Journal of Chromatography, 384 (1987) 81–90
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1027

EFFECT OF COLUMN DEGRADATION ON THE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF PEPTIDES AND PROTEINS

J. L. GLAJCH*, J. J. KIRKLAND and J. KÖHLER*
Central Research and Development Department, E. I. du Pont de Nemours and Company, Experimental Station, Wilmington, DE 19898 (U.S.A.)

SUMMARY

Many reversed-phase separations of proteins and peptides are currently performed in acidic mobile phases, *e.g.*, 0.1% trifluoroacteic acid in water (pH 2) with organic modifiers. Such conditions are known to promote the cleavage of the silane from the silica in bonded-phase columns, especially for monomeric stationary phases. The stability of some columns commonly used for proteins and peptides has been examined, and it has been shown by both chromatographic and elemental analysis that degradation occurs very rapidly with fresh, "totally covered" column materials. Despite the loss of over half of the bonded phase in some cases, certain columns still exhibit adequate chromatographic performance, although reproducibility can be affected. The implications of these results with respect to both bonded-phase synthesis and mechanistic interpretation of chromatographic data is discussed.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) for the separation and purification of unmodified or unprotected peptides and proteins has increased dramatically in the past ten years. Although a number of different modes of chromatography have been used, such as size-exclusion, ion-exchange, and affinity chromatography, the focus has been on reversed-phase separations. The introduction of trifluoroacetic acid or heptafluorobutyric acid[1,2] into aqueous–organic solvent systems was the main reason for the explosive growth in the reversed-phase separations of proteins and peptides. These reagents have been important in minimizing mixed-mode effects in HPLC, *i.e.*, avoiding secondary equilibrium effects due to the partial ionization of amine or carboxylic acid groups prevalent in peptides and proteins. The more common mobile phase systems for separating peptides and proteins have a relatively low pH of 2–3. These systems generally are preferred, because of

* Permanent address: Max-Planck-Institut für Kohlenforschung, Kaiser-Wilhelm-Platz 1, 4330 Mulheim/Ruhr, F.R.G.

(1) increased solubility of the peptides/proteins at low pH, (2) complete ionization of the amino groups, and (3) easy removal of the reagents for subsequent analysis.

Reversed-phase packings fall into two general classes: fully polymeric materials and silica with alkyl modifiers. Although most polymeric column packings have the advantage of wide pH stability, silica-based columns are still more widely employed, since they can be prepared with a variety of alkyl groups, *e.g.*, trimethylsilyl, C_3, C_4, C_8, C_{18}, CN, phenyl. In addition, silica-based particles are stronger than polymeric beads, and this increased strength is critical in preparative and process separations. Most importantly, silica-based packings exhibit higher column efficiencies because of their superior mass transfer characteristics.

Bonded-phase columns are a preferred media for HPLC separations because of their convenience and relatively high stability. If operated properly, bonded-phase columns provide reproducible separations under a variety of mobile phase conditions. Especially convenient is the rapid re-equilibration of the bonded stationary phase with the mobile phase, which is a great advantage in gradient elution separations. However, covalently bonded alkyl groups, formed by the reaction of chloro- or alkoxysilanes with hydroxylated silica, are not completely stable. The siloxane bond connecting the silane to the silica support is relatively easily hydrolyzed at low pH in aqueous solutions.

During optimization studies on the separation of the phenylthiohydantoin (PTH)-amino acids, we noted that C_8 reversed-phase columns rapidly deteriorated in phosphoric acid (pH 2.1)–methanol solutions[3]. Qualitatively, the same effect was observed for other acids in the same study. These results were obtained on alkyl reversed-phase columns similar to those that are widely used for peptide and protein separations at low pH. Since this is an important application, we decided to investigate this effect systematically. In particular, we were interested in (1) the rate of column deterioration under usual operating conditions, (2) possible effect of this deterioration on separations, and (3) ways to develop more stable bonded-phase columns.

