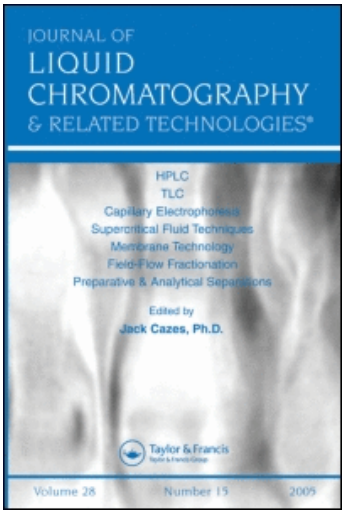

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REVERSED-PHASE LIQUID CHROMATOGRAPHY OF DANSYL AMINO ACIDS WITH MICROSPHERICAL OCTADECYL-SILICA AND OCTADECYL-ZIRCONIA BONDED STATIONARY PHASES

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

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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

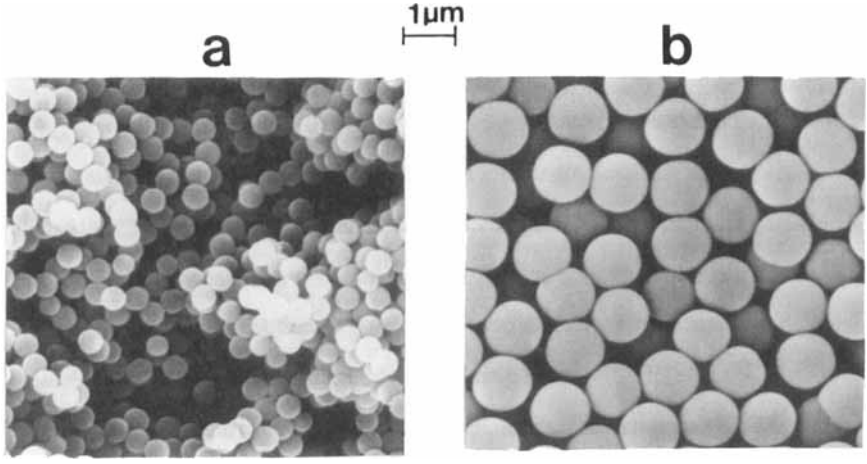

FIGURE 1. Scanning electron micrographs of the silica seed in (a) and the silica microspheres obtained by the seeded growth technique in (b).

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).

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.

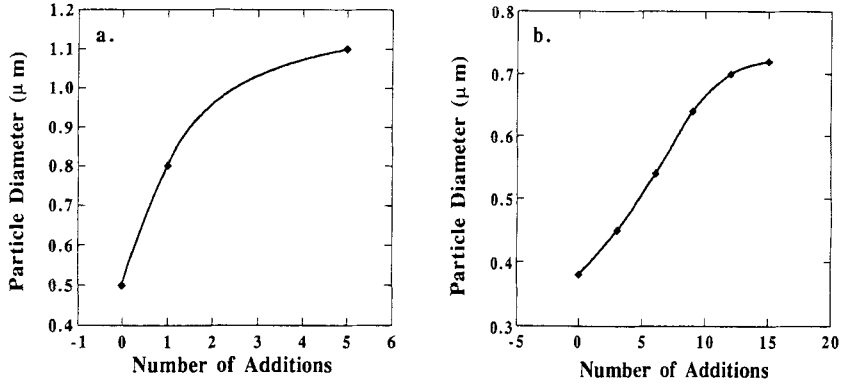

FIGURE 2. Plots of particle diameter as determined from scanning electron microscopy of the silica microspheres *versus* the number of additions of TEOS and water to the seed suspension. For experimental details see text.

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 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 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.

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

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

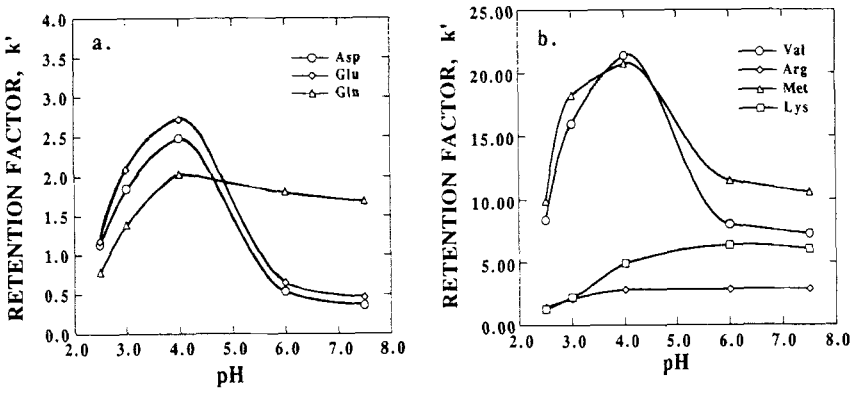

FIGURE 3. Plots of retention factor of Dns-AA *versus* pH of the eluent. Column, 30.0 x 4.6 mm, polymeric octadecyl-silica; mobile phases: 10.0 mM phosphate, pH 2.5, 3.0, 6.0, and 7.5; 10.0 mM sodium acetate, pH 4.0; all mobile phases contained 10.0% (v/v) acetonitrile; isocratic elution at 1.0 mL/min.

