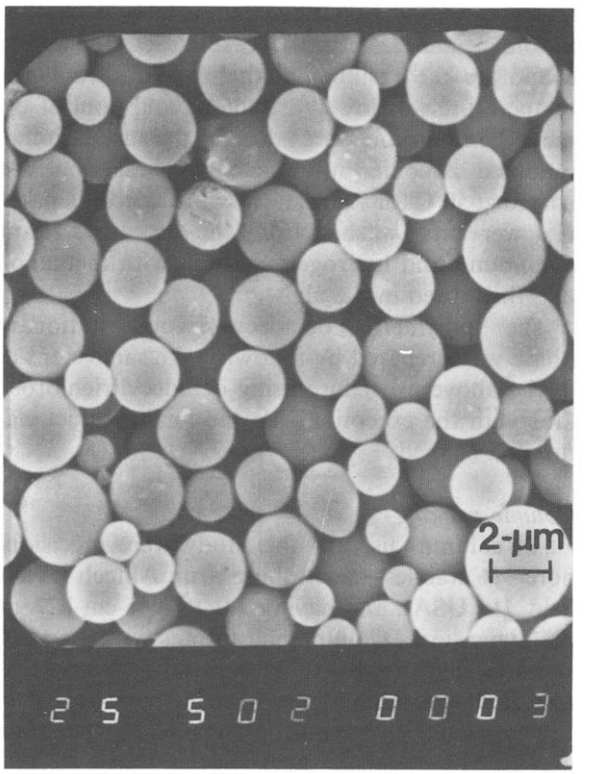


Fig. 1. Scanning electron micrograph of the zirconia microspheres.

particle size range were modified with either monomeric or "polymeric" octadecyl functions according to the following procedures. The monomeric octadecyl-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 since toluene and water form a positive heteroazeotrope with a b.p. 84.1°C [14]. Thereafter, 1 g of octadecyldimethylchlorosilane was added to the zirconia suspension. The reaction solution was stirred for 12 h 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 h. The modified zirconia particles were first washed from the unreacted silane compound and hydrogen chloride with methanol, and then dried 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 (Deerfield, IL, USA), and having 3.0 × 0.46 cm 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 Associated.

The modified zirconia microspheres were then packed from a carbon tetrachloride slurry at 8000 p.s.i. with isopropanol using a Shandon column packer instrument (Keystone Scientific, Bellefonte, PA, USA). Typically 1.5 g of surface modified zirconia microspheres were needed since zirconia is a relatively dense material (5.8 g/cm^3) [21]. 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 [22,23] 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 μmol/m^2 [24]. 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 [25]. The Lewis acid sites have been shown to undergo strong interactions with oxyanions such as phosphate containing compounds [24] 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

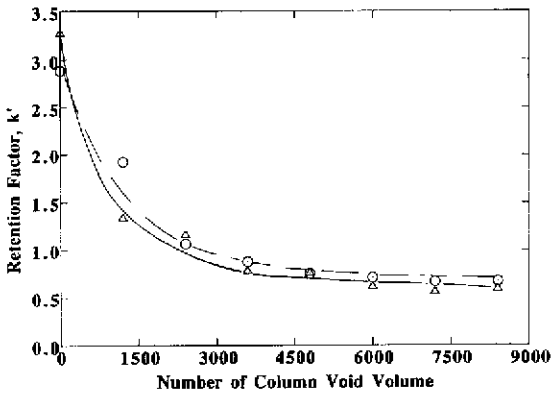


Fig. 2. Plots of retention factor of *p*-xylene *versus* the number of column void volume of solutions perfused through the column. Solutions used in the stability test, 10 m*M* NaH_2PO_4, pH 2.0 (○) and 10 m*M* Na_3PO_4, pH 12.0 (△); flow-rate, 1.0 ml/min. Column, monomeric octadecyl-zirconia with end-capping, 3.0 × 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.

solute retention in the ensuing chromatographic separation. This approach is widely practiced with silica bonded stationary phases to minimize silanophilic interactions [26].