EXPERIMENTAL

All measurements were carried out with a Model 8800 liquid chromatograph with a four-solvent pumping system, a heated column compartment, maintained at either 35 or 50°C, and a Model 860 variable-wavelength detector (Du Pont Biomedical Products Department, Wilmington, DE, U.S.A.). Samples were injected with either a 6-port sampling valve (Valco Instruments, Houston, TX, U.S.A.) or a WISP autoinjector (Millipore, Waters Chromatography Division, Milford, MA, U.S.A.).

Vydac 214TP5 (Separations Group, Hesperia, CA, U.S.A.) and Ultrapore RPSC (Beckman Instruments, Berkeley, CA, U.S.A.) columns were used as received. The Vydac columns and Ultrapore columns were 15 × 0.46 cm I.D. and 7.5 × 0.46 cm I.D., respectively. The physical characterization of packings from fresh columns was performed after unpacking an unused column from the same lot as the column used for chromatographic testing. Nucleosil columns with polymerized stationary phases were prepared by procedures detailed elsewhere[4,5]. Zorbax silica was hydroxylated by procedures described by Köhler and Kirkland[6,7] and allowed to react with silanes as described in ref. 8. All column materials were packed into 15 × 0.46

cm I.D. tubes (Alltech Associates, Deerfield, IL, U.S.A.) by conventional procedures[9].

LC-grade methanol, acetonitrile, and water (J. T. Baker, Phillipsburg, NJ, U.S.A.) were used for all studies. Trifluoroacetic acid (TFA) (Ionate grade, Pierce, Rockford, IL, U.S.A.) was used to prepare 0.1% (v/v) solutions in both water and acetonitrile. Lysozyme, ovalbumin (Sigma, St. Louis, MO, U.S.A.), and melittin (United States Biochemicals, Cleveland, OH, U.S.A.) were used as test solutes. Solutions of 0.5–1.0 mg/ml were prepared for the gradient elution studies. N,N-diethylaniline, 1-phenylpentanol, 1-phenylhexane, and 1-phenylheptane were obtained from Aldrich (Milwaukee, WI, U.S.A.) and dissolved to make solutions of 0.05–1.7 mg/ml in methanol–water (50:50) for testing. Trimethylchlorosilane was obtained from Petrarch Systems (Bristol, PA, U.S.A.).

Initial isocratic measurements were performed with methanol–water mobile phase systems ranging from 50:50 to 70:30 to obtain reasonable k' values for a specific column. Measurements of k' for the neutral 1-phenylheptane solute under isocratic conditions were used as a measure of the level of alkyl ligand remaining on the column packing during the deterioration of the stationary phase. Measurements were repeated under the same conditions after subjecting the columns to water–acetonitrile–TFA gradient elution conditions. Thus, isocratic measurements were performed on most columns with water–TFA and acetonitrile–TFA under isocratic conditions which were then alternated with the gradient elution conditions for column deterioration measurements.

Data were collected and analyzed on a Hewlett-Packard 3357 Laboratory Automation System (LAS). Chromatograms were plotted using CPLOT software and other data analysis were performed using RS/1 (BBN Research Systems, Cambridge, MA, U.S.A.) software on a VAX-11/780 computer (Digital, Maynard, MA, U.S.A.).

RESULTS AND DISCUSSION

Literature reports[10,11] indicate that certain reversed-phase columns produce better results than others in the separation of some biomolecules and basic solutes. Our work[11] confirmed this effect, and we concluded that the less active Type B silicas[11] are to be preferred for best separations and highest recoveries. All of the silicas used in this study were of Type B. These were chosen for study on the basis our experience and the experience of others that suggested that columns produced from these silicas were among the most useful for reversed-phase separations of peptides and proteins.

In order to compare various columns materials systematically, we subjected each silica and bonded-phase packing to several analyses prior to actual chromatographic testing. Ultrapore RPSC and Vydac 214TP5 were available only in prepacked columns; therefore, characterizations were performed on material from columns of the same lot to insure representative results.

