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# SIMULTANEOUS SELECTIVITY OPTIMIZATION OF MOBILE AND STATIONARY PHASES IN REVERSED-PHASE LIQUID CHROMATOGRAPHY FOR ISOCRATIC SEPARATIONS OF PHENYLTHIOHYDANTOIN AMINO ACID DERIVATIVES*

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## SUMMARY

A mixture-design statistical technique has been used to optimize simultaneously the selectivity of both mobile and the stationary phases for the isocratic high-performance liquid chromatographic separation of phenylthiohydantoin derivatives of the 20 common amino acids. This approach permits the fine tuning of selectivity to achieve the rapid separation of this relatively complex mixture with maximum resolution between the various components. An optimum isocratic reversed-phase separation has been achieved in 13 min with a minimum resolution of 1.1 for all component peak pairs. The system uses an optimum combination of four mobile phase solvents (aqueous acids, methanol, acetonitrile and tetrahydrofuran) and three stationary-phase packings ($C_8$-, CN-, phenethyl-modified silica) to obtain various separation goals.

## INTRODUCTION

The recent use of systematic strategies to optimize operating parameters in high-performance liquid chromatography (HPLC) has demonstrated great potential of this approach for improving separation resolution and/or decreasing separation time. Much of this work has concentrated on using solvent mixtures to improve separations, based on a solvent selectivity triangle classification scheme proposed by Snyder[1]. We have described a mixture-design statistical technique[2] as an experimental basis to determine the optimum solvent composition for reversed-phase LC separations. This optimization approach has been extended to include normal bonded-

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phase[3], liquid–solid (adsorption)[4], and gradient elution[5] chromatography. In addition, other investigators have described optimization approaches using effects, such as pH and ionic strength. All of these topics have been the subject of a recent review[6].

Another important variable for changing selectivity in HPLC is the stationary phase. Although altering the stationary phase by changing the column is not generally as effective in HPLC as in gas chromatography, this is often a convenient approach and can be useful as a supplement to changing selectivity by other parameters, such as mobile phase composition. In particular, the use of a different stationary phase can be effective for an especially difficult separation that cannot be optimized satisfactorily with mobile phase composition alone. Since the influence of stationary and mobile phase composition is not directly related, it should be possible to use relatively simple statistical approaches to investigate both effects independently.

Separation of the twenty phenylthiohydantoin (PTH)-amino acid derivatives is an example of a difficult and important separation that could utilize the power of such a combined optimization strategy. Current methods of choice for this separation involve gradient elution HPLC. While these separations are adequate for many applications, there are still problems with limited sensitivity, overlapping peaks, and the proliferation of separation systems that each have strengths and weaknesses. A rational approach to define a global optimum using both mobile and stationary phase selectivity in HPLC has not been attempted. This work will show how the combination of mobile and stationary phase optimization can be used to develop a superior separation of a complex mixture, using the twenty common PTH-amino acids as a model system.

## EXPERIMENTAL

All measurements were made with a Model 8800 liquid chromatograph (Du Pont, Wilmington, DE, U.S.A.), which included a Model 870 three-headed pump with a four-solvent gradient mixer, a Model 850 fixed-wavelength photometric UV detector, a heated column compartment, and a Model 4100 recording integrator. All samples were injected with a Du Pont Model 834 autoinjector using a six-port air-actuated sampling valve (Valco Instruments, Houston, TX, U.S.A.) with either a 10- or 25-$\mu$l sample loop.

Columns were prepared from two separate lots of Zorbax® silica of nominally 6-$\mu$m and 3-$\mu$m particles, respectively. Bonded phases were prepared with monofunctional silanes to make $C_8$-, CN-, benzyl-, and phenethyl-modified silica packing materials. Columns were slurry packed into tubes of 10 × 0.46 cm I.D. or 25 × 0.46 cm I.D. dimensions for the 6-$\mu$m particles and 8 × 0.62 cm I.D. and 16 × 0.62 cm I.D. dimensions for the 3-$\mu$m particles. (See ref. 7 for background on 3-$\mu$m Zorbax columns.) Table I summarizes data on the packing materials that were prepared.

Mobile phase flow-rate was measured using a volumetric flask and stopwatch. Unretained peak volumes ($V_0$) were determined by injecting a volume of mobile phase containing a small amount of deuterated water. LC-grade organic solvents used in this work were obtained from either Burdick and Jackson (Muskegon, MI, U.S.A.) or J. T. Baker (Phillipsburg, NJ, U.S.A.). Water was deionized and filtered. Solutions of phosphoric and perchloric acid were made up to a specific pH. Acetate and phosphate buffers were used to control pH and ionic strength. The 20 PTH-

TABLE I

COLUMN PACKINGS USED

| | | |
|---|---|---|
| 6-μm columns | Base particles, Zorbax | |
| | Particle size (mean) | 5.5 μm |
| | Surface area | 324 $m^2/g$ |
| | Pore diameter (mean) | 82 Å |
| | Bonded phases | |
| | Dimethyl–$C_8$ (end-capped) | 3.19 μmole/$m^2$ |
| | Dimethyl–propylnitrile | 3.92 μmole/$m^2$ |
| | Dimethyl–benzyl (end-capped) | 3.41 μmole/$m^2$ |
| 3-μm columns | Base particles, Zorbax | |
| | Particle size (mean) | 3.04 μm |
| | Surface area | 282 $m^2/g$ |
| | Pore diameter (mean) | 75 Å |
| | Bonded phases | |
| | Dimethyl–$C_8$ (end-capped) | 3.19 μmole/$m^2$ |
| | Dimethyl–propylnitrile | 3.23 μmole/$m^2$ |
| | Dimethyl–phenethyl (end-capped) | 2.88 μmole/$m^2$ |

amino acid derivatives were obtained from Sigma (St. Louis, MO, U.S.A.). Solutions were prepared in acetonitrile–water mobile phases corresponding to that used in the particular study. Solute concentrations were 7 mmol/ml. Table II lists the 20 test solutes and the one-letter abbreviation code used in this study.

