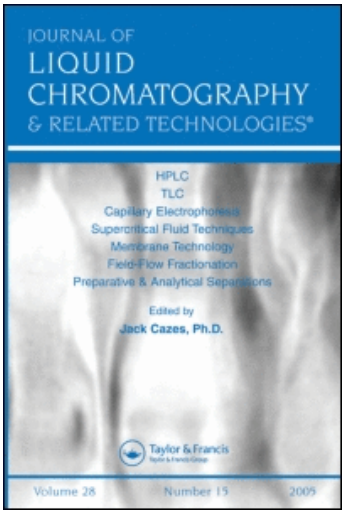

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Protein Separations on Octyl and Diphenyl Bonded Phases

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PROTEIN SEPARATIONS ON OCTYL AND DIPHENYL BONDED PHASES

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ABSTRACT

Two new packing materials specifically designed to handle high performance liquid chromatographic separations of proteins and peptides have been made. These are built on a 300 A pore silica gel to allow access of large molecular weight species, and are exhaustively bonded, first with an octyl or a diphenyl silane and final end-capping with trimethylsilyl groups. The media are called Protesil 300 Octyl and Protesil 300 Diphenyl. Their unique selectivity is shown with various samples of dipeptides and proteins. The Octyl phase has specific affinities for alkyl functionalities, the Diphenyl phase has specific affinities for aromatic functionalities. Loading and mass recovery studies have been done on these media to show their capabilities under the elution conditions shown in the various separations. Comments regarding their correct use have also been made.

INTRODUCTION

There is an ever increasing interest in separating moderate to large molecular weight biopolymers. For many years the literature has been filled with such separations on a variety of media, but the advent of high performance liquid chromatography (HPLC) has brought not only much increased speed, but more importantly, newer, more efficient and more selective media for such separations.

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Two general groups of HPLC media now exist for separating biopolymers. Those whose mode of separation is based upon size or steric exclusion and those whose mode of separation is based upon varying interaction with a bonded phase. Almost all of both groups of packings are manufactured with a silica gel backbone because its rigid nature is able to stand up to the packing and operating pressures needed for HPLC. Silica gel does have a distinct disadvantage, however, when doing biopolymer separations. Many biopolymers bond irreversibly to the silanol groups. There are, however, references to biopolymer separations being done with fair success on bare 60 or 280 A pore silica gel with aqueous mobile phases(1,2). Such mobile phases would have deactivated most of the silanol sites, but irreversible adsorption still would have occurred although this fact was substantiated in only one of the two references cited (1). Regardless of the yield, pure protein was obtained. One of these references (1) also contains comments on the effects of the pore sizes of the silica gels regarding the penetration or exclusion of various size proteins. If the pores of the gel were too small to accommodate the protein or viruses, they passed through the column easily. If the pores of the gel were larger, the same protein or viruses penetrated into the pores and became adsorbed, eluting at a later time. Such information is similar to that known for years by workers in the gel permeation and gel filtration fields.

Classically ion exchange celluloses and gels have been used to separate many proteins. The ion exchange sites were usually weak and the backbones used prevented irreversible adsorption after gradient pH or salt conditions for the separation were established. To update these media for modern HPLC use, Regnier and his coworkers (3-6) bonded glyceryl-like phases and ion exchangers to various controlled porosity

supports. Thus a combination of size exclusion and/or ion exchange modes could be used for biopolymer separations. This media research laid much of the ground work for these types of columns and packings now available commercially from a number of sources. A review of these types of packings has been written (7), as well as an excellent evaluation procedure (8).

Some interesting work on biopolymer separations on silica gel bonded with hydroxyl, amino, or cyano phases has been done (9-13). Much work remains to be done in this area, however, to differentiate between the various separation mechanisms possible. The bulk of current HPLC work on biopolymers has been published using 60 to 100 A pore silica which has been bonded with hydrocarboneous phases (C_{18}, C8, phenyl) (13-21). The references cited are only a sample of what appears in the literature. The use of these bonded phases grew naturally since many biopolymers were soluble in the largely aqueous mobile phases which are frequently used with these media, and because the media were readily available in every HPLC laboratory. The results were very successful for the most part, and their use continues for probably 80% of the biopolymer work done today.

Since the backbone for most of this media is silica gel, after initial bonding it is best to use a secondary capping reaction (with trimethylchlorosilane and related compounds) to remove as many residual silanols as possible. Although the success of such capping is variable from manufacturer to manufacturer, the media with such capping will always give better yields of any biopolymer separated. When uncapped reversed phase packings are used for biopolymer separations, various additional components are usually required in the mobile phase as is done with such separations on pure silicas. Hancock and Sparrow (22) reported that an inital wash with methanol and operation with high concentrations

(0.17 M) of triethylammonium phosphate, pH 3.2, was necessary to get efficient protein and peptide separation on an uncapped medium. Still they observed poor efficiencies when strongly basic amino acid (arginine) containing solutes were separated unless a hydroxylic solvent (methanol) was used.

A great deal of research into the chemical factors and possible mechanism of biopolymer separations on bonded reversed phase packings has been done. These constitute some of the most interesting papers in the LC literature today because they lead to a better understanding of what to expect when doing such separations, as well as new directions for media research. Some of the pertinent results will be briefly discussed since they relate to the direction taken in the development and testing of the Protesil 300 media.

Researchers (15,23,24) have noted the decreased efficiency of protein separations as compared to small molecular weight solutes separated on reversed phase media. This is due to the slower mass transfer of the larger molecular weight species, the possible presence of isomers of the solute which elute close to one another, and the inability of the biopolymers to penetrate into the small pores of some of the reversed phase media now available. Although the first two of these reasons are due to the physicochemical or chemical nature of the species involved, the latter situation is at least minimized by using larger pore diameter supports.

As with any LC column, the faster the flow rate during a protein or peptide separation, the less the efficiency (9,11,23,24). For 4.0 to 4.6 mm ID columns the flow rate should be less than 1 ml/min. This allows

more equilibration time for the slower diffusing biopolymers, resulting in better resolution. If operating isocratically, most biopolymers are extremely sensitive to the mobile phase composition. As little as a 1% change in organic/water ratio can drastically change retention and resolution, or result in no elution at all (9,13,16,25). Gradients are also very useful because of this, but also must be run slowly for best results. Jones, et al (23) recommend 2 hour gradients at 20 ml/hr flow rates. Another reason for slower flow rates, aside from better efficiency, is that the limits of detection are greater (24). Thus trace proteins or peptides can be overlooked if fast flow rates are used.

The elution conditions for biopolymers from reversed phase columns can be varied considerably. Of primary concern is the selectivity to get the separation desired. To this end the literature is filled with attempts using water combined with acetonitrile, methanol, ethanol, tetrahydrofuran, or propanol as mobile phases (16,17,24,25). Rubinstein (11) pointed out the anomoly found when doing such separations - the larger the peptide the more organic needed for elution, but many proteins or larger peptides are precipitated out of solution by high concentrations of organics. He recommended gradient work with 1-propanol (0 to 40%) to do wide range molecular weight protein separations. The disadvantage of lower efficiency and high back pressure (due to viscosity) of this mobile phase combination is offset by its versatility.

Regarding the mobile phase composition, simple organic/water ratios suffice to separate smaller non-polar di- and tri- peptides. The addition of ion pairing salts or acids, however, are generally required for the separation of larger molecular weight biopolymers (9,11,13-15,20-22,26,27). These modifiers serve two functions: a) to solubilize the solutes in the mobile phase (changing the ionic forms or

configurations of the species), and b) to condition the bonded phase. Because they become a part of the separated components, care in selection of the salts or acids must be used. Will the modifier interfere with detection (UV or MS) (9,28)? Can it be easily removed or is it compatible with the biochemical system if future work on the species is to be done (9,16)? Will it (like any part of the mobile phase) denaturize or change the biopolymer?

One of the most important variables in the separation of biopolymers on bonded reversed phase media are the media themselves. A few groups (14,16,17) have commented on the similarities of elution order when going from a C8 to a C18 bonded phase. Assuming both packings are capped and both are made from the same type reagent (monofunctional silane), then such similarities are expected. Thus only more lipophilic character is found in the C18 as compared to the C8 column. Longer retentions of non-polar solutes are found in a C18 column. Faster elution from the C18 column requires that a higher percentage of the organic solvent be used in the mobile phase. For more water soluble species, like most biopolymers, it is better to use a C8 column since the compounds elute with less organic solvent in the mobile phase. As a rule, C8 columns also equilibrate more rapidly, an important factor if various mobile phases for optimization need to be tried.

An interesting study was done by Lewis and coworkers (13) in which octyl, cyanopropyl, and diphenyl bonded phases were compared. They found similarities in the C8 and cyanopropyl bonded phases for some protein mixtures, but neither of these columns would separate all the components ($2\alpha, 1\beta, 1\gamma$) of denatured human Type I collagen except the diphenyl

bonded phase. This pointed out the potential utility of at least a combination of C8 and diphenyl bonded phases for protein separations.

A number of studies of small peptides ($\sim$ 20 residues or less) separations on reversed phase media (14,16,17,29,30) have shown a high correlation of the retention to the summing of individual lipophilic or lipophobic contributions of each amino acid (and end groups). This indicates that amino acid composition is a major factor determining retention. The small differences observed between elution orders or times points to the lesser but important role played by other factors such as polarity, sequence, size, charge distribution and conformation. Such information can be useful in looking into possible separation mechanisms when any similarities or differences in elution order or times are observed.

EXPERIMENTAL

Equipment

The liquid chromatograph used was a Perkin Elmer (Norwalk, CT) Series 3B with a Valco (Houston, TX) 7000 psig Universal inlet injector, a Laboratory Data Control (Riviera Beach, FL) Spectromonitor II (1202) and data handling was with a Hewlett Packard (Avondale, PA) Integrator 3380A and a Hewlett Packard HP2126BOEM disc based system with CIS (Computer Inquiry Systems, Englewood Cliffs, NJ) installed HP RTE2 software.

It is important that a dynamic mixing chamber be made for the gradient system since aqueous buffer and 1-propanol are being mixed.