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 m*M* phosphate buffer solutions at a flow-rate of 1.0 ml/min. At 5-h intervals, the column was first washed with water, and then equilibrated with the mobile phase. The retention of toluene, *p*-xylene, benzylamine and *trans*-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 corroborate earlier findings in that zirconium oxide was very stable in 0.1 *M* hydrochloric acid, *i.e.*, pH 1.0, and 1.0 *M* sodium hydroxide, *i.e.*, pH 14.0 [24].

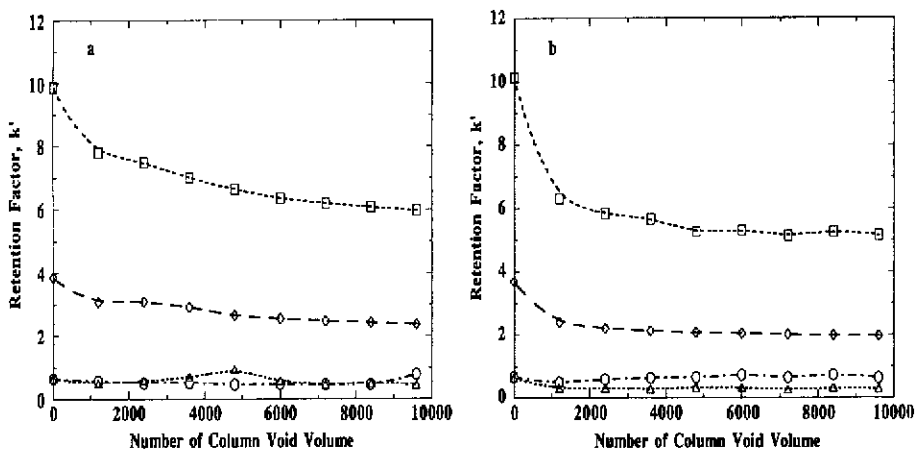


Fig. 3. Plots of retention factor of the test solutes *versus* the number of column void volume of solutions perfused through the column. Solutions used in the stability test, 10 m*M* NaH_2PO_4, pH 2.0 in (a) and 10 m*M* Na_3PO_4, pH 12.0 in (b); flow-rate, 1.0 ml/min. Column, "polymeric" octadecyl-zirconia, 3.0 × 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. ◇ = Toluene; □ = *p*-xylene; ○ = benzylamine; △ = *trans*-cinnamic acid.

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 his performance in terms of retention and separation efficiencies for a longer period of use than a monomeric octadecyl-zirconia column. The greater stability of "polymeric" octadecyl-zirconia stationary phases may be attributed to the presence of a cross linked alkylpolysiloxane layer, *i.e.*, silicon rubber, that established 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 volumes with a variety of mobile phases ranging from pH 2.0 to 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 h 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 × 0.46 cm I.D. column were evaluated with benzene, toluene, *p*-xylene and naphthalene at various acetonitrile concentrations in the mobile phase. Under these cicumstances, 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. Fig. 4a and

