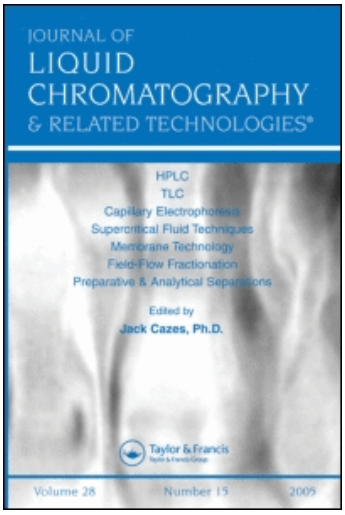

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INERTNESS AND STABILITY OF NEWLY DEVELOPED WIDE-PORE BONDED SILICA GELS

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ABSTRACT

The physically and chemically stable and batch-to-batch reproducible bonded phases of wide-pore size (300Å) were developed by using silica gels of purity over 99.99%. The chemically modified silica gels of butyl, pentyl, and octadecyl bonded phases were stable and inert for over 10,000 hours immersion in aqueous 1.0% trifluoroacetic acid (pH 1.5):CH_3CN - 50:50 and 20 mM sodium phosphate buffer (pH 10.0):CH_3CN - 50:50.

The inert and stability tests were carried out using pyridine, phenol, toluene, 8-hydroxyquinoline, and dihydroxynaphthalene. The chemical inertness and stability of these new columns have permitted the analysis of a wide variety of separations important for the pharmaceutical and biotechnological industries. The use of eluents at basic pH for peptides and enzymatic digest fragments of proteins has added a new dimension to the analysis of proteins.

INTRODUCTION

Reversed-Phase Liquid Chromatography (RPLC) is a major technique used for the separation, purification, and analysis of proteins and peptides.[1] It is commonly used as an analytical tool in biotechnology for monitoring reaction processes, determining the purity and stability of products, and studying peptide mapping.[2] The most popular columns for the separation of peptides and proteins are packed with silica-based packing materials. Especially, chemically modified packing materials from wide-pore silica gels (300Å) have become very popular for the separation of larger peptides and proteins between 10,000 and 100,000 daltons.[3] Silica gel offers good physical stability and allows a wide range of selectivities through the bonding of various phases. However, silica-based phases are generally stable only at pH between 2 and 7.5.[4]

Since chemically modified silica gels using purer silica gels were introduced,[5,6] their inertness and chemical stability have been improved. Even if the purity of silica gel is not clearly indicated, packing materials made from pure silica gels have become popular. However, the inertness and chemical stability of these phases are varied by manufacturers. The separation of polypeptides in reversed-phase liquid chromatography is based on their hydrophobicity, and their retention times can be predicted from their log P values (octanol-water partition coefficients).[7] Residual silanol groups which commonly exist in silica-based packing materials are detrimental in chromatography of peptides, giving rise to tailing peaks, excessive retention, and poor recovery. Another disadvantage of silica-based packing materials is the existence of trace metals. Many proteins and peptides are capable of metal chelation. Such secondary interactions lead to tailing peaks, low recoveries, and decreasing mass and biological activity. Metal contaminants can also indirectly contribute to peak tailing by increasing the acidity of the neighboring silanol groups.[8] The stability of bonded-phase packings is another factor of immense significance. This calls for the use of pure silica free of metal contaminations coupled with a robust bonding technique.

The stability of bonded-phase packings is largely determined by the silica support. Previous studies have shown that low pH mobile phases degrade silane stationary phases by hydrolysis[9-12] and high pH conditions influence silica support dissolution.[13,14] Consequently, most solvents which can be used on these materials

have to be within about pH 2-8.5.[15] We report here the performance of newly developed C_4, C_5 and C_{18} bonded phases prepared from pure silica gels (5μm, 300Å, over 99.99% pure). Proprietary bonding and end-capping techniques have produced inert and hydrolytically stable bonded phases. Peptide separations can now be achieved at higher pH with these stable phases. Some examples of the separations of proteins and peptides are presented.

EXPERIMENTAL

Physical Measurements

The electronmicrographs and trace element analysis of the silica gel were obtained from Ricerca (Painesville, OH) and Galbraith Laboratories (Knoxville, TN) respectively. The instruments used for particle size and surface area measurements were Mastersizer from Malvern Instruments (Southborough, MA) and ASAP 2010 from Micromeritics (Norcross, GA) respectively.

Chemicals and Instrumentation for Chromatography

All the solvents used were HPLC grade. Chemicals were purchased from Sigma Chemicals (St Louis, MO) and Aldrich (Milwaukee, WI). The pure silica gel was obtained from Phenomenex (Torrance, CA). The columns used for the comparisons, stability tests and other applications were obtained from different sources. Nucleosil C_4 and C_{18}, and Hypersil C_4 columns were obtained from Phenomenex (Torrance, CA). Vydac C_4 and C_{18} columns were purchased from Vydac (Hesperia, CA). YMC-Pack C_4-AP and YMC-Pack ODS-AP columns were purchased from YMC (Wilmington, NC). SynChropak RP-4 and SynChropak RP-P columns were purchased from SynChrom (Lafayette, IN). Zorbax 300 SB-C_{18} column was purchased from Rockland Technologies (Newport, DE). Capcell Pak C_{18} SG column was purchased from Shiseido (Japan). All columns were 250 x 4.6 mm I.D. except Capcell Pak which was 150 x 4.6 mm I.D. All the columns compared had a pore size of 300Å and a particle size of 5 μm except SynChropak columns which used 6 μm particles.

The carbon loads of these various phases were as follows: Nucleosil C_4 and C_{18} - 1 and 6% respectively, Hypersil C_4 - 2%, Vydac C_4 and C_{18} - 3 and 8% respectively, YMC-Pack C_4-AP and YMC-Pack ODS-AP - 3 and 6% respectively, and Zorbax 300 SB-C_{18} was 2.8%. The carbon loads of SynChropak RP-4, SynChropak RP-P, and Capcell Pak C_{18} SG were not reported. The reversed-phase peptide standards (RPS-I0020) were purchased from *Synthetic Peptides Inc.* (University of Alberta, Alberta, Canada).

