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SYNTHESIS OF AN ADSORBED REVERSED-PHASE PACKING MATERIAL FOR THE SEPARATION OF PROTEINS AND PEPTIDES*

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SUMMARY

We have described the preparation and chromatographic evaluation of an adsorbed hydrophobic stationary phase suitable for reversed-phase chromatography of proteins and peptides. The synthetic procedure involves three steps: (1) the adsorption of a polyamine to the silica surface; (2) crosslinking of the adsorbed polyamine layer with a bis-phenyl difunctional epoxide; and (3) the benzoylation of the remaining accessible amino groups. Performance of this chromatographic material compared favorably with Synchropak RP-8 silica (SynChrom, Linden, IN, U.S.A.) and was stable to 40% formic acid. Good separations were obtained between the components of sample mixtures containing proteins or the cyanogen bromide fragments of sperm whale myoglobin. However, in both cases, the adsorbed hydrophobic stationary phase was less retentive. Furthermore, this medium exhibited slightly different selectivity. Whereas the heme which was present in the cyanogen bromide digest of myoglobin desorbed as the second peak from the RP-8 column, it eluted last from the adsorbed stationary phase. Comparable performance, acid stability and alternate selectivity suggest that this material is an interesting alternative to organosilane reversed-phase coatings.

INTRODUCTION

High-performance reversed-phase chromatography (RPC) has become a pivotal technique in the isolation of natural and synthetic polypeptides[1,2]. With the development of efficient wide pore macroparticulate media[3] and mobile phases capable of dissolving large denatured polypeptides[4,5], peptide fragments can be isolated in sufficient purity for sequence analysis. In many cases, this chromatographic technique is the only method available.

The stationary phases most commonly used in silica-based RPC columns are normal chain alkanes of four to eighteen carbon atoms. These alkyl ligands are covalently coupled to silanol groups on the silica surface by reaction with an alkylchlorosilane to form a siloxane bond[6–9]. However, owing to steric limitations, the

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addition of bulky alkyl silane groups at the silica surface[10] is not quantitative and a number of free silanols remain. These residual silanols may be partially sequestered in a second "end capping" reaction with cloromethylsilanes.

Recent papers by Heukeshoven and Dernick[11,12] have shown that in the RPC of some membrane proteins the addition of 40–60% formic acid to the mobile phase is required to solubilize these very hydrophobic polypeptides. The use of strongly acidic mobile phases presents a problem with the current generation of silica-based RPC columns. Since the Si–C bond is more susceptible to attack by electrophiles than is a C–C bond[13], strong acids, *e.g.* formic, can gradually cleave the Si–C bond between the silica support and the stationary phase. Apparently, this is not a noticeable problem when columns are exposed to 60% formic acid for only a short period of time because the mobile phase suppresses the ionization of silanols generated. However, it has been noted that RPC columns which have been used with very acidic mobile phases behave quite differently once they are again operated at neutral pH[14].

In this work, the polyethyleneimine (PEI) coating technology introduced by Alpert and Regnier[15] was utilized as an alternative method for coupling a hydrophobic stationary phase. Although PEI coatings were initially utilized for the preparation of anion-exchange media, they can also be used in the preparation of other packing materials. In brief, the process uses adsorption to form a polyamine layer on the silica surface; this layer is then crosslinked into a continuous film with a hydrophilic multifunctional epoxide. Kopaciewicz *et al.*[16] have demonstrated that the hydrophobicity of the anion-exchange coating may be increased either through the use of hydrophobic crosslinking reagents or by further derivatization with suitable monofunctional epoxides. The amount of hydrophobicity introduced in these cases was relatively small so that the stationary phase still performed predominantly as an ion-exchange medium.

