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OPTIMIZATION OF COMPLEX SEPARATIONS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION TO PHENYLTHIOHYDANTOIN AMINO ACIDS

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SUMMARY

Graphical methods are described for determining suitable experimental conditions for optimum chromatographic resolution of complex mixtures. Plotting the logarithm of retention time for individual components or retention time ratios for close eluite pairs as a function of an experimental variable such as solvent strength or temperature allows direct visual evaluation of the best resolution attainable with a given column of known efficiency. Optimization of temperature and solvent strength for the separation of phenylthiohydantoin amino acids is demonstrated for two reversed-phase columns.

INTRODUCTION

The "window diagram" technique of Laub and Purnell[1] is a powerful tool for determining appropriate conditions for chromatographic separation of complex mixtures. Originally developed for finding optimum combinations of stationary phases in gas chromatography, it is applicable to any method of separation in which the relative mobility of sample components changes as a function of one or more experimental variables, such as temperature, pH, or mobile phase composition[2–5].

The phenylthiohydantoin (PTH) amino acids constitute such a system. These derivatives, which are produced in the Edman degradation for determining protein amino acid sequence[6], are commonly analyzed by reversed-phase high-performance liquid chromatography (HPLC). This analysis requires separation of at least eighteen PTH-amino acids, some of which are difficult to resolve or tend to change elution order with changing temperature and mobile phase composition. Many HPLC methods, involving both isocratic and gradient elution, have been recommended for their separation[7]. The multiplicity of published procedures and the variation in retention properties of reversed-phase HPLC column packings from different manufacturers[8] suggest that an efficient experimental procedure for establishing conditions for adequate resolution of these compounds on a particular column would be advantageous.

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The present paper demonstrates the utility of window diagram and related graphical approaches to devising HPLC separations of the PTH-amino acids. Improvements on the original graphical methods are shown, allowing direct visual identification of conditions that produce optimum resolution on a given HPLC column.

THEORY

Chromatographic resolution, R_s, of a pair of peaks is commonly defined as the ratio of the difference in their retention times (t) to the average baseline peakwidth (w):

$$R_s = \frac{t_2 - t_1}{1/2\,(w_2 + w_1)}$$

Introduction of expressions for the number of theoretical plates, $N = 16\,(t/w)^2$, capacity factor, $k'_n = (t_n - t_0)/t_0$, and separation factor, $\alpha = k'_2/k'_1$, leads to the other commonly used equation for resolution:

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_2}{1 + k'_2}\right)$$

The derivation includes the assumption that two close peaks are being considered, so that $k'_1 \approx k'_2$.

In the original window diagram technique[1–3], α or its logarithm is plotted as a function of one or more experimental variables for each pair of close eluite peaks. If the elution order of two peaks is reversed, their ratio in the calculation of α is inverted, so that the plotted line reflects back from the $\alpha = 1$ or $\log \alpha = 0$ x-axis. For any value of the experimental variable represented by the x-axis, the lowest line on the diagram corresponds to the pair of eluites having the poorest separation factor. The term "window" refers to the spaces below the lowest lines, and the highest window shows the best attainable α value for the eluite pairs which delineate it; all other pairs of eluites will have higher values of α at that value of the experimental variable. The number of theoretical plates necessary to produce the desired degree of resolution, N_{req}, may then be calculated. A difficulty arises in the case of low values of k'; such rapidly eluted peaks will require far more theoretical plates to produce a given resolution than will later peaks having the same value of α. Laub[4] has suggested plotting $1/N_{req}$ instead of α or $\log \alpha$, a practical solution for systems in which column length is easily adjusted.

In HPLC, however, one often wishes to evaluate the suitability of a particular column of known N for a particular separation and to avoid the expense and higher pressure requirements of additional columns, if possible. In the course of the present study, manipulation of the expressions for resolution and theoretical plates led to the equations

$$\frac{t_2}{t_1} = \frac{\sqrt{N} + 2R_s}{\sqrt{N} - 2R_s}$$

or for close eluite peaks:

$$\frac{t_2}{t_1} \approx \frac{\sqrt{N}}{\sqrt{N} - 4R_s}$$

It follows that

$$\log t_2 - \log t_1 = \log \frac{\sqrt{N} + 2R_s}{\sqrt{N} - 2R_s} \approx \log \frac{\sqrt{N}}{\sqrt{N} - 4R_s}$$

From this it can be seen that at constant N, if $\log t$ values for the sample components are plotted, a given vertical separation between lines always corresponds to the same value of R_s. In fact, for typical values of $\sqrt{N}$ and R_s the logarithmic expressions shown, and therefore the separations between $\log t$ lines, are almost exactly proportional to R_s. To construct a window diagram, t_2/t_1 is plotted. A given window height now likewise corresponds to a given resolution, so a scale of R_s values for a column of known N can be placed on the y-axis.