Because of the great viscosity difference, no static mixer (T or coil) was found to work. A suitable mixing chamber was made from a 9.4 mm x 5 cm tube with appropriate fittings and a magnetic stir bar. The device was placed just before the injector and held to pressures of 3000 psi.

Reagents

Bovine serum albumin (fraction V powder), aldolase (type X from rabbit muscle), insulin (from bovine pancreas) and cytochrome C (from horse heart) were purchased from Sigma (St. Louis, MO); lysozyme (from hen egg white) and hemoglobin (human) from Calbiochem (La Jolla, CA); human serum (lyophilized) and all dipeptides from U.S. Biochemical Corp. (Cleveland, OH); and β-lactoglobulin from Polysciences (Warrington, PA).

The 1-propanol was reagent grade from Mallinckrodt (St. Louis, MO), the triethylamine and glacial acetic acid were reagent grade from J.T. Baker (Phillipsburg, NJ). The water was deionized.

Protesil 300 Octyl, Protesil 300 Diphenyl, Partisil 10 CCS/C_8, Partisil 10 ODS-3, Partisil 10 ODS-2 and CoPell ODS are available from Whatman Inc. (Clifton, NJ). All Protesil and Partisil columns used are 4.6 mm x 250 mm and were off the shelf. The various characteristics of all the reversed phase media mentioned in this work are given in Table I.

The initial work on the Protesil 300 columns was done using only methanol/water combinations. With a simple mobile phase such as this, explanation of observations regarding selectivity would be much easier.

TABLE I

MEDIA CHARACTERISTICS

(all are 10 μm, irregular)

	Pore Diam A	Surf Area m^2/g	Phase	%C [a]	Reag. Funct. [b]	End Cap	% SiOH Coverage [c]
Protesil 300 Octyl	300	250	C8	7.5	mono-	Yes	95+
Protesil 300 Diphenyl	300	250	Ph_2	8.0	di-	Yes	95+
Partisil 10 CCS/C_8	80	375	C8	9.0	mono-	Yes	95+
Partisil 10 ODS-3	80	375	C18	10	tri-	Yes	95+
Partisil 10 ODS-2	80	375	C18	15	tri-	No	75

a) % C is the weight % carbon as determined after all bonding is completed. Usually 90-95% of this value is the weight % due to the initial bonding reagent, with the capping reagent accounting for the balance.

b) The functionality of the initial bonding reagent; the second bonding reagent (for end-capping) is always Me_3SiX, where may be halide, alkoxy, or amino.

c) Approximate % coverage of available silanols base upon retentions as measured with a nitrobenzene sample with heptane mobile phase, and/or an aniline/phenol sample with a methanol/water (60:40) mobile phase.

Additional modifiers in the mobile phase may help the separation, but complicate the explanation of the possible mechanism of such separations.

Many successful protein separations are done with gradients to allow the best peak capacity and speed. The gradient simplifies finding the right elution conditions since, as was pointed out, the correct ratio of organic/water is critical to any protein elution and the reproducibility of this elution time. High sensitivity using the triethylammonium acetate at 210 and 280 nm has been reported in the literature (9) and was the buffer of choice. The buffer solution was made by adding triethylamine to 0.5 M acetic acid until the desired pH is reached. After being made, the buffer was passed through a Partisil 10 ODS-2 (15%C, not end-capped) column to remove impurities that might elute to give baseline drift or spurious peaks during gradient runs. A treatment of similar buffers has been reported (22,31). The organic solvent used in the gradient runs was 1-propanol, as reported by Rubinstein (11), so that maximum solubility of the proteins throughout the separation could be maintained.

Protesil 300 Quality Control

To ensure reproducible columns, extensive quality control on the base silica gel, the bonded product, and the finished column is done. Before bonding, the silica is tested for pore size distribution, particle size distribution, surface area and exclusion limits. After bonding and after % carbon is assured to be within the specified range, the diphenyl or octyl chromatographic batch tests are performed. Several columns are packed and are given the standard column Q.C. test to check bed uniformity. A sample containing benzene and phenanthrene is separated

and capacity factors, efficiencies, and asymmetry ratios are measured. After assuring that the batch test columns are well packed, they are tested by polystyrene SEC for total pore volume and exclusion limits. Next the columns are tested for protein separation with cytochrome C and bovine serum albumin under gradient conditions after which capacity factors and resolution are calculated. The final batch test is a check for residual silanols with an aniline and phenol sample. If properly capped, the aniline will elute before phenol with a methanol/water mobile phase. A mass recovery test is performed using lysozyme because of its susceptability to surface effects (8,12,32). The procedure requires comparison of the area of lysozyme peaks with a packed and with an empty column under isocratic conditions. Generally the amount of lysozyme retained on the column is minimal and the percent recovery increases as the mass of lysozyme injected on the column is increased.

After a Protesil 300 batch has passed all the tests, it is released for packing of the commercial columns. Each of these columns is tested only for bed uniformity by measurement of capacity factors and efficiency with the benzene and phenanthrene sample. If the column passed this final test it is released for sale. It is important to note that any Protesil column sold has never been subjected to a protein which might interfere with subsequent work done on this new column.

RESULTS AND DISCUSSION

In an attempt to develop column media that would allow improved biopolymer separations, many of the findings mentioned previously were taken into account by the people in our research group. Thus, the media needed larger pores to accommodate the larger molecular weight species.

It also had to be bonded with different groups to get unique selectivities and be well end-capped to prevent irreversible adsorption. A low carbon percentage of the bonded phase was also important so that elution would be accomplished with high water content mobile phases, minimizing the possibility of biopolymer precipitation by high organic content mobile phases.

The initial result of these considerations are two new biopolymer separation columns called Protesil 300 Octyl and Protesil 300 Diphenyl. They are carefully sized 10um irregular silica with 300 A pores bonded and end-capped with 7.5% carbon for the octyl phase and with 8.0% carbon for the diphenyl phase.

The level of carbon on the Protesil 300 bonded phases is low to moderate as compared to other bonded reversed phases on the market (which range from 5 to 24% carbon). This, again, was to allow higher water containing mobile phases to be used, thus keeping increased solubility of the biopolymers. The carbon level was also kept about the same on each so that the same mobile phase composition as derived from one type of Protesil 300 column could be used on the second type of Protesil 300 column. Thus the different selectivities of the two bonded phases can be observed without reoptimization of the mobile phase. In other words, the same polarity mobile phase will elute components of the sample with either Protesil 300 column, yet the selectivities will be different on each.

The octyl and diphenyl phases were chosen to be bonded because these had been pointed out to have the most different selectivities by Lewis and his coworkers (13), and was substantiated by work in our laboratory.

As mentioned previously, a few research groups found that only elution time seemed to change from a C8 to a C18 bonded phase - assuming each was made on the same backbone gel and with the same bonding chemistry.

An investigation was begun with the Protesil 300 Octyl and Diphenyl columns to show their unique selectivities. The initial results are reported here. On occasion, Partisil 10 CCS/C_8 column (80 A pores, 9% carbon, end-capped) was used for comparison.

Selectivity Studies

The dipeptides seen in Figure 1 were used to investigate the selectivities of the Protesil 300 Octyl and Diphenyl media. These simplified structures allow the substituent and end groups to be more easily compared. The numbers in parentheses after each compound name are the Rekker lipophilic substituent summations (without correction for terminally located residue) (33). The compounds elute in the expected order on both columns based upon relative lipophilicities. Looking at the chromatograms of each, Figures 2 and 3, there are unique differences. The Protesil 300 Octyl (Figure 2) does separate the aliphatic substituent compounds better than the Diphenyl column. There is no separation of the aromatic substituent compounds, however, on the Octyl column. On the Protesil 300 Diphenyl column (Figure 3), however, separation of these aromatic containing compounds is easily accomplished. At the same time resolution of the aliphatic dipeptides is lost. Complete separation of the aliphatic species on the Octyl column and of the aromatic species on the Diphenyl is possible with a more water rich mobile phase or gradient. The same mixture of dipeptides is completely resolvable on the Partisil 10 CCS/C_8 (Figure 4). Thus short chain or low molecular weight species

DIPEPTIDE STRUCTURES AND ELUTION ORDER
(Rekker's constant)

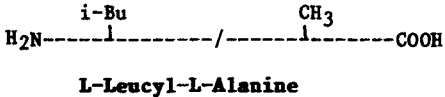
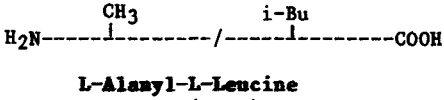
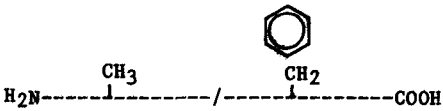
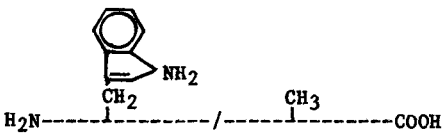
PROTESIL 300 OCTYL		PROTESIL 300 DIPHENYL
1		1
	L-Alanyl-L-Tyrosine (2.23)	and
2		2
	L-Leucyl-L-Alanine (2.52)	
3		3
	L-Alanyl-L-Leucine (2.52)	
4		4
	L-Alanyl-L-Phenylalanine (2.77)	
and		
5		5
	L-Tryptophyl-L-Alanine (2.84)	

Figure 1 Structure and elution order of dipeptides on Protesil 300 Octyl and Protesil 300 Diphenyl. Drawings show substituents only. The numbers in parentheses are the summation figures from the Rekker (33) lipophilicities of each amino acid.

