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Chromatographic evaluation of alkyl-silicas prepared through a hydride-silica intermediate

Maria Cristina Montes
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hydride-silica intermediate**

Montes, Maria Cristina, M.S.

San Jose State University, 1992

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Ann Arbor, MI 48106**

Chromatographic Evaluation of Alkyl-Silicas Prepared
through a Hydride-Silica Intermediate

A Thesis Presented to
the Faculty of the Department of Chemistry
San Jose State University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

By
Maria Cristina Montes
December, 1992

APPROVED FOR THE DEPARTMENT OF CHEMISTRY

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ABSTRACT

CHROMATOGRAPHIC EVALUATION OF ALKYL-SILICAS PREPARED THROUGH A HYDRIDE-SILICA INTERMEDIATE

by Maria Cristina Montes

The chromatographic characterization of alkyl bonded silica-based stationary phases for high performance liquid chromatography is described. A previously established method involving catalytic hydrosilation of terminal olefins on a hydride-modified intermediate was used to obtain octyl (C_8) and octadecyl (C_{18}) packings. Generally speaking, the resulting phases were found to be chromatographically equivalent (comparable retention, selectivity and column efficiency) to sorbents produced in a conventional manner using organosilanization. The new bonded phases are, however, significantly more stable toward hydrolysis than conventional bonded silicas. This highly desirable feature of the new bonded phases not only reduces the need for frequent column replacement but also provides enhanced long-term reproducibility of retention data. Finally, these highly stable bonded phases exhibit good separation of polycyclic aromatic hydrocarbons, basic solutes and polypeptides under a variety of mobile phase compositions.

TABLE OF CONTENTS

List of Tables	v
List of Figures	vi
Abstract	xiii
1. Introduction	1
1.1. Background	1
1.2. Aims of this Work	5
2. Experimental	7
2.1. Materials	7
2.2. Instrumentation	9
2.2.1. Infrared Spectrometry.....	9
2.2.2. Elemental Analysis	10
2.2.3. Liquid Chromatography	10
2.3. Procedures	10
2.3.1. Preparation of Bonded phases	11
2.3.1.1. New Bonded Phases via Hydride Intermediate	11
2.3.1.1.1. Silanization with TES	11
2.3.1.1.2. Olefin Hydrosilation	11
2.3.1.2. Conventional Bonded Phases by Organosilanization	12
2.3.1.3. Surface Coverage	13
2.3.2. Crown Compounds Retention vs Eluent	

Composition Experiments	14
2.3.3. Long-Term Hydrolysis Experiments.....	16
2.3.4. HPLC Separations	17
2.3.4.1. Separation of Proteins and Peptides on Alkyl-Silicas	17
2.3.4.2. Separation of Polycyclic Aromatic Hydrocarbons on Octadecyl-Silicas ...	19
2.3.4.3. Separation of Basic Solutes on Alkyl-Silicas	19
2.3.4.4. Separation of Alkaloids on Octadecyl- Silicas	20
3. Discussion and Results	21
3.1. Silanophilic and Solvophobic Interactions.....	21
3.2. Hydrolytic Stability	35
3.3. HPLC Separations.....	49
3.3.1. Polycyclic Aromatic Hydrocarbons	51
3.3.2. Basic Solutes	57
3.3.3. Separation of Proteins and Peptides	93
3.3.4. Separation of Alkaloids	100
4. Conclusions	102
5. Bibliography	104

LIST OF TABLES

Table I.	Native Silicas Supports Used in This Work	8
Table II.	Bonded Silicas Used in This Work	15
Table III.	Carbon Coverage and Retention Data for Octyl-Bonded Silicas (Vydac TP) during Long-Term Hydrolysis Test with TFA-Containing Aqueous Acetonitrile Mobile Phases	41
Table IV.	Retention and Selectivity Data for the Three-Component Standard SRM869 Mixture on Octadecyl Silicas	55

LIST OF FIGURES

Figure 1.	Solvent gradient used for the long-term hydrolysis test	18
Figure 2.	Schematic representation of the structures of octyl-bonded, silica based supports prepared via conventional organosilanization and olefin hydrosilation	25
Figure 3.	Plot of logarithmic retention factor of DB24C8 on conventional (not end-capped) C ₈ -Vydac TP silica as a function of the volume fraction of water in aqueous acetonitrile eluent	28
Figure 4.	Reciprocal retention factors for DB24C8 and DB18C6 measured on bare Vydac TP silica as a function of the volume fraction of water in aqueous acetonitrile eluent	29
Figure 5.	Reciprocal retention factors for DB24C8 and DB18C6 measured on Partisil-10 silica as a function of the volume fraction of water in aqueous acetonitrile eluent	30
Figure 6.	Reciprocal retention factors for DB24C8 and DB18C6 measured on Partisil-10 silica as a function of the volume fraction of water in aqueous methanol eluent	31

Figure 7.	Plots of the logarithmic retention factor of DB24C8 against the volume fraction of water in aqueous acetonitrile eluent, as a function of the surface chemistry of the sorbent	33
Figure 8.	Plots of the logarithmic retention factor of DB18C6 against the volume fraction of water in aqueous acetonitrile eluent, as a function of the surface chemistry of the sorbent	34
Figure 9.	Capacity factor (k') of 1-phenylheptane as a function of column volumes of mobile phase passed through the columns during the long-term stability test	39
Figure 10.	Relative loss of retention for 1-phenylheptane as a function of column volumes of mobile phase passed through the columns during the long-term hydrolysis test	40
Figure 11.	Alkyl surface coverage of octyl-Vydac TP as a function of hydrolysis time.....	43
Figure 12.	Relative surface coverage of octyl-Vydac TP as a function of hydrolysis time	44
Figure 13.	Conventional C_8 -Vydac TP (end-capped) before and after chromatographic long-term hydrolysis	45
Figure 14.	Conventional C_8 -Vydac TP (not end-capped) before and after chromatographic long-term	

hydrolysis	46
Figure 15. Fraction of initial retention as a function of column volumes of TFA-containing mobile phase for a commercial polymeric C ₈ Vydac TP	47
Figure 16. Structures of the three PAH components of standard mixture SRM869	52
Figure 17. Typical separation profile for the SRM869 standard mixture on a C ₁₈ phase YMC-Gel prepared via hydride	54
Figure 18. Separation of isomeric terphenyls on several C ₁₈ phases	56
Figure 19. Separation of isomeric binaphthyls on several C ₁₈ phases	58
Figure 20. Components of Standard Reference Material (SRM1647), consisting of 16 PAH's from the Environmental Protection Agency's priority pollutant list	59
Figure 21. Separation profiles for 16 PAH's (SRM1647)) on C ₁₈ phases prepared through hydride	60
Figure 22. Calculation of peak asymmetry by measuring the A/B ratio at 10% of peak height	64
Figure 23. Separation of the polar test mixture on a commercial C ₈ -Vydac TP silica	66
Figure 24. Separation of the polar test mixture on a	

home-made conventional C ₁₈ -Nucleosil silica ...	67
Figure 25. Asymmetry of polar compounds on conventional alkyl phases	68
Figure 26. Half-height column efficiency from polar compounds on conventional alkyl phases	69
Figure 27. Asymmetry-based column efficiency from polar compounds on conventional alkyl phases	70
Figure 28. Separation of the polar test mixture on C ₁₈ -bonded silicas prepared through hydride ...	72
Figure 29. Capacity factors for polar compounds on C ₁₈ YMC-Gel silica (prepared via hydride) before and after a 3-hour treatment with 50 mM phosphate pH 9.0 at room temperature	73
Figure 30. Capacity factors for polar compounds on C ₁₈ Vydac HS silica (prepared via hydride) before and after a 3-hour treatment with 50 mM phosphate pH 9.0 at room temperature	74
Figure 31. Capacity factors for polar compounds on C ₁₈ Vydac TP silica (prepared via hydride) before and after a 3-hour treatment with 50 mM phosphate pH 9.0 at room temperature	75
Figure 32. C ₁₈ -Vydac HS (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature	77

Figure 33. Asymmetry of polar compounds on C ₁₈ YMC-Gel (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature	78
Figure 34. Asymmetry of polar compounds on C ₁₈ -Vydac HS (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature	79
Figure 35. Asymmetry of polar compounds on C ₁₈ Vydac TP (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature	80
Figure 36. Half-height column efficiency from polar compounds on C ₁₈ YMC-Gel (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature	81
Figure 37. Half-height column efficiency from polar compounds on C ₁₈ Vydac HS (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature	82
Figure 38. Half-height column efficiency from polar compounds on C ₁₈ Vydac TP (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature	83

Figure 39. Asymmetry-based column efficiency from polar compounds on C ₁₈ YMC-Gel (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature	84
Figure 40. Asymmetry-based column efficiency from polar compounds on C ₁₈ Vydac HS (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature	85
Figure 41. Asymmetry-based column efficiency from polar compounds on C ₁₈ Vydac TP (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature	86
Figure 42. Elution profiles for BA, DMA, and DEA on hydride-modified YMC-Gel silica	88
Figure 43. Elution profiles for BA, DMA, and DEA on hydride-modified Vydac TP (lot 890414) silica	89
Figure 44. Elution profiles for BA, DMA, and DEA on hydride-modified Vydac TP (lot 900201) silica	90
Figure 45. Chromatographic profile for the polar mixture on C ₈ and C ₁₈ Vydac TP silicas which were darkened during olefin hydrosilation	92
Figure 46. Separation of peptides on C ₁₈ -bonded phases prepared via hydride	94