TABLE II

PHENYLTHIOHYDANTOIN (PTH)-AMINO ACID CODE

| *Name* | *One-letter code* |
|---|---|
| Alanine | A |
| Carboxymethyl cysteine | C |
| Aspartic acid | D |
| Glutamic acid | E |
| Phenylalanine | F |
| Glycine | G |
| Histidine | H |
| Isoleucine | I |
| Lysine | K |
| Leucine | L |
| Methionine | M |
| Asparagine | N |
| Proline | P |
| Glutamine | Q |
| Arginine | R |
| Serine | S |
| Threonine | T |
| Valine | V |
| Tryptophan | W |
| Tyrosine | Y |

Data were collected and analyzed on a PDP-10 computer (Digital Equipment, Maynard, MA, U.S.A.) using in-house software[8]. The optimization predictions and plotting were also carried out on the PDP-10 with networked PDP 11-60 and VAX 11-750 computers.

## RESULTS AND DISCUSSION

A special goal of this optimization study was to develop a rapid, high-resolution separation of the 20 common PTH-amino acids utilizing a single isocratic mobile phase system, rather than the commonly used gradient elution. Potential advantages of an isocratic separation include better analysis precision and higher sensitivity because of superior baseline stability, the use of less complex equipment, and faster analyses since re-equilibration of the column with the initial mobile phase is not required.

### *Acetate buffer system*

Initial studies to optimize the separation of all 20 PTH-amino acids were attempted with a single 15 × 0.46 cm column of 6-$\mu$m Zorbax-CN chromatographic packing using a buffer system reported for other PTH-amino acid studies. The approach attempted to develop an optimum isocratic mobile phase to replace the gradient elution conditions of most investigators. A sodium acetate buffer (pH 4.6) was employed as the aqueous solvent, and a seven-point mixture-design statistical experiment was carried out with methanol, acetonitrile and tetrahydrofuran (THF) as organic modifiers[2]. Although separation selectivity was found to be good with this

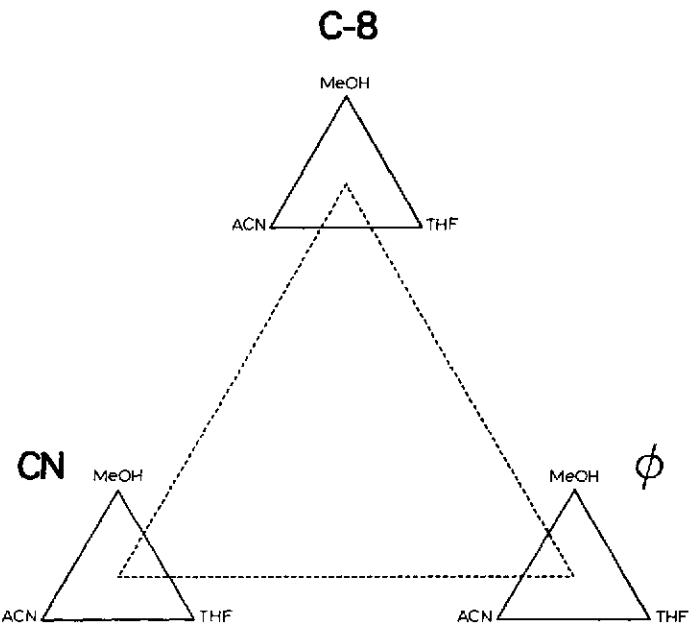
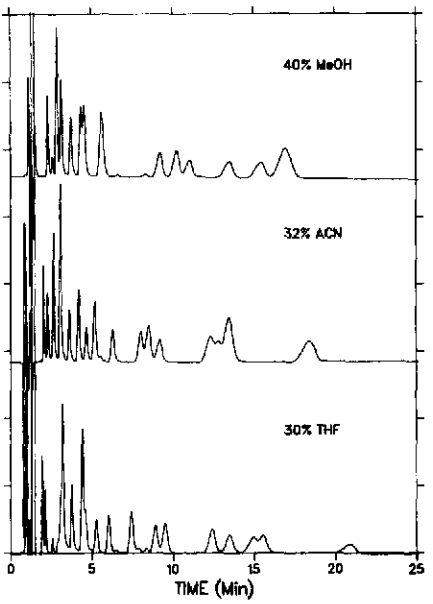



Fig. 1. Experiment design for mobile phase and stationary phase selectivity. MeOH = Methanol; ACN = acetonitrile; THF = tetrahydrofuran. Mobile phase compositions as in text.



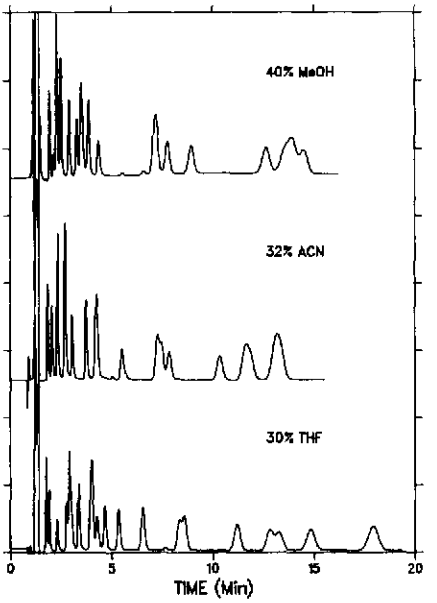




Fig. 2. Solvent selectivity on Zorbax-benzyl column, 10 × 0.46 cm I.D., packed with 6-μm particles; flow-rate, 2.0 ml/min; temperature, 50°C. Solutes are the 20 PTH-amino acid derivatives shown in Table II.

Fig. 3. Solvent selectivity on Zorbax-$C_8$ column. Conditions as in Fig. 2.

approach, the pH value of 4.6 caused certain compounds, notably lysine, arginine, and histidine, to exhibit poor peak shape. In addition, the retention range was too large under these conditions to permit a reasonable analysis time. Accordingly, other aqueous buffers were investigated to eliminate limitations of the pH 4.6 acetate buffer system.

*Phosphoric acid system*

To improve the shape of the basic PTH-amino acid peaks and to reduce the retention range of the compounds, the aqueous solvent was lowered to pH 2.1 using 10 m*M* phosphoric acid. Use of the lower pH inhibits the ionization so that selective interactions then depend largely on the composition of the organic solvent modifier, not on pH effects. It was found that the lower pH also required less organic modifier to produce a reasonable capacity factor ($k'$) range for all compounds. Initial tests on the 6-μm Zorbax-$C_8$ column demonstrated that 40% methanol, 32% acetonitrile, and 30% THF were the appropriate organic concentrations for the binary solvents in pH 2.1 phosphoric acid. These mobile phase compositions served as the apices of the solvent triangle for each column ($C_8$, CN and benzyl), and a standard seven-point mobile phase optimization[2] was carried out on all three columns, using a temperature of 50°C and a flow-rate of 2.0 ml/min. The experimental design for this approach is depicted in Fig. 1.