Utilizing a similar approach, we were able to achieve reversed-phase retention by substantially increasing stationary phase hydrophobicity. This was accomplished, in part, by the use of a bis-phenyl diepoxide crosslinking reagent. Minimization of residual positive charge in the crosslinked polyamine layer was achieved by benzoylation. This step simultaneously added phenyl groups and eliminated amines through amide bond formation. Evaluation of the resulting stationary phase showed it to be acid stable and equally effective in resolving polypeptides as SynChropak RP-8 silica (a commonly-used commercial packing material).

MATERIALS AND METHODS

Silica

Vydac 101TPB 5.5 μm (330 Å) silica used in this study was a gift from The Separations Group (Hesperia, CA, U.S.A.). SynChropak RP-8 (10 μm, 300 Å) silica was purchased from SynChrom (Linden, IN, U.S.A.).

Reagents

Benzoic anhydride, benzylamine, phenylethanol, benzoic acid, diisopropylethylamine (DIEA) and formic acid were obtained from Aldrich (Milwaukee, WI, U.S.A.). Trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) were purchased from Pierce (Rockford, IL, U.S.A.). Polyscience (Warrington, PA, U.S.A.)

supplied the polyethyleneimine-6 (PEI-6) and Epon 828. All solvents were HPLC grade or of comparable quality.

Proteins and peptides

Ovalbumin (OVA), bovine serum albumin (BSA), ribonuclease (RNase), porcine insulin, horse heart cytochrome *c* (CYTc) and sperm whale myoglobin (MYO) were purchased from Sigma (St. Louis, MO, U.S.A.). Sperm whale MYO peptides were prepared by cleaving the protein with cyanogen bromide[17]. The fragments were designateed CB_1 (residues 1–55), CB_2 (residues 56–131), and CB_3 (residues 132–153). Amino acid analyses were performed by John Cook of the Purdue University Chemistry Department.

Instrumentation

Chromatography was performed using an LDC Constametric I and IIIG system with gradient master (Laboratory Data Control, Riviera Beach, FL, U.S.A.). Absorbance at 254 and 230 nm was measured with a Spectroflow 773 detector (Kratos, Ramsey, NJ, U.S.A.). A Varian 634 UV–VIS spectrophotometer (Varian, Walnut Creek, CA, U.S.A.) was used to determine concentrations in the picric acid and protein binding assays.

Synthesis

Silica (Vydac 5.5 μm 101TPB; 1 g) was placed in 10 ml of a 1% (w/v) PEI-6–methanol solution. The suspension was degassed, agitated and allowed to stand for 30 min at room temperature. The silica was then isolated in a sintered glass funnel and dried. The dry polyamine-coated silica was transferred to a flask containing 10 ml of a 5% (v/v) Epon 828 (bisphenyl diepoxide) solution in methanol. The suspension was again degassed, agitated, and allowed to stand overnight at room temperature. At the end of this period, the flask was heated over steam for 30 min. The coated, crosslinked silica was isolated in a sintered glass funnel, washed with methanol and placed in an oven at 100°C for 1 h. The dry media was transferred to another flask containing 5 ml of dry DMF, 400 mg of benzoic anhydride and 250 μl of DIEA. After degassing and agitation, this suspension was heated in an oil bath at 60°C overnight. The benzoylated, polyamine-coated silica was isolated in a sintered glass funnel, washed thoroughly with methanol and dried in a vacuum dessicator.

Acid stability test

A 50-mg amount of packing material was placed in 5 ml of 40% (v/v) aqueous formic acid to which a few drops of DMF had been added as a wetting agent. After heating for 24 h at 60°C, test samples were reisolated on a sintered glass filter, thoroughly washed with DMF, rinsed with acetone and dried in a dessicator.

Stationary phase evaluation: static analyses

Picric acid ion-pairing capacity (IPC) assays were performed as previously described[15]. The BSA binding assay was a modification of a previously published procedure[16]. Adsorption and desorption of BSA was accomplished using 0.1% TFA and 60% acetonitrile in 0.1% TFA, respectively. A few drops of acetonitrile were required to render the hydrophobic particles wettable in the adsorption solvent. El-

emental analyses were performed by H. D. Lee (Purdue University, Chemistry Department, West Lafayette, IN, U.S.A.).