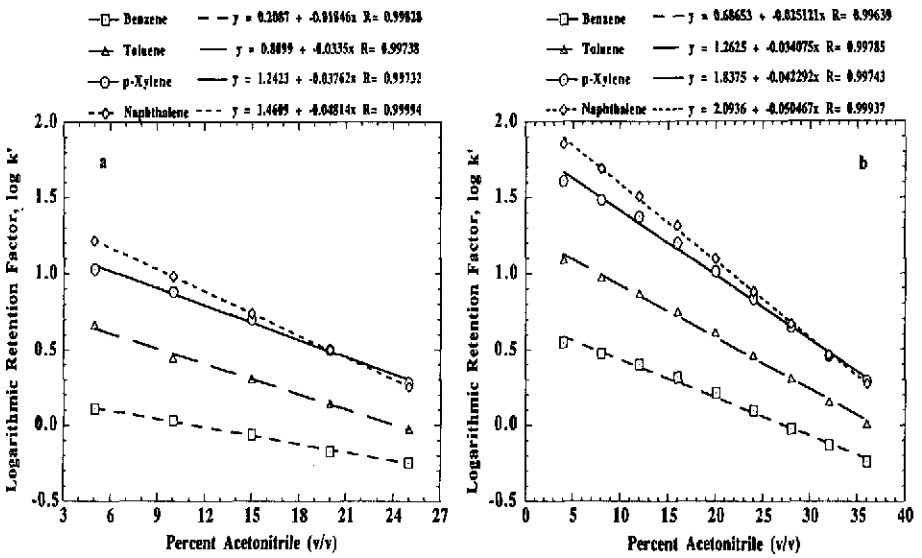


Fig. 4. Plots of logarithmic retention factor *versus* the volume percent acetonitrile in mobile phase for both monomeric (a) and "polymeric" (b) octadecyl-zirconia stationary phase. Columns, 3.0 × 0.46 cm I.D.; mobile phases, water at various volume percent acetonitrile; flow-rate, 2.0 ml/min.

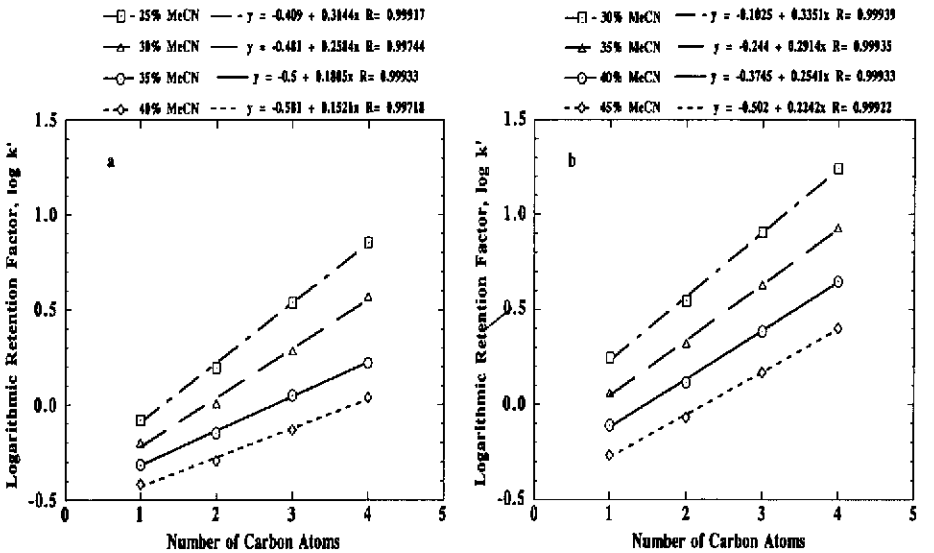


Fig. 5. Plots of logarithmic retention factor *versus* the number of carbon atoms in the alkyl chain of alkylbenzene homologous series for both monomeric (a) and "polymeric" (b) octadecyl-zirconia stationary phases. Columns, 3.0 × 0.46 cm I.D.; mobile phases, water at various volume percent acetonitrile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

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 (with end-capping) and "polymeric" octadecyl-zirconia, respectively. These plots are linear with a correlation coeffcient 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 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 obained 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 se-