Figure 47. Separation of peptides on C ₁₈ -bonded phases prepared via hydride	95
Figure 48. Typical separation of a standard protein mixture on a C ₈ Vydac TP prepared via hydride.....	96
Figure 49. Elution profiles for lysozyme, α -chymotrypsinogen A and ovalbumin on a hydride-modified Vydac TP material	99
Figure 50. RP separation of a commercial barbiturate mixture on a C ₁₈ YMC-Gel prepared via hydride	101

1. INTRODUCTION

1.1. BACKGROUND

A great deal of activity in the field of chromatography has centered around developments in bonded stationary phases for high performance liquid chromatography (HPLC). Advances in the chemical modification of adsorbents, especially silica, have led to the development of covalently-bonded phases with a wide variety of selectivities. In particular, alkyl-bonded silicas have gained great popularity for HPLC separations of solutes ranging from low molecular weight organic solutes to biological macromolecules such as proteins. Currently, the most common approach to prepare alkyl-bonded silicas involves the reaction of fully hydroxylated silica with a dimethylalkyl chlorosilane:

This reaction produces a monolayer of non-polar moieties uniformly distributed on the substrate's surface. In a related approach, "polymeric" bonded phases are prepared by reaction of trifunctional organosilanes of the type X_3SiR with silica in the presence of a measured amount of water and a

suitable solvent:

where X is an easily hydrolyzable group (e.g., halide, alkoxy, acyloxy, etc.) and Y represents -H or -Si=. Although the thickness of the attached organic layer may vary according to the reaction conditions, it has been suggested that most polymeric phases with surface coverages approaching those of materials made through reaction 1 are actually monomeric in nature (1).

Despite their wide utility, the bonded phases resulting from equations 1 and 2 suffer from two major drawbacks. Due to the steric hindrance of the R-group, residual acidic silanols remain in varying degrees on the surface of alkyl-silicas. These unreacted species often result in strong interactions with certain basic solutes and lead to non-reproducible retention and poor peak symmetry. Perhaps the most stringent limitation of commercial alkyl-silicas lies in their unsatisfactory hydrolytic stability towards certain mobile phase compositions. Chemical degradation of the bonded

phase results in decreased retention and increased exposure of silanols, which in turn leads to poor long-term precision and poor peak shape respectively.

To overcome the problems associated with the relatively poor hydrolytic stability of Si-O-Si-C linkages formed by reactions 1 and 2 above, alternate approaches involving the formation of direct Si-C linkages have been developed. The reaction of chlorinated silica with certain organometallic reagents has been used to accomplish this (2-4). Chlorination of silica is commonly carried out by reaction with thionyl chloride:

A Grignard reagent or an alkyl lithium compound is then used to complete the bonded-phase formation:

where M = MgBr or Li. Despite the hydrolytic advantage of the resulting Si-C linkage, bonded stationary phases prepared by this approach have not found wide acceptance, probably because the extreme reactivity of chlorinated silica, Grignard and

organolithium reagents makes the process extremely cumbersome to carry out at a manufacturing level.

Our research group has recently developed a completely different alternate approach for producing Si-C linkages on silica supports (5-7). In the new method, catalytic olefin hydrosilation is used as the primary bonding reaction:

The reaction involves the addition of silicon hydride to a terminal olefin. Transition metals (group VIII) and, more particularly, complexes of platinum, rhodium, ruthenium and nickel are the most commonly used catalysts. Obviously, the method requires that silicon hydride groups are already present on the silica surface. This hydride-modified silica can be prepared by either one of two methods. One approach consists of a sequential chlorination-reduction process by which the silanols from the native silica are chlorinated (see equation 3 above) and the resulting silicon chlorides are reduced, thus transforming the silanols into silicon hydrides (5):

This is a labor-intensive, time-consuming, and even dangerous method which has been abandoned. The other method is an easier one consisting of the reaction of a silica substrate with triethoxysilane (TES) in the presence of water, HCl and a solvent such as dioxane to form a monolayer of silicon hydride (6):

While the bonded materials, including the intermediate, have been previously evaluated by spectroscopic as well as chemical methods (5-7), there is still a need for a thorough evaluation of the chromatographic performance of the new stationary phases. Such an evaluation is the subject of this work.

1.2. AIMS OF THIS WORK

The main goal of this research is to evaluate the new bonded phases that result by applying the method defined by equations 5 and 7, in terms of its chromatographic properties. A comparison of the separation performance of the new bonded

material with that of conventional stationary phases is also an important objective of the present work.

The procedures used in this work emphasize the relationship between the chemical nature of the modified silica surface and its chromatographic behavior with selected analytical probes. More specifically, there are three major aspects to be considered in this work: (i) a semi-empirical study of the reversed-phase behavior of alkyl-silicas as well as the hydride intermediate under a varying mobile-phase composition; (ii) a systematic evaluation of the hydrolytic stability of alkyl-silicas under aggressive chromatographic conditions; and (iii) an assessment of the chromatographic performance of alkyl-silicas under regular separation conditions.

2. EXPERIMENTAL

2.1. MATERIALS

1-Octene (Aldrich Chemical Co.) and 1-octadecene (Sigma Chemical Co.) were used as received. When required, toluene and dioxane (EM Industries Inc.) were dried with calcium hydride and distilled before use. Polycyclic aromatic hydrocarbons were obtained from Aldrich Chemical Co. and from The National Institute of Standards and Technology (Gaithersburg, MD). Dibenzo-18-crown-6 (DB18C6), dibenzo-24-crown-8 (DB24C8), trifluoroacetic acid, 1-phenylheptane, N,N-diethylaniline, N,N-dimethylaniline and benzyl alcohol were purchased from Aldrich Chemical Co. Standard proteins and peptides were obtained from Sigma Co. (St. Louis, MO). The barbiturate mixture was obtained from Alltech Co. (Deerfield, IL). A 100 mM hexachloroplatinic acid (37.5% as Pt, Aldrich Chemical Co.) solution in 2-propanol was used as a catalyst for hydrosilation. Bonded phases were prepared from a variety of commercially available silica sorbents whose physical properties are described in Table I. The specific surface area and mean pore diameter (Brunauer-Emmett-Teller, BET, nitrogen adsorption method) were determined at Chevron Research and Technology (Richmond, Ca) with a Micromeritics

Table I. Native Silicas Supports Used in This Work.

Brand Name	Lot #	Particle diam., μm	Pore diam. $\text{\AA}$	Spec. surf. area, m^2/g	Maker

Vydac TP	890414	5.8	291 ^b	88.8 ^b	(1)
Vydac TP	900201	6.6	380 ^b	106.5 ^b	(1)
Vydac HS	900423	6.7	104 ^b	261.2 ^b	(1)
YMC-Gel	630212	10.0	120 ^a	300 ^a	(2)
Nucleosil	1021	7.0	300 ^a	100 ^a	(3)
Partisil-10	100978	10.0	75 ^a	350 ^a	(4)

^a Data supplied by manufacturer

^b B.E.T nitrogen adsorption method

(1) The Separations Group (Hesperia, CA)

(2) Yamamura Chemical Laboratories Co., LTD. (Kyoto, Japan)

(3) Macherey-Nagel (Düren, Germany)

(4) Whatman, Inc. (Clifton, NJ)

model ASAP 2400. LC-grade organic solvents were purchased from EM Science. Deionized water was obtained from a Milli-Q purification System (Millipore Corp.).

2.2. INSTRUMENTATION

2.2.1. Infrared Spectrometry

Diffuse reflectance infrared fourier transform (DRIFT) spectra were obtained with a Perkin-Elmer Model 1800 FT-IR spectrometer. A Spectra-Tech DRIFT accessory equipped with a 3-mm diameter, 2-mm depth sample cup was used. KBr was ground, dried in an oven at 110°C for several hours, and mixed with an equal amount (by weight) of silica material. Samples were filled into the DRIFT accessory's cup and then a very smooth sample surface was obtained by pressing down a microscope slide onto the cup while moving it with a circular motion. Vertical adjustment of the sample cup position was routinely used before taking a spectrum. This resulted in a reproducible positioning of the sample surface at the optimum height so that a maximum signal throughput was consistently obtained. Spectra were collected at a nominal resolution of 2 cm⁻¹, with one hundred sample scans ratioed against KBr as reference.

2.2.2. Elemental Analysis

Carbon determinations on bonded silicas were carried out with a Perkin-Elmer model 240C elemental analyzer equipped with a Perkin-Elmer model 56 recorder.

2.2.3. Liquid Chromatography

All chromatographic measurements were made with a Hewlett-Packard (Avondale, PA), model 1050 liquid chromatographic system equipped with quaternary gradient pump, automatic injector, variable wavelength photodiode array UV detector and PC data station. Columns were slurry packed into 15 cm x 0.46 cm I.D. stainless steel tubes (Alltech Co., Deerfield, IL) using a Haskel pneumatic pump at 6,000 psi with methanol as the driving solvent. When required, column temperature was controlled by a column heater Model CH-30 (Fiatron System Inc., Oconomowoc, WI). Data analysis and plotting were performed using RS/1 scientific spreadsheet software (BBN Research Systems, Cambridge, MA).