EXPERIMENTAL

Initial isocratic experiments were performed with two 25 cm × 4.6 mm I.D. Whatman Partisil ODS-2 columns connected in series, using a Glenco HPLC System I chromatograph. Later isocratic and gradient experiments were carried out with a Beckman Ultrasphere-ODS column, 25 cm × 4.6 mm I.D., with a Beckman Model 324 chromatograph. A 7 cm × 2.1 mm I.D. guard column filled with Co:Pell ODS (Whatman) was interposed between the injection valve and the Ultrasphere-ODS column. Column temperature was controlled by a circulating bath and water jacket. The detector was set at 254 nm.

Acetonitrile was Photrex (J. T. Baker) or HPLC grade (Fisher). PTH-amino acid standards were purchased from Pierce. Solutions of standards were made up in mobile phase for isocratic elution and in acetonitrile–0.01 *M* sodium acetate, pH 4.9 (40:60) for gradient elution.

Retention times of seventeen PTH-amino acids were measured at 35, 45 and 55°C for each of three or four concentrations of acetonitrile between 30 and 45%. These ranges of temperature and solvent strength were selected to give k' values less than 10, for considerations of both sensitivity and analysis time. For the Partisil ODS-2 experiments the mobile phase was 0.01 *M* sodium acetate, made by diluting 0.5 *M* stock solution (pH 4.0 until changed in later experiments) with the required volumes of water and acetonitrile. For the Ultrasphere-ODS experiments the mobile phase was mixed by the chromatograph; solvent A was 0.01 *M* sodium acetate, pH 4.9, and solvent B was acetonitrile.

PTH-Arg and -His were not included in this optimization study, since they remain in the aqueous phase during the ethyl acetate extraction which follows conversion of phenylthiazolinones to phenylthiohydantoins in the Edman procedure[6] and are therefore analyzed separately; their separation from each other by HPLC is not difficult. PTH-Ser was omitted because serine residues encountered during actual

automated sequence analysis of proteins gave no peak corresponding to the PTH-Ser standard in the chromatogram; the only peak seen was a degradation product[9] which was eluted later than all the other PTH amino acids and was well separated from them under all conditions studied.

For use in calculating capacity factors, the dead-volume elution time (t_0) was determined by injecting water or deuterium oxide[10] and measuring the time to the inflection point of the refractive index perturbation.

RESULTS

Isocratic elution

Values of k' were calculated from the retention data for the PTH-amino acids on Partisil ODS-2. Graphs were drawn of log k' *versus* acetonitrile volume fractions, φ, at constant temperature and *versus* $1/T$ at constant φ. An example is shown in Fig. 1a. All such plots displayed some reversals in elution order with changing solvent strength or temperature. PTH-Asp and -S-carboxymethyl-Cys are not shown; they were inseparable under all conditions. The log α window diagram corresponding to Fig. 1a is shown in Fig. 1b. The highest window occurs at $1/T = 3.070 \cdot 10^{-3}$ (temperature $T = 52.6°C$) and is defined by the lines for PTH-Pro/-Trp and PTH-Asn/-Glu. Although the value of α at that point is 1.057 for each pair, the calculated N_{req} for a resolution of 1.0 (good but not baseline resolution) is 9640 theoretical plates for PTH-Pro/-Trp but 104,000 theoretical plates for PTH-Asn/-Glu, the pair eluted early.