Data collected under these conditions consisted of retention time and peak width measurements for all 20 solutes in seven different mobile phases on three col-

umns. Figs. 2–5 graphically illustrate the changes in selectivity that were observed with different mobile or stationary phases. Although all the peaks are not labeled for purposes of clarity, two points are evident. First, the overall retention $k'$ ranges on each of the three column types were similar with the same mobile phases. Second, both mobile phases on a particular column (Figs. 2–4) and column type for a particular mobile phase (Fig. 5) showed significant selectivity changes. These effects are exactly those needed to provide the basis for a successful optimized separation of a complex mixture.

Separation of the twenty-component PTH-amino acid mixture was first optimized on each individual column, using the mixture design overlapping resolution mapping approach previously described[2]. In effect, a resolution map for each peak pair in the mixture is superimposed over the entire solvent selectivity triangle. Fig. 6 shows a three-dimensional view of the overlapping resolution map (ORM) for the benzyl column. The calculated optimum mobile phase of methanol–acetonitrile–THF–phosphoric acid (15.2:5.4:13.4:66.0) solution predicts a minimum resolution $R_s$ of 1.4 for limiting-resolution solutes E and A (with a 18,200 plate count column), and an actual minimum resolution value of 1.2 was observed, as shown in Fig. 7. Similar results were obtained for the $C_8$ and CN columns but are not illustrated.

Although the chromatographic results for each of the optima for each individual column was an improvement over previously reported systems, it was felt that the greatest selectivity effects and best resolution would be obtained by combining stationary and mobile phase effects. In this case we made the reasonable assumption that retention for each column would be linearly additive for any mobile phase.

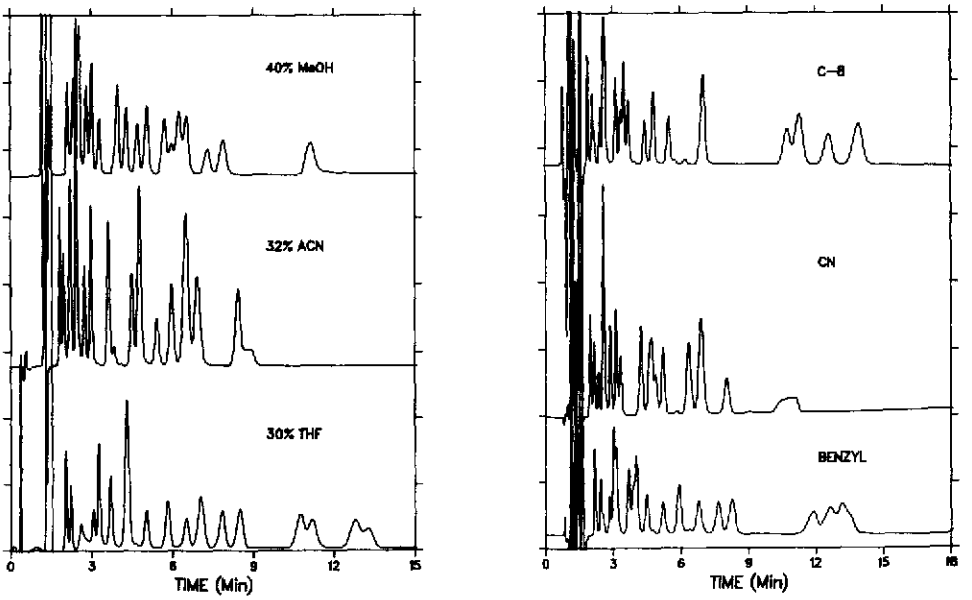



Fig. 4. Solvent selectivity on Zorbax-CN column. Conditions as in Fig. 2.

Fig. 5. Column selectivity on three different columns. Mobile phase is pH 2.1 phosphoric acid–methanol–acetonitrile–THF (66:13.3:10.7:10).

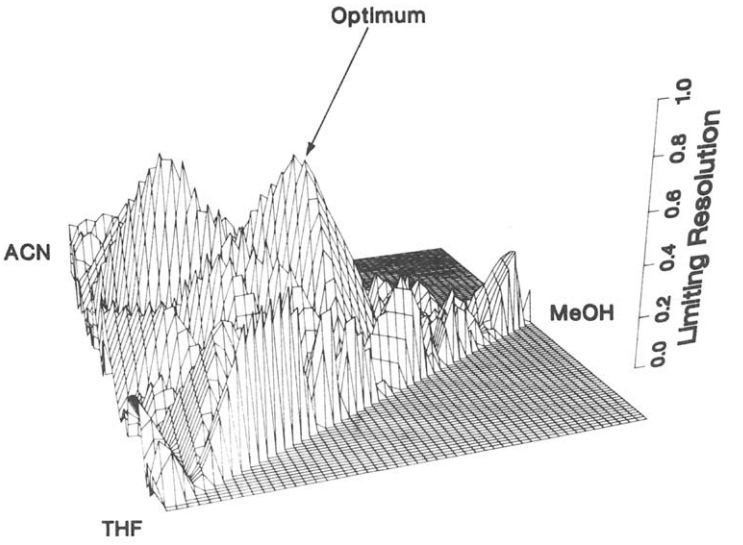



Fig. 6. Overlapping resolution map for all 20 PTH-amino acids on benzyl column.

