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STUDIES ON THE STABILIZATION OF REVERSED PHASES FOR LIQUID CHROMATOGRAPHY

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SUMMARY

This paper examines several approaches to the stabilization of silica-based reversed phases against acid-catalyzed hydrolysis. A hydrolytic mobile phase, consisting of 0.5% (v/v) trifluoroacetic acid at 27 or 50°C, was recycled through beds or columns of the various alkyl-bonded phases. Headspace gas chromatographic analysis of hydrofluoric acid digests was used to monitor the changes in bonded-phase coverage on the silica surface. Small hydrophobic and basic molecules and proteins were also used to probe changing chromatographic performance as phase hydrolysis proceeded. In this way, several characteristics of *n*-alkyl-bonded phases were evaluated for their influence on bonded-phase stability. The silica employed for bonding, the degree of coverage to which the phase is bonded, the chain length or "bulkiness" of the alkyl moiety on the silane used to react with the silica, and the reactivity of the silane employed were all shown to influence significantly the stability of the bonded phase produced. In particular, silicas of inherently high stability, bonded either with di- or tri-functionally reactive silanes, or with bulky (*e.g.*, triethyl) alkyl groups or long alkyl chains (*e.g.*, *n*-octadecyl groups), exhibited the highest stability against acid hydrolysis.

INTRODUCTION

Much research activity has focused on the characterization of silica-based bonded phases commonly used in high-performance liquid chromatography (HPLC). Many of these studies have examined reversed phases consisting of *n*-alkyl groups immobilized on silica, as these supports have seen widespread and predominant use in HPLC. Bonded-phase characterization by chromatographic[1–5], spectroscopic (*e.g.* NMR[6,7], Fourier transform IR[8]), and recently, direct chemical analysis[9–11] of the surface has provided a deeper understanding of the *n*-alkyl-modified surface relative

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to the unmodified precursor. These tools can also yield benchmark performance data with which to monitor the changes in chromatographic behavior as the support becomes degraded. Kirkland and co-workers[12–15] have provided a wealth of data on short *n*-alkyl-bonded reversed-phase quality and stability in four recent papers.

Stationary phase stability is of great concern to the enduser of chromatographic columns, particularly in preparative applications, where column degradation products can adulterate the fractions to be isolated. In fact, one recent publication documents the presence of 1,1,3,3-tetramethyl-1,3-bis(octadecyl)disiloxane (the dimer of *n*-octadecyldimethylchlorosilane) in fractions collected from a commercial C_{18} preparative column[16]. These results clearly indicate the need for stationary phases of improved stability in preparative work. A number of research groups have addressed this need, utilizing a variety of approaches, including the development of bonded phases based on improved silica gels[14] or alumina[17], various polymer-coated silicas[18,19], and polymeric supports[20–22], as well as mobile phase effects[23].

In this paper, we examine several strategies for the stabilization of bonded *n*-alkyl reversed phases based on silica to achieve extended operation under conditions commonly used for protein and peptide separations [*e.g.*, 0.1% aq. trifluoroacetic acid (TFA) with gradients of increasing acetonitrile concentration]. Two methods to assess bonded-phase stability were utilized in this work. First, a batch hydrolysis experiment was performed to prepare sizeable (*ca.* 10 g) amounts of hydrolyzed stationary phases for further characterization by headspace gas chromatography (HSGC). The second stability study was carried out on packed HPLC columns, containing the bonded silica-based material of interest. Both chemical analyses of surface coverage and sensitive chromatographic evaluations of the columns were used to monitor hydrolysis of the bonded phases as a function of silica type, bonded group type, coverage, and silane reactivity.

EXPERIMENTAL

Equipment

The liquid chromatograph was composed of Model M6000 solvent delivery pumps, a M660 gradient programmer (Waters Assoc., Milford, MA, U.S.A.), a Model 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.), a Model LC-15B UV detector (254 nm) (Perking-Elmer, Norwalk, CT, U.S.A.) and a Model BD40 recorder (Kipp & Zonen, Bohemia, NY, U.S.A.). The column temperature was maintained in a Model LC-100 column oven (Perkin-Elmer). The chromatographic data were processed with a Nelson Analytical (Cupertino, CA, U.S.A.) Model 2600 chromatography software package, used in conjunction with an IBM-PC AT (Boca Raton, FL, U.S.A.).

Batch hydrolysis experiments were performed in low-pressure glass chromatography columns (250 mm × 15 mm I.D.), supplied by Beckman Instruments (San Ramon, CA, U.S.A.). In these experiments, the column temperature was maintained by a Model 334 Hotpack (Philadelphia, PA, U.S.A.) refrigerated-bath circulator, set at 27.0°C. Alkylsilane coverages for the batch hydrolysis experiments were determined by a hydrofluoric acid derivatization–static HSGC analysis technique (HF–HSGC)[9,24]. A 12-m SE-30 capillary column of 250 μm internal diameter (Scientific Glass Engineering, Austin, TX, U.S.A.) was used in an HP 5890A gas chromato-

graph equipped with a flame ionization detector (Hewlett-Packard, Avondale, PA, U.S.A.). Gas chromatographic data were collected by an HP 3392A recording integrator.

Chemicals and materials

Trimethylsilyl chloride, triethylsilyl chloride, *n*-butyldimethylchlorosilane, *n*-butylmethyldichlorosilane, *n*-butyltrimethoxysilane, *n*-octadecyldimethylchlorosilane and *n*-octadecyltriethoxysilane were purchased from Petrarch Systems (Bristol, PA, U.S.A.) and used without further purification. Monobasic sodium phosphate, trifluoroacetic acid, orthophosphoric acid and various high-quality protein standards were obtained from Sigma (St. Louis, MO, U.S.A.) and used as received. Small-molecule standards, including caffeine, phenol, uracil, N,N-diethylaniline, 2,6-di-*tert.*-butylpyridine, 5-phenylpentanol, benzene, and C_1–C_9-*n*-alkylbenzenes were purchased from Aldrich (Milwaukee, WI, U.S.A.). Acetonitrile and TFA for the batch hydrolysis study were of "Baker Analyzed" reagent grade (J. T. Baker, Phillipsburg, NJ, U.S.A.). Other organic solvents for HPLC were from E. M. Science (Cherry Hill, NJ, U.S.A.), while HPLC-grade water was prepared in-house.