Chromatographic evaluation

Portions of coated silicas were packed into 5 × 0.41 cm I.D. columns[18]. Gradient elution was achieved with a linear gradient ranging from 0.1% acid to 0.1% in 80% acetonitrile at the indicated flow-rate. The acid was either TFA or HFBA and the gradient time was 20 or 40 min. The protein test probe consisted of selected mixtures of RNase, insulin, CYTc, OVA, and BSA. The peptide sample consisted of 300 μg of a cyanogen bromide digest of MYO. Resolution (R_s) between OVA and BSA was calculated according to the equation:

$$R_s = 2(t_{R_2} - t_{R_1})/(\Delta t_{R_1} + \Delta t_{R_2})$$

where t_R and Δt_R represent retention time and peak width in minutes, respectively. The subscripts 1 and 2 refer to the first and second peak to elute from the column.

RESULTS AND DISCUSSION

Synthesis

The first step of the synthesis was the adsorption of polyethyleneimine to the silica surface from a methanolic solution. The adsorbed polyamine was then crosslinked into a pellicle with Epon 828 (a bisphenyl diepoxide, see Fig. 1). Although this packing material was sufficiently hydrophobic to be nonwettable, chromatographic retention and resolution of the protein test mixture was unacceptable (data not shown). Apparently, residual amines in the coating convey sufficient hydrophilic character to the stationary phase that proteins are not strongly retained. In an effort to render the coating more hydrophobic, residual primary and secondary amines were derivatized with benzoic anhydride. Unfortunately, tertiary amines initially present in PEI and those generated during the crosslinking step are not acylated and will remain in the stationary phase.

Elemental analysis of the hydrophobic adsorbed PEI (PEI-Phenyl) coating indicated a 12% carbon load (Table I). This carbon loading is substantially higher than the 3.32% obtained[19] by trichlorooctylsilylation of this same Vydac 101TPB silica. These results suggest that the PEI-Phenyl stationary phase is thicker. (The SynChropak RP-8 used as a reference media had a carbon load of 2.80). Although increased layer thickness may add stability to the coating, it could also consume pore volume and surface area. This may explain the lower BSA loading capacity of the PEI-Phenyl medium compared to the SynChropak RP-8 packing material (Table II).

Acid resistance of these packing materials was investigated by subjecting both to a 40% formic acid treatment for 24 h at 60°C. Elemental carbon analysis showed this treatment to cause a 63% loss of the bonded phase from the RP-8 media while only 3.3% was removed from the PEI-Phenyl coated media (Table I). These results suggest that extended operation of organosilane reversed-phase columns under strongly acidic conditions will result in substantial loss of bonded phase from the supprt. By contrast, adsorbed coatings in which the organic phase contains only C–C or C–N bonds would be expected to be much more acid resistant.