2.3. PROCEDURES

2.3.1. Preparation of Bonded Phases

2.3.1.1. New Bonded Phases via Hydride Intermediate

2.3.1.1.1. Silanization with TES

A 5.00-g amount of silica was placed in a 3-neck, 100-mL round bottom flask equipped with a condenser, an addition funnel with equalizing tube, a heating mantle and a magnetic stirrer. A 100-mL amount of dioxane was then added, followed by 7 ml of (aqueous) 2.3 M HCl solution. The mixture was heated to about 70-80°C and then a 45-mL amount of 0.50 M TES/dioxane solution was added dropwise with vigorous stirring, over a period of 15-20 min. The mixture was then gently refluxed for about 1 hour, after which the product was centrifuged and washed consecutively with 50-mL portions of 20:80 water/THF, THF and diethyl ether (twice with each solvent). The final product was dried at room temperature and then in a vacuum oven at 110°C for 6 hours or more.

2.3.1.1.2. Olefin Hydrosilation

Prior to hydrosilation, the hydride silica was dried at 110°C for 6 hours or more. In a typical preparation of an

octyl-bonded silica, 75 mL of 1-octene (density 0.715 g/cm³, 97% purity) containing 255 μ L of 100 mM hexachloroplatinic acid solution in 2-propanol was heated to about 60-70°C while being agitated magnetically for about 1 hour (a clear solution was obtained after about 15-20 min of mixing). A 5.00-g amount of hydride substrate was then slowly added to the olefin/catalyst solution, and the reaction was allowed to proceed for 48 hours at 100 $\pm$ 2°C. The mixture was then centrifuged and the solid washed with three 40-mL portions of benzene followed by similar washing with dichloromethane and diethyl ether. After the solvent was removed, the solid was dried under vacuum at 110°C overnight. A similar procedure, this time with 1-octadecene (density 0.79 g/cm³, 97% purity) instead of 1-octene, was followed to prepare an octadecyl-bonded silica. Alternatively, an equivalent molar amount of 50 mM dicyclopentadienylplatinum(II) chloride in chloroform was used as catalyst.

2.3.1.2. Conventional Bonded Phases by Organosilanization

Octyl- and octadecyl-dimethylsilyl silicas were prepared according to the procedures described by Berendsen et al. (8). A typical procedure is as follows. A 7.50-g amount of silica was transferred to a 100-mL, three-neck, round-bottom flask

(equipped with a magnetic bar) and the solid dried at 110°C under vacuum overnight. After the solid had cooled down to room temperature, 30 mL of dry toluene, 5.0 mL of dry pyridine, and 13 mL of octyldimethylchlorosilane (approximately 50 mmol) were added. The reaction proceeded at 105±2°C for 48 hours with magnetic stirring. The product was washed in the reaction vessel three times with 25-mL portions of dry toluene (15 minutes each time), to remove excess of silane reagent. Then, the solid was transferred to a 50-mL centrifuge tube and washed consecutively with 25-mL portions of dichloromethane, dichloromethane/methanol 1:1 v/v, methanol, methanol/water 1:1 v/v (two times), methanol, and dichloromethane (two times). During washing, the suspension was magnetically stirred for 5-10 minutes with each solvent. The aqueous solvent was used to remove any base.HCl salt. Lastly, the product was allowed to dry first at room temperature for several hours, until all of the solvent was completely evaporated, and then at 110°C under vacuum overnight.

2.3.1.3. Surface Coverage

The concentration, α_R , of surface-bonded groups was obtained from the carbon content of the bonded material along

with the BET specific surface area of the native silica substrate using the equation proposed by Berendsen and De Galan (9):

$$\alpha_R(\mu\text{mol}/\text{m}^2) = 10^6 p_c / (10^2 M_c n_c - p_c M_R) S_{\text{BET}} \quad (8)$$

where p_c is the carbon percentage by weight of the bonded material (after correction from any carbon present before bonding), n_c is the number of carbon atoms in the bonded organic group, S_{BET} is the specific surface area (m^2/g) of the hydride substrate, M_R is the molecular weight of attached group, and M_c is the atomic weight of carbon. Table II contains alkyl density data as well as other chromatographically relevant information for the different bonded silica-based packings used during this study.

2.3.2. Crown Compounds Retention vs Eluent Composition Experiments.

Columns with native-, hydride- and octyl-silica were first conditioned with acetonitrile-water mixtures of decreasing acetonitrile content at 0.5 mL/min. After 10% changes the eluent composition was held constant for about 30 minutes. Total equilibration time was about 12 hours.

Table II. Bonded Silicas Used in This Work

Silica	Type	Lot #	Particle diam., μm	Pore diam., $\text{\AA}$	α_R $\mu\text{mol}/\text{m}^2$
Vydac TP	C8 (1)	890414	5.8	291	4.62
Vydac TP	C8 (1)	890414	5.8	291	3.95
Vydac TP	C8 (1)	900201	6.6	380	3.11
Vydac TP	C8 (3) (5	890414	5.8	291	1.71
Vydac TP	C8 (3) (4) (5)	890414	5.8	291	3.23
Vydac TP	C8 (2) (4) (5)	890331	10.0	300	*
Vydac TP	C18 (1)	890414	5.8	291	2.85
Vydac TP	C18 (1)	900201	6.6	380	2.66
YMC-Gel	C18 (1)	630212	10.0	120	3.53
Vydac HS	C18 (1)	900423	6.7	104	2.33
Nucleosil	C18 (3)	1021	7.0	300	2.31
Vydac TP	C18 (3)	900201	6.6	380	2.54

(1) From hydride

(2) Polymeric

(3) Conventional

(4) End-capped

(5) Commercial packing

* Unknown (packing has 4.23% carbon)

The experiment was performed using acetonitrile-water mixtures of increasing water content and measuring (by triplicate) the retention times of DB18C6 and DB24C8 after a 20-min equilibration period. Retention times were evaluated from the position of peak maxima on the chromatogram and the mobile phase hold-up time was obtained using KNO_3 as unretained marker. In all experiments, the flow-rate was 1.0 mL/min and the wavelength used was 220 nm. Solute retention was measured in terms of its capacity factor, k' , as defined by the following equation:

$$k' = (t_r - t_0)/t_0 \quad (9)$$

where t_r is the solute's retention time and t_0 is the hold-up time for KNO_3 . A non-linear least squares curve fitting routine was used for data analysis.

2.3.3. Long-Term Hydrolysis Experiments

In the degradation studies, mobile phases of 0.10 % v/v trifluoroacetic acid (TFA) in water (solvent A) and 0.10 % v/v TFA in acetonitrile (solvent B) passed through each column at 50°C for a total of about 100 hours at 1.0 mL/min. The gradient used in this process (see Figure 1) was as follows:

50% B for 20 minutes, 50-100% B ramp over 40 minutes, 100% B for 40 minutes, 100-0% B ramp over 80 minutes, 0% B for 40 minutes, 0-50% B ramp over 40 minutes, and finally 50% for 20 minutes. Isocratic retention time measurements of 1-phenylheptane (10 μ L injection of 0.1 mM solution in 50% B) were made during the initial 50% B period. After initial retention time determination, measurements were repeated under the same conditions after subjecting the columns to the mobile phase cycle described above. Column void volume was determined using KNO₃ (1 mM solution in 50% B) as an unretained marker.

2.3.4. HPLC Separations

2.3.4.1. Separation of Proteins and Peptides on Alkyl-Silicas

Proteins tested included ribonuclease A, cytochrome C, lysozyme (chicken), myoglobin, and trypsinogen in concentrations ranging 0.5-1.0 mg/mL. Polypeptides used were bradykinin, angiotensin II and oxytocin at concentrations of 1.0-4.0 mg/mL in the starting mobile phase. Other peptides used were Gly-Gly-Tyr and Gly-Phe-Ala-Ala in concentrations of 1.0 mg/mL. Linear gradients with aqueous-acetonitrile containing 0.10 % v/v of TFA were used. Flow-rates ranged 0.5

Solvent A: 0.10% v/v TFA in Acetonitrile
Solvent B: 0.10% v/v TFA in Water

Figure 1. Solvent gradient used for the long-term hydrolysis test.

to 1.5 mL/min and injection volumes ranged 2.5 to 10 μ l. All experiments were done at room temperature.

2.3.4.2. Separation of Polycyclic Aromatic Hydrocarbons on Octadecyl-Silicas

All separations were performed in acetonitrile/water at 25°C column temperature with an injection volume of 10 μ L. A variety of solutes was used to test the selectivity of octadecyl phases toward polycyclic aromatic hydrocarbons (PAH's). A 16-component PAH's mixture (SRM1647) was separated using various linear gradients of acetonitrile/water at 2 mL/min. o-, m-, p-terphenyls, 1,1'- and 2,2'-binaphthyls and a three component standard test mixture (SRM869) were chromatographed isocratically at several mobile phase compositions.

2.3.4.3. Separation of Basic Solutes on Alkyl-Silicas

0.1 mM solutions of benzyl alcohol as a polar non-basic solute along with N,N-dimethylaniline (DMA) and N,N-diethylaniline (DEA) as basic probes were separated isocratically on different types of alkyl-silicas. Solute were dissolved in the same composition of mobile phase which

was 60% v/v acetonitrile in water. Flow-rate was 1 mL/min, injection volume was 5 μ L, and detection wavelength was 200 nm.

Some selected columns were subjected to a treatment with 50% of 15 mM phosphate at pH 9.0 and 50% acetonitrile for 3 hours at 0.8 mL/min at room temperature.