The surface area for each column packing was measured by the Brunauer-Emmett-Teller (BET) method, using nitrogen adsorption. Pore-size-distributions were determined by mercury porosimetry. Bonded-phase materials were analyzed for carbon, hydrogen and nitrogen by elemental analyses both prior to use and after gradient elution testing with the TFA–water–acetonitrile mobile phases. These data are summarized in Table I.

TABLE I

COLUMN PACKING CHARACTERIZATION

Nominal particle size, 7–8 μm; k' measured in acetonitrile–water (50:50) with 0.1% TFA in both solvents except as noted.

Column packing	*Surface area* (m^2/g)	*Average pore size* (Å)	*Organic ligand*	*Carbon analysis*		*k' of 1-phenyl heptane*		*Purge column volumes*
				%C initial	*%C final*	*Initial*	*Final*	
Nucleosil®-Cl	86	248	C_1	1.3	0.3	7.95*	<0.1*	3663
Nucleosil®-PMSC1	86	248	C_1 (polymer)	9.0	1.7	7.42**	2.82**	3639
Ultrapore® RPSC	81	228	C_3	1.7	1.4	3.58	1.06	7906
Vydac® 214TP5	78	312***	C_4	3.3	2.6	5.62	3.55	3459
Zorbax®-Cl	58	280	C_1	1.0	0.4	15.51	0.80	785

* k' measured in methanol–water (50:50).
** k' measured in methanol–water (70:30).
*** Pore size is actually bimodal (150 Å, 435 Å).

Scanning electron micrographs were obtained on each of the silicas to determine an approximate particle size and shape. Micrographs of various particles are shown in Fig. 1. It appears that these four column packings represent at least three different silica types. Nucleosil is spherical and appears to have an outer shell around each particle. Zorbax is also spherical and shows the characteristic appearance of a particle made by the coascervation of collodial silica. Ultrapore and Vydac materials are both more irregular in shape; each encompasses a wider particle size and shape distribution than the Nucleosil and Zorbax packings. In our experience, columns of Nucleosil and Vydac are less stable to high pressure, and columns made of these materials exhibit higher back-pressures than Zorbax columns.