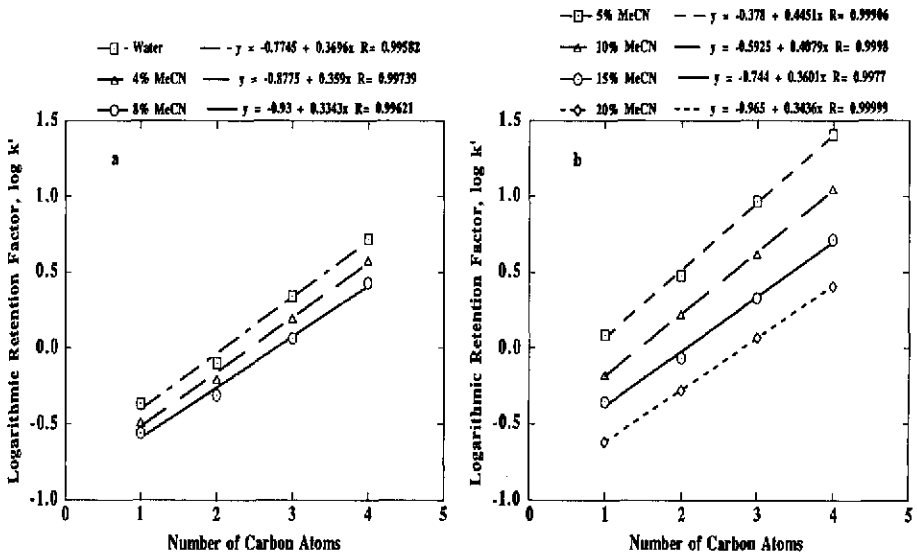


Fig. 6. Plots of logarithmic retention factor *versus* the number of carbon atoms in the alkyl chains of phenylalkylalcohol homologous series for both monomeric (a) and "polymeric" (b) octadecyl-zirconia stationary phases. Columns, 3.0 × 0.46 cm I.D.; mobile phase, water at various volume percent acetonitrile (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.

lectivity 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 [27,28] 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 energetic of solute retention on different columns. If the Gibbs free energies for a given solute are identical in both columns, *i.e.*,

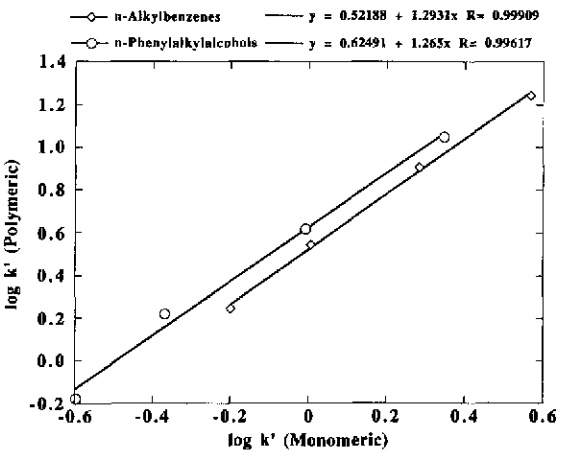


Fig. 7. Plots of logarithmic retention factor of phenylalkylalcohol and alkylbenzene homologous series on the "polymeric" phase *versus* that on the monomeric phase. Columns, 3.0 × 0.46 cm I.D.; mobile phase, water at 30% acetonitrile (v/v) for alkylbenzenes and 10% acetonitrile (v/v) for phenylalkylalcohols; 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.

the retention is homoenergetic, then plots of log k'–log k' obtained on the two stationary phases yield straight line with unit slope and the intercept is the logarithmic quotient of two columns phase ratios [26]. 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 homoenergetic [27].

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.

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 energetic 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 [29], 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 energetic 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 ra-

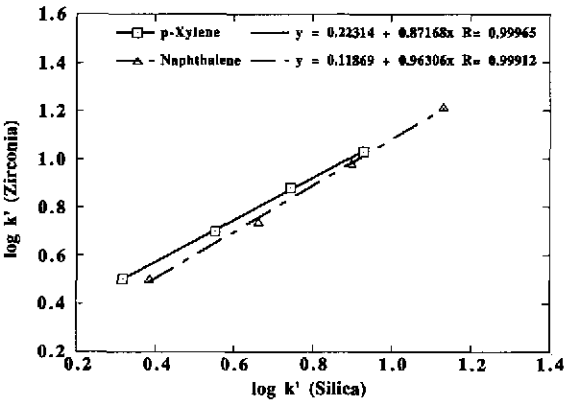


Fig. 8. Plots of logarithmic retention factor of *p*-xylene and naphthalene on octadecyl-zirconia *versus* that on octadecyl-silica. Columns, monomeric with end-capping, 3.0 × 0.46 cm I.D.; mobile phases, water at various volume percent of acetonitrile, 1, 2, 5, 10, 15 and 20% (v/v).