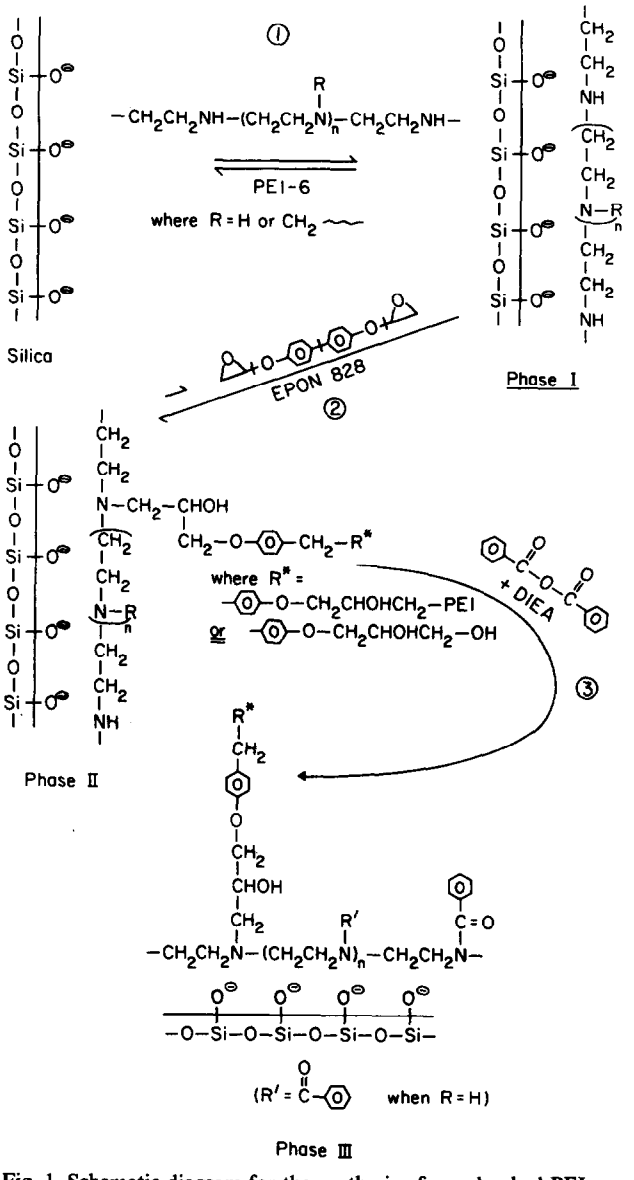

Fig. 1. Schematic diagram for the synthesis of an adsorbed PEI reversed-phase stationary phase. Arabic and Roman numerals denote reaction steps and products, respectively.

The number of phenyl groups per square meter on the PEI-Phenyl coating was calculated as 12 μmole/m^2 from elemental analysis data. Pearson[19] has shown the density of octylsilane moieties to be 2.9 μmole/m^2 for C_8-derivatized Vydac silica. Although the density of phenyl groups is obviously higher, the adsorbed stationary phase was more hydrophilic. Reduced hydrophobicity either results from the higher polarity of the phenyl group or the polymer coating itself.

TABLE I

ELEMENTAL ANALYSIS DATA

Packing material	*C (%)*	*C (%)**	*Net loss (mg C/g support)***	*Total mass lost (%)*
SynChropak RP-8	2.8	1.04	1.76	63.0
PEI-Phenyl	12.0	11.6	0.4	3.3

* Analysis of media after treatment with 40% formic acid for 24 h at 60°C.

** Obtained by subtracting the carbon content after formic acid treatment from the initial carbon load.

TABLE II

COMPARISON OF THE PEI-PHENYL SILICA TO SYNCHROPAK RP-8 MEDIA

Packing material	*IPC (μmol amine/g)**	*L_C, BSA (mg/g)***	*t_R, BSA (min)****	*R_s (BSA/OVA)§*
SynChropak RP-8	–	169	15.4	2.9
PEI-Phenyl	170	84	11.0	2.8

* Picric acid ion-pairing capacity; correlates with the amount of residual amines.

** Static loading capacity of bovine serum albumin per gram coated silica.

*** Chromatographic retention on a 5 × 0.41 cm I.D. column during a 20-min linear gradient from 0.1% TFA to 60% acetonitrile in 0.1% TFA at a flow-rate of 1 ml/min.

§ Resolutions between bovine serum albumin and ovalbumin under the chromatographic conditions stated above.

TABLE III

ISOCRATIC CHROMATOGRAPHY OF SMALL MOLECULES

Chromatography was performed on 5 × 0.41 cm I.D. columns using a methanol–water mobile phase (1:9, pH 7). The retention of phenylethanol in 100% methanol was used as t_0. A flow-rate of 0.5 ml/min was maintained throughout.