Fig. 1c and d represent the same data, now displayed as a plot of log t *versus* T and as a t_2/t_1 *versus* T window diagram. (Since straight lines are no longer expected, it is easier and more directly informative to plot temperature in degrees centrigrade rather than the reciprocal of absolute temperature.) The highest window is now at 50°C and is still defined by the pairs PTH-Pro/-Trp and PTH-Asn/-Glu. It can now be seen that the resolution of each of these pairs on the particular column would be only *ca.* 0.6, which is inadequate. However, a better window for all pairs except PTH-Asn/-Glu occurs at *ca.* 54.5°C. The separation of the PTH-Asn/-Glu pair could be improved without detriment to the other separations by raising the pH of the sodium acetate stock solution used to prepare the mobile phase to 4.8. The mobilities of PTH-Glu, -Asp, and -S-carboxymethyl-Cys were thereby reduced while all others remained unchanged. PTH-S-methyl-Cys was substituted for PTH-S-carboxymethyl-Cys, which had remained inadequately resolved from PTH-Asp. Their isocratic separation at 54.3°C is shown in Fig. 2. (This particular column had been used a great deal before these experiments; its efficiency should not be taken as representative of that of new columns of the same type.)

Optimization of temperature and solvent strength for PTH-amino acid separation was repeated with an Ultrasphere-ODS column. The most promising retention time plot and its t_2/t_1 window diagram are shown in Fig. 3a and b. They led to the isocratic separation shown in Fig. 4.

Gradient elution

A gradient elution scheme was devised for the Ultrasphere-ODS column, using Fig. 3a as a guide to experimentation. It showed that a continuous linear gradient

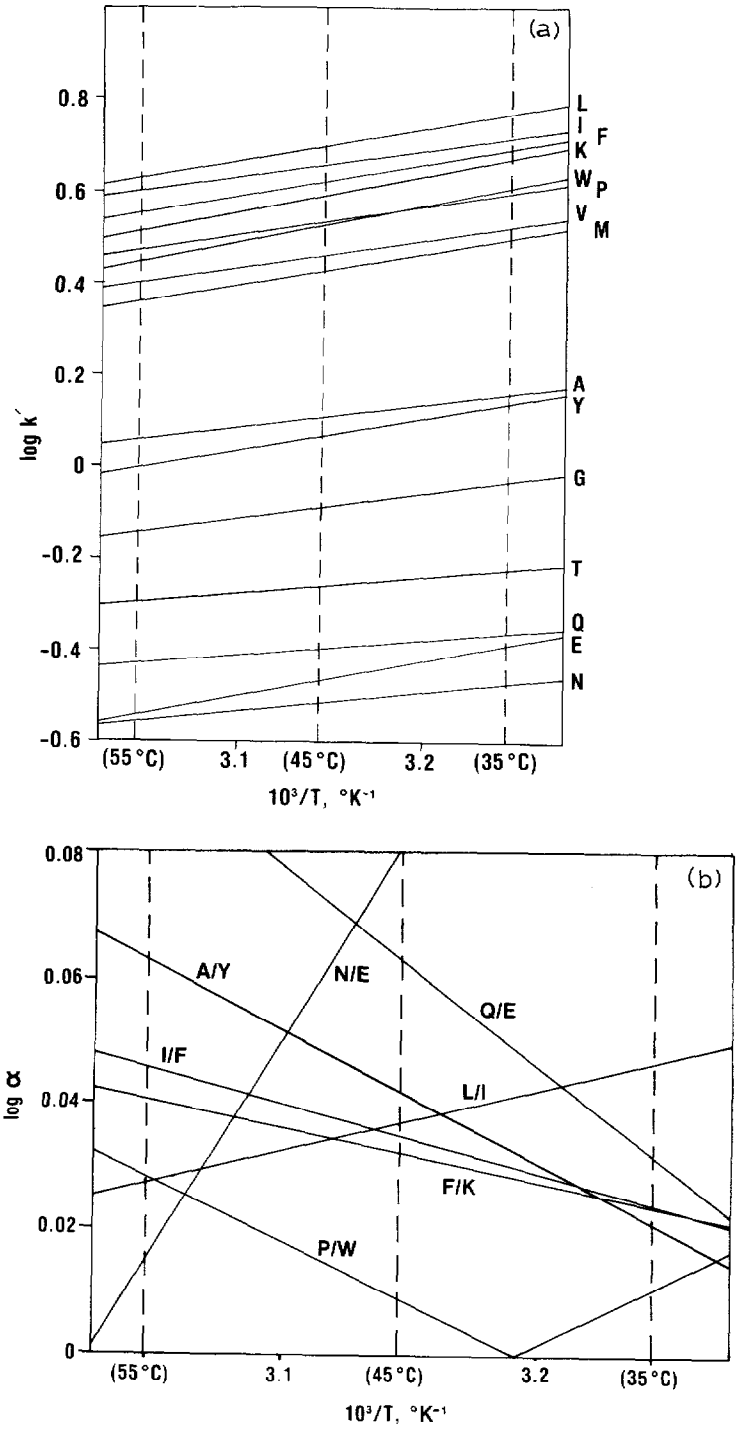

Fig. 1.