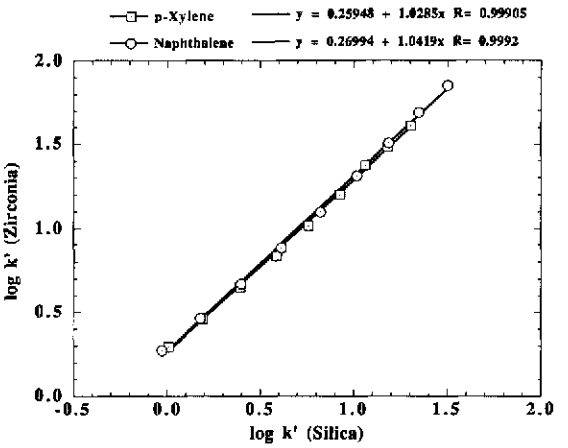


Fig. 9. Plots of logarithmic retention factor of *p*-xylene and naphthalene on octadecyl-zirconia *versus* that on octadecyl-silica. Columns, "polymeric" octadecyl-zirconia and octadecyl-silica, 3.0 × 0.46 cm I.D.; mobile phase water at various volume percent of acetonitrile, from 4 to 36% (v/v) with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

tio 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 [19]. The packing density of the zirconia particles used in this study was *ca.* 5.6 g/ml. Based on literature data [19], a non-porous silica of 0.8 μm particle diameter similar to that used in this study would have a specific surface area of 3.4 m^2/g, whereas the specific surface area of the non-porous 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.

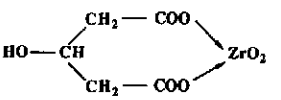
As expected, "polymeric" bonded octadecyl-zirconia stationary phases compared favorably with the silica-based stationary phases (Fig. 9). The zirconia surface was well covered with octadecyl functions in this modification process. The antilog of the intercepts of log k'–log k' plots for *p*-xylene and 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 *trans*-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 m*M* 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 [30–32].

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 [24,30–32]. The isoelectric point of the ampholytic surface can range form below 3 to above 10 depending on the source and the type of zirconia support [33]. Whereas the ligand exchange property of zirconia is the result of the presence of coordinatively unsaturated zirconium sites, the anion- and cation-exchange behavior is thought to arise from the protonation and deprotonation of surface hydroxyl groups [34], respectively.

When tartrate 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 tartrate ions may reduce the residual ion-exchange of the zirconia matrix.

In the absence of tartrate, 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

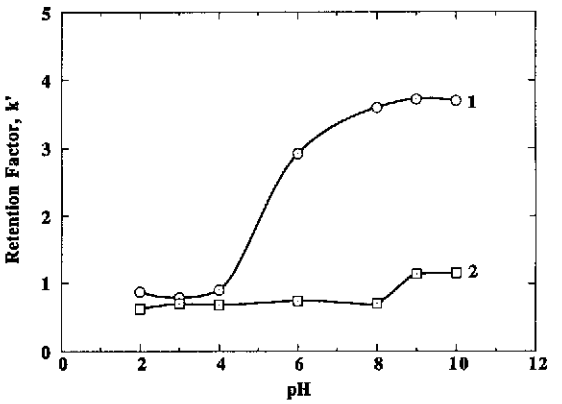


Fig. 10. Plots of the retention factor of benzylamine *versus* the pH of the mobile phase. Column, monomeric bonded octadecyl-zirconia with end-capping, 3.0 × 0.46 cm I.D.; mobile phases: curve 1, 1% (v/v) acetonitrile in all the buffer solutions; curve 2, same mobile phases as in curve 1 in the presence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

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, is 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 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 tartrate 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 tartrate to the mobile phase, the chromatographic retention was mainly due to hydrophobic interaction.