Therefore, retention behavior for any serially connected column in any mobile phase could be calculated by simply using a linear combination of the PTH-amino acid retention for each individual column. Using the $k'$ values measured for each solute in each mobile phase–stationary phase combination (21 in all), a grid-search approach with iterative interpolation of the data was used on the VAX-11/750 to predict an optimum column of 28% CN, 72% benzyl with an optimum mobile phase of methanol–acetonitrile–THF–phosphoric acid (9.2:10.6:13:67.2) solution. To test the value of this approach, a column of this composition was closely approximated by connecting a 10-cm length of CN column to a 25-cm length of benzyl column. The

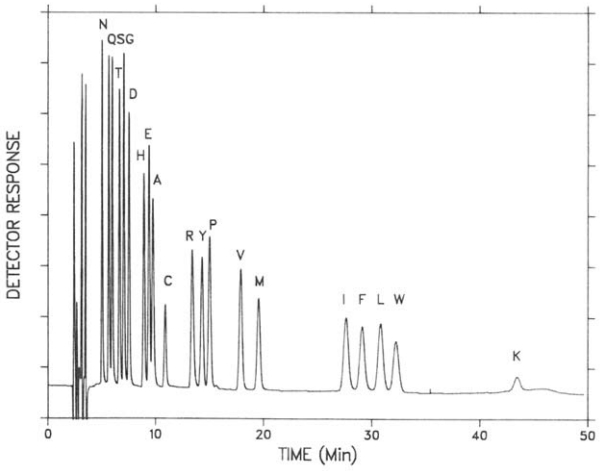



Fig. 7. Optimum separation for all 20 PTH-amino acids on benzyl column. Mobile phase: methanol–acetonitrile–THF–pH 2.1 phosphoric acid (15.2:5.4:13.4:66.0).

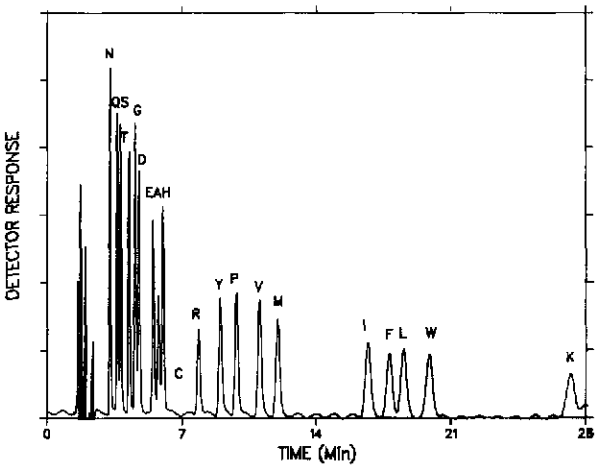



Fig. 8. Serially-connected columns optimum stationary and mobile phases. Combination column of 10 × 0.46 cm I.D. benzyl plus 25 × 0.46 cm I.D. CN columns. Mobile phase: methanol–acetonitrile–THF–pH 2.1 phosphoric acid (9.2:10.6:13.0:67.2). Other conditions as in Fig. 2.

separation in Fig. 8 shows the resulting chromatogram obtained with this serially-connected column. A predicted minimum resolution of 1.3 matched very well with the measured value of 1.2 for the E,A peak pair.

At this point, the interesting question arose as to whether a single column containing a mixed bed of CN and benzyl bonded phase packings in the proper ratios would produce the same results as the serially-connected columns just discussed. It should be noted that the predicted resolution in a mixed bed column cannot be based on the standard resolution equation given in eqn. 1:

$$R_s = \frac{(t_2 - t_1)}{2(\sigma_1 + \sigma_2)} \tag{1}$$

where $t_1$ and $t_2$ are the retention times and $\sigma_1$ and $\sigma_2$ the standard deviations for compounds 1 and 2. The reason for this is that, although retention times of each solute can be predicted as a linear function of the different packings, the standard deviation of each peak cannot. Therefore, it is necessary in predicting the resolution of mixed-bed column systems to assume a constant plate count $N$ for all components and use eqn. 2, which is another form of eqn. 1:

$$R_s = \frac{\sqrt{N}(k_2 - k_1)}{2\,(2 + k_1 + k_2)} \tag{2}$$

where $k_1$ and $k_2$ are the $k'$ values for compounds 1 and 2.

The mixed bed column of 0.28 CN/0.72 benzyl was prepared, and the chromatogram in Fig. 9 shows the result of the separation of all twenty compounds. The selectivity of this mixed-bed column is very similar but not identical to the serially-connected column (*cf.* Fig. 8). The reason for the slightly different selectivities ex-

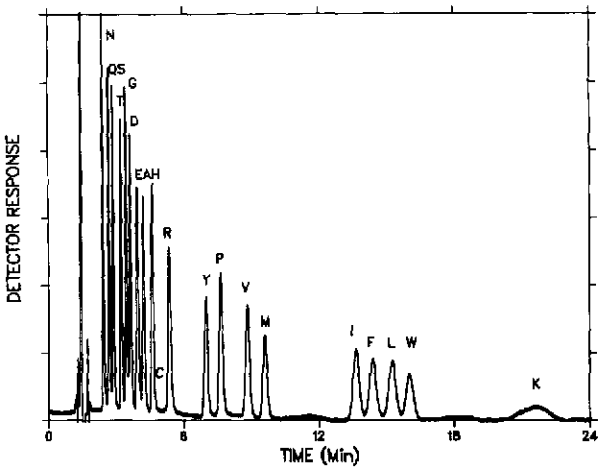



Fig. 9. Optimum mixed-bed column separation. Column: 28% CN, 72% benzyl, 25 × 0.46 cm I.D. Other conditions as in Fig. 8.

hibited for the serially-connected and mixed-bed columns is still unknown but is believed to be associated with localized solvent non-equilibration effects for particles within the mixed-bed column.

Unfortunately, a relatively serious disadvantage to using the pH 2.1 phosphoric acid system developed. All three columns showed a loss of retention as a function of use-time, although column efficiency was maintained. This observation strongly suggested that the bonded phases were being removed from the silica support under the low pH conditions used for the separations. Loss of bonded phase was confirmed by the fact that thermogravimetric analysis carried out on a benzyl column showed a 30% weight-loss in bonded phase after a few weeks of use, a loss which corresponded closely to the concomitant loss in $k'$ values for the solutes.

This decrease in $k'$ was investigated further on a fresh $C_8$ column. As shown in Fig. 10, $k'$ values for tryptophan steadily decreased during 13-h use-period, from an initial value of 7.1 to a final value of 6.2. This loss of bonded phase clearly was unacceptable for a practical analytical system.

A number of factors could accelerate column degradation of this type, such as the influence of the higher column temperature, low pH, and type of anion present. The effect of anion type was studied briefly by maintaining the pH at 2.1 but replacing phosphoric acid with perchloric acid. Other work has suggested that the solubility of silica might be less in the presence of perchlorate ion[9]. Replacing phosphate with perchlorate did appear to reduce column bonded phase loss, but the rate of packing degradation was still unacceptable.