Column hardware (blanks, fittings, etc.) was obtained from Extrudehone (Irwin, PA, U.S.A.) and Valco Instruments (Houston, TX, U.S.A.). Three experimental silica gel batches (A, B and C) with the characteristics listed in Table I (data reported

TABLE I

CHARACTERISTICS OF SILICA GELS STUDIED

	Batch A	*Batch B*	*Batch C*
Date of manufacture	March, 1984	February, 1986	May, 1986
Pore volume (ml/g)*	1.3	1.21	1.39
Surface area (m^2/g)*	261	238	254
Median pore diameter (Å)*	186	197	188
Particle size properties			
Dv, 50 (μm)**	19.2	17.0	16.4
Dv, 50/Dp, 50**	1.09	1.16	1.18
Dv, 10/Dv, 90**	1.55	1.76	1.72
Metals, anions (ppm)***			
Al^{3+}	45	50	88
Ca^{2+}	38	7	<25
Fe^{2+}	53	33	50
Na^+	10	31	41
SO_4^{2-}	25	<25	31
Cl^-	27	<25	17
pH§	4.0	5.2	4.7
Loss on drying§§	<5	4.2	4.4

* Porisimetry determined by nitrogen sorption (BET method).

** Particle-size analysis by Coulter counter.

*** Cation analysis was by hydrofluoric acid digestion of silica followed by flame atomic absorption spectroscopy for Na and inductively coupled plasma spectroscopy for Al, Ca, Fe, and Mg determination. Anion analysis was by ion chromatography of water extracts[29].

§ pH measured of a 10% slurry of silica in water.

§§ Loss on drying determined from the weight change of the silica after heating at 105°C for 2 h.

by the manufacturer) were obtained from The PQ Corporation (Valley Forge, PA, U.S.A.) and used as received.

Synthesis

The silica samples for the batch hydrolysis study were bonded using the desired silanes according to a modification of the procedure of Unger and co-workers[25,26] with dichloromethane as the solvent and imidazole as an acid scavenger (Table II). Bonded alkyl-ligand coverage was determined by the previously developed HF–HSGC method[24]. In this procedure, a quantity of the bonded phase (*e.g.*, the *n*-butyl-bonded phase) is weighed into a PTFE vial as a 1:1 molar mixture with a quantity of a *n*-propyldimethylsilyl-bonded silica, which serves as an internal standard. The hydrofluoric acid reagent [HF–water–acetone, 1:1:2 (v/v/v)] is added with stirring and dissolution of the packing material in 5–60 min. The headspace is sampled by GC, and quantitation is performed by comparison with a calibration curve, determined with the appropriate standards. The internal standard serves to correct for variable recoveries, while the peaks were identified by mass spectrometry[24]. The precision of this method was *ca.* 2–3% relative standard deviation (R.S.D.).

For the subsequent stability study of stationary phases, prepared from the mono-functionally reactive silanes *vs.* tri-functionally reactive silanes, the column packings were synthesized following the procedure of Kinkel and Unger[25] for chlorosilanes and a modified procedure of Miller *et al.*[27] for trialkoxysilanes. The characteristics of these phases are indicated in Table III. Elemental analysis for surface coverage was performed by Galbraith Labs. (Knoxville, TN, U.S.A.) with a precision of percent carbon (% C) data for a given C_{18} bonded phase, *e.g.*, of *ca.* 0.6% R.S.D. A correction is made for the *ca.* 0.21% C found in the blank silica gel.

Batch hydrolysis procedure

Samples (5 g) of bonded silica were placed in each of three low-pressure chro-

TABLE II

CHARACTERISTICS OF BONDED PHASES USED IN BATCH HYDROLYSIS EXPERIMENTS

Experiment	*Bonded phase*	*Silica batch**	*Silane used*	*Coverage*** *(μmol/m²)*
Effect of coverage	I	A	*n*-Butyldimethylchlorosilane	1.95
	II	A	*n*-Butyldimethylchlorosilane	3.00
	III	A	*n*-Butyldimethylchlorosilane	3.67
Effect of silica	III	A	*n*-Butyldimethylchlorosilane	3.67
	IV	B	*n*-Butyldimethylchlorosilane	2.83
	V	C	*n*-Butyldimethylchlorosilane	4.04
Effect of silyl alkyl group	III	A	*n*-Butyldimethylchlorosilane	3.67
	VI	A	Triethylchlorosilane	2.85
Effect of silane reactivity	III	A	*n*-Butyldimethylchlorosilane	3.67
	VII	A	*n*-Butylmethyldichlorosilane	3.15

* See Table I.

** Coverage determined by HF–HSGC (see Experimental section).

TABLE III

CHARACTERISTICS OF *n*-BUTYL AND *n*-OCTADECYL REVERSED PHASES USED IN THE ACCELERATED HYDROLYSIS STUDY

For hydrolysis conditions, see text.

Reversed phases	*Silica batch**	*Silane used*	*Coverage (μmol/m²)*	*% C***	
				Initial§*	*Final*§*
Mono-C_4*** (IV)	B	*n*-Butyldimethylchlorosilane	2.83§§	4.48	3.10
Tri-C_4 (VIII)	C	*n*-Butyltrimethoxysilane	7.22	7.52	7.53
Mono-C_{18} (IX)	C	*n*-Octadecyldimethylchlorosilane	3.11§§§	16.90	16.80
Tri-C_{18} (X)	C	*n*-Octadecyltriethoxysilane	5.33	21.01	19.02

* See Table II.

** Elemental analysis data were corrected for background carbon in the unbonded silica.

*** The mono-C_4 bonded phase was not end-capped.

§ Initial % C values on the sorbents after synthesis, final % C on the phases after passage of 1436 column volumes of the hydrolytic mobile phase for the mono-C_4 column, after 2948 column volumes passed through the tri-C_4 column, and after 3169 column volumes passed through each of the mono-C_{18} and tri-C_{18} columns.

§§ The mono-C_4 bonded phase (V) synthesized from batch C silica yielded a coverage of 4.0 $\mu mol/m^2$.

§§§ The mono-C_{18} bonded phase was end-capped with trimethylchlorosilane in a second reaction. Coverage was calculated by using the non-end-capped % C value of 16.65.

matography columns, which were then placed into the controlled temperature bath (held at 27.0°C). The hydrolytic mobile phase used in all batch studies was 32% *aq.* acetonitrile, the aqueous component being 10 m*M* phosphoric acid and, in the final solution, the TFA concentration being 0.5% (v/v). The aqueous component was adjusted to a pH of 1.6, using potassium hydroxide prior to the addition of acetonitrile. A 7-l volume of the solution was pumped in a continuous recycle arrangement throughout the time of the study with a total flow-rate of 14 ml/min through the three columns connected in parallel. The solvent was recirculated with a Model 26740 air driven pump (Haskel, Burbank, CA, U.S.A.). At various times, small aliquots (*ca.* 250 mg) of the bonded silica were sampled for alkylsilane coverage determination.