In the absence of tartrate, 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 [24]. When the pH reached 9.0 and 10.0, benzylamine peak became less broad indicating that residual adsorptivity from the support matrix was still operating. When tartrate was added to the mobile phase, slight peak tailing appeared only at pH 4.0 and 6.0.

Fig. 11 shows the behavior of benzylamine on polymeric octadecyl-zirconia in the absence and presence of tartrate in the mobile phase at different acetonitrile concentrations. As can be seen in Fig. 11, in the absence of tartrate 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

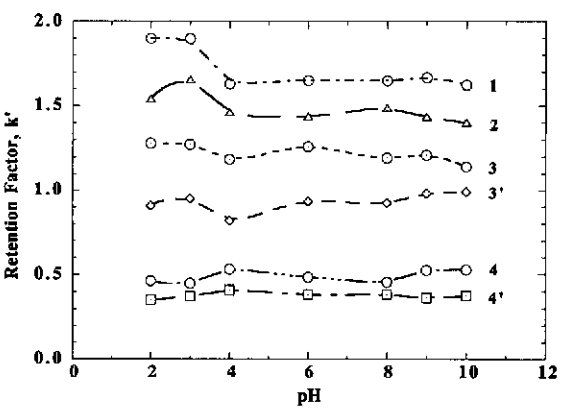


Fig. 11. Plots of the retention factor of benzylamine *versus* the pH of the mobile phase. Column, "polymeric" bonded octadecyl-zirconia, 3.0 × 0.46 cm I.D.; mobile phases: 1 = aqueous buffer solutions; 2, 3 and 4 = 1, 2 and 14% (v/v) acetonitrile, respectively, in the same buffers as in 1; 3′ and 4′ = 50 m*M* tatrate in the same buffers as in 3 and 4. Buffers and flow-rate as in Fig. 10.

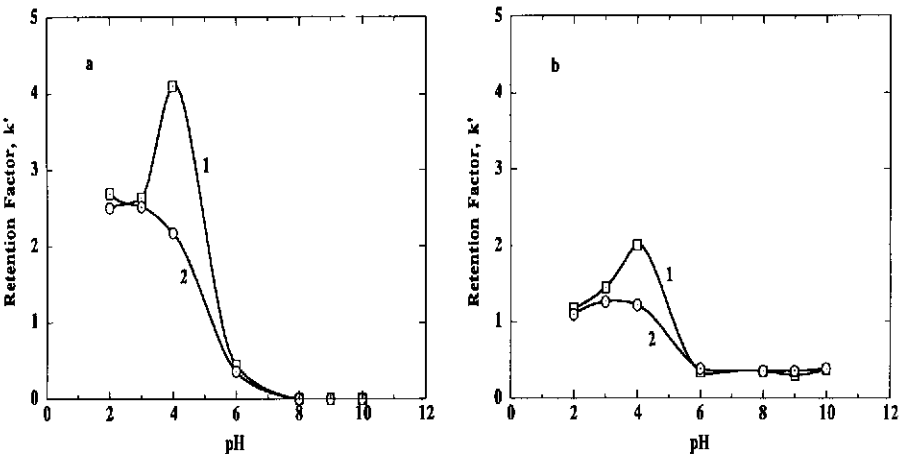


Fig. 12. Plots of retention factor of *trans*-cinnamic acid *versus* the pH of the mobile phase. Column, monomeric octadecyl-zirconia with end-capping (a) and "polymeric" octadecyl-zirconia (b), 3.0 × 0.46 cm I.D., Mobile phases: (a) 1% acetonitrile and 50 m*M* NaCl in all the buffer solutions in the absence (1) and presence of 50 m*M* tartrate (2); (b) 15% (v/v) acetonitrile and 50 m*M* NaCl in all buffer solutions in the absence (1) and presence of 50 m*M* tartrate (2). Buffers as in Fig. 10; flow-rate, 1.0 ml/min.