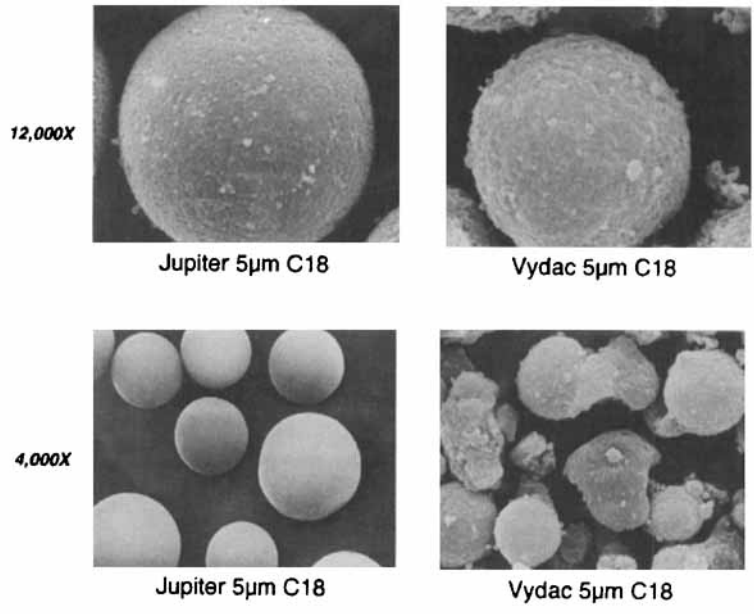

Figure 1. The electronmicrographs of bonded silica gels, Jupiter 5 µm C_{18} and Vydac 5 µm C_{18}.

The liquid chromatographs used were Models 1050, 1090, and 1100 purchased from Hewlett Packard (Waldbronn, Germany). The Hewlett Packard Chemstation was used for controlling instruments and data analysis, including measurements of column efficiency and peak asymmetry.

The properties of the developed octadecyl (Jupiter C_{18}), pentyl (Jupiter C_5), and butyl (Jupiter C_4) were studied by their chromatographic behavior of pyridine, 8-hydroxyquinoline,[16,17] and dihydroxynaphthalene.[18]

Stability tests were performed on the columns to monitor the theoretical plate number, retention factor of toluene, peak asymmetry of pyridine and toluene. The columns were exposed for over 10,000 hours to acidic and basic conditions by immersion in aqueous 1.0% trifluoroacetic acid (pH 1.5):CH_3CN - 50:50 and 20 mM sodium phosphate buffer (pH 10.0):CH_3CN - 50:50 respectively and tested under neutral conditions. These columns immersed in acidic and basic solutions were further exposed to continuous flushing using the same solutions for over 3,500 hours.

RESULTS AND DISCUSSION

The particle size of silica gel (Jupiter) was measured by using laser diffraction, and the average particle size was 5.15 ± 0.20 μm. The pore size and surface area were measured by using nitrogen adsorption, and the average pore size was 340 ± 40 Å and the average surface area was 170 ± 30 m^2/g. The amount of trace metals was measured by using inductively coupled plasma. The major metals, Fe, Na, and Ca, were 11.2, 13.4, and 8.9 ppm respectively. The total metal content was less than 37 ppm. The contents of trace metals was very little compared to that of a commercially available silica gel whose metal content is about 600 ppm.[6]

The electronmicrographs of bonded silica gels (Jupiter C_{18} and Vydac C_{18}) are shown in Fig.1. The surface of Jupiter C_{18} was smoother compared to that of Vydac C_{18}. Furthermore, the particle shape of Jupiter C_{18} was very uniform compared to that of Vydac C_{18}. The pure silica gel, Jupiter C_{18}, contained less fine particles, which in turn stabilize column back pressure and increase column performance over the long term.

The % carbon loading of these modified silica gels were measured by elemental analysis, and those of octadecyl (Jupiter C_{18}), pentyl (Jupiter C_5), and butyl (Jupiter C_4) silica gels were 13.25, 5.50, and 5.00 % respectively.

Under acidic conditions, the majority of silanol groups (Si-OH) on the silica surface are protonated. The low pH helps to suppress any undesirable ionic interactions between the solute and packing due to the presence of non-derivatized silanols.[19,20] Hence, they do not contribute significantly to any peak tailing. On the other hand, above pH > 8, silanols are ionized and capable of giving rise to acid-base interactions. Such interactions generally cause peak tailing.

The existence of ionized silanols can be detected by chromatographic behavior of basic compounds. Aniline and toluidine isomers were used to measure the existence of such silanols,[21] however, pyridine was more sensitive to detect such residual silanols.[22] When the surface of bonded silica gels is completely covered by alkyl groups, pyridine elutes as a sharp and symmetrical peak.[16,17] When the surface coverage is not sufficient, pyridine elutes as a tailing peak, typically co-eluting with phenol or eluting after phenol. If the interactions are strong, pyridine peak cannot be detected within a short time.

The comparable chromatograms of a mixture of pyridine, phenol, and toluene on butyl and pentyl bonded phases of 300Å pore size are shown in Fig.2A where toluene was used to measure the column efficiency, (theoretical plate number). Under identical chromatographic conditions, Jupiter C_5 and Jupiter C_4 demonstrated high peak asymmetry of pyridine compared to ordinary bonded