Chromatographic procedures

The stationary phases were packed into 4.6-mm I.D. stainless-steel tubes, 10 or 15 cm long, following standard slurry procedures, with methanol as the driving solvent with a Model DSTV 122 air-driven pump (Haskel). Mobile phases were prepared by adding the correct volumes of degassed organic solvent and water to volumetric flasks. The pH was adjusted as necessary using the appropriate reagents. A precolumn (50 × 4.6 mm I.D.), containing the unbonded silica gel, was placed between the pump and the injector when chromatography was performed. In the sta-

bility study, the precolumn was removed, and the hydrolytic mobile phase described above was recycled through the analytical column at a rate of 1 ml/min and a temperature of 50°C for a given time. Multiple columns were operated in parallel. The hydrolytic mobile phase was prepared fresh at frequent intervals to prevent any equilibration with the bonded phase during the hydrolysis experiment. The column was removed periodically and examined by small-molecule chromatography of caffeine, phenol[28], N,N-diethylaniline[12–14], and *n*-alkylbenzenes, using the appropriate percentage of methanol in water according to Köhler *et al.*[12] at either 25 or 50°C. For larger-molecule chromatography of ribonuclease A, insulin, cytochrome *c* and bovine serum albumin, a 30-min gradient from 10–70% B (eluent B = 0.1% TFA in acetonitrile) in A (eluent A = 0.1% aq. TFA) was used at a flow-rate of 1.0 ml/min and 25°C.

RESULTS AND DISCUSSION

The overall goal of this paper was to evaluate some of the available options for the preparation of acid-stable alkyl-bonded reversed phases exhibiting acceptable chromatographic performance. Our first investigation involved the chemical characterization of reversed phases subjected to hydrolysis over time in batch experiments. Bonded *n*-alkylsilicas were prepared and subjected to the hydrolytic mobile phase by flow recycling, as described in the Experimental section. The hydrolytic mobile phase consisted of 32% aq. acetonitrile, a typical organic solvent concentration used for protein elution from reversed phases, while a five times greater acid (TFA) concentration was used than that needed in typical protein reversed-phase liquid chromatography (RPLC). Chemical analysis of the sorbents involved hydrofluoric acid digestion, followed by HSGC analysis (see Experimental). Full details of this method are reported elsewhere[9,24]. In this way, the stationary phases could be analyzed periodically to quantitate the alkyl group hydrolysis (*i.e.*, changing bonded-phase coverage was determined).

Table I provides the chemical and physical characteristics of several silica batches used in this study, as analyzed by a variety of indicated procedures[29]. These nominal 20-μm particle diameter, 200-Å pore diameter materials were made over the course of three years under varying reaction conditions. However, they possess similar porisimetry and particle-size distribution properties. Analysis of metals and anions in the gels revealed low levels of these impurities with some variability in the aluminum, calcium, iron and sodium content.

The batch-mode experiments were carried out on various bonded phases, based on batches A, B or C to examine the influence of several parameters on the stability of the bonded-alkyl phase, including (1) bonded-alkyl-group coverage of the silica, (2) silica gel selected for bonding, (3) alkyl functionality used in the silane, and (4) the number of reactive functionalities in the silane.

Table II shows the silane used and coverage characteristics of bonded silicas A, B and C, evaluated in the above experiments. In the next sections, we shall discuss the results of the batch hydrolysis of these stationary phases from the point of view of changes in bonded-phase coverage. We will then turn to the analysis of stability of stationary phases prepared from mono- and tri-functional silanes by small- and large-molecule chromatography. Taken together, the results of these studies provide

a variety of directions to be taken for the stabilization of bonded reversed phases, based on silica, under acidic mobile phase conditions.

Effect of alkylsilane coverage

This study was performed to examine the effect of surface coverage on the stability of the prepared bonded phase for a given ligand and silica. As indicated in Table II, three samples of batch A silica were bonded to the indicated coverages by reaction of the silica with variable concentrations of *n*-butyldimethylchlorosilane. Coverages of 1.95, 3.00 and 3.67 μmol/m^2 of *n*-butyldimethylsilylgroups on stationary phases I, II and III, respectively, were determined by the HF–HSGC method. The three materials were then exposed to the hydrolytic mobile phase at 27°C, samples of the material being examined periodically for loss of coverage. Stationary phase hydrolysis is presumed to occur by cleavage of siloxane bonds under acidic conditions[9,10].

Fig. 1 presents the results of this experiment and indicates that the high-coverage (3.67 μmol/m^2) stationary phase III possessed the greatest resistance to hydrolysis under these conditions, as compared to the stationary phases I and II of lesser coverage (1.95 and 3.00 μmol/m^2, respectively). Interestingly, all of the materials showed a rapid loss of coverage (over *ca.* 20 h), followed by a more gradual loss of the bonded phase. This result may suggest the presence of two differentially hydrolyzed populations of siloxanes on the surface. The converse observation of two differential reacting groups of silanols has been postulated by Köhler *et al.*[12]. The lower-coverage stationary phases I and II exhibit a similar extent of alkyl-group loss of *ca.* 1.5 μmol/m^2 at *ca.* 50 h. This also indicates that the initial sites of bonding on the silica are more susceptible to acid hydrolysis than is the case after further bonding (*i.e.*, the high-coverage 3.67 μmol/m^2 stationary phase III). This effect may also be due to greater shielding of the acid-labile alkylsilyl sites when the coverage exceeds a certain value[12]. However, this interpretation does not explain the lack of more rapid hydrolysis after *ca.* 50 h in the order: stationary phase I > II > III, when coverages are 0.45, 1.5 and 2.87 μmol/m^2, respectively, and the shielding effect should

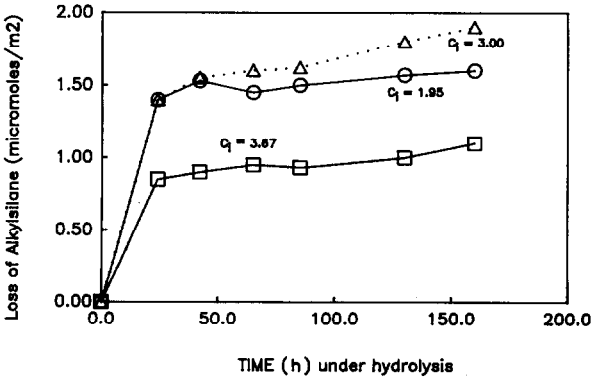

Fig. 1. Hydrolysis response of batch A silica, bonded with *n*-butyldimethylchlorosilane to different coverages. Bonded phases I (○), II (△) and III (□) (Table II) of 1.95, 3.00 and 3.67 μmol/m^2 were evaluated. For conditions, see Experimental and text.

be less important. Interestingly, stationary phase I exhibits greater stability than stationary phase II after *ca.* 50 h. This result may reflect the variable uniformity of the alkylated surfaces. These issues are under further study in our laboratories.