As a compromise, it was then decided to raise the mobile phase pH as high as possible, consistent with reasonable peak shape and retention range. It was found that with a pH 3.2 phosphate buffer (12 m$M$) combined with a lower column temperature of 35°C, bonded phase loss was substantially reduced to the point where the stability of all three columns was acceptable. Curiously, even under these conditions, a slow decrease in $k'$ with time was noted when methanol-rich mobile phases were used. Columns were stable in systems containing only acetonitrile and THF, or if

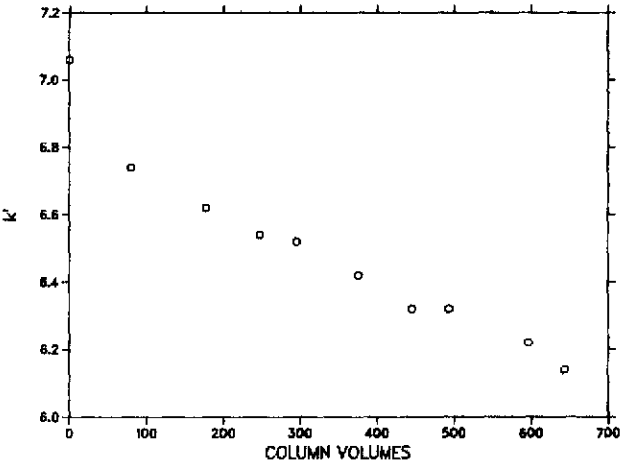



Fig. 10. Loss of tryptophan capacity factor ($k'$): $C_8$ column. Column: freshly prepared $C_8$ material, 25 × 0.46 cm I.D. Mobile phase: methanol–pH 2.1 phosphoric acid (40:60); column temperature, 50°C; flow-rate, 2.0 ml/min.

only low concentrations of methanol were present. We postulate that in high methanol concentrations at pH 3.2 (and particularly at higher temperatures), there may be a slow methanolysis of the bonded phase packing. Fortunately, as discussed below, we found that optimum mobile phase systems for the separation of PTH-amino acids contain little or no methanol so that our columns were stable for extended periods.

These studies resulted in a stable column mobile phase system that was amenable to a careful optimization strategy. First, the benzyl bonded phase column was replaced with a recent commercially available phenethyl bonded phase column of similar properties. Second, columns were prepared from a single lot of 3-$\mu$m particles which offered equivalent resolution to the 6-$\mu$m columns but in one-half the separation time. It should be noted that the larger diameter (0.62 cm) of these 3-$\mu$m columns resulted in peak volumes that were consistent with use in conventional HPLC instruments without significant band broadening effects[7]. Finally, the organic composition of the mobile phases had to be re-optimized in light of the new phenethyl bonded phase column and the temperature and pH changes. In this final system, 50% methanol, 36% acetonitrile and 33% THF were the corresponding values for the three apices of the solvent triangle for each column.

Data for this optimization were obtained in the manner described above. Retention was measured for all 20 compounds with each of 21 separate mobile phase–stationary phase combinations. Data are shown in Tables III–V for each of the three 8 × 0.62 cm columns of 3-$\mu$m particles operated with a flow-rate of 2.0 ml/min. To ensure internal consistency for all mobile phases and stationary phases during this study, solvents from one bottle of each organic solvent and a single preparation of the phosphate buffer were used throughout the study. These liquids were then mixed by the instrument to obtain the desired mobile phase composition. In addition, certain runs were repeated for each column at the conclusion of the original data collection to ensure retention repeatability and verify that there was no loss of retention due to column degradation.

Data in Tables III–V were first used to calculate an optimum mobile phase composition for each column, as shown by the first three entries in Table VI. It was found that the separation with the $C_8$ column was unacceptable even with an optimum mobile phase, since a limiting resolution of 0.38 was predicted for the separation of D,G for a column with a plate count of 10,000. The phenethyl column exhibited a limiting resolution of 0.78 for the peak pair, E,C. The best single column, however, is the CN column for which was predicted a resolution of 0.85 (for a 10,000 plate column) for the limiting peak pair of D,T for optimum organic modifier conditions, as shown in Fig. 11.

The CN column also had an important practical advantage, only acetonitrile and THF were required as organic modifiers. This fact not only simplifies the experimental aspects of the separation and allowed the separation to be performed on systems having only ternary solvent mixing capability, but also eliminates the column degradation problem associated with methanol-rich solvent system, as discussed above.

TABLE III

CAPACITY FACTORS ($k'$) ON $C_8$ COLUMN

Solutes as in Table II. Column, 8.0 × 0.62 cm I.D., packed with Zorbax-$C_8$, 3-$\mu$m particles; flow-rate, 2.0 ml/min; temperature, 35°C; mobile phases with pH 3.2 phosphate buffer (12 m*M*).