Packing material	*k' value**		
	Benzylamine	*Phenylethanol*	*Benzoic acid*
SynChropak RP-8	2.93	0.12	0
PEI-Phenyl	0.27	0.52	5.97

* $k' = (t_R - t_0)/t_R$, where t_R is the solute retention time, and t_0 is the void time.

The picric acid assay indicated that the PEI-Phenyl coating contained 170 μmoles of ion-pairable amines per gram of silica (Table II) corresponding to 2.2 μmoles of amine per square metre. This residual charge on the PEI-Phenyl media appears to be similar to RPC silica, although opposite in sign. Erard and Kováts[20] have calculated that there are 7.8 μmoles hydroxyl groups per square metre on Carbosil silica (surface area equals 100 m^2/g). If one assumes a similar surface structure and reactivity for Carbosil and Vydac, the number of residual hydroxyl moieties can be estimated; *i.e.* 2.9 μmole/m^2 of the 7.8 μmole/m^2 of silanols will be consumed leaving *ca.* 4.9 μmole of residual hydroxyl groups.

Chromatographic comparison

Small molecules. Benzylamine, phenylethanol and benzoic acid were chromatographed isocratically on a SynChropak RP-8 and the PEI-Phenyl column (Table III). At neutral pH, the residual charge in the column and its sign are indicated by the exaggerated retention characteristics of either the aromatic base or acid. As expected (using a mobile phase of methanol–water, 1:9), benzylamine was retained on the SynChropak RP-8 column, probably as the result of residual silanols. In contrast, benzoic acid was preferentially retained on the PEI-Phenyl column. The latter phenomenon apparently results from the interaction of the carboxylic acid with residual tertiary amines in the bonded phase. Under these conditions, phenylethanol was weakly retained on both stationary phases.

Proteins and peptides. The separation of ovalbumin and bovine serum albuminon the SynChropak RP-8 and the PEI-Phenyl column was compared (Fig. 2). On the basis of selectivity and resolution, the two were essentially equivalent. However, the PEI-Phenyl column was less retentive. Both proteins eluted several minutes earlier than on the RP-8 media. An additional separation of a five-protein mixture consisting of RNase, insulin, CYTc, BSA and OVA was performed on the PEI-Phenyl column to further test its effectiveness (Fig. 3). The separation achieved was virtually identical to that shown by Pearson *et al.*[19] on C_8-derivatized Vydac 101TPB silica.

A comparison was conducted using the products of the cyanogen bromide treatment of sperm whale myoglobin. Myoglobin, which contains two methionine

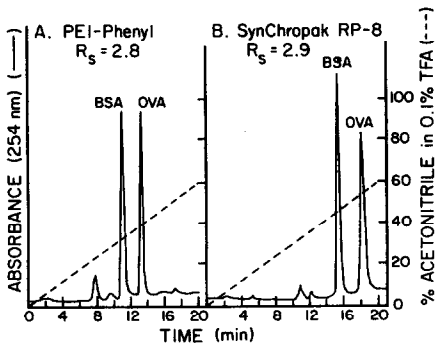

Fig. 2. Comparative protein separations. A mixture of OVA (140 μg) and BSA (100 μg) was chromatographed on (5 × 0.41 cm I.D.) columns packed with either PEI-Phenyl material (A) or SynChropak RP-8 silica (B). Adsorbed proteins were eluted during a 20-min linear gradient from 0.1% TFA to 60% acetonitrile in 0.1% TFA using a 1 ml/min flow-rate.