Effect of silica used

We next examined the role of the silica support used in preparing the bonded phase on sorbent stability. Three prototype batches of silica gel were made under different reaction conditions over the course of three years (Table I). Samples of these batches were made to react to nominally maximum coverage with *n*-butyldimethylchlorosilane. Table II lists coverages of 3.67, 2.83 and 4.04 μmol/m^2 for batches A, B and C, respectively. No correlations are apparent between these results and the characteristics listed in Table I. Results obtained by other investigators[12] have prompted us to examine silica surface phenomena as a possible source of variability. Attempts to verify and quantitate these differences spectroscopically are now underway in our laboratories.

Fig. 2 presents the analysis of changes in bonded-group coverage of these materials as a function of time. Both stationary phases IV and V on silicas B and C, respectively, exhibit losses of *ca.* 0.1 μmol/m^2 relative to the less stable (*ca.* 0.8 μmol/m^2 loss) bonded phase III, based on silica A. The "shielding" effect discussed above does not explain the relatively greater stability of bonded phase IV *vs.* bonded phase III. Rather, the results indicate the differential reactivity of the siloxanes on these bonded surfaces. Further work is underway to characterize more fully the silanol chemistry of each of the silica materials.

The above experiments indicate that the silica used as support can determine the stability of the resultant bonded phase in chromatographic practice. This conclusion has also been drawn by other researchers[12]. While improved bonding methods can serve to increase stability, it is obviously an advantage to use a silica (*i.e.*, batch B or C) that provides alkylated surfaces of greater stability. We next examined the use of improved (*i.e.*, for extended stability) bonding schemes on the silica batch that exhibited the poorest stability (*i.e.*, batch A) to explore avenues for stabilization.

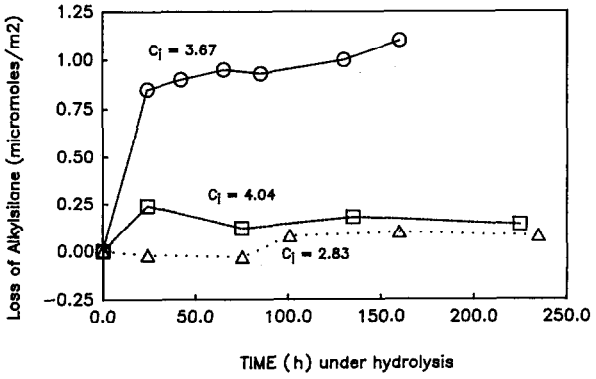

Fig. 2.Hydrolysis response of batch A, B and C silicas, bonded with *n*-butyldimethylchlorosilane. Bonded phases III (○), IV (△) and V (□) (Table II) of coverages 3.67, 2.83 and 4.04 μmol/m^2 were evaluated. For conditions, see Experimental and text.

Effect of alkyl group on the silane

One approach to stabilizing the alkyl-bonded stationary phase is the use of bulky silanes as bonding reagents. Accordingly, silica batch A was bonded with triethylsilyl chloride to yield bonded phase VI with a coverage of 2.85 μmol/m^2 (Table II). A reduction in the maximum bonded coverage obtainable by using bulky silanes has been noted by others[26]. We expect the bonded triethyl moiety to hinder hydrolysis of the bonded phase, both by virtue of forming a thermodynamically more stable bond with the silica gel matrix, and by a possible shielding effect of using a larger-"footprint" alkylsilyl ligand[30]. The concept of a more stable bond is given support by the work of Ackerman[31], in which the stability against hydrolysis increased fifty-fold on progressing from a trimethyl- to a triethylphenoxysilane.

Fig. 3 shows the expected increased stability of this triethylsilyl-bonded phase VI, relative to the *n*-butyldimethylsilyl-bonded phase III in spite of the lower coverage on the former support. As both ligand chains contain the same number of carbons, albeit in different configurations, this approach indicates the value of a "bulky" ligand for improved phase stability. Protein chromatography was similar to that on the *n*-butyldimethylsilyl-bonded phase III[32–34]. Thus, the triethylsilyl-bonded phase VII was quite suitable for protein RPLC.

Effect of silane reactivity

A second approach to extended column lifetime is to increase the number of siloxane bonds between the alkylsilane and the silica gel matrix (*i.e.*, the use of multi-functional silanes[35]). The bonding agent selected was *n*-butylmethyldichlorosilane, since this bonded phase would be expected to be chromatographically similar to that made with the *n*-butyldimethylsilyl chloride, while easy quantitation of the stationary phase is expected by the HF–HSGC method. Table II indicates coverage of the *n*-butylmethylsilyl-bonded phase VII to be 3.15 μmol/m^2, while Fig. 3 demonstrates the improved stability relative to the monofunctional silyl-bonded packing. Chromatographic evaluation of bonded phase VII indicated performance equivalent to

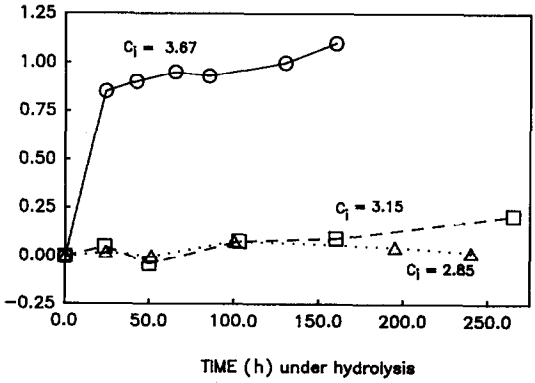

Fig. 3. Hydrolysis response of batch A silica, bonded with various silanes. Sorbent III (○) was prepared by reaction of *n*-butyldimethylchlorosilane to 3.67 μmol/m^2 coverage. Sorbent VI (△) was prepared by reaction of triethylchlorosilane to 2.85 μmol/m^2 coverage. Sorbent VII (□) was prepared by reaction of *n*-butylmethyldichlorosilane to 3.15 μmol/m^2 coverage. For conditions, see Experimental and text.

that of the *n*-butyldimethylsilyl-bonded phase III[32,33]. We next extended the multifunctionally reactive silane approach to an examination of stationary phases produced by reaction of the silica with trialkoxysilanes. In this approach, the trialkoxysilane is capable of forming a cross-linked polymeric layer on the surface, which can serve to improve the resistance of the stationary phase to hydrolysis[35]. Of course, these materials can be irreproducible and require careful control of reaction conditions[28]. In these experiments, we also evaluated the effect of chain length (*i.e.*, *n*-butyl *vs.* *n*-octadecyl) on stationary phase stability.