| *Solute* | *Mobile phase* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* | *7* |
| N | 1.09 | 1.04 | 1.04 | 1.14 | 1.04 | 1.08 | 1.07 |
| Q | 1.19 | 1.12 | 1.11 | 1.28 | 1.10 | 1.18 | 1.17 |
| S | 1.17 | 1.16 | 1.30 | 1.24 | 1.20 | 1.21 | 1.21 |
| D | 1.13 | 1.23 | 1.69 | 1.26 | 1.38 | 1.32 | 1.30 |
| T | 1.24 | 1.27 | 1.50 | 1.32 | 1.32 | 1.29 | 1.30 |
| E | 1.39 | 1.43 | 2.07 | 1.55 | 1.66 | 1.65 | 1.56 |
| G | 1.30 | 1.49 | 1.60 | 1.46 | 1.42 | 1.38 | 1.38 |
| H | 1.21 | 1.26 | 1.28 | 1.27 | 1.18 | 1.11 | 1.17 |
| C | 1.30 | 1.47 | 2.12 | 1.54 | 1.67 | 1.59 | 1.57 |
| R | 1.35 | 1.51 | 1.66 | 1.44 | 1.45 | 1.30 | 1.33 |
| A | 1.60 | 1.95 | 2.13 | 1.89 | 1.83 | 1.73 | 1.74 |
| Y | 1.55 | 2.15 | 3.20 | 2.06 | 2.40 | 2.16 | 2.16 |
| V | 2.84 | 3.59 | 4.24 | 3.57 | 3.30 | 3.03 | 3.15 |
| M | 2.61 | 3.53 | 4.37 | 3.49 | 3.32 | 3.16 | 3.14 |
| P | 2.65 | 3.80 | 2.69 | 3.47 | 2.61 | 2.34 | 2.54 |
| I | 3.97 | 5.43 | 6.49 | 5.51 | 5.03 | 4.60 | 4.79 |
| W | 4.48 | 4.79 | 6.51 | 4.64 | 4.73 | 4.63 | 4.60 |
| F | 3.76 | 5.31 | 5.54 | 5.49 | 4.53 | 4.32 | 4.54 |
| L | 4.30 | 6.03 | 7.63 | 6.08 | 5.65 | 5.12 | 5.28 |
| K | 3.56 | 5.83 | 8.78 | 6.18 | 5.88 | 5.45 | 5.44 |
| *Composition of mobile phase (%)* | | | | | | | |
| Buffer | 50.0 | 64.0 | 67.0 | 57.0 | 65.5 | 58.5 | 60.3 |
| Methanol | 50.0 | – | – | 25.0 | – | 25.0 | 16.7 |
| Acetonitrile | – | 36.0 | – | 18.0 | 18.0 | – | 12.0 |
| THF | – | – | 33.0 | – | 16.5 | 16.5 | 11.0 |

TABLE IV

CAPACITY FACTORS ON PHENETHYL COLUMN

Solutes as in Table II. Column, 8.0 × 0.62 cm I.D. packed with Zorbax-phenethyl, 3-μm particles; flow-rate, 2.0 ml/min; temperature, 35°C; mobile phases with pH 3.2 phosphate buffer (12 m*M*).

| *Solute* | *Mobile phase* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* | *7* |
| N | 1.43 | 1.23 | 1.30 | 1.51 | 1.26 | 1.39 | 1.37 |
| Q | 1.72 | 1.33 | 1.41 | 1.73 | 1.36 | 1.57 | 1.52 |
| S | 1.57 | 1.40 | 1.66 | 1.68 | 1.51 | 1.64 | 1.60 |
| D | 1.57 | 1.52 | 2.19 | 1.75 | 1.78 | 1.85 | 1.79 |
| T | 1.73 | 1.57 | 1.92 | 1.83 | 1.71 | 1.79 | 1.76 |
| E | 2.13 | 1.79 | 2.73 | 2.25 | 2.14 | 2.40 | 2.25 |
| G | 1.97 | 1.90 | 2.05 | 2.12 | 1.85 | 1.95 | 1.92 |
| H | 1.97 | 1.91 | 1.80 | 2.05 | 1.73 | 1.67 | 1.72 |
| C | 2.06 | 1.87 | 2.84 | 2.30 | 2.24 | 2.44 | 2.32 |
| R | 2.30 | 2.49 | 2.61 | 2.55 | 2.33 | 2.21 | 2.24 |
| A | 2.48 | 2.49 | 2.71 | 2.74 | 2.41 | 2.49 | 2.48 |
| Y | 2.93 | 2.94 | 4.35 | 3.45 | 3.44 | 3.63 | 3.49 |
| V | 4.67 | 4.53 | 5.16 | 5.06 | 4.43 | 4.53 | 4.57 |
| M | 5.52 | 4.88 | 5.69 | 5.92 | 4.75 | 5.33 | 5.12 |
| P | 6.12 | 5.38 | 3.55 | 6.22 | 3.82 | 3.91 | 4.25 |
| I | 7.46 | 6.72 | 7.76 | 7.84 | 6.62 | 7.03 | 6.99 |
| W | 6.48 | 6.93 | 8.66 | 8.58 | 7.16 | 8.30 | 7.96 |
| F | 8.01 | 7.23 | 7.22 | 9.00 | 6.54 | 7.30 | 7.39 |
| L | 8.27 | 7.47 | 8.91 | 8.72 | 7.35 | 7.98 | 7.82 |
| K | 13.16 | 9.18 | 12.51 | 14.17 | 9.57 | 11.79 | 11.19 |
| *Composition of mobile phase (%)* | | | | | | | |
| Buffer | 50.0 | 64.0 | 67.0 | 57.0 | 65.5 | 58.5 | 60.3 |
| Methanol | 50.0 | – | – | 25.0 | – | 25.0 | 16.7 |
| Acetonitrile | – | 36.0 | – | 18.0 | 18.0 | – | 12.0 |
| THF | – | – | 33.0 | – | 16.5 | 16.5 | 11.0 |

The data in Tables III–V also were used to predict an optimum stationary phase–mobile phase combination for the 20 PTH amino acids. To calculate an optimum mixed-bed column, the reasonable assumption again was made that the retention behavior of a solute on a mixed bed would be a linear combination of the retention of that solute on each individual packing under the same mobile phase conditions. For example, the $k'$ value for M on a mixed-bed column of 0.20 $C_8$/0.30 phenethyl (PE)/0.50 CN in mobile phase No. 2 would be 0.2 times the $k'$ value of M on the $C_8$ column plus 0.3 times the $k'$ value on the PE column, plus 0.5 times the $k'$ value on the CN column. This approach was used to computer-grid a large number of potential mixed-bed column systems to determine the optimum packing mixture for the best separation of the PTH-amino acid mixture. A grid resolution of 2% for stationary phase and 1% for pseudo-component mobile phase was used in these calculations. Analysis of all of the data resulted in an optimum mixed-bed column of 0.22 PE/0.78 CN, as shown by the fourth entry in Table VI. However, the predicted resolution for the limiting peak pair (in this case, D,T) was 0.86 (for *ca.* 10,000 plate

TABLE V

CAPACITY FACTORS ON CN COLUMN

Solutes as in Table II. Column, 8.0 × 0.62 cm I.D. packed with Zorbax-CN, 3-μm particles; flow-rate, 2.0 ml/min; temperature, 35°C; mobile phases with pH 3.2 phosphate buffer (12 m*M*).