Before using each column for the gradient elution separation of peptides and

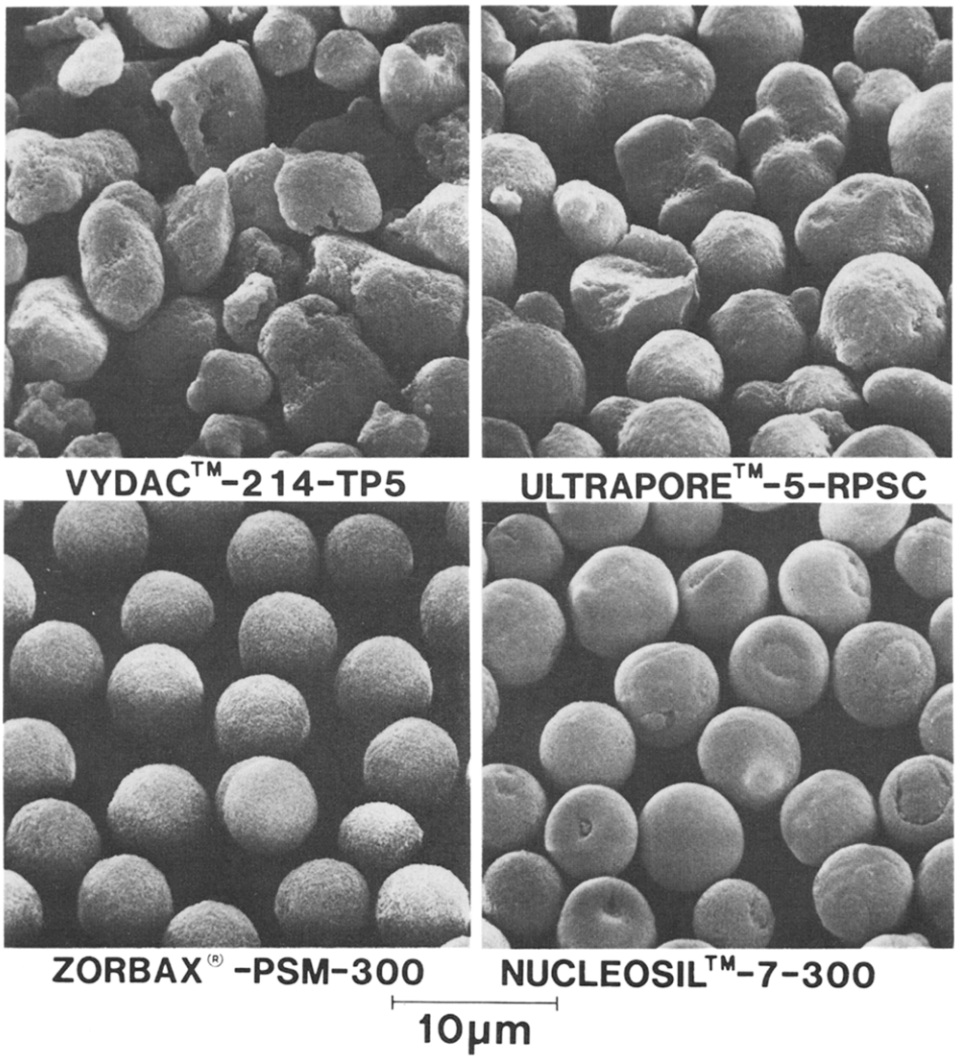

Fig. 1. Scanning electron micrographs of silica particles used in this study.

proteins, we measured the isocratic retention of a mixture of 1-phenylpentanol (unretained), 1-phenylhexane, 1-phenylheptane, and N,N-diethylaniline (N,N-DEA) using methanol–water mobile phases ranging in composition from 50:50 to 70:30, depending on the bonded phase being examined. N,N-DEA has previously been shown to be a useful indicator of undesirable acidic sites on adsorptive silica packings[11]; however, none of the Type B silica columns in this work exhibited any adsorptive properties toward this basic solute. 1-Phenylheptane was used to ascertain the amount of bonded phase on the silica after exposure to the gradient elution process, as a measure of bonded-phase loss. Values obtained in the column deterioration study are shown in Table I. Similar information might have been obtained from elemental carbon analysis, but unfortunately, this method is imprecise at the low carbon levels on the packings of interest.

In the column deterioration studies, each column was kept in contact with solution for a total of 96 h at a flow-rate of 1.0 ml/min, with 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B) mobile phases at 50°C. The gradient used was generally 0 to 100% B in 80 min with isocratic steps at 100% B and 0% B for equilibration. Measurements of the retention time of 1-phenylheptane under isocratic conditions were performed at 50% B so that the average mobile phase composition contacting any column during the entire 96 h testing period was always 50% B.

Capacity factor (k') data for 1-phenylheptane in these tests are shown in Fig. 2 for three of the columns studied. These data show that each column significantly loses retention for the neutral test sample during the relatively short study period. The three complete plots in Fig. 2 are for Zorbax-C_1. Ultrapore RPSC, and Vydac 214TP5 columns, and represent retentions measured with the TFA–water–acetonitrile system. The two-point (start/final) measurements in methanol–water (in Table I but not shown in Fig. 2) on the covalently bonded and polymer-coated Nucleosil columns are in general agreement with the full curves for the other columns.