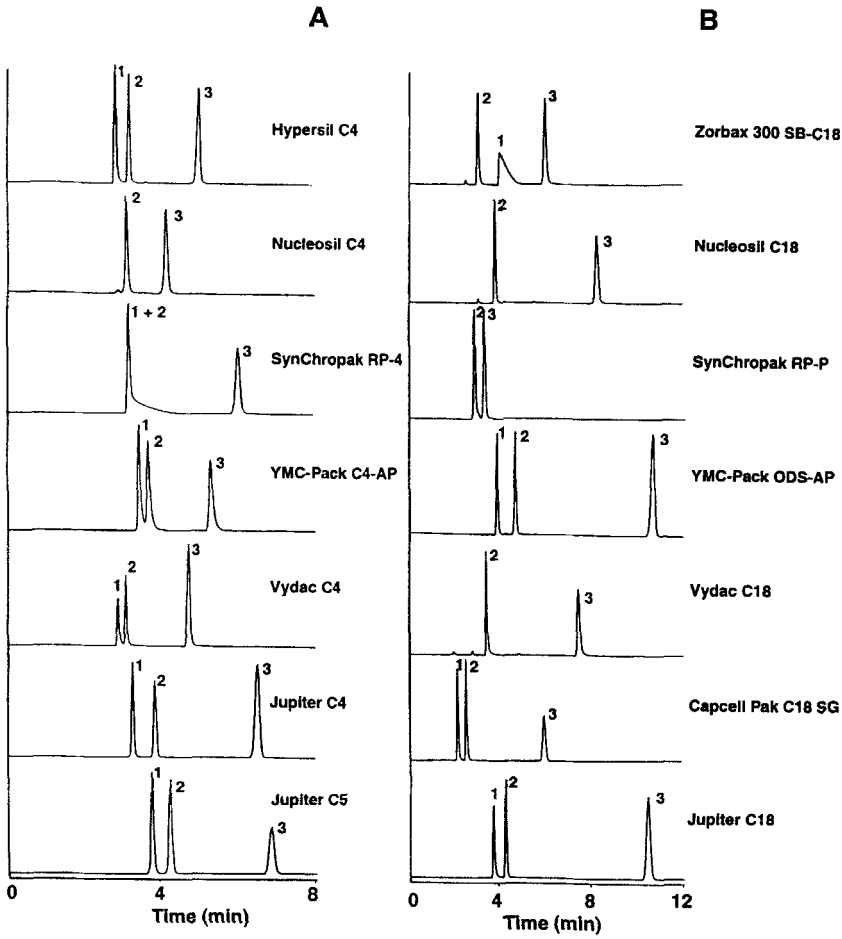

Figure 2. Chromatograms of inert test mixture on various chemically modified silica gels of (A) short alkyl chains, and (B) octadecyl bonded phases. Chromatographic conditions: Sample: solution of pyridine, phenol, and toluene in acetonitrile; flow-rate: 1.0 mL/min; detection: UV @ 254 nm; eluent: 50% aqueous acetonitrile; peaks 1, 2, and 3: pyridine, phenol, and toluene.

phases made from wide-pore silica. However, the retention time of toluene indicated differences in retention capacity due to their hydrophobicity. The values varied from 4.8 to 7.2 min. Significant differences can be observed from the chromatographic behavior of pyridine. When the peak shape of pyridine is sharp, depicts less tailing, and elutes before phenol, the activity of silanol groups is negligible.[17] These chromatograms can be classified by the order of inertness

Table 1

Inertness and Metal Sensitive Tests of Chemically Modified Silica Gels

Column	Pyridine As	Pyridine tR, (min)	Toluene As	Toluene tR, (min)	HDQ As	DHN As	DERT N_1/N_2
Jupiter C4	1.23	3.63	0.85	7.22	1.33	1.35	0.94
Jupiter C5	1.06	3.82	0.93	6.92	1.59	1.32	0.92
Vydac C4	1.52	3.31	0.96	5.35	1.64	1.43	0.55
YMC-Pack C4-AP	1.54	3.77	1.23	5.78	1.85	2.86	7.84
SynChropak RP-4	a	a	1.01	6.96	2.94	1.37	0.92
Nucleosil C4	nd	nd	0.96	4.79	nd	nd	nd
Hypersil C4	1.85	3.21	0.74	5.69	3.13	3.23	2.29
Jupiter C18	1.47	3.78	1.08	10.49	1.18	1.41	0.86
Vydac C18	nd	nd	1.32	7.49	1.69	1.33	0.91
YMC-Pack ODS-AP	1.43	3.61	0.83	9.66	1.85	1.54	0.91
SynChropak RP-P	nd	nd	1.16	3.42	2.78	b	b
Zorbax 300 SB-C18	nd	nd	1.16	8.35	2.01	nd	nd
Nucleosil C18	1.45	2.22	1.01	5.96	1.67	1.54	0.83
Capcell Pak C18 SG	9.09	4.28	1.41	6.23	5.88	1.64	0.85

HDQ - 8-Hydroxyquinoline; DHN- 2,3-Dihydroxynaphthalene; a - Co-elution with phenol; b - Co-elution of two isomers; nd - Not detected within a certain time.

against pyridine, Jupiter C_5, Jupiter C_4 > Vydac C_4, YMC-Pack C4-AP, Hypersil C4 > SynChropak RP-4 >> Nucleosil C_4. The separation between pyridine and phenol became poor on YMC-Pack C4-AP, and pyridine peak showed a very long tailing and co-elution with phenol on SynChropak RP-4.

However, pyridine peak could not be observed within 10 min on Nucleosil C_4. It may be bound with active silanol groups. The values of peak asymmetry and retention time of pyridine and toluene are summarized in Table 1.

The same test was carried out on octadecyl bonded silica gels of wide-pore size (300Å) (Fig.2B). The retention factor of toluene varied from 0.10 to 2.53. A short retention time of 3.4 min was indicative of a column with low retention capacity. Pyridine eluted after phenol with long tailing even if the retention time of toluene was 8.3 min on Nucleosil C_{18}. The retention time of toluene was short (3.4-6.2 min) on SynChropak RP-P and Zorbax 300 SB-C_{18}. Pyridine eluted after phenol on SynChropak RP-P and Zorbax 300 SB-C_{18}, especially, pyridine peak could not be observed within a short time from Vydac C_{18}. The values of peak asymmetry and retention time of pyridine and toluene are summarized in Table 1. Only columns Jupiter C_{18}, YMC-Pack ODS-AP, and Capcell Pak C_{18} SG were acceptable for chromatographic separation of very basic compounds among these octadecyl bonded silica gel columns.