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

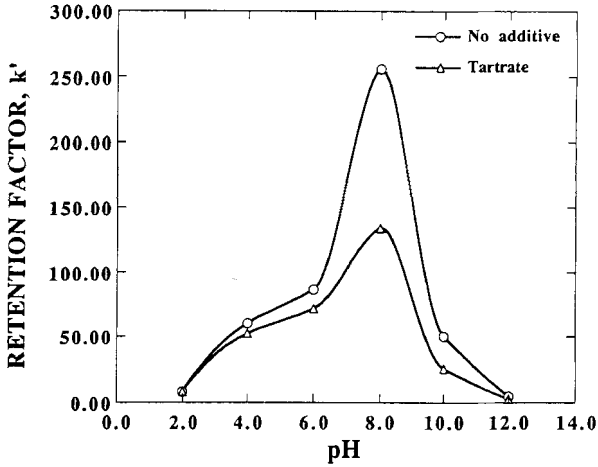

FIGURE 4. Plots of retention factor of Dns-lysine *versus* the pH of the eluent in the presence and absence of tartrate. Column, 30.0 x 4.6 mm, polymeric octadecyl-zirconia; mobile phases: 10.0 mM sodium phosphate, pH 2.0, 6.0, 8.0, 10.0 and 12.0; 10.0 mM each sodium phosphate and sodium acetate, pH 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.

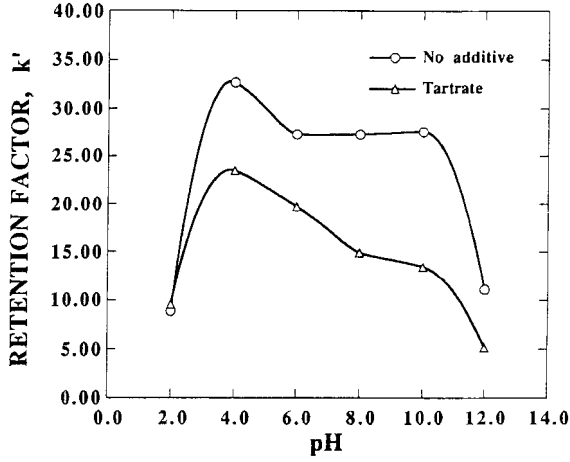

FIGURE 5. Plots of retention factor of Dns-arginine *versus* pH of the eluent in the presence or absence of tartrate. Conditions are as in Fig. 4.

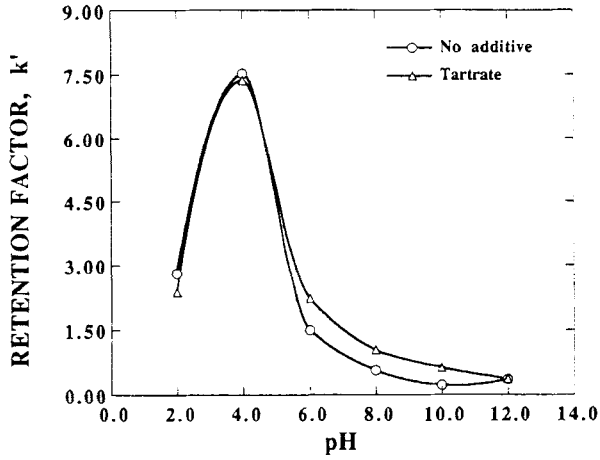

FIGURE 6. Plots of retention factor of Dns-glutamate *versus* pH of the eluent in the presence or absence of tartrate. Conditions are as in Fig. 4.

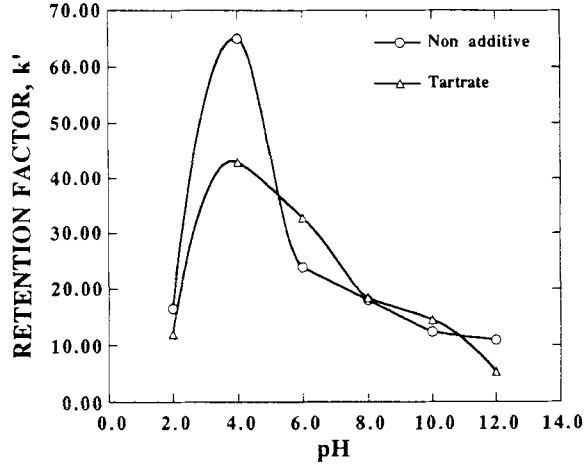

FIGURE 7. Plots of retention factor of Dns-methionine *versus* pH of the eluent in the presence or absence of tartrate. Conditions are as in Fig. 4.