2.3.4.4. Separation of Alkaloids on Octadecyl-Silicas

A commercially available mixture containing five barbital derivatives (butobarbital, amobarbital, secobarbital, hexobarbital, and phenobarbital) was used as a typical pharmaceutical sample. The mixture was isocratically separated using several alkyl-bonded phases. The flow rate was 1 mL/min, the injection volume was 10 μ L, and detection wavelength was 220 nm.

3. DISCUSSION AND RESULTS

3.1. Silanophilic and Solvophobic Interactions

Reversed-phase (RP) HPLC separations are generally characterized by a mobile phase which is more polar than the stationary phase. In the early years of chromatography bare (native) silica was used as stationary phase, with a non polar solvent as mobile phase. This mode of chromatography was known as "normal"-phase. In RP chromatography the silica surface is derivatized in such a way that it is less polar than the water-rich mobile phase used. Typically, the stationary phase surface consists of a uniform non-polar layer of covalently bonded alkyl moieties on a silica substrate. Common solvents in reversed-phase include aqueous tetrahydrofuran, methanol, acetonitrile, isopropanol, etc. The order of solute elution in RP is generally reversed with respect to that obtained in normal phase.

It was proposed by Horvath and co-workers in 1981 (10,11) that under regular reversed phase conditions, (i. e., a mobile which is more polar than the stationary phase), retention is due to the superimposed contribution of two mechanisms, characterized by different modes of solute binding to the alkyl-derivatized silica surface: (i) regular "solvophobic"

interactions and (ii) silanophilic interactions. According to the solvophobic theory, the capacity factor k' is related to the mobile phase composition by the equation

$$k'_{\text{solvophobic}} = Ae^{B\psi} \quad (10)$$

where ψ is the volumetric fraction of water in the mobile phase, A is the limiting retention factor when neat acetonitrile is the mobile phase ($\psi = 0$) and B is a measure of the binding molecular contact area between the solute and the surface alkyl groups. What this means is that a plot of logarithmic k' vs ψ will give a straight line, A and B being obtainable from the intercept and slope of the linear plot, respectively. In "normal"-phase chromatography, the capacity factor is related to the eluent composition by the equation

$$k'_{\text{silanophilic}} = 1/(C + D\psi) \quad (11)$$

where C is the inverse of the limiting retention factor when neat acetonitrile is the mobile phase ($\psi = 0$), and D is an inverse measure of the surface silanol density. That is, under "normal"-phase conditions if one plots $1/k'$ vs ψ , a straight line results.

What Horvath has proposed is that, in practical RP, the global capacity factor is the sum of the capacity factors due to solvophobic and silanophilic interactions:

$$k' = k'_{\text{solvophobic}} + k'_{\text{silanophilic}} \quad (12)$$

therefore,

$$k' = Ae^{B\psi} + 1/(C+D\psi) \quad (13)$$

Application of this model to several typical stationary phases with probes which were known to be particularly sensitive to silanophilic interactions satisfactory explained the irregular (non-linear logarithmic k' vs ψ plots) behavior with certain solutes. Crown ethers in their most energetically-favorable conformation exhibit a sort of oxygen "cluster" in the center of the molecule. Such a geometry appears to be responsible for the very strong interactions with the surface silanols through hydrogen bonds. Logarithmic k' - ψ plots were found to pass through a minimum. According to the dual mechanism model, silanophilic interactions are predominant when the mobile phase is very rich in the organic modifier (small ψ). On the other hand, when one has a mobile phase that is water-rich the prevalent mechanism is solvophobic. An important consequence is that the polarity of the mobile phase can be adjusted by changing the amount of water, and thus switched

between solvophobic and silanophilic interactions. This behavior is not only exhibited by crown ethers. They are used just as model probes. The practical importance of the model is that it rationalizes the unusual behavior observed with some biologically important solutes. For instance, many peptides which have free amino groups exhibit this behavior.

As shown in Figure 2 for octyl-phases, there are several fundamental differences between conventional monomeric bonded phases (made via equation 1) and those resulting from olefin hydrosilation on hydride-silicas (equation 5). Differences in the anchored moieties lies on the dimethylsiloxane linkages at the bottom of the attached group. Solute interactions with these bridging groups are rather weak. From a solvophobic or reversed-phased point of view, the hydrocarbonaceous layers on the two alkyl-bonded silicas are more or less equivalent. In a conventional bonded phase a significant fraction of the original silanols is left unreacted. In fact, not more than about 50% of the silanols react during their preparation. On the other hand, in the new bonded phase we find mostly SiH groups underneath the alkyl moieties. The low polarity of such SiH species decreases even further the overall polarity of the bonded material. Thus, generally speaking, we expect to have a much more hydrophobic bonded phase than the conventional one, and therefore solvophobic interactions

Figure 2. Schematic representation of the structures of octyl-bonded, silica-based supports prepared via conventional organosilanization (A) and olefin hydrosilylation (B). Adapted from reference (12).

should govern retention on these new bonded phases. This observation, however, does not necessarily mean that on the new bonded phase there is an absence of silanols. Although during the reaction of the silica with TES (see equation 7) most of the silanetriols formed upon hydrolysis are completely condensed, there are a few silanols that are not totally removed as evidenced by IR and NMR (7).

In order to explore the possibility of obtaining some information on the extent at which silanophilic and solvophobic interactions are present in both RP phases, the dual mechanism model of Horvath's was comparatively applied to conventional and new octyl-bonded materials prepared from the same silica substrate. Thus, we should be able to estimate the relative amount of silanophilic activity by comparing the corresponding values for the model's A-D parameters (equation 13). A single batch of silica was used to eliminate any difference arising from any batch-to-batch substrate variation.

Although, generally speaking, the retention behavior of the crown ethers measured with C₈-Vydac TP resembles that found by Horvath with C₁₈-Partisil (i. e., k' increases with both low as well as high volume fraction of water in acetonitrile), the curves appear to be seriously "distorted" in the area where $0.6 > \psi > 0.1$. This was observed for both

conventional and new C_8 bonded phases as shown in Figure 3 for DB24C8. In the figure, the dashed curve corresponds to a function described by equation 13 in which the A, B and C, D values were calculated from the first and last set of three data points which respectively corresponds to regions where silanophilic and solvophobic interactions are supposed to prevail.

To further investigate this apparent deviation from the dual-mechanism model, the reciprocal retention factors of the two crown ethers measured on bare silica were plotted against the volumetric fraction of water in the acetonitrile-water mobile phase over the range between 0 and 0.4, as shown in Figure 4. Clearly, a non-linear relationship is obtained indicating an irregular "normal-phase" behavior. This relationship does not agree with that obtained by Horvath on Partisil-10, using similar conditions. We decided to check whether the effect was due to differences in the silica or the fact that acetonitrile instead of methanol was used in the mobile phase. The results, shown in Figures 5 and 6, clearly confirm the fact that a linear relationship is obtained only for ψ -values not greater than 0.1. Above this value, the curves consistently tend to level off, indicating a greater than expected retention compared to that obtained if the straight line was extrapolated. Unfortunately, the effect

Figure 3. Plot of logarithmic retention factor of DB24C8 on conventional (not end-capped) C_8 -Vydac TP silica as a function of the volume fraction of water in aqueous acetonitrile eluent. The dashed line was calculated considering only the first and last set of three data points on which equations 10 and 11 were respectively applied.

Figure 4. Reciprocal retention factors for DB24C8 and DB18C6 measured on bare Vydac TP silica as a function of the volume fraction of water in aqueous acetonitrile eluent.

Figure 5. Reciprocal retention factors for DB24C8 and DB18C6 measured on Partisil-10 silica as a function of the volume fraction of water in aqueous acetonitrile eluent.

Figure 6. Reciprocal retention factors for DB24C8 and DB18C6 measured on Partisil-10 silica as a function of the volume fraction of water in aqueous methanol eluent.

cannot be explained by just the introduction of the solvophobic component in equation 13. This is evidenced by the fact that application of a curve fitting routine on the experimental data failed to produce an acceptable fitting. These results indicate that, under the conditions used, equation 13 does not seem to appropriately describe the experimental dependance of the total k' value on eluent composition. Despite this, some important qualitative observations can still be made about the data. Figures 7 and 8 summarize the experimental results for DB24C8 and DB18C6, respectively. Clearly, bare silica exhibits the lowest retention when water is the major component of the mobile phase ($\psi > 0.4$), in agreement with its essentially hydrophilic (silanol-populated) surface. On the other hand, surprisingly, the hydride and not the bare material shows the greatest retention at low water concentration in the mobile phase ($\psi < 0.1$), particularly in the case of DB18C6. Evidently, some very active sites are still present on the hydride surface and are accessible for interaction with the crown ring. If it is assumed that isolated silanols on bare silica are more readily removed during silanization (equation 7), partially condensed species of the type $=SiH(OH)$ might be responsible for the unusually high retention on the hydride material. Surprisingly again, at high water concentration in

Figure 7. Plots of the logarithmic retention factor of DB24C8 against the volume fraction of water in aqueous acetonitrile eluent, as a function of the surface chemistry of the sorbent.