Metal contaminants can prevent maximum bonded phase coverage of the silica surface, indirectly contribute to sample peak tailing by increasing the acidic nature of the neighboring silanol groups,[8] and furthermore form complexes with a variety of compounds resulting in poor peak shapes or adsorption of compounds during the liquid chromatographic process. The existence of metals on the surface of silica-based packing materials can be detected by the chromatographic behavior of chelating agents such as acetylacetone, catechol, salicylaldehyde, 8-hydroxyquinoline, α-benzoinoxomine, and 2,3-dihydroxynaphthalene.[18,23,24]

Euerby et.al. proposed that 2,3-dihydroxynaphthalene was a suitable reagent to detect the existence of metals.[18] 2,3- and 2,7-Dihydroxynaphthalene efficiency ratio test (DERT) was designed to determine the surface metal contamination of silica gels. The DERT value compares the peak efficiency of two regioisomers, 2,3- and 2,7-dihydroxynaphthalene. The former possesses the ability to chelate with metals while the latter does not. On a lower metal content bonded phase, the 2,7-dihydroxynaphthalene should elute before the 2,3-dihydroxynaphthalene and the ratios of peak efficiencies for the 2,7/2,3-dihydroxynaphthalene (DERT value) should be close to unity. On the other hand, a value of infinity means that the 2,3-dihydroxynaphthalene analyte elutes very slowly over a long time, effectively losing the peak in the baseline noise, or that the analyte binds strongly to the metals and fails to be eluted.[25] Metal contaminated low-purity phases typically yield DERT values much greater than unity, infinity (cannot be detected within a certain time), or show co-elution of the two isomers. Figs.3A and B show some comparisons between Jupiter bonded phases and ordinary bonded phases using 2,3- and 2,7- dihydroxynaphthalene. The results are summarized in Table 1. The peak asymmetry of 2,3-dihydroxynaphthalene on Jupiter C_{18} and comparable C_{18} bonded phases varied from 1.33 to 1.64 except SynChropak RP-P and Nucleosil C_{18}. There was no significant difference among these columns tested except SynChropak RP-P and Nucleosil C_{18}. 2,3-Dihydroxynaphthalene and 2,7-dihydroxynaphthalene co-eluted on SynChropak RP-P and 2,3-dihydroxynaphthalene appears to be adsorbed on Nucleosil C_{18}. The plate number ratio of 2,7-dihydroxynaphthalene to 2,3-dihydroxynaphthalene (N_1/N_2) was also identical among these columns except

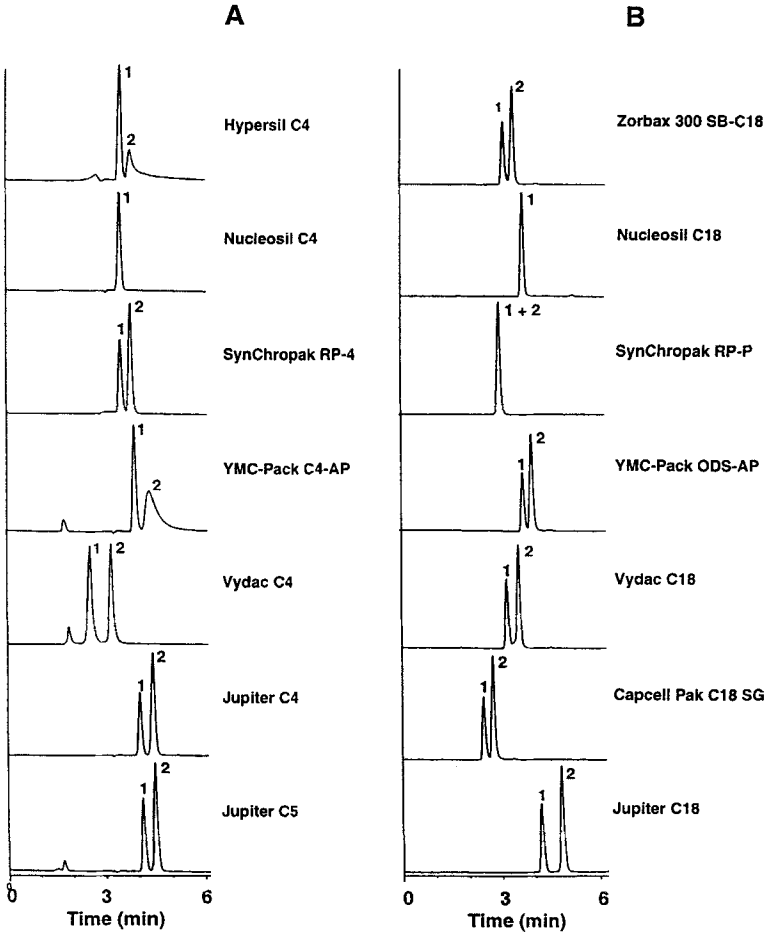

Figure 3. Chromatograms of 2,3- and 2,7-dihydroxynaphthalene on various chemically modified silica gels of (A) short alkyl chains, and (B) octadecyl bonded phases. Chromatographic conditions: Sample: solution of 2,3- and 2,7-dihydroxynaphthalene in acetonitrile; flow-rate: 1.0 mL/min; detection:UV @ 230 nm; eluent: 50% aqueous acetonitrile; peaks 1 and 2: 2,7- and 2,3-dihydroxynaphthalene.

SynChropak RP-P and Nucleosil C_{18}. The theoretical plate number and resolution between these two compounds showed variations. However, on comparing the DERT values of these columns, 2,3-dihydroxynaphthalene seemed to be a less sensitive reagent than 8-hydroxyquinoline to detect trace metals on the surface of packing materials (Table 1).