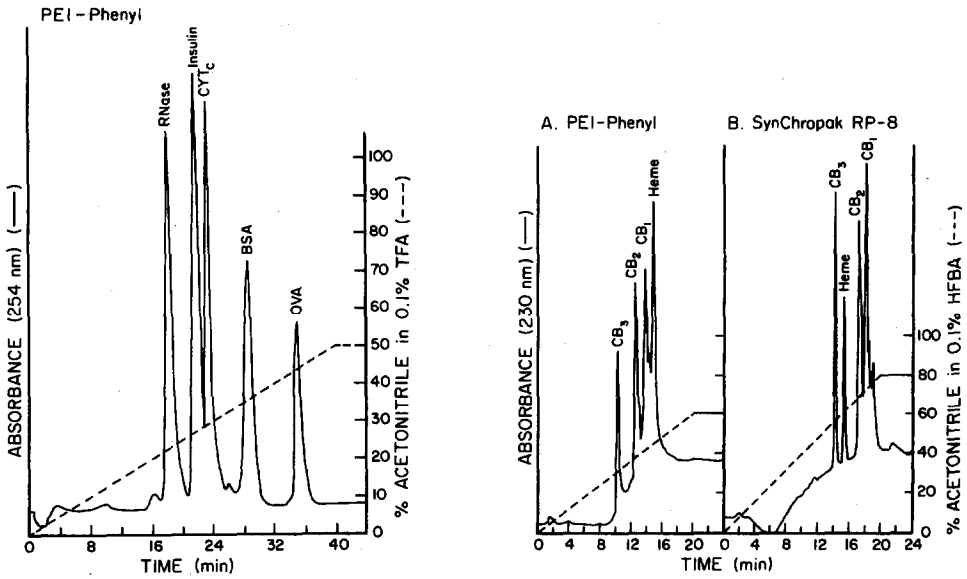

Fig. 3. Separation of a five-protein mixture. A sample containing RNase (75 μg), insulin (75 μg), CYTc (40 μg), BSA (75 μg) and OVA (125 μg) was chromatographed on the PEI-Phenyl column (5 × 0.41 cm I.D.). Proteins were eluted during a 40-min linear gradient from 0.1% TFA to 50% acetonitrile in 0.1% TFA at a 0.5 ml/min flow-rate.

Fig. 4. Comparative peptide separations. Components of a sperm whale myoglobin cyanogen bromide digest (300 μg) were chromatographed on (5 × 0.41 cm I.D.) columns packed with either the PEI-Phenyl stationary phase (A) or SynChropak RP-8 silica (B). Peaks were identified by amino acid composition and/or spectral data. Peptides were eluted during a 20-min linear gradient from 0.1% HFBA to 60% or 80% acetonitrile in 0.1% HFBA at a 1 ml/min flow-rate.

residues and a heme, yields three peptide fragments and a porphyrin ring. Heptafluorobutyric acid was used as the ion-pairing agent, because TFA provided insufficient retention on the PEI-Phenyl column. Both stationary phases exhibited good resolution and retention charcteristics in separating in major sample components (Fig. 4). The PEI-Phenyl packing material was again less retentive requiring only 50% acetonitrile to elute the last peak, while 80% acetonitrile was needed on the RP-8 column. Interestingly, the PEI-Phenyl support offered a slightly different selectivity for chromatography of the heme moiety. It eluted as the second peak from the RP-8 column and as the last peak from the PEI-Phenyl column. The delayed elution probably resulted from stacking interactions between stationary phase aromatic groups and the porphyrin ring.

CONCLUSIONS

An adsorbed hydrophobic stationary phase has been synthesized which offered good resolution of proteins and peptides when placed on a wide-pore microparticulate silica. Hydrophobicity of the coating resulted primarily from the phenyl moieties which were added via crosslinking and acylation. There were several differences

in both the chemical composition and chromatographic properties of this material compared with an organosilane stationary phase. Elemental analysis data indicated that the adsorbed phase is much thicker. Although this is advantageous in sequestering surface silanols, pore volume and surface area are consumed, thereby decreasing mass loading. Isocratic chromatography of benzylamine and benzoic acid suggested that the adsorbed coating contains positively-charged groups resulting from tertiary amines, as opposed to the negatively-charged residual silanols in conventional RPC materials. However, the density of residual charge appears to be similar.