The full deterioration curves of Fig. 2 suggest an exponential retention time decay or a first-order rate loss of the bonded phase under these conditions. However,

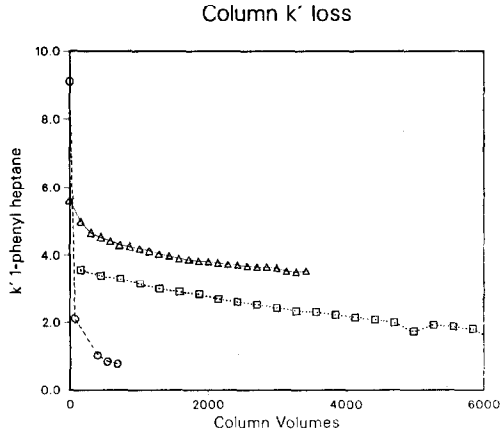

Fig. 2. Capacity factor (k') of 1-phenylheptane as a function of volume of mobile phase passed through the column in the gradient elution testing. Conditions given in the text. △ = Vydac C_4, □ = Ultrapore C_3, ○ = Zorbax C_1.

simple analysis of the data is not possible. Both the Vydac (C_4) and Ultrapore (C_3) columns are known to be endcapped with trimethylchlorosilane. It is well known that trimethylsilyl groups are most easily hydrolyzed and lost from silica surfaces[11]. Thus, it is likely that two species (trimethyl, and C_3 or C_4) are being lost from these columns simultaneously at different rates. Further studies are underway in our laboratories to clarify this point.

Further evidence that bonded phase is being lost from these columns can be seen by the %C data in Table I. In all cases, a significant fraction of the bonded phase is lost as a result of the gradient elution with acetonitrile–water–TFA. As anticipated from a previous study[11], the Nucleosil- and Zorbax-TMS columns lost organic ligand more rapidly than Ultrapore or Vydac. However, the data in Table I show that a significant loss of organic ligand also takes place from the latter materials. It is obvious that none of these packings remained fully covered after four days of gradient elution under typical conditions for the separation of peptides and proteins. Nucleosil-PMSC1, having a polymer coating prepared by cross-linking polysiloxane[4,5] appears somewhat more stable than Nucleosil-C1. From the data in Fig. 2 and Table I, one would expect that the TMS columns have no practical utility, but the discussion below indicates otherwise.

An important consequence of the loss of bonded phase during use is that significant organic material must be eluted from the column during gradient elution. Estimations of ligand loss for each of the columns during the 96 h period show that 8–38 μmol of silane are lost (depending on the ligand) in the 1300–6700 ml of mobile phase used in the tests. It is unlikely that the loss is evenly distributed. We speculate that ligand degradation (hydrolysis) occurs preferentially in mobile phases with a low organic solvent content, while the organic ligand is eluted from the column preferentially at higher organic solvent concentrations. We estimate the average ligand loss during the experiments to be 1–25 nmol/ml of mobile phase. Obviously, elution of such contaminating organic ligand material at this level would have serious consequences for both analytical and preparative or process separations. The level of contaminating silane in the mobile phase could actually be higher than the level of the peptide or protein being chromatographed. This fact can make the identification of isolates by mass spectrometry and other techniques very difficult. We speculate that some problems regarding recovery and reproducibility of peptide and protein separations could actually be due to column degradation and contamination of the eluent with silane; however this point requires further study.

It is instructive to look at the retention of a peptide–protein mixture on both "fresh" and "used" columns, *i.e.*, when a significant amount of bonded phase has been stripped from the column. Lysozyme, melittin, and ovalbumin were chosen as test solutes because these compounds represent "easy" (lysozyme), strongly basic (melittin, 2 peaks), and "difficult" (ovalbumin) compounds for reversed-phase HPLC, based on experiences in our laboratories and outside reports. Fig. 3 show the generally satisfactory separations after the first and last (24th) injection for gradient elution separations of these three solutes with an Ultrapore RPSC column. Clearly, retention for all of the peaks has decreased during column use, and there is some loss in resolution, especially for the second melittin peak and ovalbumin peak. However, this resolution loss is not as great as anticipated from the isocratic retention data, which showed that *ca.* 40% of the organic bonded phase had been removed from the column.