retention factor of benzylamine decreased slightly upon adding tartrate to the eluents, and again showed no dependence on pH (see curves 3′ and 4′ in Fig. 11). The addition of small amount of tartrate would enhance the ionic atmosphere about the solute molecules, which would cause solute interaction with the hydrophobic phase to decrease [26]. It should be noted that no peak tailing was observed for benzylamine on "polymeric" octadecyl-zirconia column in the presence or absence of tartrate ions in the eluent.

trans-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 tartrate, *trans*-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. *trans*-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 tartrate, 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, *trans*-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 tartrate the solute–support interaction was minimized, and *trans*-cinnamic acid was retained primarily by hydrophobic interactions (see curves 2 in Fig. 12a and b). At pH higher than 6.0, *trans*-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 tartrate in the mobile phase (see Fig. 12a and b).

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

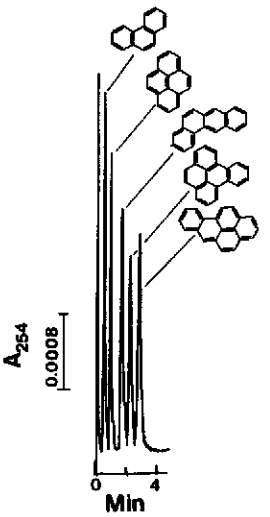


Fig. 13. Separation of polycyclic aromatic hydrocarbons. Column, "polymeric" octadecyl-zirconia, 3.0 × 0.46 cm I.D. Linear gradient in 6 min from 40 to 70% (v/v) acetonitrile in water; flow-rate, 2.0 ml/min. Solutes from left to right: phenanthrene, pyrene, benz[*a*]anthracene, benzo[*e*]pyrene and benzo[*a*]pyrene.

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 hydrocarbons (PAHs) (for recent reviews see refs. 35 and 36). It is well documented [35] 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.*, PAHs having the same number of aromatic rings, the breadth-to-length ratio (B/L) determined the relative retention of the isomers. For pyrene and benzo[*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 and 1.50 [35]. As can be seen in Fig. 13 the isomer of higher B/L was more retarded. This is identical to that reported [37] 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 re-

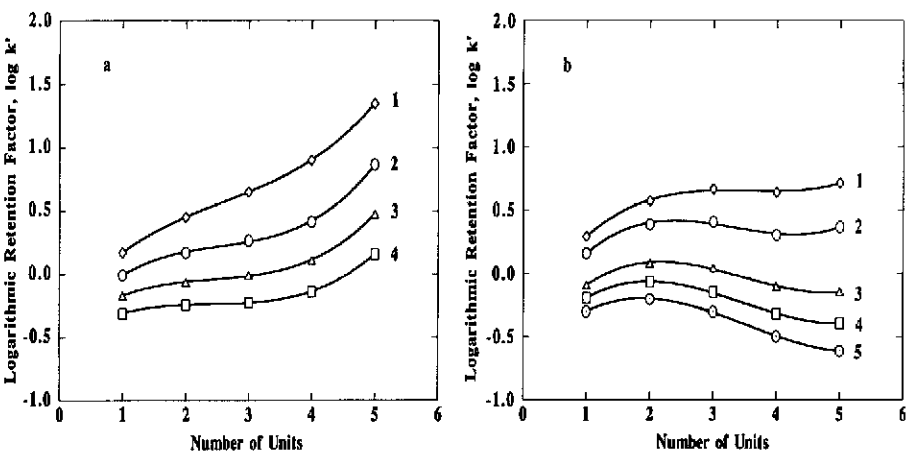


Fig. 14. Plots of logarithmic retention factor of *p*-nitrophenyl derivatives of maltooligosaccharides *versus* the number of glucose units in the homologous series. Column, "polymeric" octadecyl-zirconia (a) and "polymeric" octadecyl-silica (b), 3 × 0.46 cm I.D. Mobile phases, 0.05% trifluoroacetic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