| *Solute* | *Mobile phase* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* | *7* |
| N | 1.36 | 1.19 | 1.39 | 1.27 | 1.26 | 1.37 | 1.29 |
| Q | 1.48 | 1.25 | 1.51 | 1.36 | 1.33 | 1.48 | 1.37 |
| S | 1.41 | 1.31 | 1.72 | 1.34 | 1.43 | 1.51 | 1.40 |
| D | 1.43 | 1.41 | 2.26 | 1.39 | 1.62 | 1.64 | 1.51 |
| T | 1.45 | 1.41 | 1.96 | 1.39 | 1.55 | 1.57 | 1.48 |
| E | 1.67 | 1.58 | 2.68 | 1.58 | 1.84 | 1.94 | 1.74 |
| G | 1.60 | 1.62 | 2.12 | 1.55 | 1.70 | 1.74 | 1.63 |
| H | 2.12 | 1.97 | 3.00 | 1.95 | 2.14 | 2.25 | 2.03 |
| C | 1.66 | 1.62 | 2.81 | 1.58 | 1.91 | 1.96 | 1.76 |
| R | 2.46 | 2.46 | 4.68 | 2.31 | 2.85 | 2.97 | 2.57 |
| A | 1.80 | 1.93 | 2.67 | 1.76 | 2.05 | 2.05 | 1.89 |
| Y | 2.15 | 2.30 | 4.11 | 2.10 | 2.71 | 2.68 | 2.40 |
| V | 2.28 | 2.80 | 4.50 | 2.33 | 3.03 | 2.81 | 2.62 |
| M | 2.74 | 3.00 | 4.96 | 2.65 | 3.28 | 3.28 | 2.95 |
| P | 2.61 | 2.99 | 3.47 | 2.57 | 2.85 | 2.79 | 2.65 |
| I | 2.76 | 3.62 | 6.24 | 2.91 | 3.97 | 3.64 | 3.34 |
| W | 3.81 | 4.19 | 7.50 | 3.68 | 4.74 | 4.88 | 4.26 |
| F | 3.14 | 3.92 | 6.02 | 3.27 | 4.11 | 3.96 | 3.64 |
| L | 3.05 | 3.97 | 7.12 | 3.18 | 4.36 | 4.05 | 3.67 |
| K | 5.15 | 4.97 | 11.09 | 4.59 | 5.96 | 6.77 | 5.41 |
| *Composition of mobile phase (%)* | | | | | | | |
| Buffer | 50.0 | 64.0 | 67.0 | 57.0 | 65.5 | 58.5 | 60.3 |
| Methanol | 50.0 | – | – | 25.0 | – | 25.0 | 16.7 |
| Acetonitrile | – | 36.0 | – | 18.0 | 18.0 | – | 12.0 |
| THF | – | – | 33.0 | – | 16.5 | 16.5 | 11.0 |

column), which was not significantly better than the CN column alone. In addition, the optimum mixed-bed column required a quaternary mobile phase, which is not as convenient as the ternary system required for the CN column. Based only on this consideration, therefore, we propose that the CN column forms the basis for the best compromise in separating the 20 PTH-amino acids with a simple isocratic mobile phase.

In the process of acquiring data to predict this optimum mobile phase, we noticed that a change in the ionic strength of the mobile phase (keeping pH and organic modifier compositions constant) did not significantly affect the retention of most of the PTH-amino acids. On the other hand, large changes were observed in the retention behavior of H, R and C, as might be expected for these highly ionic, basic compounds. This fact presented an attractive option to further improve the PTH-amino acid separation, by optimizing for the other 17 compounds and then adjusting ionic strength to place compounds H, R and C in favorable locations in the chromatogram. This was an especially effective alternative since one of these three

TABLE VI

OPTIMUM MOBILE PHASE SYSTEMS (3-μm COLUMNS)

| *Column* | *Pseudo component values* | | | *Solvent compositions (%)* | | | | *Min* $R_s$* | *Limiting peak pair* |
|---|---|---|---|---|---|---|---|---|---|
| | *Methanol* | *Acetonitrile* | *THF* | *Buffer* | *Methanol* | *Acetonitrile* | *THF* | | |
| PE | 0.30 | 0.22 | 0.48 | 61.3 | 15.0 | 7.9 | 15.8 | 0.78 | E,C |
| $C_8$ | 0.36 | 0.12 | 0.52 | 60.5 | 18.0 | 4.3 | 17.2 | 0.38 | D,R |
| CN | 0.00 | 0.56 | 0.44 | 65.3 | 0.0 | 20.2 | 14.5 | 0.85 | D,T |
| 0.22 PE/ 0.78 CN | 0.06 | 0.50 | 0.44 | 64.5 | 3.0 | 18.0 | 14.5 | 0.86 | D,T |
| *17 Peaks only* | | | | | | | | | |
| CN | 0.08 | 0.42 | 0.50 | 64.4 | 4.0 | 15.1 | 16.5 | 1.05 | I,F |
| 0.27 PE/ 0.73 CN | 0.24 | 0.26 | 0.50 | 62.1 | 12.0 | 9.4 | 16.5 | 1.11 | D,T |
| *17 Peaks, no methanol* | | | | | | | | | |
| CN | 0.00 | 0.54 | 0.46 | 65.4 | 0.0 | 19.4 | 15.2 | 0.93 | D,T |
| 0.67 PE/ 0.33 $C_8$ | 0.00 | 0.52 | 0.48 | 65.5 | 0.0 | 18.7 | 15.8 | 0.92 | D,G |

* For plate count assumed = 10,000. Other operating conditions as in Table III.

compounds often was involved in the limiting peak pair which defined the optimum separation.