Table III provides data on four bonded phases, including *n*-butyl (mono-C_4, bonded phase IV) and *n*-octadecyl (mono-C_{18}, bonded phase IX) packings prepared from the monochlorodimethylsilanes and the corresponding stationary phases (tri-C_4, bonded phase VIII and tri-C_{18}, bonded phase X) prepared from the trialkoxysilanes. The tri-C_4 and tri-C_{18} packing materials show greater carbon content and

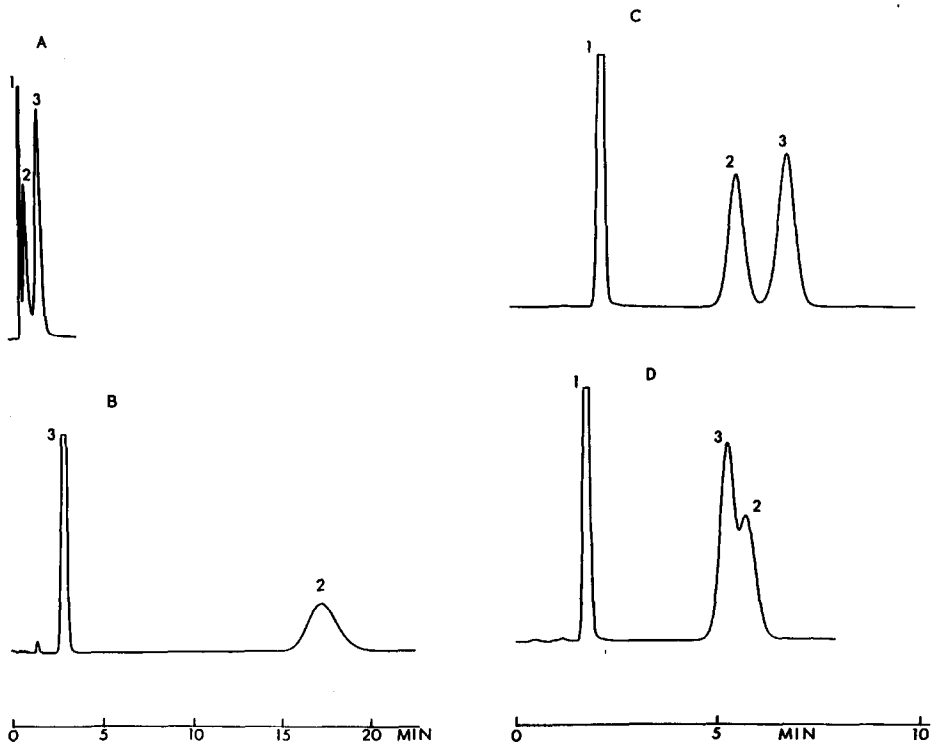

Fig. 4. Chromatography of caffeine and phenol on *n*-butyl-bonded columns before and after column stability experiments. (A) 100 × 4.6 mm I.D. column packed with mono-C_4 sorbent IV (prepared from the *n*-butyldimethylchlorosilane), see Table III. Uracil (1), caffeine (2) and phenol (3) were used as solutes with a mobile phase of 100% water at 1.0 ml/min and 25°C. Column evaluated immediately after packing. (B) Same column as in A after passage of 1436 column volumes of the hydrolytic mobile phase (see Experimental section) at 50°C. (C) Column (150 × 4.6 mm I.D.) packed with tri-C_4 sorbent VIII (prepared from the trimethoxysilane) (Table III). Uracil (1), caffeine (2), and phenol (3) were used as solutes with a mobile phase of 20% aq. (v/v) methanol at 1.0 ml/min and 25°C. Column evaluated immediately after packing. (D) Same column as in C after passage of 2948 column volumes of the hydrolytic mobile phase (see Experimental section) at 50°C.

higher coverages relative to the mono-packings and suggest the formation of a polymeric layer. In the case of the tri-bonded phases, the coverages were calculated assuming the three alkoxy functions have reacted. The validity of this assumption is under further investigation in our laboratories.

Note that the mono-C_4 bonded phase prepared on batch B silica yielded a lower coverage (*i.e.*, 2.83 μmol/m^2) relative to the mono-C_{18} bonded phase on batch C silica for the same bonding procedure. As shown in Table II, the *n*-butyldimethylsilyl-bonded phase V was prepared on batch C silica, using the same bonding procedure to yield a coverage of *ca.* 4.0 μmol/m^2. Despite this difference in coverage, which may reflect differences in surface silanol reactivity, Fig. 2 indicates the two *n*-butyldimethylsilyl-bonded Phases IV and V to show identical high stability under the test conditions employed. On the same batch of silica, the longer-chain C_{18} stationary phases show less coverage relative to the shorter-chain C_4 stationary phases, which may reflect a steric constraint in favor of the shorter chains for higher coverage[26]. We decided to examine the lower-coverage mono-C_4 bonded phase, based on batch B silica, in comparison with the tri-C_4 bonded phase in an accelerated