Dipeptide separation of Protesil 300 Octyl

Conditions:

Mobile phase: MeOH/H_2O (25/75)

Flow rate: 0.5 ml/min

Back pressure: 600 psi

Detection: RI

Sample conc.: 10 mg/ml

Sample volume: 20 µl

Peak identification:

1. L-alanyl-L-tyrosine 7.9 min
2. L-leucyl-L-alanine 8.2
3. L-alanyl-L-leucine 9.0
4. L-alanyl-L-phenylalanine 10.6
5. L-tryptophyl-L-alanine

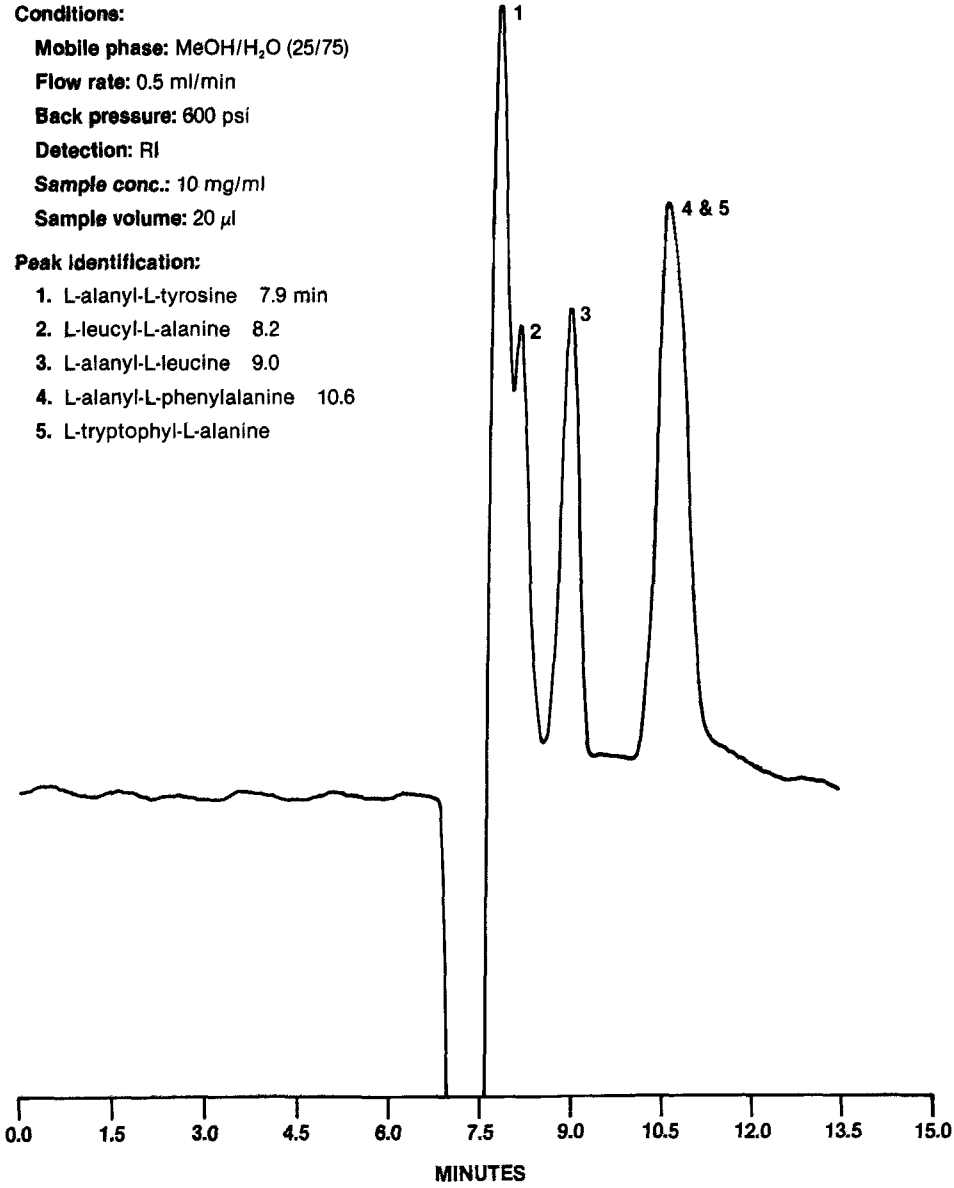


Figure 2

Dipeptide separation on Protesil 300 Diphenyl

Conditions:

Mobile phase: MeOH/H_2O (25/75)
Flow rate: 0.5 ml/min
Back pressure: 600 psi
Detection: RI
Sample conc.: 10 mg/ml
Sample volume: 20 μl

Peak identification:

1. L-alanyl-L-tyrosine 7.6 min
2. L-leucyl-L-alanine
3. L-alanyl-L-leucine 7.9
4. L-alanyl-L-phenylalanine 9.4
5. L-tryptophyl-L-alanine 9.9

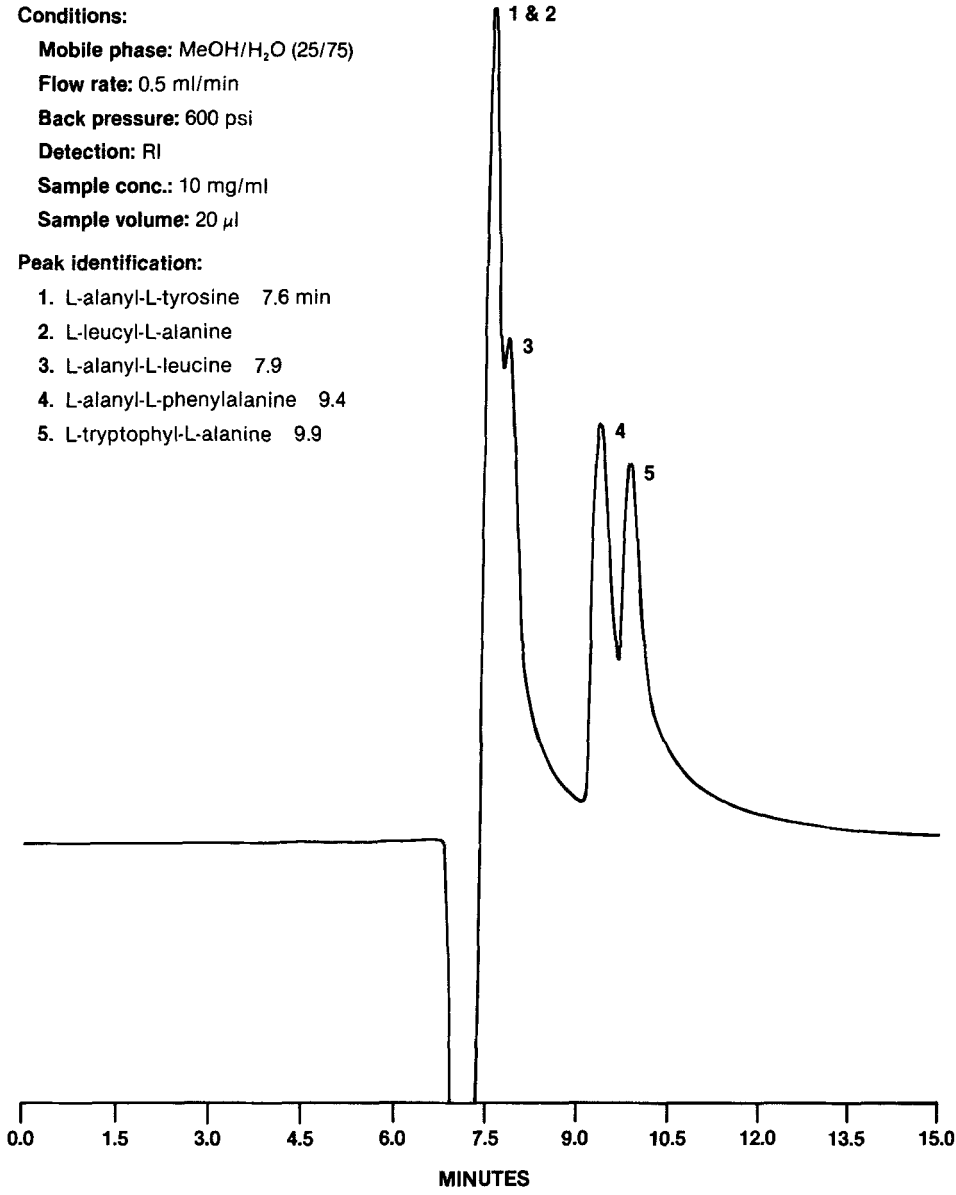


Figure 3

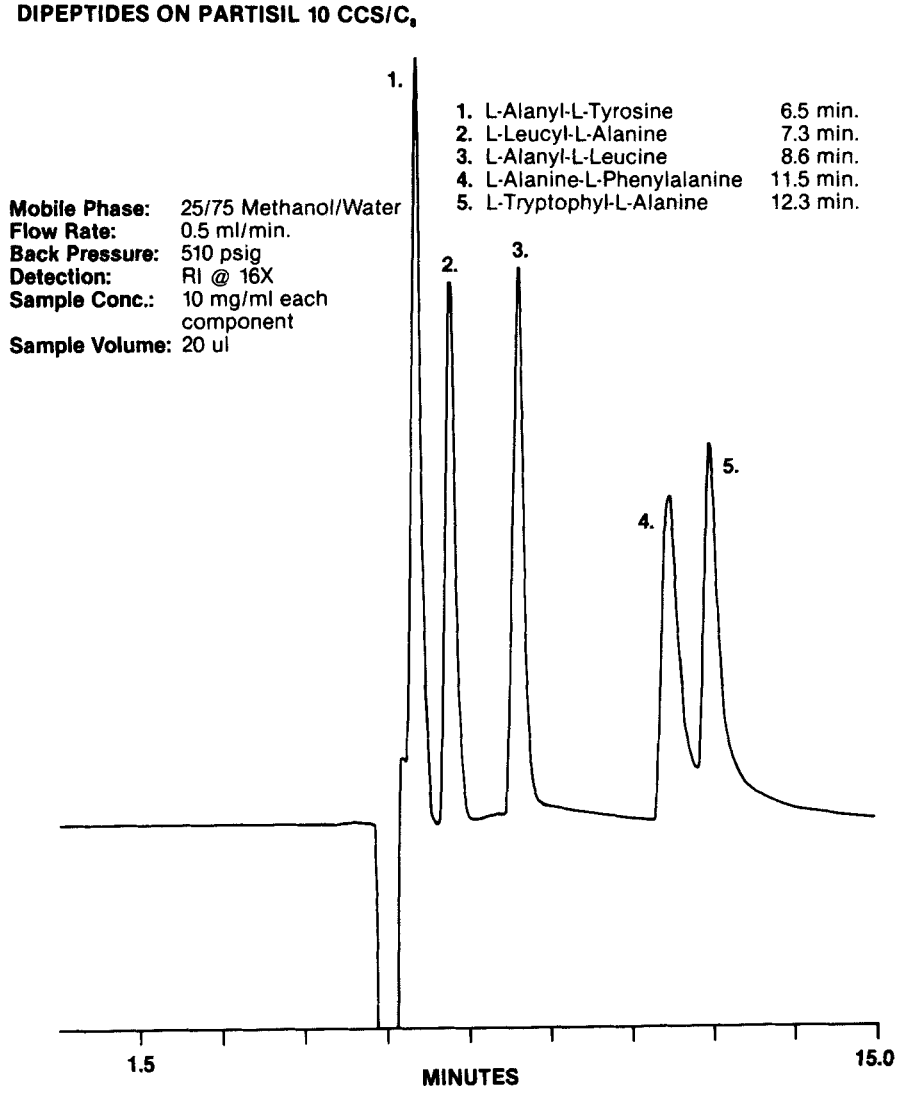


Figure 4

are able to interact with the 80 A, C8 bonded phase to get a better separation, probably because of the greater interaction with C8 due to the closer spacing of these groups on the silica. Larger molecular weight species, however, do not separate well on a small pore bonded phase, as has been mentioned, but are better separated on large pore media as will be shown.