Figure 8. Plots of the logarithmic retention factor of DB18C6 against the volume fraction of water in aqueous acetonitrile eluent, as a function of the surface chemistry of the sorbent.

the eluent ($\psi > 0.8$) the hydride exhibits solvophobic retention for crown ethers as strong as that with the conventional C₈-bonded silica. Due to the greater alkyl coverage, larger k'-values are shown by the new C₈ phase, as expected. Finally, it should be recalled that the "anomalous" behavior of the two C₈-bonded materials is more pronounced in the 0.1 to 0.6 ψ -range, particularly in the case of the conventional phase which exhibits a significant departure from the dual model (see Figure 3). Curiously enough, the four data points in this ψ -range show a great degree of linearity (see Figures 7 and 8). It can be concluded that although a simple analysis of the data was not possible, the general pattern observed with silanol-sensitive probes such as crown ethers is qualitatively followed in our case; that is, the logarithmic retention factor is larger at low as well as high water concentrations in the eluent, and a minimum is exhibited at intermediate compositions.

3.2. Hydrolytic Stability

In a vast majority of HPLC applications hydrolytic stability of the bonded-phase packing is not regarded as a problem. Most separations are carried out with aqueous/organic mobile phases, at room temperature and a pH

ranging from 4 to 7. In recent years, however, RP-HPLC has found extensive use in the separation of a great variety of basic solutes, particularly biomolecules. For instance, to achieve satisfactory reversed-phase separations of certain proteins, it is often desirable to incorporate in the mobile phase dilute aqueous solutions such as phosphate at pH 2-3, or trifluoroacetic acid. Such mobile phase compositions have been found to be very aggressive toward the bonded ligand and invariably led to the gradual loss of the bonded material along with an increase of silanol exposure. Short column lifetime, poor long-term precision and potential fraction contamination are the most evident deleterious effects arising from phase deterioration. Fraction contamination can be particularly disadvantageous in the case of preparative separations.

The limited stability of the siloxane (Si-O-Si-C) linkage formed through equation 1 has been recognized as the major source of hydrolytic instability of conventional silica-based stationary phases used in HPLC bioseparations. The improved stability obtained through the formation of direct surface-to-carbon linkages (see equation 4) strongly suggests that the siloxane and not the Si-C bond is the most hydrolytically labile portion of the organosiloxane structure.

Recently, there has been a considerable activity aimed at evaluating stationary phase degradation (13-16). Kirkland and coworkers, in their attempts to develop more stable silica-based separation materials, have studied degradation of bonded phases in a systematic way (14,16). Capacity factor values for 1-phenylheptane were used as a measure of the alkyl coverage remaining on the bonded phase as it becomes degraded. 1-phenylheptane was selected as a probe because its retention was shown to be directly proportional to the support's alkyl coverage.

It was decided to use Kirkland's approach to evaluate the new bonded phases because a purely chromatographic parameter, retention, is used as a measure of degradation of the column performance. In doing so, the solvent gradient depicted in Figure 1 is adapted from the information provided in references 14 and 16. Selected bonded materials prepared from a single batch of Vydac TP silica were subjected to the TFA-containing acetonitrile-water gradient at 1 mL/min and 50°C, and the retention of 1-phenylheptane was measured at the beginning of each cycle. It should be pointed out that the retention of the probe was always measured under isocratic conditions at 50% solvent A. Additionally, the average composition of the mobile phase during the whole test was always 50% solvent A. Furthermore, it should be noticed that

each cycle involves two 40-minute periods during which 100% solvent B and 100% solvent A passed through the column packing. It has been suggested (14) that the hydrolytic process most likely occurs in mobile phases approaching 100% solvent B while the degradation byproducts are eluted from the column in mobile phases approaching 100% solvent A.

Capacity factor (k') data for 1-phenylheptane during the tests as a function of the number of column volumes are shown in Figure 9 for three columns tested. It is clearly seen that the silica modified via hydrosilation keeps most of its retention capabilities while both end-capped and not end-capped conventional packings undergo almost full deterioration. Since the packings shown have different levels of coverages which result in correspondingly different initial k' -values, normalized retention in the form of fraction of remaining k' seems to be more appropriate, as shown in Figure 10. The loss of retention for the new phase was about 12% during the entire test period, significantly better than either of the conventional phases which lost more than 90% of their retention. Additional evidence that the bonded alkyl groups were actually lost from the tested packings can be obtained from the percent carbon data in Table III. Again, it is quite clear that in the case of the conventional materials, significant fraction of the bonded phase is lost

Figure 9. Capacity factor (k') of 1-phenylheptane as a function of column volumes of mobile phase passed through the columns during the long-term stability test.

Figure 10. Relative loss of retention for 1-phenylheptane as a function of column volumes of mobile phase passed through the columns during the long-term hydrolysis test.

Table III. Carbon Coverage and Retention Data for Octyl-Bonded Silicas (Vydac TP) during Long-Term Hydrolysis Test with TFA-Containing Aqueous Acetonitrile Mobile Phases.

Bonded Phase	Carbon Content % by Weight		Capacity Factor (k') of 1-phenylheptane		Column Volumes
	Initial	Final	Initial	Final	
Octyl-Vydac TP (from hydride)	4.14	3.88	5.79	5.11	2842
Octyldimethyl- silyl-Vydac TP (end-capped ¹)	2.13	0.81	2.99	0.22	2927
Octyldimethyl- silyl-Vydac TP (not end-capped)	1.78	0.65	1.93	0.10	2813
"Polymeric" octyl-Vydac TP ^{2,3}	4.23	4.00	7.57	6.88	2628

¹ Encapped with trimethylchlorosilane

² Commercial sample from a different batch of silica

³ Encapped with 2-propyldimethylsilane

as a result of the long-term elution with the TFA-containing eluent. In contrast, the bonded phase prepared by hydrosilation via hydride maintains most of its carbon content upon the same hydrolysis regime. The present data also confirms the results from a previous study (6), in which elemental analysis was used to monitor the effects of phase degradation. The results, shown in Figures 11 and 12 were obtained from a non-chromatographic, "static" test. To further substantiate our findings, DRIFT spectra were taken on the hydrolyzed packings removed from the columns. This is shown in Figures 13 and 14 for the end-capped and not end-capped commercial products, respectively. In both cases the results are consistent with the removal of the anchored alkyl groups, as evidenced by the almost complete loss of C-H stretching band in the hydrolyzed product.

It seems interesting to include at this point the data obtained from a commercially available "polymeric" octyl-phase prepared from a similar silica substrate (Vydac TP), although from a different batch. The results, shown in Figure 15, indicate that this polymeric phase is a very stable material under the conditions used. Whether the stationary phase owes its stability to a relatively dense alkyl coverage or to the end-capping process was not possible to determine because of the limited information provided by the manufacturer. The

Figure 11. Alkyl surface coverage of octyl-Vydac TP (same lot as that of Figures 9 and 10) as a function of hydrolysis time. Long-term exposure of the bonded phase with aqueous 0.10% v/v TFA containing 20% v/v of dioxane, at room temperature ($26 \pm 2^\circ\text{C}$). Taken from reference 6 with permission from the authors.

Figure 12. Relative surface coverage of octyl-Vydac TP (same lot as that of Figures 9 and 10) as a function of hydrolysis time. Long-term exposure of the bonded phase with aqueous 0.10% v/v TFA containing 20% v/v of dioxane, at room temperature ($26 \pm 2^\circ\text{C}$). Taken from reference 5 with permission from the authors.

Figure 13. Conventional C₈-Vydac TP (end-capped) before (upper curve) and after chromatographic long-term hydrolysis.

Figure 14. Conventional C₈-Vydac TP (not end-capped) before (upper curve) and after chromatographic long-term hydrolysis.

Figure 15. Fraction of initial retention as a function of column volumes of TFA-containing mobile phase for a commercial polymeric C_8 Vydac TP.

result indicates, however, that there are other alternatives to obtain superior hydrolytic stability.

It can be concluded that olefin hydrosilation on an SiH-containing substrate produces a very stable bonded phase. It is claimed that the superior stability of the new product is a direct result of the greater hydrolytic strength of the Si-C linkage as compared to the siloxane Si-O-Si-C linkage of conventional bonded phases. The practical importance of this hydrolytic advantage is quite evident: (1) After several thousands of column volumes of a mobile phase containing aqueous TFA, a conventional bonded silica loses its anchored organic material to such an extent that the column has to be replaced by a new one. Because of the superior hydrolytic stability of the Si-C linkage (the predominant species in the new bonded packings) the useful lifetime of the column will be significantly increased. Obviously, the hydrolytic advantage means lower operating costs for HPLC separations. (2) From an analytical point of view, the long-term precision of retention is compromised in the case of a conventional bonded phase used under the conditions described above. Under the same conditions, the new bonded phases also degrade, but much more slowly and therefore the long-term precision should not be limited. (3) Another practical consequence of the loss of bonded phase during use is that significant organosiliceous

material must be washed off the column during gradient elution. Elution of such hydrolytic degradation byproducts would have deleterious consequences, particularly for preparative or process separations where fraction contamination may become a serious problem.

3.3. HPLC Separations

The ultimate purpose for preparing bonded-phase materials is performing separations under regular conditions. It is a well known fact that differences between individual stationary phases cannot only be attributed to the varying properties of the silica substrate but also to differences in the preparation of the bonded-phase product. We have already emphasized two fundamental structure differences between the new product and their conventional counterparts: (i) the formation of an Si-alkyl linkage as opposed to a Si-O-SiMe₂alkyl structure and (ii) the presence of a predominantly hydrophobic (SiH-populated) surface underneath the organic layer, contrasting with a hydrophilic (SiOH-populated) surface. These differences will be highly relevant when comparing separation performances. The actual performance of a RP material is a function of several factors, such as the porosity and particle size of the silica substrate, the

surface concentration of bonded alkyl groups, and the concentration and accessibility of remaining surface silanols.