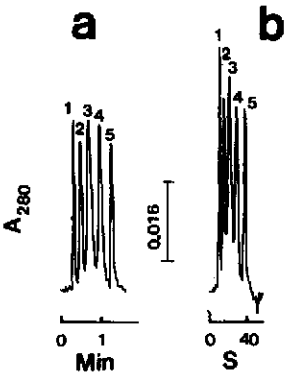


Fig. 15. Chromatograms of *p*-nitrophenyl maltooligosaccharides. Column, "polymeric" octadecyl-zirconia, 3.0 × 0.46 cm I.D., Linear gradient in 1 min (a) and 0.5 min (b) from 0 to 20% (v/v) acetonitrile in water at 0.05% (v/v) trifluoroacetic acid; Flow-rates, 1.0 ml/min (a) and 4.0 ml/min (b). Solutes: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

tention 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 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 non-porous 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 [38].

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.

Fig. 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 s 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 *trans*-cinnamic acid was greatly attenuated at high pH and/or in the presence of competing agent such as tartrate. 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 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

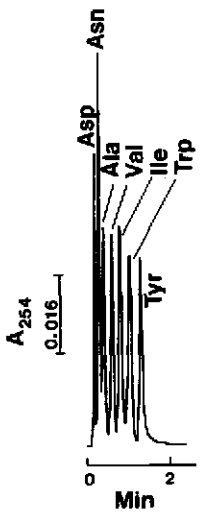


Fig. 16. Chromatogram of dansyl-amino acids. Column, "polymeric" octadecyl-zirconia, 3.0 × 0.46 cm I.D. Consecutive linear gradients, 0.8 min from 0 to 19.5%, and 0.1 min from 19.5 to 30% followed by isocratic elution for 0.2 min with 30% (v/v) acetonitrile in 5 m*M* Na_3PO_4, pH 11.0; flow-rate, 2.0 ml/min. Solutes: Asp = aspartic acid; Asn = asparagine; Ala = alanine; Val = valine; Ile = isoleucine; Trp = tryptophan; Tyr = tyrosine.

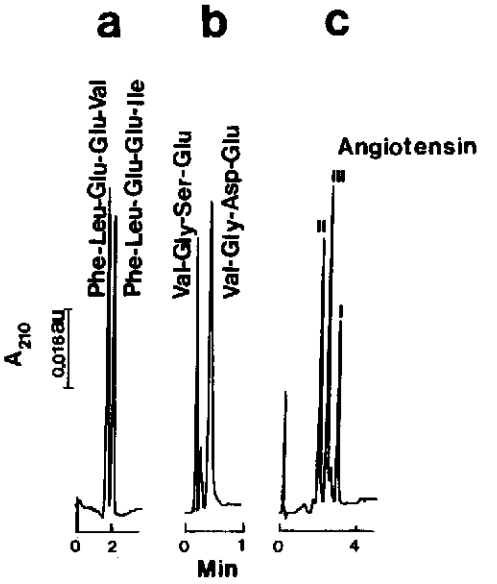


Fig. 17. Chromatograms of peptides. Column, "polymeric" octadecyl-zirconia, 3.0 × 0.46 cm I.D. Linear gradients: (a) 2 min from 0 to 20% (v/v) acetonitrile in 5 m*M* phosphate, pH 2.0; (b) 2 min from 0 to 20% (v/v) acetonitrile in 5 m*M* sodium phosphate containing 10 m*M* decyltrimethylammonium bromide,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

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 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

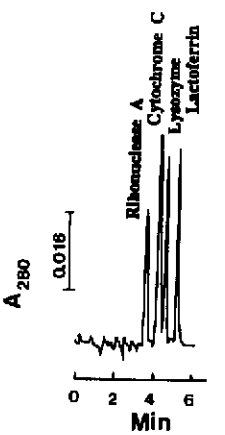


Fig. 18. Chromatogram of proteins. Column, monomeric octadecyl-zirconia, 3.0 × 0.46 cm I.D. Linear gradient in 7.0 min from 0 to 70% (v/v) acetonitrile in water at 0.05% (v/v) trifluoroacetic acid; flow-rate, 1.0 ml/min.

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, high-molecular-mass 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 is 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.

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