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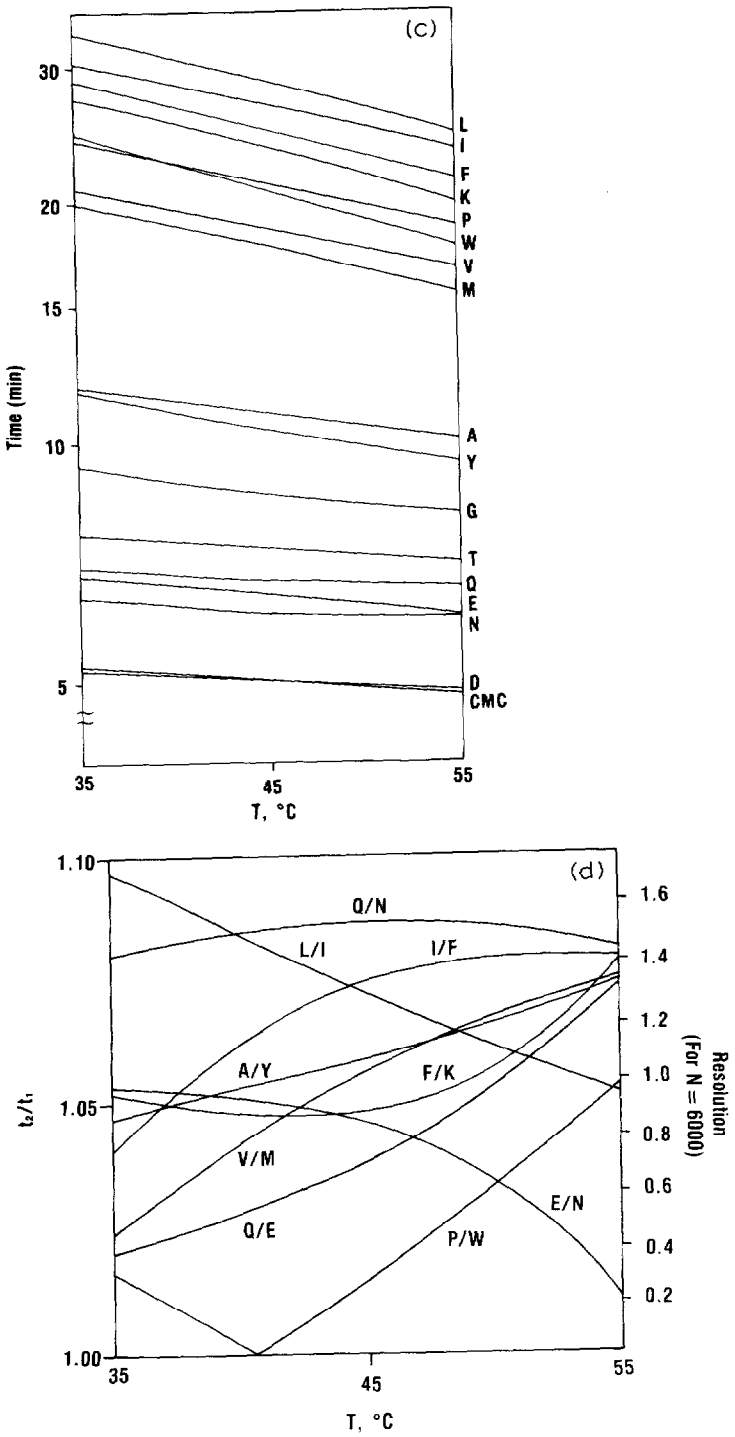

Fig. 1. (a) Plot of log k' *vs.* $10^3\ T^{-1}$ for PTH-amino acids on Partisil ODS-2, 50 cm × 4.6 mm I.D. Mobile phase, 0.01 *M* sodium acetate in acetonitrile–water (40:60), measured pH 4.9; flow-rate, 1.0 ml/min. Single-letter code for PTH-amino acids: N = Asn; E = Glu; T = Thr; G = Gly; Y = Tyr; A = Ala; M = Met; V = Val; P = Pro; W = Trp; K = Lys; F = Phe; I = Ile; L = Leu. (b) Window diagram, log α *vs.* $10^3\ T^{-1}$, for the data shown in (a). (c) Plot of log retention time *vs.* $10^3\ T^{-1}$ for same experiments as in (a). (d) Window diagram, t_2/t_1 *vs.* temperature, for the data shown in (c).