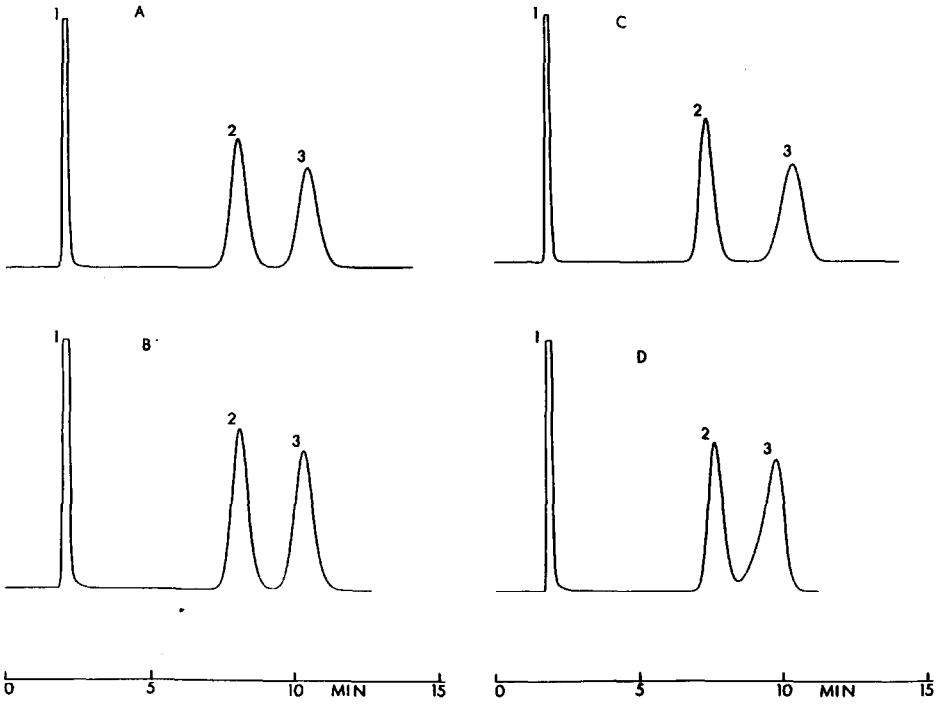

Fig. 5. Chromatography of uracil (1), caffeine (2), and phenol (3) on *n*-octadecyl-bonded columns before and after column stability experiments. (A) Column (150 × 4.6 mm I.D.) packed with mono-C_{18} sorbent IX (prepared by reaction of *n*-octadecyldimethylchlorosilane), see Table III. Column evaluated immediately after packing. All other conditions as Fig. 4C. (B) Same column as in A, evaluated after 3169 column volumes of use (see Experimental section). (C) Column (150 × 4.6 mm I.D.) packed with tri-C_{18} sorbent X (prepared by reaction of the *n*-octadecyltriethoxysilane) (Table III). Column evaluated immediately after packing. All other conditions as Fig. 4C. (D) Same column as in C, evaluated after 3169 column volumes of use (see Experimental section.)

hydrolysis evaluation, using a new batch of the hydrolysis reagent used earlier but at a higher temperature of 50°C and at a flow-rate of 1 ml/min. This accelerated hydrolysis test was also conducted on the mono-C_{18} and tri-C_{18} packings.

Chromatographic characterization

Several tests were employed to monitor the changing chromatographic behavior of the stationary phases throughout this stability evaluation. Fig. 4 provides illustrative chromatograms of uracil, caffeine and phenol for the mono-C_4 and tri-C_4 columns before and after passage of 1436 and 2948 column volumes of the hydrolysis reagent. Note that the mono-C_4 column was used with a mobile phase of 100% water while the more hydrophobic tri-C_4 column required a mobile phase of 20% methanol in water. Caffeine is expected to undergo hydrogen-bonding to neutral silanols[36] under these mobile phase conditions. Thus, the increased retention of caffeine is indicative of poor stability. While inversion of elution order is observed for the tri-C_4 column, the effect is less severe and occurs after significantly greater usage (twice that of the mono-C_4 bonded phase) of the column. Interestingly, as shown in Fig. 5, the mono-C_{18} and tri-C_{18} columns show little change with use in this test. These

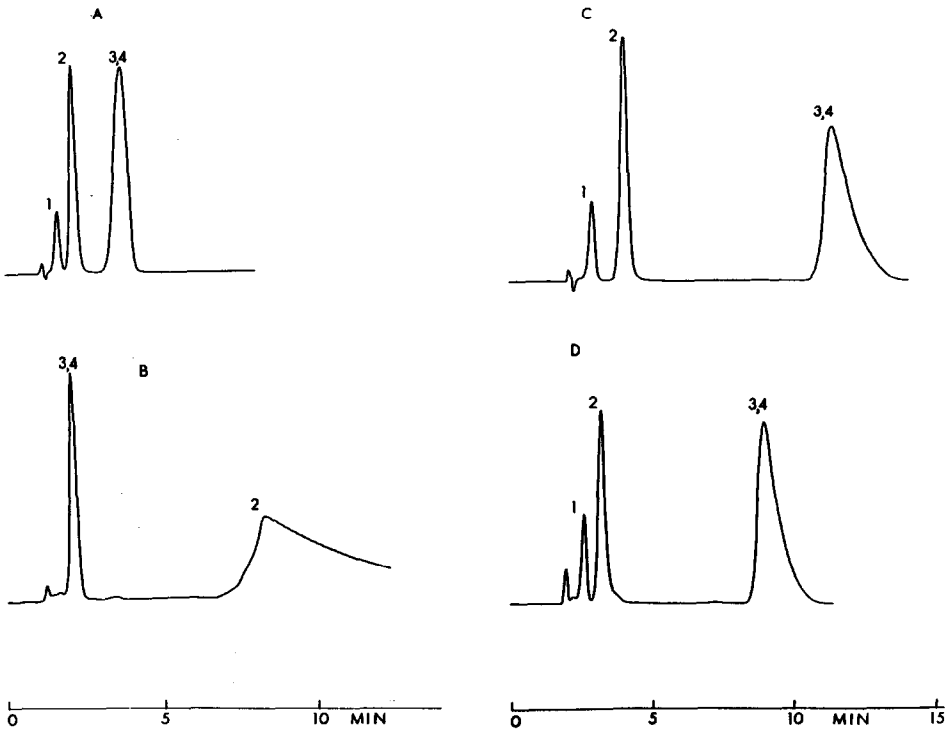

Fig. 6. Chromatography of 5-phenylpentanol (1), N,N-diethylaniline (2), 2,6-di-*tert.*-butylpyridine (3), and 1-phenylheptane (4) on *n*-butyl-bonded columns before and after stability experiments. The stated solutes were chromatographed in a mobile phase of 70% (v/v) aq. methanol at 1.0 ml/min and 50°C. (A) New mono-C_4 column. (B) Mono-C_4 column after 1436 column volumes of use. (C) New tri-C_4 column. (D) Tri-C_4 column after 2948 column volumes of use.

results suggest that long-chain bonded phases are inherently more stable under these conditions, in agreement with the literature[12].

We next examined retention of the four-component mixture (5-phenylpentanol, N,N-diethylaniline, 2,6-di-*tert.*-butylpyridine, and 1-phenylheptane), proposed by Köhler *et al.*[12] for measuring bonded-phase quality as a function of column use for the four columns. In particular, N,N-diethylaniline is expected to show increased retention and peak tailing by interaction with silanols as stationary phase hydrolysis proceeds, while decreased k' should result for the hydrophobic probe 1-phenylheptane as the bonded-phase coverage decreases. Fig. 6 provides chromatograms of this mixture on the mono-C_4 and tri-C_4 columns before and after passage of the hydrolytic mobile phase at 50°C. The 2,6-di-*tert.*-butylpyridine and 1-phenylhetane peaks are not resolved on either C_4 column. The mono-C_4 column is degraded throughout the 1436 column volumes of use. Thus, N,N-diethylaniline is well retained and tails while the simultaneously eluted peaks lose retention. The tri-C_4 column exhibits greater stability with a *ca.* 20% loss in retention of the later-eluted peaks after twice the number of column volumes passed through the mono-C_4 column. This result further illustrates the stabilizing influence of the trialkoxysilane. Fig. 7 shows results