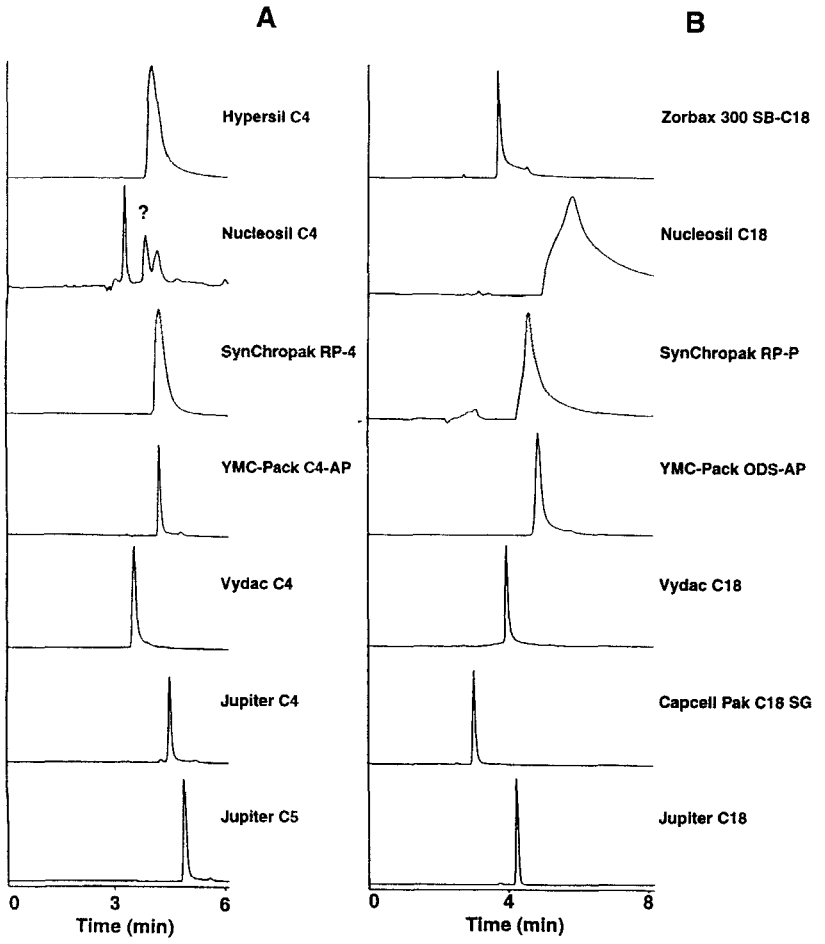

Figure 4. Chromatograms of 8-hydroxyquinoline on various chemically modified silica gels of (A) short alkyl chains, and (B) octadecyl bonded phases. Chromatographic conditions: Sample: 8-hydroxyquinoline in acetonitrile; flow-rate: 1.0 mL/min; detection: UV @ 220 nm; eluent: 50% aqueous acetonitrile.

The N_1/N_2 ratio on Jupiter C_{18} and non-endcapped Jupiter C_{18} were identical. This implies that the silica gel (Jupiter) was very pure according to the DERT value. The same test was carried out on Jupiter C_4, Jupiter C_5, and other C_4 bonded phases (Fig.3A). The values of peak asymmetry of 2,3-dihydroxynaphthalene varied from 1.32 to 3.23 except Nucleosil C_4 on which the chelating agent seemed to be adsorbed. The plate number ratio of 2,7-dihydroxynaphthalene to 2,3-dihydroxynaphthalene (N_1/N_2) was very poor on Vydac C_4, YMC-Pack C4-AP,

and Hypersil C_4. These results on C_4 bonded phases using dihydroxynaphthalene isomers indicated that the DERT value can differentiate heavy metal contaminated silica gels from others. When compared to earlier data on C_{18} bonded phases, the DERT values on C_4 bonded phases were more indicative of metal contamination. This is due to less hydrophobicity of C_4 phases than C_{18} phases.

In comparison to 2,3-dihydroxynaphthalene, which is not sensitive enough to detect metal contaminants, 8-hydroxyquinoline commonly used as a reagent to extract trace metals from water, is a very active chelating reagent and an excellent probe to detect trace amounts of heavy metals. 8-Hydroxyquinoline was also used to detect trace metals on the silica surface of butyl, pentyl, and octadecyl bonded phases. The peak asymmetry of 8-hydroxyquinoline was measured on a variety of columns and the values are summarized in Table 1. The order of peak asymmetry of 8-hydroxyquinoline on Jupiter C_4, Jupiter C_5, and other C_4 bonded phases was Jupiter C_4 > Jupiter C_5 > Vydac C_4 > YMC-Pack C_4-AP > SynChropak RP-4 > Hypersil C_4 >> Nucleosil C_4 as shown in Fig.4A. The values of peak asymmetry of 8-hydroxyquinoline on these columns varied from 1.33 to 3.13 except Nucleosil C_4. 8-Hydroxyquinoline may be adsorbed on Nucleosil C_4 as the peak was not observed within a short time. If the original silica gels of these bonded phases were completely endcapped, peak tailing indicates the existence of trace metals on the surface which interrupt the bonding process. On the other hand, if these silica gels were the same, peak tailing indicates variations in the degree of bonding.

Examples of chromatograms of 8-hydroxyquinoline run on octadecyl bonded silica gels are shown in Fig.4B and the results are summarized in Table 1. The peak asymmetry of 8-hydroxyquinoline on bonded silica gels made from pure silica gels was better than that obtained on ordinary bonded silica gels. The peak asymmetry of Jupiter C_{18} (1.18) was better than Jupiter C_4 (1.33) and Jupiter C_5 (1.59) even when the same bonding technology was used.

Both the inert and metal sensitive tests were carried out using one batch of commercially available columns and three batches each of Jupiter C_4, C_5, and C_{18} columns. The reproducibility of Jupiter C_4, C_5, and C_{18} columns was quite good. The relative standard deviation of inertness for pyridine and 8-hydroxyquinoline was less than 5% (n = 3).

Jupiter C_4, C_5, and C_{18} were stable and inert for over 10,000 hours immersion in solutions of mixtures of aqueous 1.0% trifluoroacetic acid (pH 1.5) and acetonitrile (50:50) and that of 20 mM sodium phosphate buffer (pH 10.0) and acetonitrile (50:50). Jupiter C_5 columns were further exposed for over 3,500 hours (35,000 column volumes) under continuous flushing in the same solutions. The theoretical plate number and the peak asymmetry of toluene were constant. The retention factor of toluene and peak symmetry of pyridine were also constant as seen in Fig.5 for Jupiter C_{18} columns.