The PEI-Phenyl stationary phase was as effective in resolving protein and peptide test mixtures as the SynChropak RP-8 column, but it was less retentive. Since the surface phenyl density of the adsorbed coating appears to be greater than the alkylsilane density, decreased retention probably results from the greater polarity of the phenyl ligand. Reduced retention may be advantageous with large polypeptides that have limited solubility in organic solvents. Specifically, elution may be achieved at a lower organic solvent concentration. In case where it is disadvantageous, decreased retention may be circumvented by the use of a more hydrophobic ion-pairing agent (*e.g.* heptafluorobutyric acid).

Although further study is required, initial findings indicate that the greatest advantage of adsorbed hydrophobic stationary phases over an organosilane bonded phase may be their improved stability at acidic pH. Increased acid resistance would allow the use of strongly acidic mobile phases without fear of stripping the bonded phase. Furthermore, such acids could be used routinely for column cleanup and depyrogenation.

Adsorbed polyamines have now been used to generate the full spectrum of modern high-performance liquid chromatographic columns for proteins. The reversed-phase medium described here is an additional application of a synthetic method which has already been used to prepare anion-exchange[15,16], cation-exchange[21], hydrophobic interaction[22], and affinity[23] stationary phases.

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REFERENCES

1 M. J. O'Hare and E. C. Nice, *J. Chromatogr.*, 171 (1979) 209.
2 B. Larsen, J. Viswanatha, S. Y. Chang and V. Hruby, *J. Chromatogr. Sci.*, 16 (1975) 207.
3 J. D. Pearson, W. C. Mahoney, M. A. Hermodson and F. E. Regnier, *J. Chromatogr.*, 207 (1981) 325.
4 W. C. Mahoney and M. A. Hermodson, *J. Biol. Chem.*, 255 (1980) 11,199.
5 H. P. J. Bennett, *J. Chromatogr.*, 266 (1983) 501.
6 J. D. Pearson and F. E. Regnier, *J. Liquid Chromatogr.*, 6 (1983) 497.
7 R. J. Lewis, A. Fallon, S. Stein, K. D. Gibson and S. Udenfreind, *Anal. Biochem.*, 104 (1980) 153.
8 G. E. Berendsen, K. A. Pikaart and L. de Galan, *J. Liquid Chromatogr.*, 3 (1980) 1437.
9 M. B. Evans, A. D. Dale and C. J. Little, *Chromatographia*, 13 (1980) 5.

10 J. L. M. van de Verne, J. P. M. Rindt, G. J. M. M. Coenen and C. A. M. G. Cramers, *Chromatographia*, 13 (1981) 11.
11 J. Heukeshoven and R. Dernick, *J. Chromatogr.*, 252 (1982) 241.
12 J. Heukeshoven and R. Dernick, *Protides Biol. Fluids*, 32 (1985) 1085.
13 J. D. Roberts and M. J. Caserio, *Basic Principles of Organic Chemistry*, W. A. Benjamin, New York, 1965, p. 1185.
14 Geng Xindu, personal communication.
15 A. J. Alpert and F. E. Regnier, *J. Chromatogr.*, 185 (1979) 375.
16 W. Kopaciewicz, M. A. Rounds and F. E. Regnier, *J. Chromatogr.*, 318 (1985) 157.
17 E. Gross, *Methods Enzymol.*, 11 (1967) 238.
18 G. Vanecek and F. E. Regnier, *Anal. Biochem.*, 121 (1982) 156.
19 J. D. Pearson, N. T. Lin and F. E. Regnier, *Anal. Biochem.*, 124 (1982) 217.
20 J. Erard and E. Kováts, *Anal. Chem.*, 54 (1982) 193.
21 W. Kopaciewicz and F. E. Regnier, *J. Chromatogr.*, 358 (1986) 107.
22 L. A. Kennedy, W. Kopaciewicz and F. E. Regnier, *J. Chromatogr.*, 359 (1986) 73.
23 M. A. Rounds, W. Kopaciewicz and F. E. Regnier, in preparation.