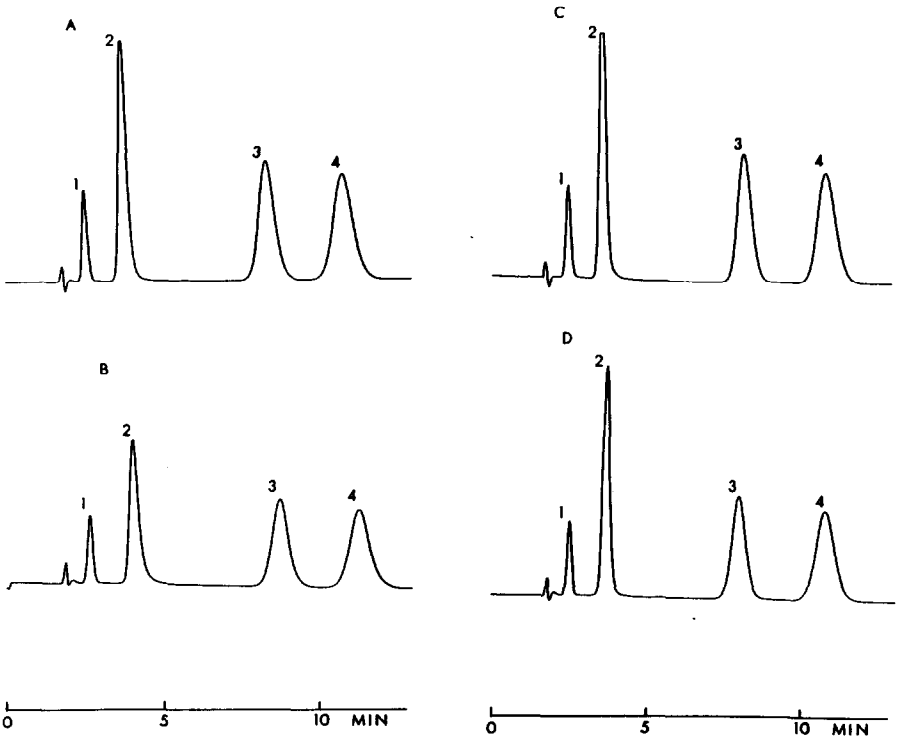

Fig. 7. Chromatography of 5-phenylpentanol (1), N,N-diethylaniline (2), 2,6-di-*tert.*-butylpyridine (3), and 1-phenylheptane (4) on *n*-octadecyl-bonded columns before and after stability experiments. The stated solutes were chromatographed in a mobile phase of 80% (v/v) aq. methanol at 1.0 ml/min and 50°C. (A) New mono-C_{18} column. (B) Mono-C_{18} column after 3169 column volumes of use. (C) New tri-C_{18} column. (D) Tri-C_{18} column after 3169 column volumes of use.

of the same test on the mono-C_{18} and tri-C_{18} columns before and after passage of *ca.* 3200 column volumes of the hydrolytic mobile phase. Minimal change is seen in these chromatograms. This illustrates the stabilizing influence of the longer *n*-alkyl chain against sorbent degradation.

Surprisingly, evaluation of the C_4 and C_{18} bonded phases with the basic drugs procainamide and N-acetylprocainamide and an aqueous buffer of pH 7.6 as the mobile phase failed to show major changes in the retention and peak shape of these solutes during the stability experiment. This observation may have relevance as to the type and/or distribution of silanols present on the surface during stationary phase hydrolysis. Further work is needed to clarify and understand this result.

A more rigorous evaluation of hydrophobic selectivity lies in the elution of the *n*-alkylbenzene homologous series as a function of column use. Figs. 8 and 9 show chromatograms of these solutes on the C_4 and C_{18} columns before and after passage of the hydrolytic mobile phase. Note the decreased k' and α for these solutes on the mono-C_4 column, suggesting serious stationary-phase degradation, while the tri-C_4 column showed less of this effect after greater use. Interestingly, increased band tailing with increased k' was noted on the tri-C_4 column, suggesting a heterogeneity of

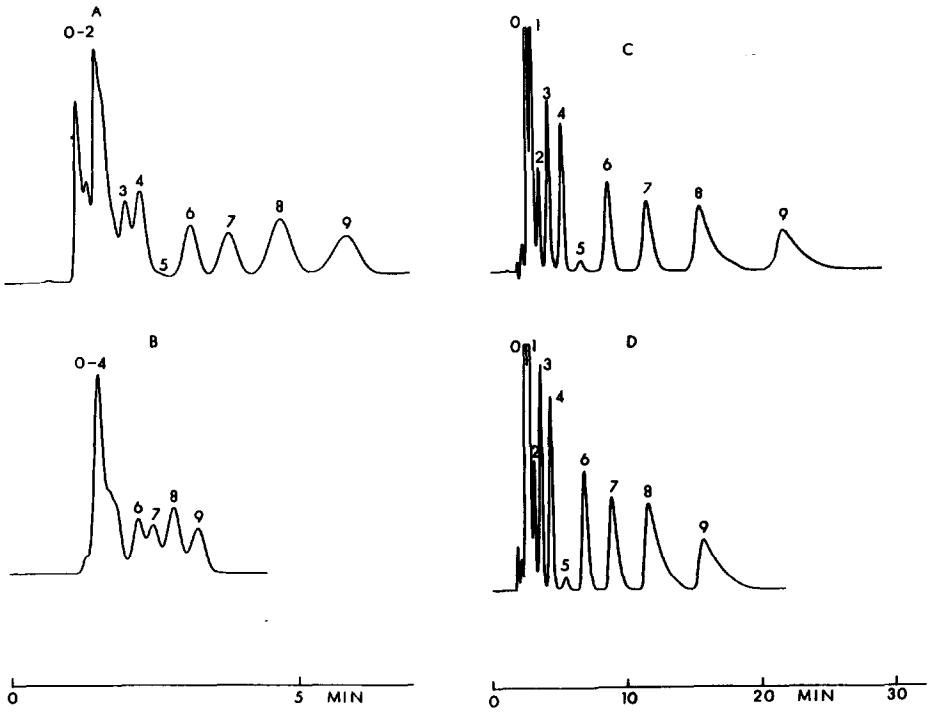

Fig. 8. Chromatography of *n*-alkylbenzenes on *n*-butyl-bonded columns before and after stability experiments. Benzene (0) and *n*-alkylbenzenes [toluene (1), *n*-ethylbenzene (2), *n*-propylbenzene (3), *n*-butylbenzene (4), 1-phenylpentane (5), 1-phenylhexane (6), 1-phenylheptane (7), 1-phenyloctane (8), and 1-phenylnonane (9)] were chromatographed in 70% (v/v) aq.methanol at 1.0 ml/min and 50°C. (A) New mono-C_4 column. (B) Mono-C_4 column after 1436 columns of use. (C) New tri-C_4 column. (D) Tri-C_4 column after 2948 column volumes.