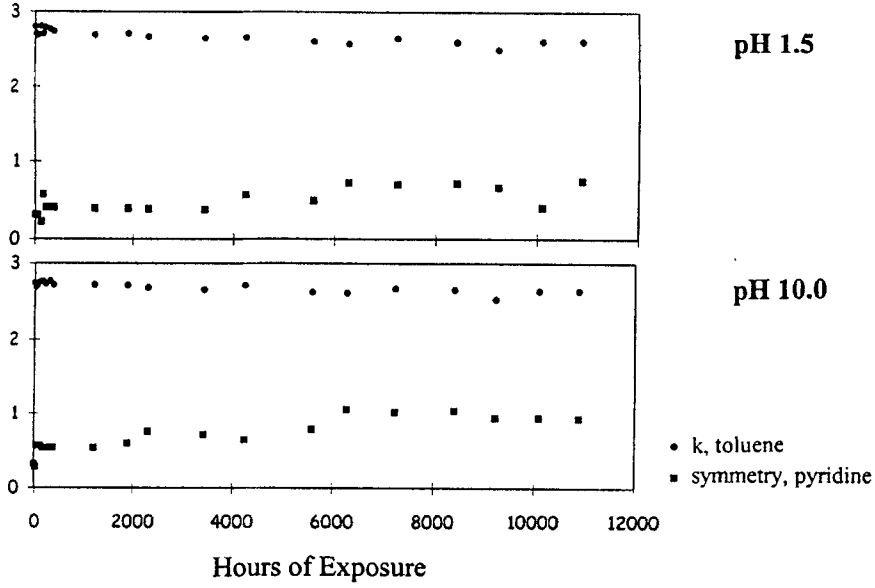

Figure 5. Stability of Jupiter 5 μm C_{18} columns for over 10,000 hours immersion in acidic (pH 1.5) and basic (pH 10.0) solutions. (See conditions in text).

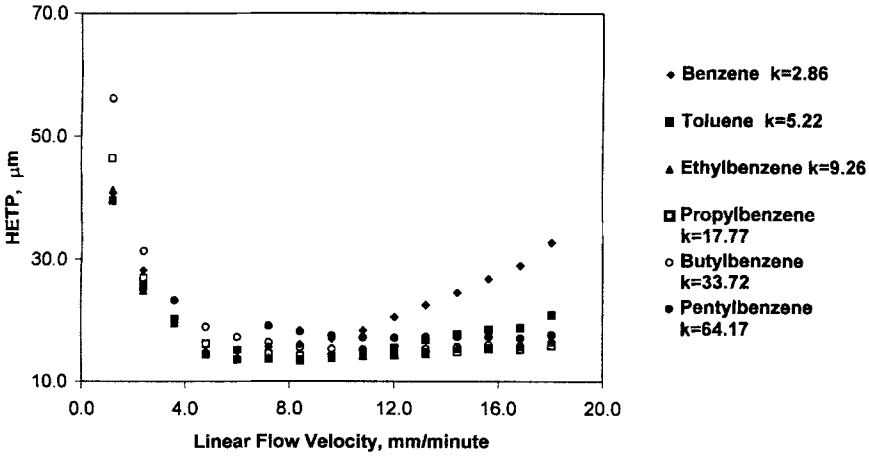

Figure 6. HETP vs. linear flow velocity of Jupiter 5 μm C18. Chromatographic conditions: Column size: 50 x 4.6 mm I.D.; sample: mixture of alkylbenzenes (k: 2.86 to 64.17) in acetonitrile; detection: UV @ 260 nm; eluent: 40% aqueous acetonitrile.

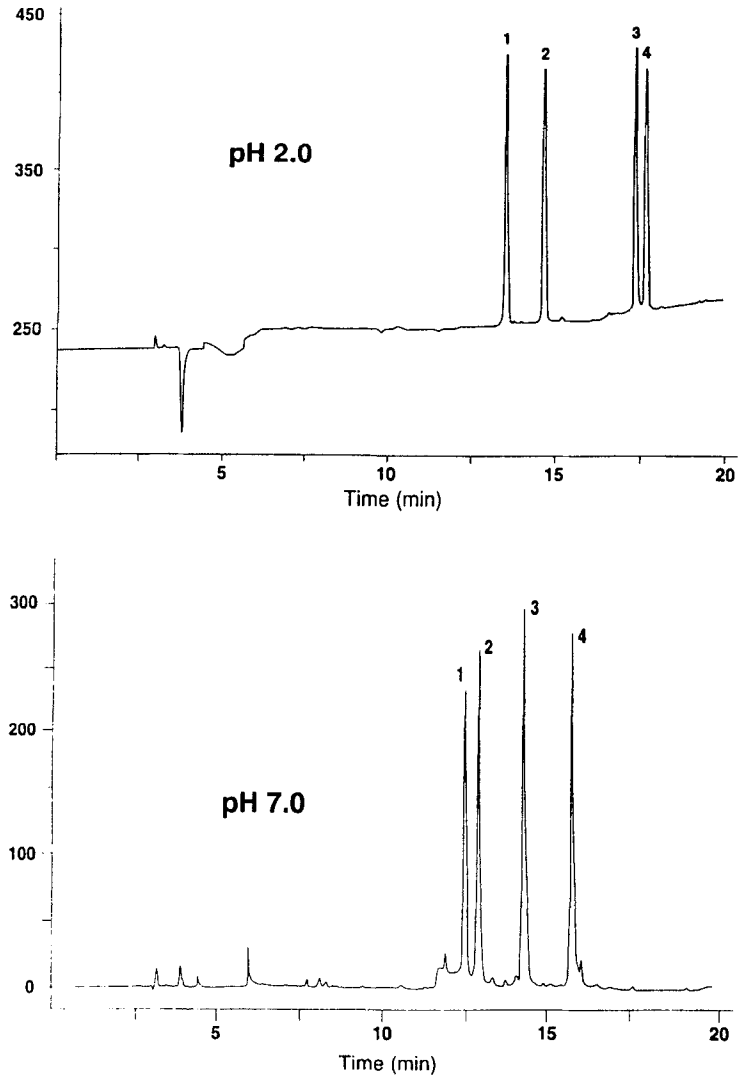

Figure 7. pH effect on separation of peptide standards on Jupiter 5 μm C_{18}. Chromatographic conditions: Column size: 250 x 4.6 mm I.D.; sample: four peptides of the following sequences: 1. **+1** Ac-Gly-Gly-Gly-Leu-Gly-Gly-Ala-Gly-Gly-Leu-**Lys**-Amide, 2. **+2** Ac-**Lys**-Tyr-Gly-Leu-Gly-Gly-Ala-Gly-Gly-Leu-**Lys**-Amide, 3. **+3** Ac-Gly-Gly-Ala-Leu-**Lys**-Ala-Leu-**Lys**-Gly-Leu-**Lys**-Amide, and 4. **+4** Ac-**Lys**-Tyr-Ala-Leu-**Lys**-Ala-Leu-**Lys**-Gly-Leu-**Lys**-Amide; flow-rate: 1.0 mL/min; detection: UV @ 210 nm; eluent: (a) pH 2.0: A: 0.1% TFA in H_2O, B: 0.1% TFA in CH_3CN, (b) pH 7.0: A: 20 mM phosphate buffer, pH 7.0, B: CH_3CN, for both pH, linear gradients run from 0 - 40% B in 20 min (2% B/min).