A mixture of moderate molecular weight proteins was separated on these same three columns. These chromatograms are shown in Figures 5,6, and 7. On all columns these proteins seem to separate well, but with different relative retentions. On each column, the higher molecular weight BSA (bovine serum albumin) elutes before the β - lactoglobulin of 1/3 the molecular weight. Such anomolous elutions are often seen in biopolymer separations on reversed phase media where various lipophilic interactions are occurring. The conformation of each protein species, as well as its composition of amino acids, will determine its elution characteristics in each combination of column (type of bonded phase) and mobile phase. In each chromatogram all the peaks are fairly symmetrical except that of BSA which apparently is beginning to be separated into its dimeric and trimeric forms (34).

The Partisil 10 CCS/C_8 separation (Figure 7) is interesting in that even through this product has the largest percent carbon (9 versus 7.5 or 8) of the three columns, the elution time is the shortest. This is probably a result of the smaller pores not allowing much interaction with the bonded phase in these pores, thus giving faster elution of all the components.

PROTEIN TEST MIX ON PROTESIL 300 OCTYL

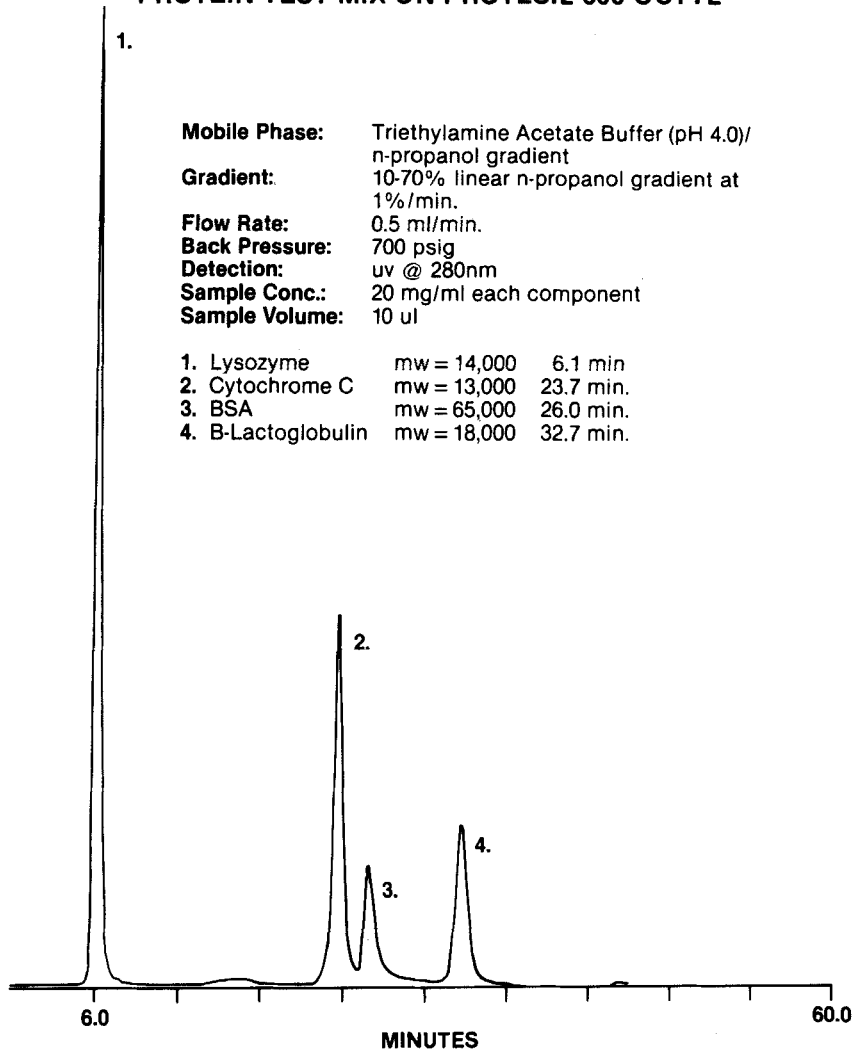


Figure 5

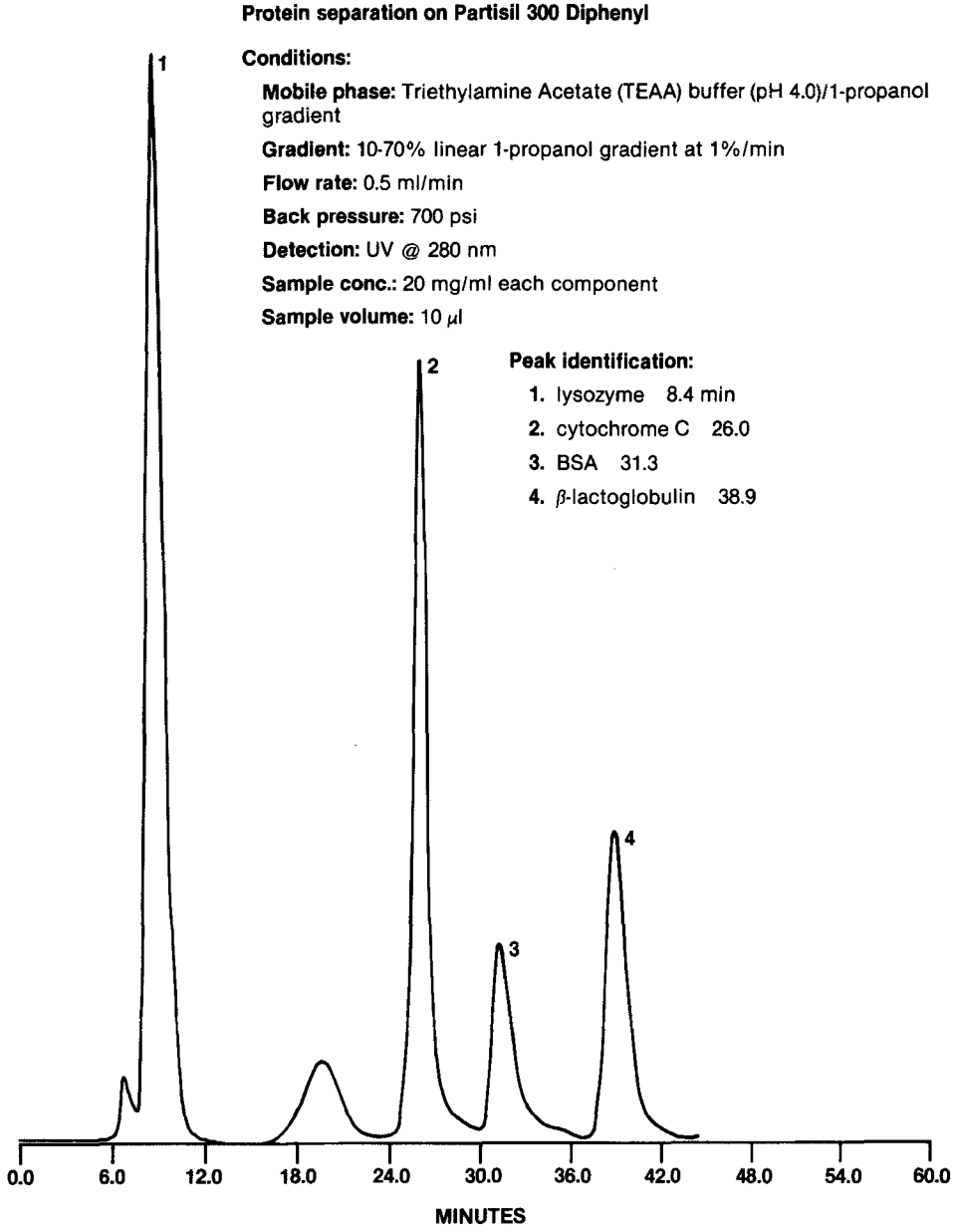


Figure 6

Protein separation on Partisil-10 C8

Conditions:

Mobile phase: Triethylamine Acetate (TEAA) buffer (pH 4.0)/1-propanol gradient

Gradient: 10-70% linear 1-propanol gradient at 1%/min

Flow rate: 0.5 ml/min

Back pressure: 700 psi

Detection: UV @ 280 nm

Sample conc.: 20 mg/ml each component

Sample volume: 10 μl

Peak identification:

1. lysozyme 4.2 min
2. cytochrome C 21.4
3. BSA 23.4
4. β-lactoglobulin 29.9

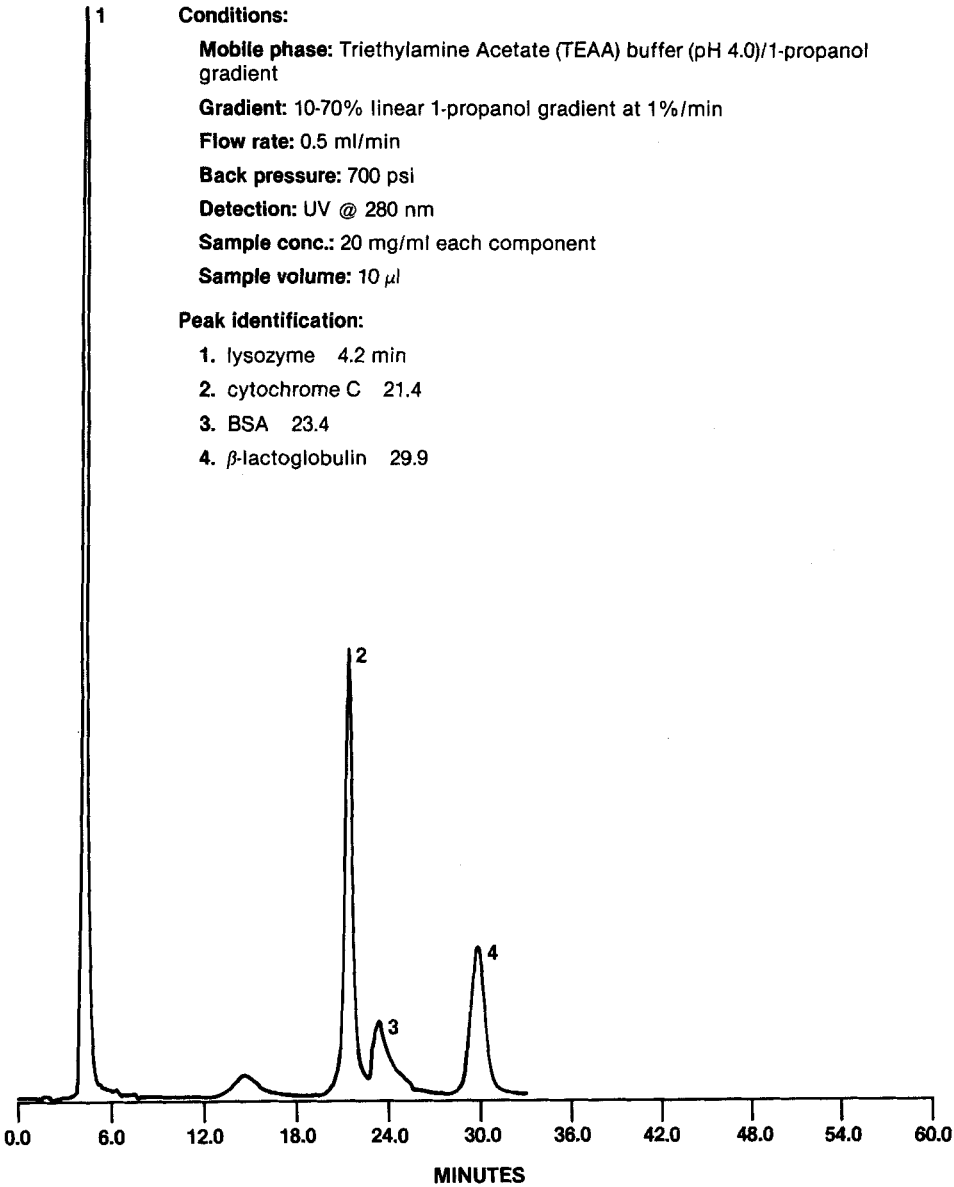


Figure 7

A second mixture of low to high molecular weight proteins was also separated on the Protesil 300 columns (Figures 8 and 9). These chromatograms are even more interesting. Even though the elution order for these proteins is the same on both columns, the Protesil 300 Octyl gives two peaks for hemoglobin (confirmed with a pure sample). An investigation as to the identity of these peaks is now being undertaken. The initial supposition is that perhaps the mobile phase is causing the heme and the globin to be separated and these are seen eluting as two peaks on the Octyl column. The same breakdown of hemoglobin would occur in the Diphenyl column because the mobile phase is the same, but the aromatic interaction of the diphenyl groups with the pyrrole rings of the heme are too strong to allow elution under the conditions employed. If such is the case, a different mobile phase (possibly close to neutrality) might be suggested to allow unmodified hemoglobin to be separated.

Since the conformations of these various proteins in the mobile phases used are not known, no definite comments on their elution order with regard to the bonded phase can be made. Work with smaller peptides whose structures are known and whose conformations would be less complex are now being done to confirm the unique selectivities found with the dipeptides.

An additional test of the selectivity differences on Protesil 300 Octyl and Diphenyl comes from samples of human serum separated on both. These profiles are seen in Figures 10 and 11. All peaks are unidentified at this time, but the largest peak is probably albumin. When the end point of the gradient is held for one hour, no further peaks eluted from either column.

PROTEIN STANDARDS ON PROTESIL 300 OCTYL

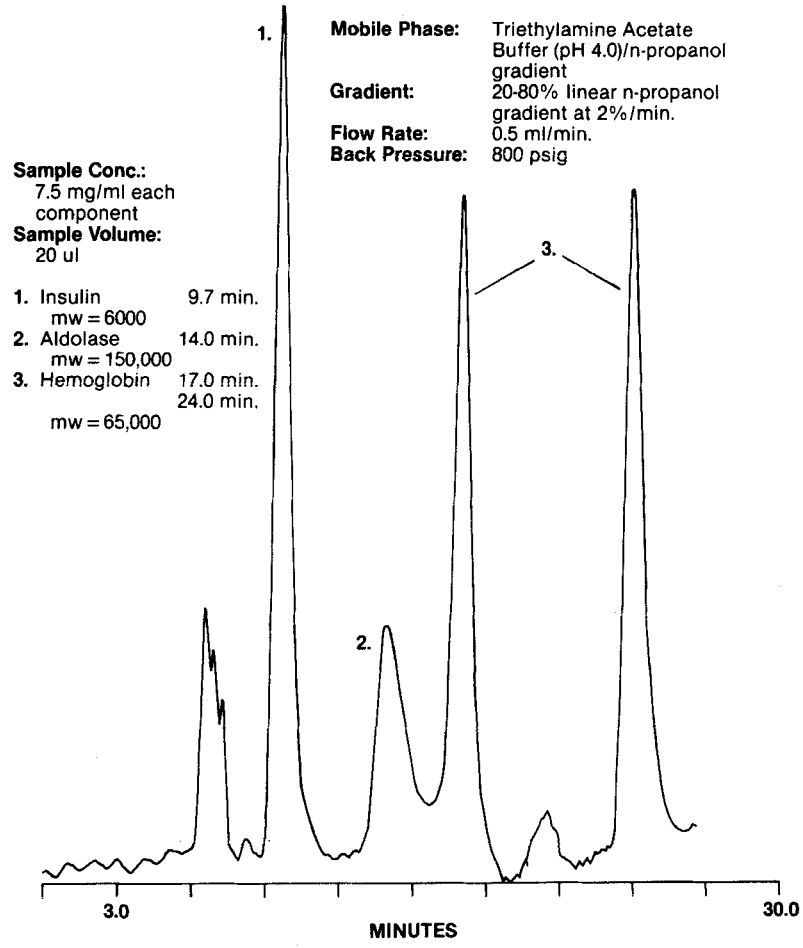


Figure 8

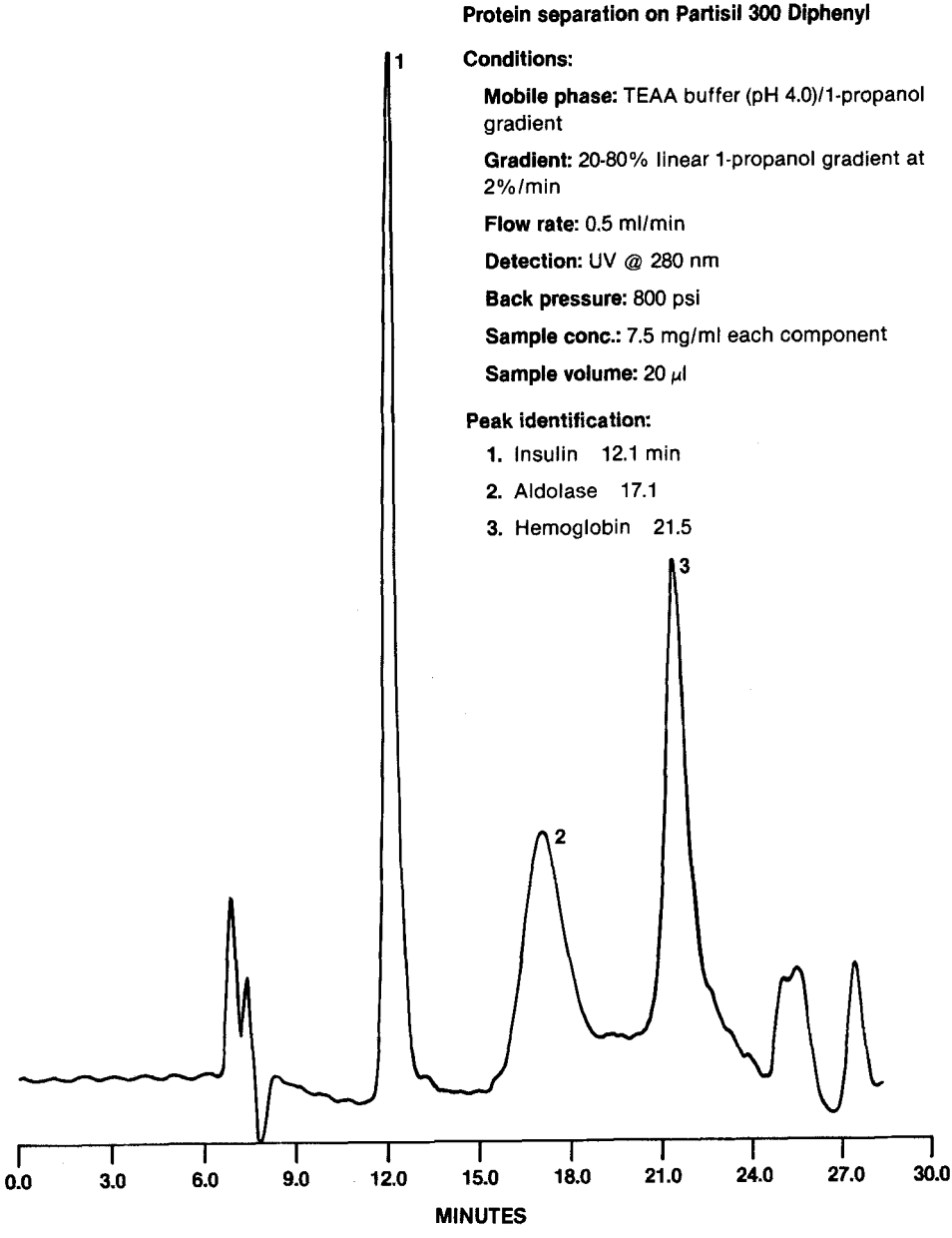


Figure 9

Human serum profile on Protesil 300 Octyl

Conditions:

Mobile phase: TEAA buffer (pH 4.0)/1-propanol gradient

Gradient: 0-100% linear 1-propanol gradient at 2%/min with a 10 min hold at 100% 1-propanol

Flow rate: 0.5 ml/min

Back pressure: 800 psi

Detection: UV @ 280 nm

Sample conc.: 4 g/20 ml

Sample volume: 50 μl

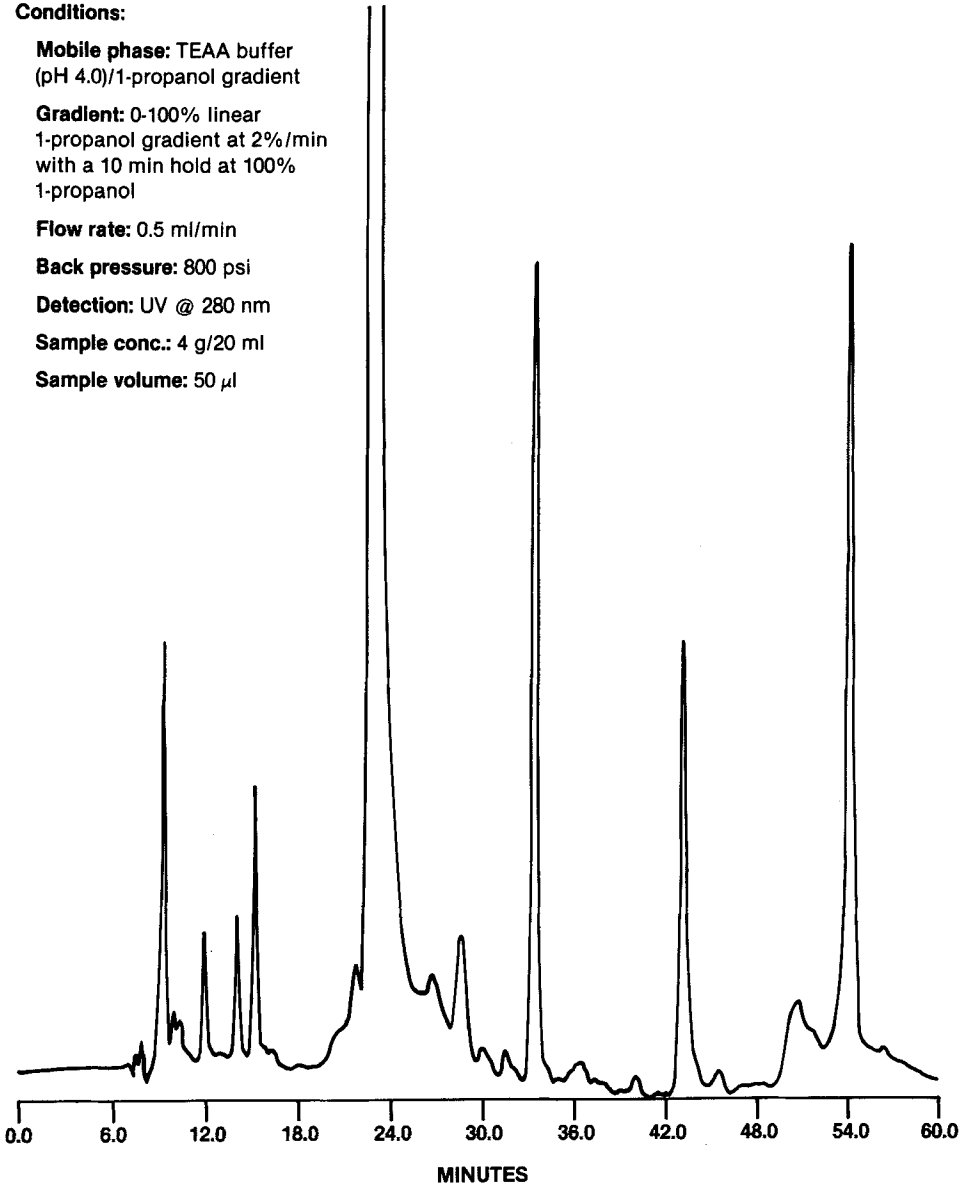


Figure 10

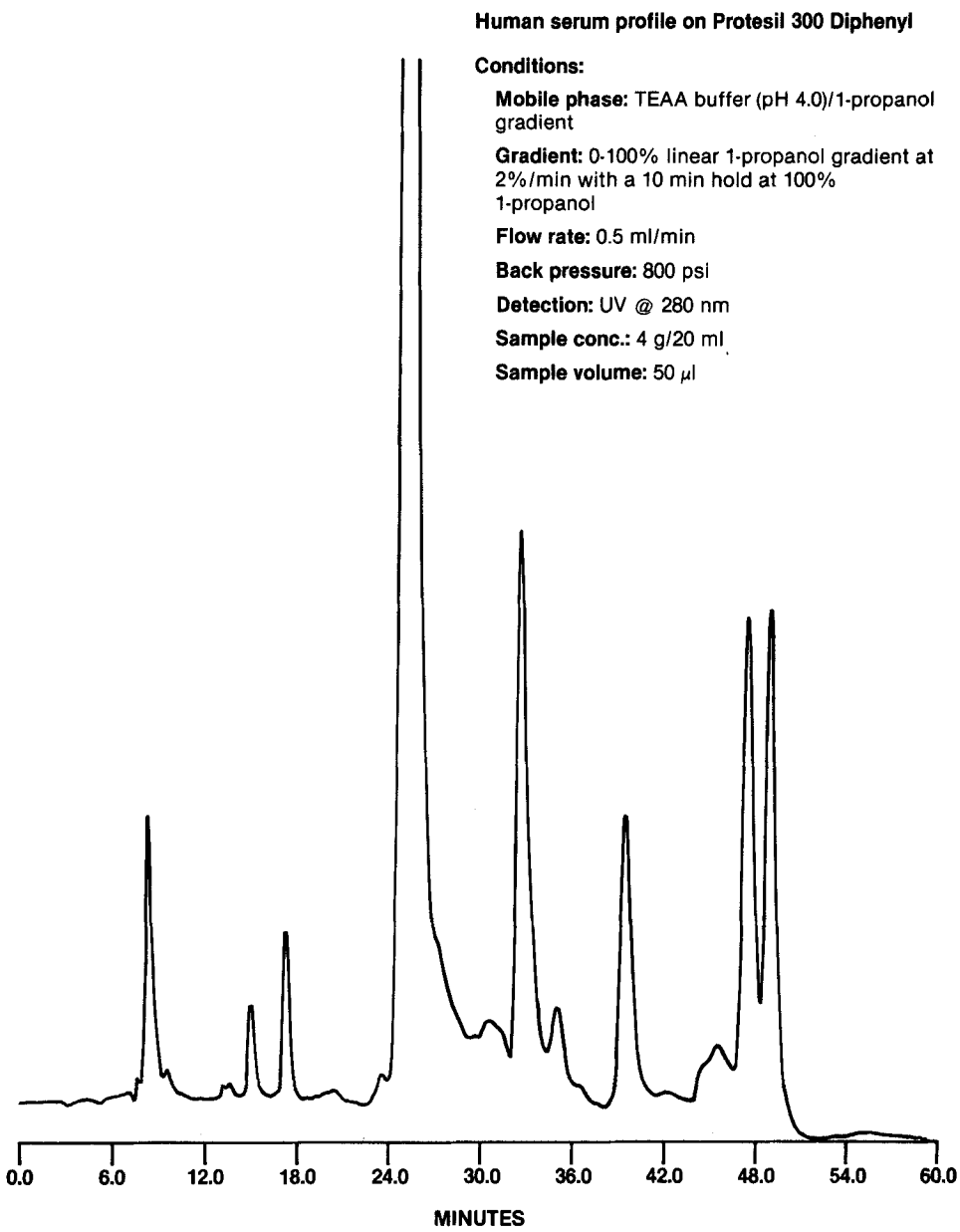


Figure 11

Loadability Studies

Since many protein separations will have to be scaled up to produce more of each species, a study into the loadability of these columns was done. The Partisil 10 CCS/C_8 was compared to the Protesil 300 Octyl and Diphenyl. Figure 12 shows tailing peaks and complete merging of the cytochrome C and BSA peaks in going from 2.5 mg of each protein in 50 ul to 3.75 mg in 75 ul injection on the Partisil 10 CCS/C_8. Figure 13 shows that the large sample is still able to be separated on the Protesil 300 Octyl column. Similarly, Figure 14 shows up to 5 mg each in 100 ul can be separated on the Protesil 300 Diphenyl column with no loss in resolution. These latter two chromatograms show that the maximum load is still higher because the peak retention is the same (indicating no mass overload) and the peak shape is symmetrical (indicating no volume overload) (35). The higher loadability of large pore bonded reversed phase media for biopolymers has been noted previously (13). Gradient elution of proteins has also been shown to give improved capacity (11).

Mass Recovery

Quantities of lysozyme were injected into Protesil 300 Octyl and Diphenyl and Partisil CCS/C_8 and ODS-3 columns and into an empty column to study mass recovery. A guard column (2.1 mm x 7 cm) filled with CoPell ODS was also put in line to see if additional lysozyme might be lost due to irreversible adsorption on the guard column media. The results are shown in Table II. As is usually found with such studies, the larger the quantity injected, the higher the recovery. In all of the columns the recovery is quite good as expected, since all of the media shown are end-capped.