Although neutral solutes such as hydrocarbons do not cause elution problems on any column, in some cases, particularly with closely related polycyclic aromatic hydrocarbons, separations are difficult and sometimes impossible. A fundamental difficulty in HPLC separations arises, however, from basic solutes which are frequently eluted with asymmetric peaks. Another critical problem stems from certain macromolecular solutes such as proteins which, due to their structural complexities, appear to be particularly susceptible to poor reproducibility in retention, band tailing, low recovery or, worse, irreversible adsorption.

A great variety of test procedures has been developed to evaluate the performance of RP material. We have already used a long-term hydrolysis test to confirm the superior stability of the new bonded phases. Then a wide variety of solutes were selected to assess the separation capabilities of the products. Neutral compounds, mainly PAH's, were used to evaluate the packing selectivity. Polar solutes including those containing basic functional groups were considered to establish the presence of chromatographically accessible silanols. Selected peptides and proteins were used to illustrate typical bioseparations.

3.3.1. Polycyclic Aromatic Hydrocarbons

One very important application of reversed phase is in the separation of polycyclic aromatic hydrocarbons (PAH). Chemically bonded octadecyl (C_{18}) phases are the most popular packings for the separation of PAH's.

A simple empirical HPLC test has been developed by Sander and Wise (1,17-19) to assess the monomeric or polymeric nature of a reversed-phase material. The test mixture (known as SRM869) is an acetonitrile solution of three PAH's: benzo[a]pyrene (BaP), 1,2:3,4:5,6:7,8-tetrabenzonaphthalene (TBN) also known as dibenzo[g,p]chrysene, and phenanthro[3,4-c]phenanthrene (PhPh). The structures are shown in Figure 16. The elution order of the mixture is thought to be strongly dependent on the type of phase. Under isocratic conditions of 85% v/v acetonitrile/water at $25 \pm 2^\circ\text{C}$ and 2.0 mL/min, monomeric C_{18} phases (see equation 1) have been shown to produce the elution order $\text{BaP} \leq \text{PhPh} < \text{TBN}$, while polymeric phases (see equation 2) give the elution order $\text{PhPh} < \text{TBN} \leq \text{BaP}$. Phases with intermediate properties (that is, heavily loaded monomeric or lightly loaded polymeric C_{18} phases) show elution order $\text{PhPh} < \text{BaP} < \text{TBN}$. Semiquantitative comparisons between different C_{18} phases can be accomplished by calculating the selectivity factor of TBN with respect to BaP, which is

BENZO[a]PYRENE

TETRABENZONAPHTHALENE

PHENANTRO[3,4-C]PHENANTHRENE

Figure 16. Structures of the three PAH components of standard mixture SRM869.

defined as

$$\alpha_{\text{TBN/BaP}} = k'_{\text{TBN}}/k'_{\text{BaP}} \quad (14)$$

where k' is defined by equation 9. Polymeric phases show $\alpha_{\text{TBN/BaP}}$ values less than one, while monomeric phases have $\alpha_{\text{TBN/BaP}}$ greater than 1.7.

Figure 17 shows a typical separation profile for the SRM869 mixture on a C_{18} YMC-Gel bonded-phase prepared via hydride. Table IV summarizes the results obtained from several columns tested. It can be seen that all columns exhibit a selectivity toward the three-component mixture which was typical of monomeric phases (i. e., $\alpha_{\text{TBN/BaP}} > 1.7$), as expected.

Octadecyl-phases prepared from different silica substrates were also examined by using other PAH's mixtures. Representative separations of a three-component mixture containing o-, m- and p-terphenyls are illustrated in Figure 18. The selectivities among the different phases do not seem to change significantly with pore size or substrate. The elution order of the terphenyls isomers is invariable. Resolution of each mixture is somewhat better with the smaller pore silica (panel B) since the retention is greater. Similar observations can be made for a two-component mixture

Figure 17. Typical separation profile for the SRM869 standard mixture on a C_{18} phase YMC-Gel prepared via hydride. The mixture was chromatographed isocratically with 85% acetonitrile/water at 2 mL/min, and UV detection at 254nm. Component identification: 1, PhPh; 2, BaP; 3, TBN.

Table IV. Retention and Selectivity Data for the Three-Component Standard SRM869 Mixture on Octadecyl Silicas

Silica (Type)	BaP k'	PhPh k'	TBN k'	$\alpha_{\text{TBN/BaP}}$
Vydac TP (conventional)	1.30	1.30	2.31	1.78
Nucleosil-300 (conventional)	1.45	1.54	2.74	1.90
Vydac TP (from hydride)	0.89	1.01	1.57	1.76
Vydac TP (from hydride)	0.58	0.69	1.10	1.88
Vydac HS (from hydride)	2.13	2.69	3.96	1.86
YMC-Gel (from hydride)	3.26	3.99	5.71	1.75

Figure 18. Separation of isomeric terphenyls on several C₁₈ silicas. (A) Vydac TP (via hydride, 6.6 μm, 380 Å); (B) YMC-Gel (via hydride, 10 μm, 120 Å); (C) Nucleosil (conventional, 7 μm, 300 Å); (D) Vydac TP (conventional, 6.6 μm, 380 Å). Isocratic separation at 70% acetonitrile/water, 1.0 mL/min with detection at 211 nm. Component identification: (1) o-terphenyl; (2) m-terphenyl; (3) p-terphenyl.

consisting of 1,1'- and 2,2'-binaphthyls, as shown in Figure 19.

Typical separations of a more complex mixture consisting of sixteen priority pollutant PAH's (see Figure 20 for structures) on several C_{18} phases are shown in Figure 21. It can be seen that, in all cases, only partial separation of closely related isomers was achieved, a behavior which appears to be characteristic of monomeric phases (7). It seems reasonable to conclude that selectivity of the octadecyl bonded phases prepared from olefin hydrosilation is comparable to that exhibited by their conventional octadecyl-dimethylsilyl counterparts.

3.3.2. Basic Solutes

The fundamental limitation of many RP sorbents lies in the separation of solutes containing basic nitrogen functionalities. They are frequently eluted with varying degrees of asymmetry depending on the accessibility of certain acidic sites on the silica surface. Such active groups have been identified as surface silanols which remained after the substrate derivatization or those that were exposed as a result of hydrolytic removal of the organic ligands. Recently, Nawrocki (20,21) has reviewed this issue in a very

Figure 19. Separation of isomeric binaphthyls on several C_{18} phases. (A) Vydac TP (via hydride, $6.6\ \mu\text{m}$, $380\ \text{\AA}$); (B) YMC-Gel (via hydride, $10\ \mu\text{m}$, $120\ \text{\AA}$); (C) Nucleosil (conventional, $7\ \mu\text{m}$, $300\ \text{\AA}$); (D) Vydac TP (conventional, $6.6\ \mu\text{m}$, $380\ \text{\AA}$). Isocratic separation at 80% acetonitrile/water, $2.0\ \text{mL/min}$ with detection at $211\ \text{nm}$. Component identification: (1) 1,1'-binaphthyl; (2) 2,2'-binaphthyl.

Figure 20. Components of Standard Reference Material (SRM1647), consisting of 16 PAH's from the Environmental Protection Agency's priority pollutant list.

Figure 21. Separation profiles for 16 PAH's (SRM1647) on C₁₈-bonded phases prepared via hydride. (A) Vydac TP (6.6 μm, 380 Å); (B) YMC-Gel (10 μm, 120 Å). Separation with gradient elution, 45-100% acetonitrile in water over 30 min at 1.5 mL/min, and UV detection at 254 nm.

thorough manner.

Not all surface silanols necessarily have deleterious effects on the separation of basic solutes. It has been shown (22,23) that as long as the silanols are hydrogen-bonded, they will show little activity toward basic solutes. Strongly adsorbing activity has been attributed to the presence of isolated single silanols and/or geminal silanols (silanediols). It has been suggested (20) that these species exhibit sharp IR absorption bands which, presumably, overlap with each other at about 3749 cm^{-1} . In the discussion that follows the presence of this strong band in the DRIFT spectra of silica materials will be stressed.

In order to evaluate the separation performance of the bonded phases, a three-component mix containing benzyl alcohol (BA), N,N-dimethylaniline (DMA, $K_b = 7.1 \times 10^{-6}$) and N,N-diethylaniline (DEA, $K_b = 2.5 \times 10^{-7}$) was used. BA was included as a neutral, poorly retained solute which has a polar, non-basic group and therefore should not exhibit any strong acid-base interaction with the stationary phase. In this way, any peak broadening and/or asymmetry arising from other sources will affect all solutes, while silanophilic interactions affect only the anilines.