The fifth entry in Table VI shows the result of optimizing the organic solvent modifier for the other 17 compounds. Again, the CN bonded phase was found to be the best single column. In this case the optimum mobile phase composition contained methanol–acetonitrile–THF–buffer (4.0:15.1:16.5:64.4). The limiting peak pair has now changed to I,F one of the later pairs in the chromatogram. An even better separation could be obtained with a mixed-bed column of 0.27 PE/0.73 CN using

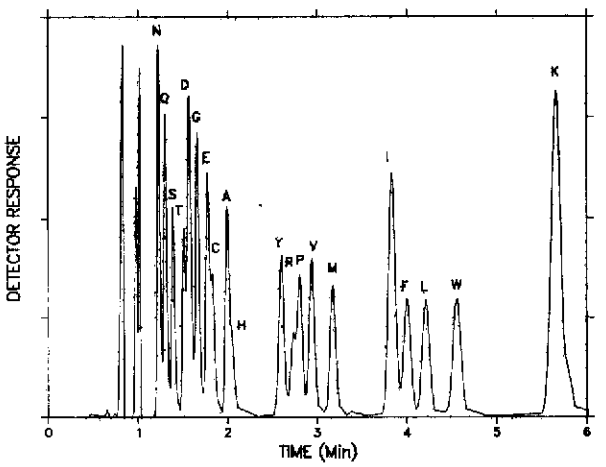



Fig. 11. First optimum separation on 3-μm Zorbax-CN column 8.0 × 0.62 cm I.D. Column temperature, 35°C; flow-rate, 2.0 ml/min; mobile phase is acetonitrile–THF–pH 3.2 phosphate buffer (ionic strength of 12 μ*M*) (20.2:14.5:67.3).

12.0% methanol, 9.4% acetonitrile and 16.5% THF in 62.1% buffer, where the limiting peak pair of D,T is predicted to give a resolution of 1.1 for a 10,000 plate count column. The disadvantage of both of these column systems is that a quaternary solvent with methanol is specified. As described above, a mobile phase system without methanol is preferred to minimize the potential for column degradation.

Therefore, another interesting option is the utilization of retention data to optimize for a mobile phase that contains no methanol so that maximum column stability is obtained. Results of this approach are shown in the seventh and eight entries of Table VI. The CN bonded phase remained the best single-phase column, showing a limiting resolution of 0.93 for D,T with only acetonitrile and THF as modifiers in the buffer. The best mixed-bed column, 0.67 PE/0.33 $C_8$, provides an optimum resolution that was essentially identical to that of the CN column. With this mixed-bed column, the D,G peak pair was limiting, with a resolution of 0.92 for a 10,000 plate count column using a ternary solvent.

Although the columns just discussed are optimum for the 17 PTH-amino acids we found that these 17 are relatively unaffected by changes in ionic strength. However, in the system of prime interest, it is desirable to separate all 20 compounds (although C largely is missing in many standard protein sequencing schemes). In both the single CN column and the mixed bed column, the retention of the H, R, and C peaks can be changed by varying the buffer ionic strength. In this study, the initial retention data were obtained with an ionic strength of 12 m*M* phosphate. It was determined that a decrease in ionic strength was required to increase the retention of the three compounds H, R and C for the desired separation. On a single CN column of 3-$\mu$m particles, the best separation was obtained with a mobile phase of 8 m*M* phosphate buffer–acetonitrile–THF (65.5:18.0:16.5) as shown in Fig. 12. All 20 peaks are separated in 13 min with the limiting resolution of $R_s = 1$ for peaks E and C.

The $k'$ solvent selectivity maps obtained by the solvent-triangle statistical de-

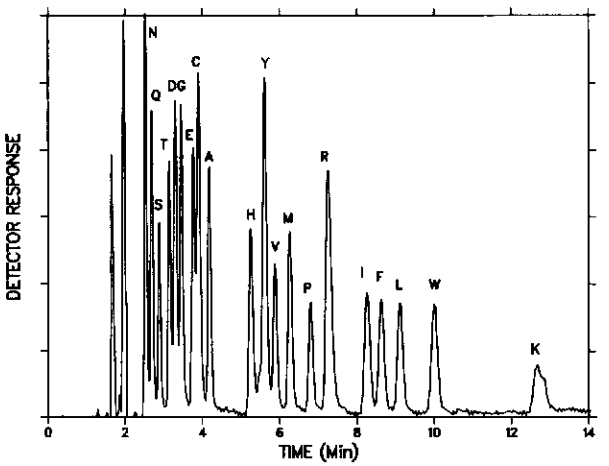



Fig. 12. Optimum separation of 20 PTH-amino acids on 3-$\mu$m Zorbax-CN column. Column, 8.0 × 0.62 cm I.D.; column temperature, 35°C. Mobile phase: acetonitrile–THF–pH 3.2 phosphate buffer (ionic strength, 8 m*M*) (18.0:16.5:65.5).

sign experiments provide important additional flexibility in optimizing LC separations. For example, the optimum mixed-bed separation with 3-$\mu$m particles (0.67 PE/0.33 $C_8$) was determined to be with 5.1 m*M* phosphate buffer–acetonitrile–THF (65.0:20.4:14.5). A resolution of 1.0 occurred for the limiting peak pair, F,I. In this case, the organic modifier composition is slightly different from the predicted optimum shown in Table VI. The reason for this slight difference is that the prediction for optimum mobile and stationary phase compositions were based on the assumption of constant plate-count columns for all peaks in the system. In fact, plate-count is usually lower for early eluting peaks, and the separation of T and G was not as large as expected. However, the $k'$ maps for these peaks revealed that a slight change in the mobile phase to a slightly higher concentration of acetonitrile would improve this separation. The final solvent composition reflects a compromise between the best T,G separation and that of F,I.

## CONCLUSIONS

This study has demonstrated the power of utilizing both stationary phase and mobile phase optimization for designing the separation of complex mixtures with minimum experimentation. It is interesting to note that the final optimum conditions use only three or four of the six phase variables (methanol, acetonitrile, THF, $C_8$, PE, and CN) that were studied. This is a strong indication that the limit of the capability to alter selectivity was being approached, at least with the variables used in this study. It is also important to note that selectivity improvement was achieved by combining the proper proportions either of mobile phase (constant column) or stationary phase (with constant mobile phase). For a very difficult separation such as the PTH-amino acids, it could be necessary to optimize both mobile and stationary phases. However, in most cases involving less demanding separations, a simple mobile phase optimization using a single-column stationary phase is adequate and preferred. Our experience suggests that about most of the available selectivity optimization can be achieved by solvent optimization alone for typical systems.

Finally, the suggested optimum system for separating the 20 PTH-amino acids is a 16-cm, 3-$\mu$m CN bonded phase column. A viable but less desirable alternative is an appropriate mixed-bed of $C_8$/PE. Both systems have the advantage of using only three mobile phase solvents (phosphate buffer, acetonitrile and THF) and therefore, can be used in ternary solvent delivery systems. Fortunately, methanol is absent in both so that long-term column stability is excellent.

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