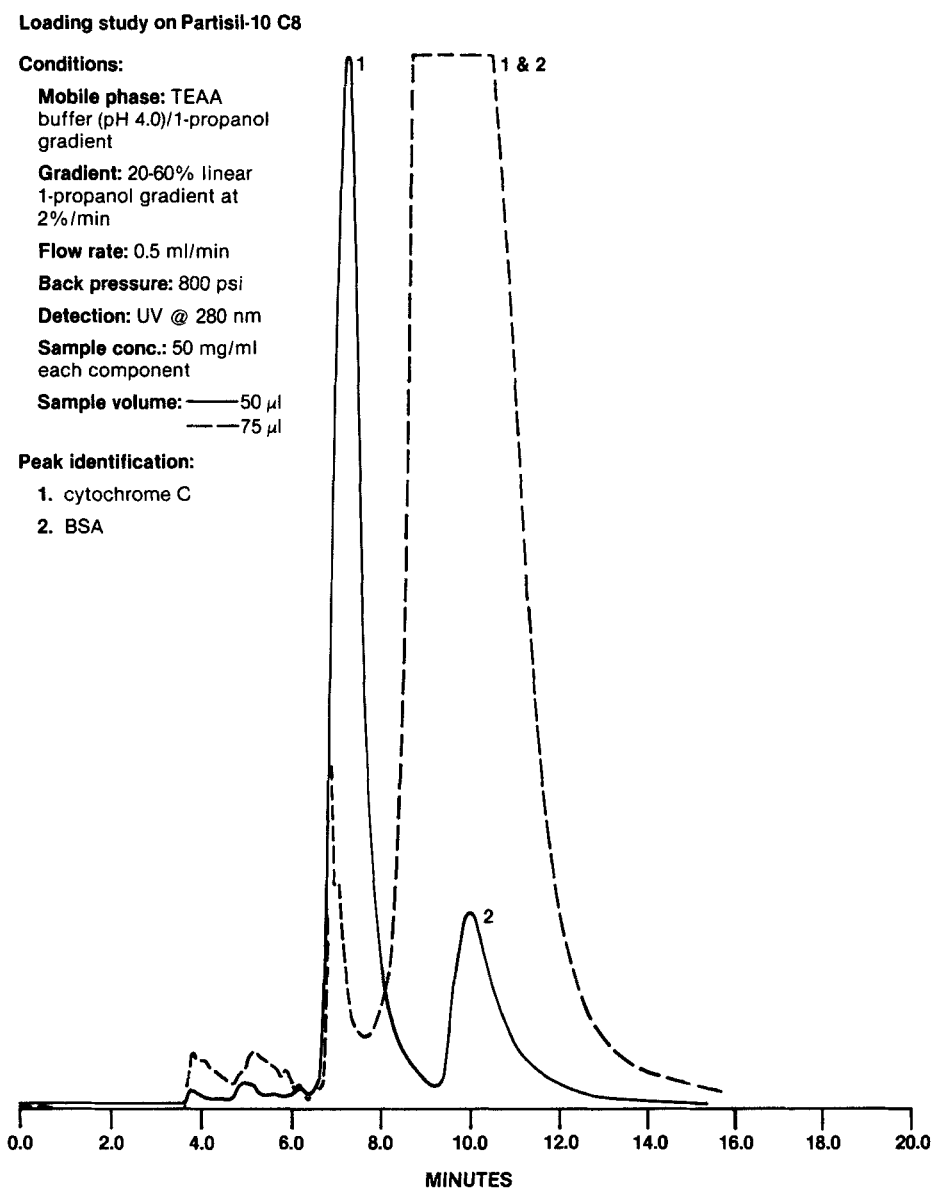


Figure 12

Loading study on Protesil 300 Octyl

Conditions:

Mobile phase: TEAA buffer (pH 4.0)/ 1-propanol gradient

Gradient: 20-60% linear 1-propanol gradient at 2%/min

Flow rate: 0.5 ml/min

Back pressure: 800 psi

Detection: UV @ 280 nm

Sample conc.: 50 mg/ml each component

Sample volume: ——50 µl
— —75 µl

Peak identification:

1. cytochrome C
2. BSA

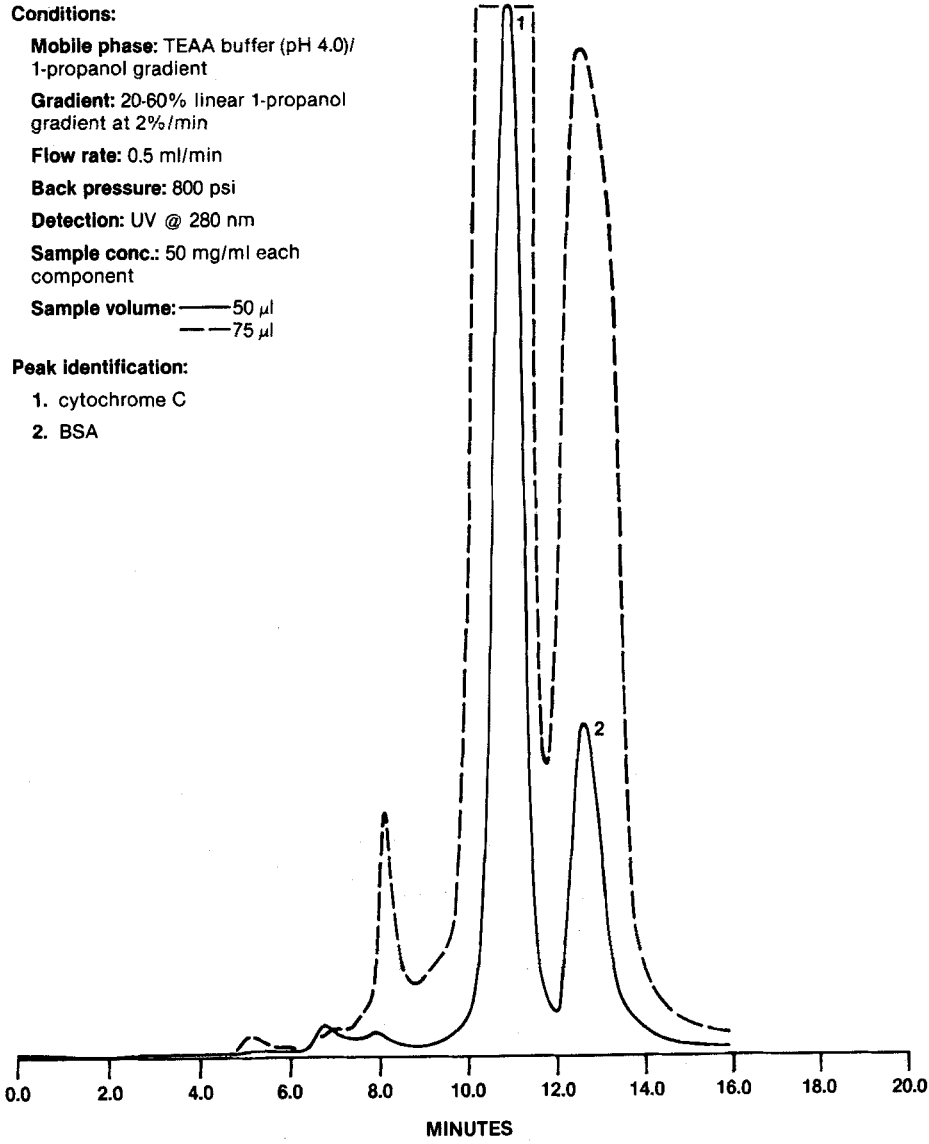


Figure 13

Loading study on Protesil 300 Diphenyl

Conditions:

Mobile phase: TEAA buffer (pH 4.0)/ 1-propanol gradient

Gradient: 20-60% linear 1-propanol gradient at 2%/min

Flow rate: 0.5 ml/min

Back pressure: 800 psi

Detection: UV @ 280 nm

Sample conc.: ——— 1 mg/ml

Sample volume: ——— 100 µl

Sample conc.: — — 50 mg/ml

Sample volume: — — 100 µl

Peak Identification:

1. cytochrome C
2. BSA

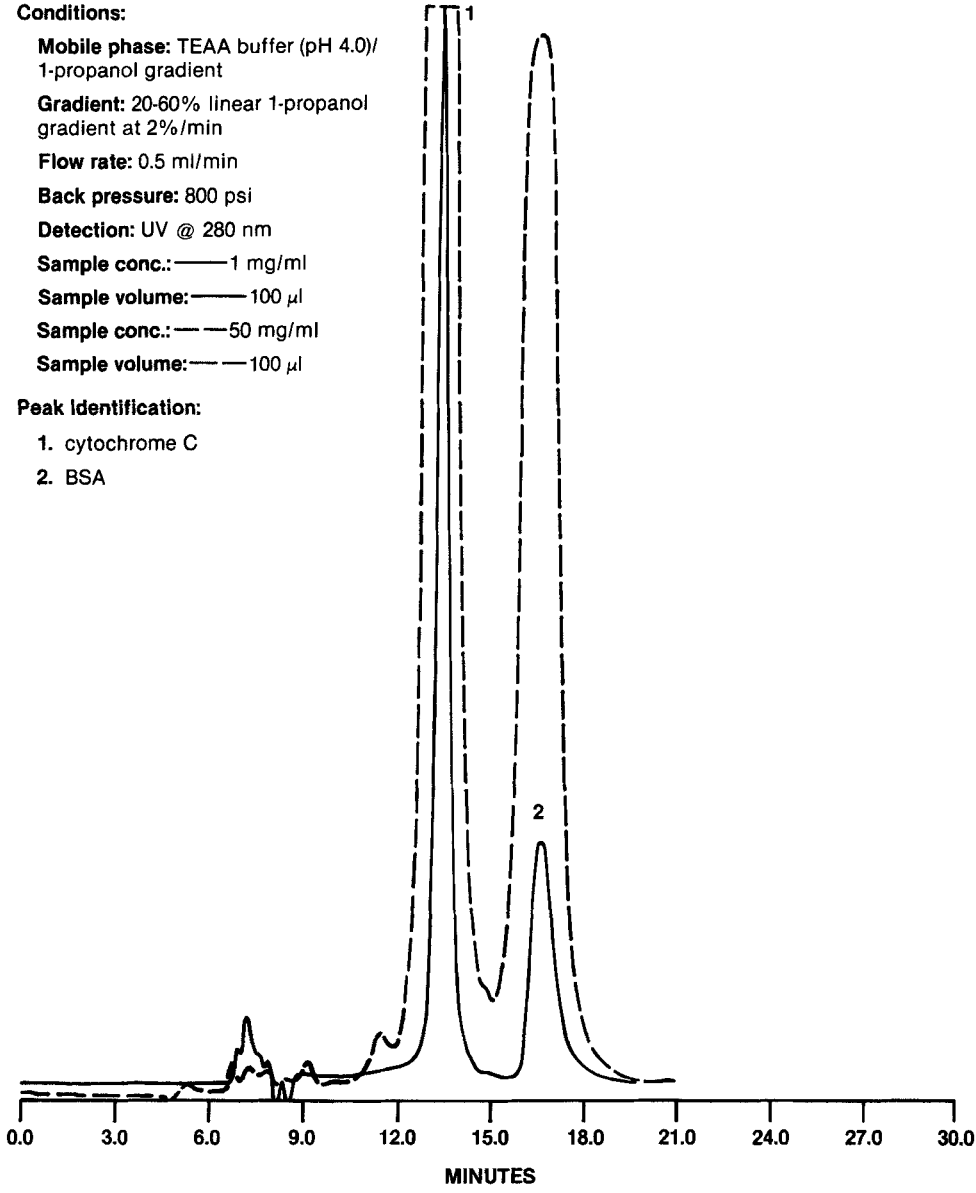


Figure 14

TABLE II

MASS RECOVERY OF LYSOZYME

Mass (ug)	Pr 300 Diphenyl	Pr 300 Octyl	Pa 10 CCS/C8	Pa 10 ODS-3	Gd Col (CoPell ODS) & Pr 300 Diphenyl
100	99	94	96	86	87
50	93	86	92	77	80
20	84	78	83	71	72
10	81	78	80	65	68

Abbreviations: Pr 300 = Protesil 300
Pa 10 = Partisil 10
Gd Col = Guard Column

With the guard column added to the system, there is some additional loss of protein. This is, however, a small price to pay for the extra life such guard columns often give to the analytical column.

The recovery studies based upon biological activities of proteins passed through the Protesil 300 columns are now underway and will be reported separately.

Comments

It is important for each company to provide as much information as possible on its columns and their use, so that the chromatographer gets the maximum potential from each column. Although a more thorough treatment of the general care of columns is available (36), a few more specific comments will be made here as a precautionary measure.

Any sample going onto a Protesil column should be cleaned up in some manner, if it is other than a standard. This may involve passing through mini-columns of ion exchange celluloses, size exclusion media or other. Filtration and/or a good centrifugation of the sample will also prevent particulates from getting into the LC system. Both Rubinstein (11) and Nice and his coworkers (15) have commented on sample preparation prior to HPLC separation.

Since the buffer salts or acids often used in biopolymer separations are not available usually as ´chromatographic grades´, they should be monitored carefully (UV scan of 1 M solutions possibly) and purified if necessary. Many such reagents can be purified easily by passing through columns of silica gel, alumina, polymer, or reversed phase bonded media.

When preparing buffer solutions, the pH of the aqueous portion is adjusted to that specified, then the organic is added. There is a shift to higher ´apparent pH´ (pH*) values on addition of organic (36). This apparent pH should not be allowed to become too high (no greater than 7 or 7.5) or the backbone of any bonded silica will be short lived.

Both pre-columns (before the injector) and guard columns (after the injector) are recommended for longest column life. Each serves a particular function and their use has been discussed (36,37).

It is critical that when any biopolymer separation is to be attempted that the sample solution be completely miscible with the isocratic mobile phase or the extremes of a gradient mobile phase. This will prevent high molecular weight species from being inadvertently precipitated at the head of the Protesil column.

If work with different biopolymers is to be done, it is recommended that 'dedicated' columns be considered to prevent cross contamination (15,16).

As was pointed out by Su and his coworkers (30), with the larger peptides the secondary and tertiary structures will become more important and 'the choice of mobile phase composition will have a profound effect on resolution and sample recovery.' Thus judicious and varied choices for mobile phase composition should be tried as well as the different Protesil 300 columns for optimum separation results.

Summary

Two new HPLC column packings have been designed for use as biopolymer separation media. Information derived from the work of many research groups has lead to what could be considered the optimal combination of bonded phase types, percent bonded phase, end-capping, and pore diameter. Various studies with dipeptide and protein mixtures have been done to show the distinct differences in selectivity and loadability of the Protesil 300 Octyl and Diphenyl in comparison with each other, and with Partisil 10 CCS/C_8, a similar bonded phase but on a smaller pore size silica gel. Each new Protesil packing appears to have unique selectivities, related to the differing hydrophobicities (or lipophilicities) of the bonded phases and their interaction with such sites on protein and peptide structures. Mass recovery has been shown to be excellent. With the usual column care, these columns will be long lived and will be a useful addition to the separation arsenal of the biochemist.

REFERENCES

1. Kielev, A.V., Khokhlova, T.D., and Eltekov, Y.A. in Column Chromatography, 5th International Symposium on Separation Methods, Lausanne, Oct. 7-10, 1969 (Kovats, E., editor), p. 124-125, Swiss Chemists Association, publisher, available from Saulerlander AG, CH-5001 Aarau, Switzerland.

2. Pickart, L.R. and Thaler, M.M., Prep. Biochem., 5, 397-412 (1975).

3. Chang, S.H., Gooding, K.M., and Regnier, F.E., J. Chromatogr., 125, 103-114 (1976).

4. Chang, S.H., Noel, R., and Regnier, F.E., Anal. Chem.,48, 1839-1845 (1976).

5. Schlabach, T.D., Chang, S.H., Gooding, K.M., and Regnier, F.E., J. Chromatogr., 134, 91-106 (1977).

6. Gooding, D.L., Chatfield, C., and Coffin, B., Am. Lab., Aug. 1980,48-61.

7. Barth, H.G., J. Chrom. Sci., 18, 409-429 (1980).

8. Pfannkoch, E., Lu, K.C., Regnier, F.E., and Barth, H.G., J. Chrom. Sci., 18, 430-441 (1980).

9. Rivier, J.E., J. Liq. Chrom., 1, 343-366 (1978).

10. Becker, N. and Unger, K.K., Chromatographia 12, 539-544 (1979).

11. Rubinstein, M., Anal. Biochem., 98, 1-7 (1979).

12. Schmidt, D.E., Giese, R.W., Connor,D., and Karger, B.L., Anal. Chem., 52, 177-182 (1980).

13. Lewis, R.V., Fallon, A., Stein, S., Gibson, K.D., and Udenfriend, S., Anal. Biochem., 104, 153-159 (1980).

14. Molnar, I. and Horvath, C., J. Chromatogr., 142, 623-640 (1977).

15. Nice, E.C., Capp, M., and O'Hare, M.J., J. Chromatogr., 185, 413-427 (1979).

16. O'Hare, M.J. and Nice, E.C., J. Chromatogr., 171, 209-226 (1979).

17. Blevins, D.D., Burke, M.F., and Hruby, V.J., Anal. Chem., 52, 420-424 (1980).

18. Margolis, S.A., and Longenback, P.J. in The Role of Peptides in Neuronal Function (Barker, J.L. and Smith, Jr., T.G., editors), Dekker, New York (1980).

19. Schroeder, W.A., Shelton, J.B., and Shelton, J.R., Hemoglobin 4, 551-559 (1980).

20. Bennett, H.R.J., Solomon, S., and Glotzman, D., Biochem. J., 197, 391-400 (1981).

21. Stricker, M.P., Gemski, M.J., and Doctor, B.P., J. Liq. Chrom., 4, 1765-1775 (1981).

22. Hancock, W.S. and Sparrow, J.T., J. Chromatogr.,206, 71-82 (1981).

23. Jones, B.N., Lewis, R.V., Paabo, S., Kojima, K., Kimura, S., and Stein, S., J. Liq. Chrom., 3, 1373-1383 (1980).

24. Meek, J.L. and Rossetti, Z.L., J. Chromatogr., 211, 15-28 (1981).

25. Stoklosa, J.T., Ayi, B.V., Shearer, C.M., and DeAngelis, N.J., Anal. Letters, B11, 889-899 (1978).

26. Hancock, W.S., Bishop, C.A., Prestidge, R.L., Harding, D.R.K., and Hearn, M.T.W., J. Chromatogr., 153, 391-398 (1978).

27. Hearn, M.T.W., Grego, B., and Hancock, W.S., J. Chromatogr., 185, 429-444 (1979).

28. Desiderio, D.M. and Cunningham, M.D., J. Liq. Chrom., 4, 721-733 (1981).

29. Meek, J.L., Proc. Nat'l. Acad. Sci. USA, 77, 1632-1636 (1980).

30. Su, S.J., Grego, B., Niven, B., and hearn, M.T.W., J. Liq. Chrom., 4, 1745-1764 (1981).

31. Gloor, R. and Johnson, E.L., J. Chrom. Sci., 15, 413-423 (1977).

32. Gruber, K.A., Whitaker, J.M., and Morris, M., Anal. Biochem., 97, 176-183 (1979).

33. Rekker, R. in The Hydrophobic Fragmental Constant, p. 301, Elsevier, New York (1977).

34. Welinder, B.S., J. Liq. Chrom. 3, 1399-1416 (1980).

35. Scott, R.P.W. and Kucera, P., J. Chromatogr., 119, 467-482 (1976).

36. Rabel, F.M., J. Chrom. Sci., 18, 394-408 (1980).

37. Atwood, J.G., Schmidt, G.J., and Slavin, W., J. Chromatogr., 171, 109-115 (1977).