Peak efficiency and asymmetry are used to quantitatively compare the different packings tested. Efficiency or plate

count is a generally accepted parameter to assess "how good" a column is. The general formula for calculating column efficiency N (in units of theoretical plates) is defined as

$$N = a (t_r/W)^2 \quad (14)$$

where t_r is the retention time for the peak, W is its width measured in time units, and the parameter a depends on the height from the peak's baseline at which the width is measured. The half-height measure of W ($a = 5.54$) is the most commonly used method to calculate N . The height equivalent to a theoretical plate (HETP or, simply, H) is sometimes a better way to express column efficiency in terms of length units per theoretical plate:

$$H = L/N \quad (15)$$

where L is the column length. H can be conveniently expressed as a multiple of the particle diameter of the packing material. The minimum plate height (maximum N) in a good column should be between 2 and 4 particle diameters measured at optimum mobile phase velocity (24). Unfortunately, in practice, the efficiency value is not only a function of how well a column has been packed, but also depends on other

factors such as the solutes being separated, the mobile phase composition and linear velocity, and the method selected for calculation. Silanophilic interactions leading to peak asymmetry have been found to be responsible for large differences in efficiencies measured at varying heights from the peak baseline. Peak asymmetry, denoted as A/B, is defined as the ratio of the width after the peak apex (A) to that before the peak apex (B), both measured at 10% peak height. This is shown in Figure 22. It has been suggested that a "good" column should have an asymmetry ratio smaller than 1.5 under mobile phase composition not exceeding 50% v/v in water content (25). Foley and Dorsey (26) developed an equation for column efficiency which takes into account peak asymmetry:

$$N_{sys} = 41.7(t_r/W_{0.1})^2 / (A/B + 1.25) \quad (16)$$

Here, N_{sys} denotes plate count for the chromatographic system and $W_{0.1}$ is the width at peak height fraction of 0.1 (or 10%). This calculation method, being very sensitive to peak asymmetry, appears to give a more realistic measure of practical peak efficiencies than those based on idealized Gaussian peak profiles (27).

Freshly prepared bonded phases were initially conditioned with neat organic modifier (acetonitrile or methanol) for

Figure 22. Calculation of peak asymmetry by measuring the A/B ratio at 10% of peak height. The peak shown corresponds to N,N-dimethyl-aniline on a C₁₈ Vydac TP at 60% acetonitrile/water and 1 mL/min. UV detection at 200 nm.

about 1 hour at 1 mL/min and then with 60% v/v organic modifier in water for 6-12 hours at the same flow rate. Retention times, k' values, peak asymmetry and plate counts for the different solute bands were determined from each chromatogram. Figures 23 and 24 show the separation profile of the three polar components mixture on two conventional alkyltrimethylsilyl silicas. A cursory comparison of the two chromatograms indicate good selectivity in both cases but better peak shape with C_{18} -Nucleosil. Triplicate measurements on similar chromatograms are summarized in Figures 25 to 26. Clearly, all three peaks exhibit similarly poor symmetry in the case of C_8 -Vydac TP (Figure 25). However, when the asymmetry value of each base is ratioed against that of BA, a value close to unity is obtained. This indicates that a factor other than silanophilic effects are causing this problem. Notice that both packing materials exhibit a half-height plate count of about 5,000 (Figure 26). However, peak asymmetry appears to affect the N_{sys} value of C_8 -Vydac in a larger extent as shown in Figure 27.

The same test was performed on three C_{18} -bonded silicas modified by hydrosilation. In addition, we wanted to examine the effect of treating these phases with an alkaline buffer solution. In doing so, a 50 mM phosphate solution of pH 9.0 was passed through each column for 3 hours at room temperature

Figure 23. Separation of the polar test mixture on a commercial C₈-Vydac TP silica (end-capped, 5.8 μm, 334 Å). Mobile phase: 60% v/v acetonitrile in water at 1.0 mL/min. UV absorbance at 200 nm. Sample injection was 5 μL of 0.1 mM solution, each solute. Sample components: 1, benzyl alcohol (BA); 2, N,N-dimethyl aniline (DMA); 3, N,N-diethyl aniline (DEA).

Figure 24. Separation of the polar test mixture on a home-made conventional C₁₈-Nucleosil silica (not end-capped, 7 μ m, 300 Å). Remaining conditions as in Figure 23.

Figure 25. Asymmetry of polar compounds on conventional alkyl phases.

Figure 26. Half-height column efficiency from polar compounds on conventional alkyl phases.

Figure 27. Asymmetry-based column efficiency from polar compounds on conventional alkyl phases.

(26±2°C). Upon alkaline treatment, silane groups underneath the anchored octyls might be hydrolyzed to silanols according to the reaction

Triplicate retention and symmetry measurements were carried out before and after this treatment. Typical chromatograms are shown in Figure 28 for C₁₈ Vydac HS and TP. No visual differences with respect to the original separation profiles were observed after the treatment. Figures 29 to 31 show that retention of various C₁₈-silicas, as measured by capacity factors, were affected in different extent. k'-values for YMC-Gel silica increased somewhat, while those for Vydac HS remained almost constant and those for Vydac TP decreased, particularly for DEA. Notice that k'-values for benzyl alcohol remained unchanged. It seems likely that some silane groups were hydrolyzed to silanols resulting in changed retention due to the added silanophilic interaction in the case of YMC-Gel silica. The decreased retention observed in the case of DEA on Vydac TP is more difficult to explain. Hydrolytic cleavage of C₁₈ ligands does not seem likely due to the mild treatment conditions.

In order to further substantiate this observation, the

Figure 28. Separation of the polar test mixture on C_{18} -bonded silicas prepared through hydride. (A) Vydac HS ($6.7 \mu\text{m}$, $104 \text{ \AA}$); (B) Vydac TP ($5.8 \mu\text{m}$, $291 \text{ \AA}$). Chromatographic conditions as in Figure 23.

Figure 29. Capacity factors for polar compounds on C_{18} YMC-Gel silica (prepared via hydride) before and after a 3-hour treatment with 50 mM phosphate pH 9.0 at room temperature.

Figure 30. Capacity factors for polar compounds on C_{18} Vydac HS silica (prepared via hydride) before and after a 3-hour treatment with 50 mM phosphate pH 9.0 at room temperature.

Figure 31. Capacity factors for polar compounds on C_{18} Vydac TP silica (prepared via hydride) before and after a 3-hour treatment with 50 mM phosphate pH 9.0 at room temperature.

base-treated packings were removed from the columns and tested by DRIFT. A typical comparison is shown in Figure 32 for Vydac HS. Clearly, no signs of deterioration are evident: a sharp SiO-H stretching peak due to isolated silanols is virtually absent and no apparent decrease in the intensity of the Si-H stretching peak is apparent. This observation is particularly important because it indicates that the anchored octadecyl groups efficiently shield the underlying SiH from the aqueous hydroxyl-containing treating solution. The other two C₁₈-phases (not shown) also exhibit identical DRIFT profiles before and after treatment. Perhaps more importantly, peak symmetry does not appear to deteriorate significantly upon alkaline treatment, as shown in Figures 33 to 35. Both the absolute symmetry ratio as well as the one referred to BA remained very close to unity. Similarly, the plate count value appears not to be affected by the treatment, as shown in Figures 36 to 41. These results strongly suggest that, under the conditions used, no deleterious effects result from the short-term exposure of the octyl-bonded phases to alkaline buffer.

The hydride intermediate was also evaluated with respect to its adsorptivity toward basic solutes. A total of five packings were tested; one was YMC-Gel and the other four were Vydac TP silicas from two different batches. Four of these

Figure 32. C₁₈-Vydac HS (prepared via hydride) before (upper curve) and after treatment with 50 mM phosphate pH 9.0 at room temperature.

Figure 33. Asymmetry of polar compounds on C_{18} YMC-Gel (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature.

Figure 34. Asymmetry of polar compounds on C₁₈-Vydac HS (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature.

Figure 35. Asymmetry of polar compounds on C_{18} Vydac TP (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature.

Figure 36. Half-height column efficiency from polar compounds on C_{18} YMC-Gel (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature.

Figure 37. Half-height column efficiency from polar compounds on C₁₈ Vydac HS (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature.

Figure 38. Half-height column efficiency from polar compounds on C₁₈ Vydac TP (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature.

Figure 39. Asymmetry-based column efficiency from polar compounds on C₁₈ YMC-Gel (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature.

Figure 40. Asymmetry-based column efficiency from polar compounds on C₁₈ Vydac HS (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature.

Figure 41. Asymmetry-based column efficiency from polar compounds on C₁₈ Vydac TP (prepared via hydride) before and after treatment with 50 mM phosphate pH 9.0 at room temperature.

hydride-modified packings exhibit similarly low capacity factors of about 0.40 ± 0.05 for all the three components, indicating very poor selectivity toward these solutes, as expected from hydrophobic bonded phases with a zero-length "alkyl" chain. In general, the chromatograms showed broad and asymmetric peaks, including that for BA (see Figures 42 and 43). Two of the remaining packings (Vydac TP's, one from each batch) showed increased retention times for the organic bases, with worse band broadening and tailing (see Figure 44). Additionally, the DEA and in a lesser extent the DMA peaks showed decreasing retention times upon consecutive injections. In one case (see Figure 44) this compound eluted with very low recovery (as evidenced by peak area comparisons). These observations strongly suggest that DEA is adsorbed on a relatively small number of highly active sites, until they are rendered inactive by strong binding with the organic amine. It is possible that one of these active sites on hydride-modified silicas correspond to surface silicon atoms bearing both a hydride and a hydroxyl group:

Similarly with geminal silanols on native silicas, these

Figure 42. Elution profiles for (A) BA, (B) DMA, and (C) DEA on hydride-modified YMC-Gel silica. Chromatographic conditions as in Figure 23.