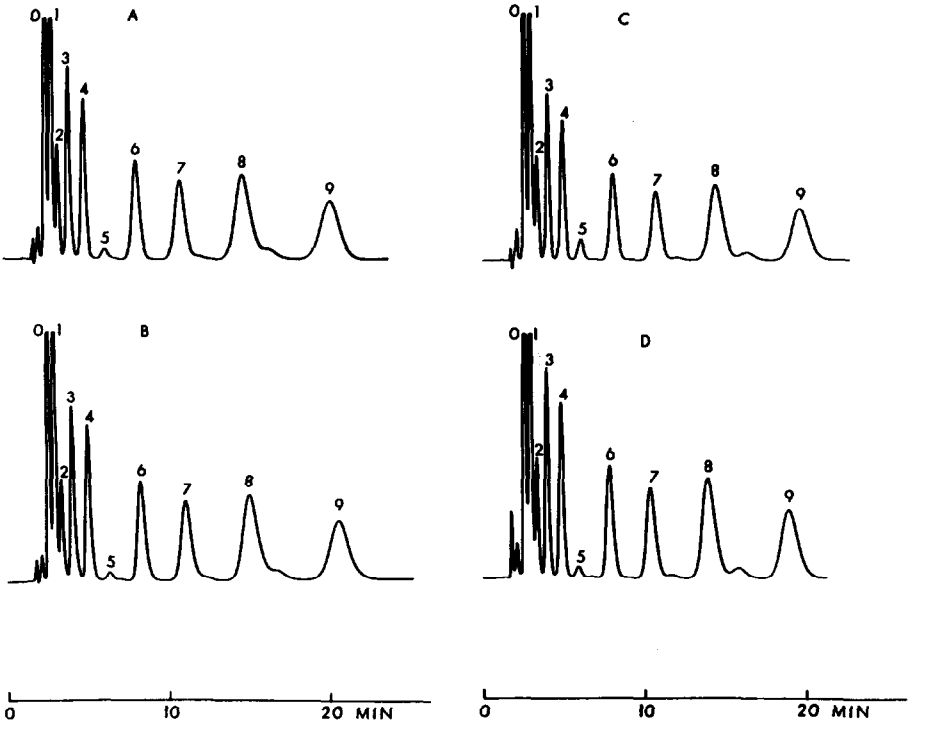

Fig. 9. Chromatography of *n*-alkylbenzenes on *n*-octadecyl-bonded columns before and after stability experiments. Same solutes as in Fig. 8 were chromatographed in 80% (v/v) aq. methanol at 1.0 ml/min and 50°C. (A) New mono-C_{18} column. (B) Mono-C_{18} column after 3169 column volumes of use. (C) New tri-C_{18} column. (D) Tri-C_{18} column after 3169 column volumes of use.

sites in the polymeric C_4 bonded layer[37]. As expected from the previous experiments, the results in Fig. 9 indicate minor changes in hydrophobic retention or selectivity on the longer-chain C_{18} bonded phases.

Gradient elution of proteins was also used to evaluate the mono-C_4 and tri-C_4 columns after hydrolysis. As shown in Fig. 10A, the mono-C_4 column provided acceptable biopolymer elution even with the first injection, despite the deterioration of this column demonstrated by the small-molecule LC tests. This is not surprising, since it is well established that chromatographic performance in gradient RPLC of proteins is predominantly influenced by mobile phase variables and less influenced by column variables[38]. Furthermore, in a separate study[39], it was shown that typical protein RPLC conditions (*i.e.*, 0.1% TFA in 32% aq. acetonitrile) prevent interactions between the test proteins used and unbonded silica gel. As expected, acceptable protein resolution was obtained on the more stable tri-C_4 column (Fig. 10B). Portions of both of the C_4 columns (and the C_{18} columns), submitted for elemental carbon analysis, gave the values shown in Table III. Note that after passage of *ca.* 1440 column volumes through the mono-C_4 column, the % C value or coverage decreased by *ca.* 31% while the tri-C_4 bonded phase shows no change in % C over the passage of *ca.* 2950 column volumes. Thus, acceptable protein resolution is obtained on the

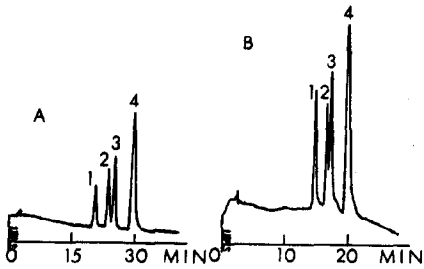

Fig. 10. Chromatography of proteins on *n*-butyl-bonded columns after stability experiments. Proteins (1 = ribonuclease A; 2 = insulin; 3 = cytochrome *c*; 4 = bovine serum albumin) were chromatographed at a flow-rate of 1.0 ml/min using a 30-min gradient at 25°C from 10 to 70% B (eluent B = 0.1% TFA in acetonitrite) in A (eluent A = 0.1% TFA in water). Detector, 254 nm. (A) Mono-C_4 column after 1436 column volumes of use. (B) Tri-C_4 column after 2948 column volumes of use.

mono-C_4 column, despite the loss of *ca.* 31% of the bonded phase. This result has also been obtained by Glajch and Kirkland[15] and illustrates that the chromatographic behavior of small and larger molecules on alkyl reversed phases is different. The observed minor changes in the small-molecule chromatograms on the tri-C_4 column over time may be due to minor changes in the polymeric layer which are not reflected in the % C value. Likewise, the mono-C_{18} bonded phases show small changes in % C during the passage of *ca.* 3200 column volumes, and the results correlate well with the absence of change observed in the small-molecule chromatograms over the course of the stability study.

CONCLUSIONS

This paper examined several strategies to make and test silica-based reversed phases that are resistant to acid hydrolysis. Batch hydrolysis of bonded phases followed by headspace GC analysis offers a useful and quantitative method to evaluate bonded phase stability. Chromatography of appropriate test probes proved to be a sensitive and convenient method to qualitatively evaluate bonded phase stability. The role of the silica chosen for bonding has a major influence on stability of the bonded phase, however, the contribution of the underlying silanol chemistry is not completely understood. Improvements in bonding chemistry including high coverage, multi-reactive silanes, and/or the use of bulky (*e.g.*, triethyl) or long chain (*e.g.*, C_{18}) groups in the silane showed significant stabilizing effects over the use of a short chain monofunctionally reactive silane. The use of improved bonding schemes on a silica of acceptable stability should provide greater confidence in the preparation of stable stationary phases suitable for protein separation. Further studies are underway in our laboratories to clarify the influence of silanol chemistry present on the silica on the stability of the resultant bonded phase.

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