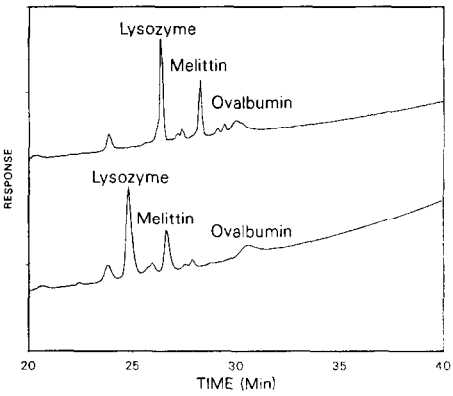

Fig. 3. Separation of lysozyme, melittin and ovalbumin on Ultrapore C_3 column. Top chromatogram is first gradient injection; bottom chromatogram is after 6000 column volumes of gradient use.

Data from the other columns were similar to that for Ultrapore-C_3, although the absolute rate of bonded phase loss was dependent on the substituent ligand. These results confirm our general hypothesis that aqueous–organic solvent systems at low pH gradients are deleterious to bonded-phase stability. Note, however, that full coverage of the surface apparently is not required for good peak shape and convenient separation of biomolecules, provided columns are based on the less adsorptive Type B silicas. Bonded-phase loss in TFA-containing mobile phases occurs fairly rapidly with fresh columns (see results for the first 1000 ml of mobile phase for each column in Fig. 2). Thus, it appears that most reversed-phse protein separations are now probably performed on silicas that are only partially covered with ligand. This result is in contrast to the frequently-held position that a fully bonded packing is required to prevent deleterious interaction of proteins and other macromolecules with residual silanols on the packing surface.

To further demonstrate the effectiveness of protein reversed-phase separations on partially covered bonded-phase silicas, we prepared two additional columns of silica, modified with trimethylchlorosilane (C_1 phase). The first column contained a silica which had been totally rehydroxylated[6,7]. The second column contained the same silica which was incompletely rehydroxylated and this silica was subjected to the identical reaction with trimethylchlorosilane. Isocratic retention and subsequent %C data showed that the columns were similar in retention for a non-polar solute. However, the initial peptide/protein chromatograms in Fig. 4 clearly demonstrate that the column made from completely rehydroxylated silica gave an adequate separation, while the column made from incompletely rehydroxylated silica became totally unusable after the first injection. The latter column was immediately retested with 1-phenylheptane in the isocratic test. It showed that only 5–10% of the bonded phase had been lost from the original column. This level of degradation was subsequently confirmed by %C analysis.

Finally, a third C_1 column, prepared from completely rehydroxylated silica, was tested initially with a melittin mixture and used intermittently for one month for both synthetic test mixtures and unknown samples for protein sequencing. Although C_1 phases are known to be relatively unstable, this column, nonetheless, was useful,

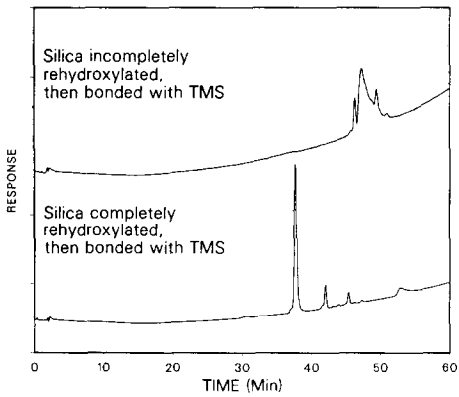

Fig. 4. Effect of silica support treatment on separation of lysozyme, melittin, and ovalbumin. Top chromatogram is silica which was incompletely rehydroxylated and then modified with trimethylchlorosilane. Bottom chromatogram is silica which had been completely rehydroxylated and then modified with trimethylchlorosilane.

especially for very hydrophobic proteins that are difficult to separate on other column types[12]. During this period of application, the column was retested with a melittin mixture and, as shown in Fig. 5, it exhibited retention and separation characteristics similar to those measured when the column was initially prepared. Isocratic measurements performed with 1-phenylheptane showed a significant loss of retention (> 50%) when the second melittin sample was run. Subsequent elemental analysis confirmed that the column had only *ca.* 50% of the initial carbon content remaining on the surface; despite this, the separation was still satisfactory.