Figure 43. Elution profiles for (A) BA, (B) DMA, and (C) DEA on hydride-modified Vydac TP (lot 890414) silica. Chromatographic conditions as in Figure 23.

Figure 44. Elution profiles for (A) BA, (B) DMA, and (C) DEA on hydride-modified Vydac TP (lot 900201) silica. Chromatographic conditions as in Figure 23.

groups should show an O-H IR stretching band corresponding to an isolated silanol (3740 cm^{-1}). The presence of the H-Si(OH)(OSi=)₂ species has been confirmed by ²⁹Si CP-MAS NMR (6,7).

The three-component polar mixture was also used to evaluate the effect of darkening of the bonded silica product during olefin hydrosilation (6). Product darkening is more pronounced at relatively high temperatures (above 110°C) and has been attributed to the reduction of the Pt(II) catalyst complex to elemental platinum by the surface SiH groups:

Catalytic activity of platinum is obviously decreased or completely lost as a result of this reaction. The polar mixture was injected into columns containing darkened C₈- and C₁₈-silicas. Typical chromatograms are shown in Figure 45 where strong tailing for DEA is quite evident. Similar to the hydride-modified sorbents, the retention time of this solute (and to a lesser extent that of DMA) decreased upon repeated injections and peak shape was consecutively improved. This effect also indicates adsorption of the organic amine(s). Clearly, product darkening should be avoided by careful choice of the catalyst and/or the reaction conditions.

Figure 45. Chromatographic profile for the polar mixture on C_8 (A) and C_{18} (B) Vydac TP silicas which were darkened during olefin hydrosilylation. Separation conditions and solutes as in Figure 23.

3.3.3. Separations of Proteins and Peptides

Another very important application of RP-HPLC is the separation of proteins, or in general biomolecules. Proteins are large, complex molecules which under certain conditions might be tenaciously adsorbed on many reversed phases. The incorporation of trifluoroacetic acid (TFA) or low pH phosphate buffers into aqueous acetonitrile or 2-propanol solvent mixtures has resulted in improved peak shapes. In some cases, particularly in preparative separations, TFA is preferred because it is easily removed from the separated fraction by lyophilization. We have already demonstrated that the new bonded phases which are the object of this study are very resistant to hydrolytic deterioration under these conditions. Gradient elution on C_4 - and C_8 -bonded wide-pore silicas appears to provide satisfactory separation conditions for proteins. Smaller polypeptides are more commonly separated on narrow-pore C_{18} -phases. In all cases, particle sizes below 10 μm are almost mandatory.

The potential of two C_{18} phases for the separation of two tripeptides is shown in Figure 46. Another example of the separation of somewhat larger peptides is depicted in Figure 47. Baseline separation of all peptides was accomplished in all cases. As illustrated in Figure 48, a standard protein

Figure 46. RP-HPLC separation of peptides on C_{18} phases prepared via hydride. (A) Vydac HS, $6.7 \mu\text{m}$, $104 \text{ \AA}$; (B) YMC-Gel, $10 \mu\text{m}$; $120 \text{ \AA}$. Mobile phase: solvent A: 0.10% v/v TFA in acetonitrile; solvent B: 0.10 v/v TFA in water. Linear gradient 20-45% A in 10 min. Flow rate: 1.3 mL/min. UV detection at 215 nm. Injection: $5 \mu\text{L}$ of 1.0 mg/mL of each peptide. Sample peaks: 1, Gly-Gly-Tyr; 2, Gly-Phe-Ala-Ala.

Figure 47. Separation of peptides on C₁₈ phases prepared via hydride. (A) Vydac HS, 6.7 μm , 104 Å; (B) YMC-Gel, 10 μm , 120 Å. Chromatographic conditions as in Figure 46. Sample peaks: 1, bradykinin; 2, oxytocin; 3, angiotensin II.

Figure 48. Typical separation of a standard protein mixture on a C₈ Vydac TP (5.8 μ m, 291 Å) prepared via hydride. Mobile phase: solvent A: 0.10% v/v TFA in acetonitrile, solvent B: 0.10% v/v TFA in water. Gradient: 24-96% A in 30 min at 1.5 mL/min. Sample injection: 5.0 μ L of 1.0 mg/mL each standard protein. Detection at 220 nm. Sample peaks: 1, ribonuclease B; 2, cytochrome C; 3, lysozyme; 4, myoglobin.

mixture of ribonuclease B, cytochrome C (horse heart), lysozyme (chicken) and myoglobin (horse) can be separated with a C₈-Vydac TP phase. The secondary peak shown with ribonuclease B might be due to some contamination with ribonuclease S. Other peaks shown in the chromatogram are unknown impurities that were present in the proteins standards.

It should be noted that attempts to separate other proteins such as α -chymotrypsinogen A and ovalbumin were precluded by irreversible adsorption of these proteins on the C₈ phase, under the conditions used. The same proteins were easily separated on a commercially available end-capped C₈-Vydac TP support. It is known that these proteins are very hydrophobic and low recoveries are common even with relatively short columns. It is possible that the higher coverage of the new C₈ phase on relation to the commercial product is the major reason for the irreversible adsorption of these and other proteins.

A likely solution to this problem is the use of a C₄ or even shorter alkyl chain. Unfortunately, the corresponding olefins, namely butene (b.p. -6.3°C), propene (b.p. -47.7°C) and ethylene (b.p. -104°C) are gases at room temperature and therefore would require special high-pressure reactors to realize the relatively high temperatures required for

hydrosilation. It should also be pointed out that the same commercial C₈-Vydac TP material that neither showed good shape with our polar mixture (BA, DMA, and DEA) nor exhibited acceptable hydrolytic stability, was able to perform better with respect to protein separation of hydrophobic proteins. More work is obviously required to obtain improved protein recovery with the new bonded phases. The later observation is an indication that solvophobic interactions, rather than silanophilic binding, are the reason for low protein recovery. Despite the fact that some silanophilic activity toward basic solutes is still present (see Figures 42-44), the hydride intermediate should provide a less hydrophobic surface than that of a C₈ phase prepared from it. This is illustrated in Figure 49 for lysozyme, α -chymotrypsinogen A and ovalbumin where virtually complete solute recovery is obtained with acceptable peak shape. Clearly, the surface SiH species do not interact strongly with the proteins and therefore the proteins coelute. However, the results confirm that a control of surface hydrophobicity should be possible by carefully choosing the optimum size of the anchored alkyl group, so that hydrophobic interactions are strong enough to provide good selectivity but not so much as to result in irreversible binding.

Figure 49. Elution profiles for (A) lysozyme, (B) α -chymotrypsinogen A and (C) ovalbumin on a hydride-modified Vydac TP material. Mobile phase: solvent A: 0.10% v/v TFA in acetonitrile, solvent B: 0.10% v/v TFA in water. Gradient: 20-35% of A in 15 minutes. Flow rate: 0.5 mL/min. UV detection at 220 nm. Injection: 5.0 μ L of 0.8-1.0 mg/mL of each protein.

3.3.4. Separation of Alkaloids

The ability of a C₁₈-bonded narrow pore silica for the separation of a mixture containing five barbitol derivatives is shown in Figure 50. Clearly, baseline separation of all sample components with excellent peak shape is obtained in a little more than ten minutes. This separation is a typical example of the applicability of the new bonded phases to pharmaceutical analysis.

Figure 50. RP separation of a commercial barbiturate mixture on a C₁₈ YMC-Gel (prepared via hydride). Mobile phase: 50% methanol/water at 1.0 mL/min. 10 μ L sample injection. UV detection at 220 nm. Sample peaks: 1, unknown impurity; 2, butabarbital; 3, amobarbital; 4, secobarbital; 5, phenobarbital; 6, hexobarbital.

4. CONCLUSIONS

Evaluation of the alkyl-bonded phases prepared via olefin hydrosilation on hydride-modified silica supports showed that, generally speaking, the selectivity of the new chromatographic phases resembles that of their conventional counterparts, alkyltrimethylsilyl-bonded phases. Compared to conventional phases under particularly aggressive eluent conditions, more specifically those involving TFA-containing aqueous-organic solvents, the new bonded materials showed superior hydrolytic stability, as expected from a direct Si-C linkage formed upon olefin addition. Besides improving column longevity, the hydrolytic advantage reduces to a minimum the fraction contamination that might result from coelution of phase degradation byproducts with the analyte(s) of interest.

The inherently deactivated surface underneath the anchored alkyl ligands provides for symmetric solute peaks. Treatment of alkyl-silicas with an alkaline pH buffer for several hours did not seem to significantly deteriorate peak shape, indicating effective shielding of SiH species by the organic moieties. Nevertheless, darkening of bonded silicas during olefin silanization appears to have deleterious effect on the separation of organic amines, resulting in some cases in irreversible adsorption of certain solutes.

Data presented here show that the irregular retention behavior of silanol-sensitive probes such as certain crown ethers cannot be described in the light of a dual-mechanism model.

Finally, the separating potential of C_8 and C_{18} phases prepared by the new method was demonstrated. A special PAH mixture was used to confirm the monomeric nature of the new phases. Separation profiles of complex PAH mixtures indicate selectivity typical of monomeric stationary phases. Separation of alkaloids, polypeptides and proteins clearly confirmed the applicability of the new phases to the separation of a wide variety of "real world" samples. Similar to conventional phases, extensive method development is sometimes required to accomplish a particular separation.

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