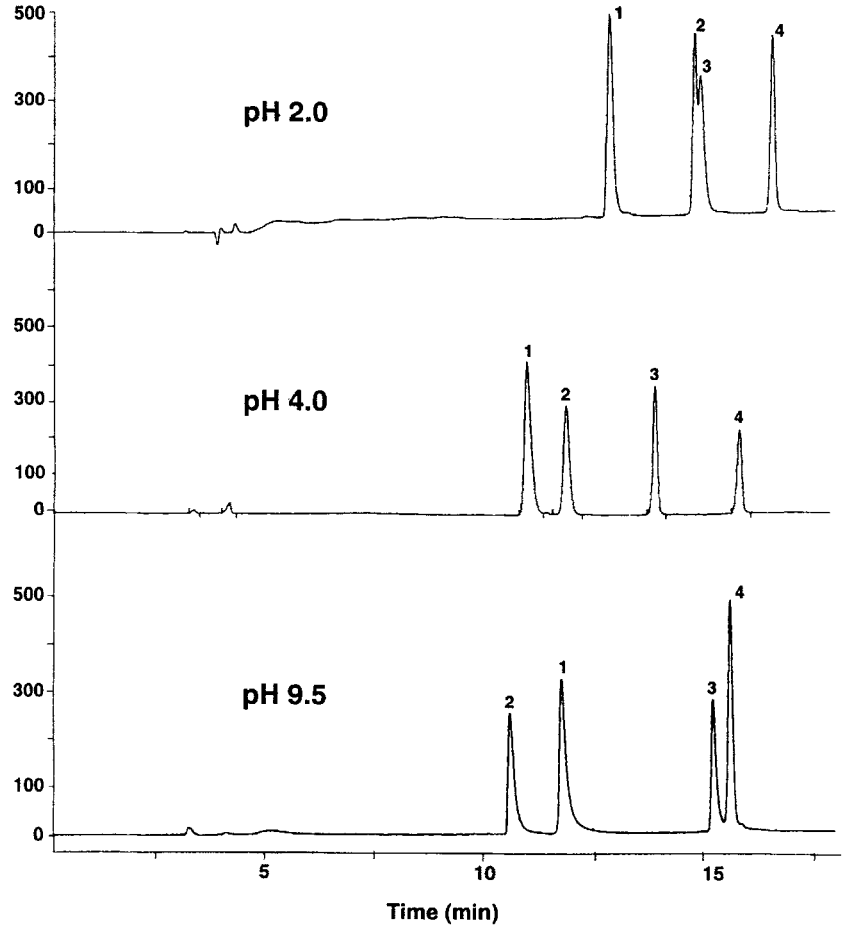

Figure 8. pH effect on pepide selectivity on Jupiter 5 μm C_{18}. Chromatographic conditions: Column size: 250 x 4.6 mm I.D.; sample: bioactive peptides 1, 2, 3, and 4: bradykinin, neurotensin, bombesin, and eledoisin; flow-rate: 1.0 mL/min; detection: UV @ 214 nm; eluent: (a) pH 2.0: A: 0.1% TFA in H_2O, B: 0.1% TFA in CH_3CN, (b) pH 4.0 and 9.5: A: 20 mM potassium phosphate buffer, pH 4.0 or 9.5, B: CH_3CN, for all three pH, step gradients run from 5-20% in 5 min followed up to 40% B in 13 min.

For LC-MS compatibility, the stability tests were also carried out by exposing Jupiter C_{18} columns for over 300 hours (13,408 column volumes) of continuous flush in aqueous 30 mM ammonium hydroxide (pH 10.0) and acetonitrile (50:50) mixture. The retention factor of toluene and the peak asymmetry of toluene and pyridine were constant.

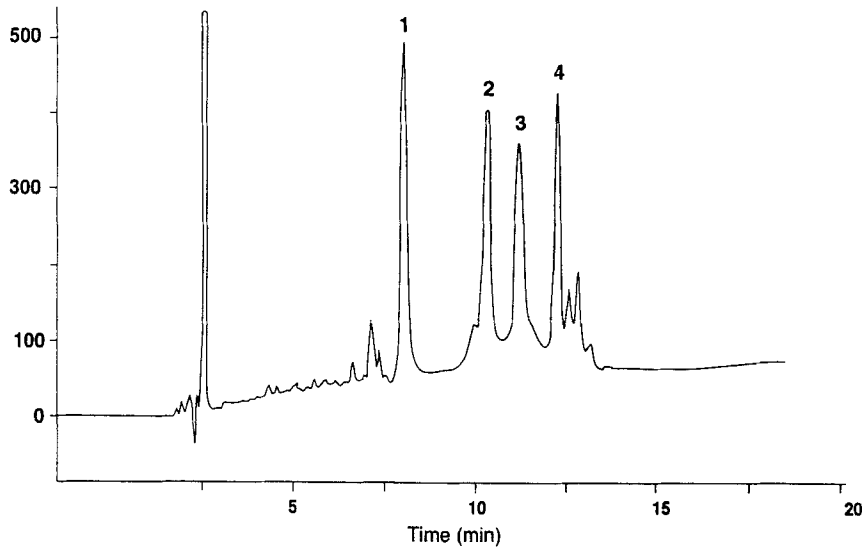

Figure 9. Separation of proteins on Jupiter 5 μm C5. Chromatographic conditions: Column size: 250 x 4.6 mm I.D.; sample: peaks 1, 2, 3, and 4: bovine serum albumin, peroxidase (horse-radish), catalase (beef liver), and carbonic anhydrase (bovine); flow-rate: 1.0 mL/min; detection: UV @ 220 nm; eluent: A: 0.1% TFA in H_2O, B: 0.1% TFA in CH_3CN, step gradients run from 10-40% B in 5 min (6% B/min) followed upto 65% B in 15 min (1.67% B/min).