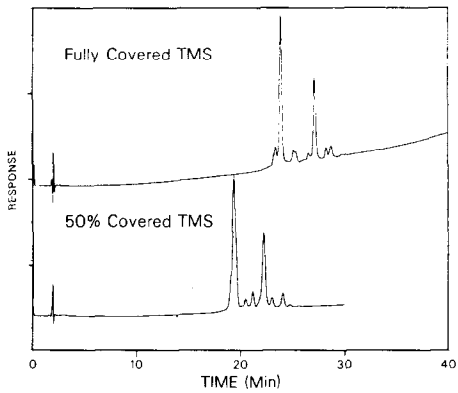

Fig. 5. Effect of bonded-phase loss on the separation of an impure melittin sample. Top chromatogram is a fresh, fully-reacted TMS column. Bottom chromatogram is a used column which has lost *ca.* 50% of the TMS bonded-phase.

CONCLUSIONS

From the data in this study, we can draw some important conclusions about reversed-phase columns as applied to protein separations. First, the type and state of silica support for the packing is important to insure a satisfactory initial separation and repeatable results. This effect has been previously described[10,11]. However, we now know the factors that are necessary to insure the desired results: complete re-hydroxylation of the silica support surface and generation of a homogeneous population of silanol groups prior to bonding.

Second, all of the columns examined lost significant amounts of bonded phase during use with TFA–water–acetonitrile gradients. However, all columns performed well in the separation of certain proteins, even when 50% of the original covalently bonded stationary phase was lost from the packing. This result implies that complete coverage of the proper silica support is not required for protein separations. However, it may be concluded, that a stable, full coverage of the support could be more desirable for reproducible separations by true reversed-partition.

Finally, although longer chain-length bonded-phase materials appear to be more stable than short-chain length ligands, *e.g.*, $C_4 > C_3 > C_1$, C_1 columns exhibit significant utility despite relative instability of the organic ligand. Certainly, improved column stability would be desirable, and we are currently working on this aspect of reversed-phase columns.

ACKNOWLEDGEMENTS

We are grateful to H. Czisch for the preparation of the Nucleosil columns, and to G. R. Wooler and J. B. Marshall for their help with the experiments.

REFERENCES

1 H. J. P. Bennett, A. M. Hudson, C. McMartin and G. E. Purdon, *Biochem. J.*, 168 (1977) 9.
2 H. J. P. Bennett, C. A. Browne and S. Solomon, *J. Liq. Chromatogr.*, 3 (1980) 1353.
3 J. L. Glajch, J. C. Gluckman, J. G. Charikofsky, J. M. Minor and J. J. Kirkland, *J. Chromatogr.*, 318 (1985) 23.
4 U. Bien-Vogelsang, A. Deege, H. Figge, J. Köhler and G. Schomburg, *Chromatographia*, 19 (1984) 170.
5 H. Figge, A. Deege, J. Köhler and G. Schomburg, *J. Chromatogr.*, 351 (1986) 393.
6 J. Kohler and J. J. Kirkland, *U.S. Pat. Appl. No. 798,332* (1985).
7 J. Kohler and J. J. Kirkland, 385 (1987) 125.
8 L. R. Snyder and J. J. Kirkland, *An Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 2nd ed., 1979, Ch. 7.
9 L. R. Snyder and J. J. Kirkland, *An Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 2nd ed., 1979, Ch. 5.
10 J. D. Pearson, N. T. Lin and F. E. Regnier, *Anal. Biochem.*, 124 (1982) 217.
11 J. Köhler, D. B. Chase, R. D. Farlee, A. J. Vega and J. J. Kirkland, *J. Chromatogr.*, 352 (1986) 275.
12 J. L. Glajch, R. M. Kutny and J. E. Shively, in preparation.