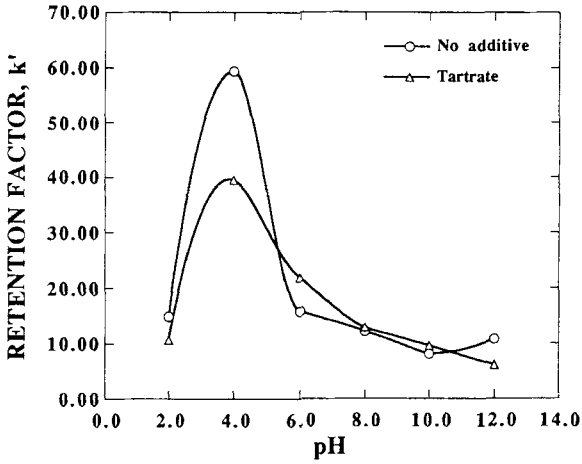

FIGURE 8. Plots of retention factor of Dns-valine *versus* pH of the eluent in the presence or absence of tartrate. Conditions are as in Fig. 4.

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 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 has 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 acquire a net negative charge at such high pH, conditions which may further contributed 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 reason for which 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 largely 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,

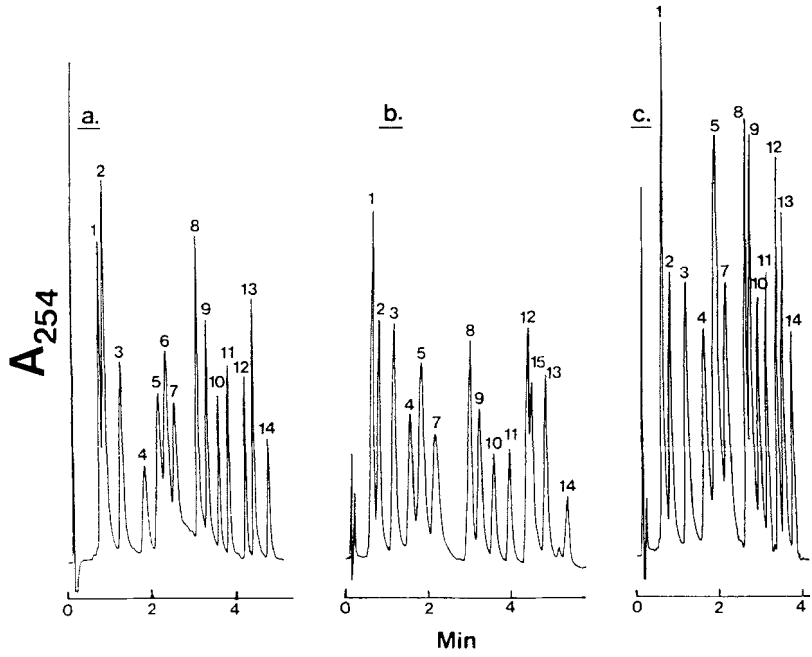

FIGURE 9. Chromatograms of Dns-AA obtained on polymeric octadecyl-silica stationary phase. Column, 30.0 x 4.6 mm; (a): consecutive linear gradients, 0.70 min from 5.0 to 6.5% (v/v), 0.10 min from 6.5 to 10.0% and 6.2 min from 10.0 to 40.0% (v/v) acetonitrile in 10.0 mM sodium phosphate containing 0.50 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.

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

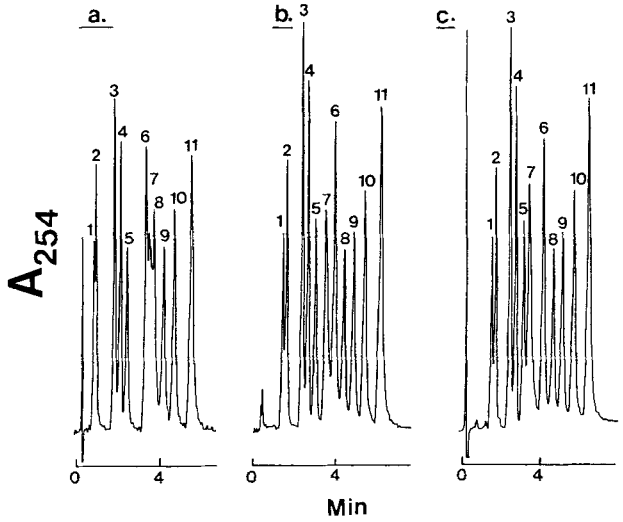

FIGURE 10. Chromatograms of Dns-AA obtained on polymeric octadecyl-zirconia stationary phase. Column, 50.0 x 4.6 mm; consecutive linear gradient, 2.0 min from 6.0 to 14.0%, 9.0 min from 14.0 to 36.0% (v/v) acetonitrile in 20.0 mM ammonium phosphate containing 0 in (a) 50.0 in (b) and 100.0 mM tartrate 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.

phosphate mobile phase system. With a steeper gradient of acetonitrile in 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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