The column performance of Jupiter C_{18} was measured from HETP of alkylbenzenes in 40% aqueous acetonitrile. The column size was 50 x 4.6 mm I.D. The results are shown in Fig.6. The HETP value was minimum at a linear flow velocity of 7.0 mm/min. The HETP values were about 15 μm between a linear flow velocity of 5.0 ~ 16.0 mm/min for components having retention factors 5 ~ 34. Such low HETP values at high flow rates can reduce analysis time.

The above results indicated that Jupiter C_4, C_5, and C_{18} were chemically very stable and suitable for the separation of proteins and peptides in acidic and basic conditions. The examples of liquid chromatographic separations of peptides influenced by pH of eluents are shown in Figs.7 and 8. In Fig.7, a mixture of four synthetic peptide standards, peptides 1 to 4 containing, respectively, 1 to 4 basic residues (lysine residues) was used for monitoring silanol activity of Jupiter C_{18}. At pH 2.0, silanol ionization is suppressed and, hence, sharp, well-defined peaks are expected. However, at neutral pH, ionized, free silanols are capable of influencing chromatography of basic solutes. Under these conditions, all four standards were well resolved on this column, exhibiting good peak shape and thereby, demonstrating an inert bonded phase. The slightly longer retention times of the

peptides in the pH 2.0 mobile phase compared to the pH 7.0 system is due to the more hydrophobic nature of the TFA anionic counterion compared to the phosphate ion.[26] Eluent pH can have a dramatic effect on peptide selectivity as seen in Fig.8. Mobile phase pH can thus be modulated to achieve improved separations at higher pH. An example of protein separation on Jupiter C_5 is shown in Fig.9. Jupiter C_5 demonstrated identical selectivity as that obtained on Jupiter C_4, however, the chemical stability of Jupiter C_5 was equivalent to that of Jupiter C_{18}. Jupiter C_5 can be used for a variety of applications generally performed on butyl bonded phases.

CONCLUSIONS

Newly developed, butyl, pentyl, and octadecyl bonded silica gels of pore-size 300Å and purity of over 99.99% were inert for nitrogen-containing and metal-sensitive compounds. Furthermore, pentyl and octadecyl bonded silica gels were very stable for long term operations in 50% acetonitrile solutions of 1.0% trifluoroacetic acid (pH 1.5) and 20 mM sodium phosphate buffer (pH 10.0). These packing materials were suitable for the analysis of proteins.

REFERENCES

1. F. E. Regnier, LC/GC, **5(5)**, 392 (1987).

2. M. Kunitani, D. Johnson, L. R. Snyder, J. Chromatogr., **371**, 313 (1986).

3. W. G. Burton, K. D. Nugent, T. K. Slattery, B. R. Summers, J. Chromatogr., **443**, 363 (1988).

4. N. Nagae, D. Ishii, Am. Lab., March (1995).

5. T. Hanai, M. Ohhira, T. Tamura, LC-GC, **6(10)**, 922 (1988).

6. M. Ohhira, F. Ohmura, T. Hanai, J. Liq. Chromatogr., **12(6)**, 1065-1074 (1989).

7. M. J. O'Hare, E. C. Nice, J. Chromatogr., **171**, 209 (1979).

8. R. K. Iler, in **The Chemistry of Silica**, R. K. Iler, ed. John Wiley & Sons, New York, 1979, p. 668.

9. J. L. Glajch, J. C. Gluckman, J. G. Charlkofsky, J. M. Minor, J. J. Kirkland, J. Chromatogr., **318**, 23-29 (1985).

10. J. L. Glajch, J. J. Kirkland, U.S. Pat. 4, 705, 725 (1987).

11. J. L. Glajch, J. J. Kirkland, LC-GC, **8**, 140-150 (1990).

12. J. J. Kirkland, J. L. Glajch, R. D. Farlee, Anal. Chem., **61**, 2-11 (1988).

13. J. J. Kirkland, M. A. van Straten, H. A. Claessens, J. Chromatogr., **691**, 3 (1995).

14. J. J. Kirkland, J. W. Henderson, J. Chromatogr. Sci., **32**, 473 (1994).

15. **Introduction to Modern Liquid Chromatography**, 2nd Ed.,L. R. Snyder, J. J. Kirkland, eds., John Wiley & Sons, New York, 1979, p. 281.

16. T. Hanai, **Encyclopedia of Analytical Science**, Academic Press, London, 1995, pp. 2558-2567.

17. **Advances in Liquid Chromatography**, T. Hanai, H. Hatano, eds., World Scientific, Singapore, London, New York, 1996.

18. M. R. Euerby, C. M. Johnson, I. D. Rushin, D. A. S. Sakunthala Tennekoon, J. Chromatogr. A, **705**, 229-245 (1995).

19. D. Guo, C. T. Mant, A. K. Taneja, J. M. R. Parker, R. S. Hodges, J. Chromatogr., **359**, 499 (1986).

20. **High Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis, and Conformation**, C. T. Mant, R. S. Hodges, eds., CRC Press, Boca Raton, 1991, p. 289.

21. H. Engelhardt, H. Low, W. Gotzinger, J. Chromatogr., **544**, 371 (1991).

22. Y. Sudo, J. Chromatogr. A, **737**, 139 (1996).

23. K. M. Mackay, R. A. Mackay, **Introduction to Modern Inorganic Chemistry**, 2nd ed., Intext Education Publishers, New York, 1973.

24. M. Verzele, D. Dewaele, Chromatographia, **18**, 84 (1984).

25. R. Vespalec, J. Neca, J. Chromatogr., **281**, 35-47 (1983).

26. **High Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis, and Conformation**, C. T. Mant, R. S. Hodges, eds., CRC Press, Boca Raton, 1991, p. 302.

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