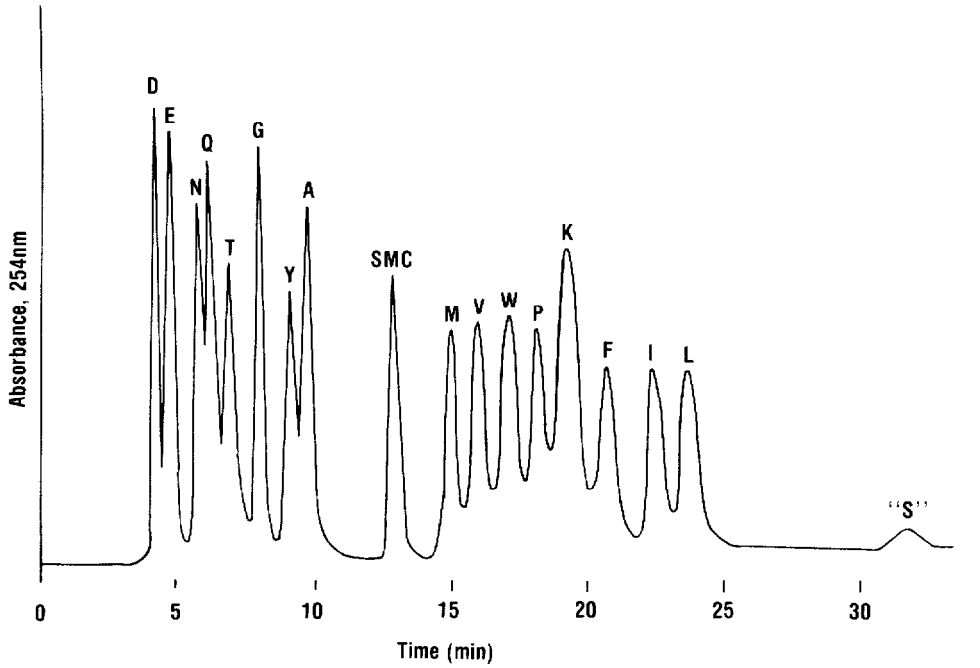

Fig. 2. Separation of PTH amino acids on Partisil ODS-2, 50 cm × 4.6 mm I.D. Mobile phase, 0.01 *M* sodium acetate in acetonitrile–water (40:60), measured pH 5.4; flow-rate, 1.0 ml/min; T = 54.3°C; D = Asp; SMC = S-methylCys; "S" = degradation product of PTH-Ser.

would not allow simultaneous resolution of PTH-Val/-Pro and PTH-Phe/-Lys/-Ile. Therefore, a break in the gradient was used to adjust the retention of the latter three peaks. Fig. 5 shows the chromatogram obtained. (Although the solvent program contains a short drop in percent acetonitrile, when gradient elution was performed with acetone-spiked solvent, it was found that actually only a plateau in solvent strength reached the column because of the damping effect of the solvent mixing chamber.) Total analysis time, including allowance for system re-equilibration and delay between start of program and sample injection, was 35 min. The increased sensitivity of detection for the PTH-Ser degradation product was particularly helpful in protein sequence determinations and was a major reason for using this gradient procedure in routine analysis rather than the simpler and slightly faster isocratic elution. Although it appears from Fig. 3a that faster gradient elution might be achieved by rapidly raising the solvent strength after elution of PTH-Pro, this was found not practical in actual sequence determinations, since two peaks of byproducts from the sequencer are then eluted together with PTH-amino acid peaks.

DISCUSSION

In the studies reported here, simple graphical treatment of retention as a function of solvent strength and temperature led quickly to the selection of isocratic and gradient elution conditions for routine analysis of PTH-amino acids, requiring only a single organic component in the mobile phase. It happened that for each of the

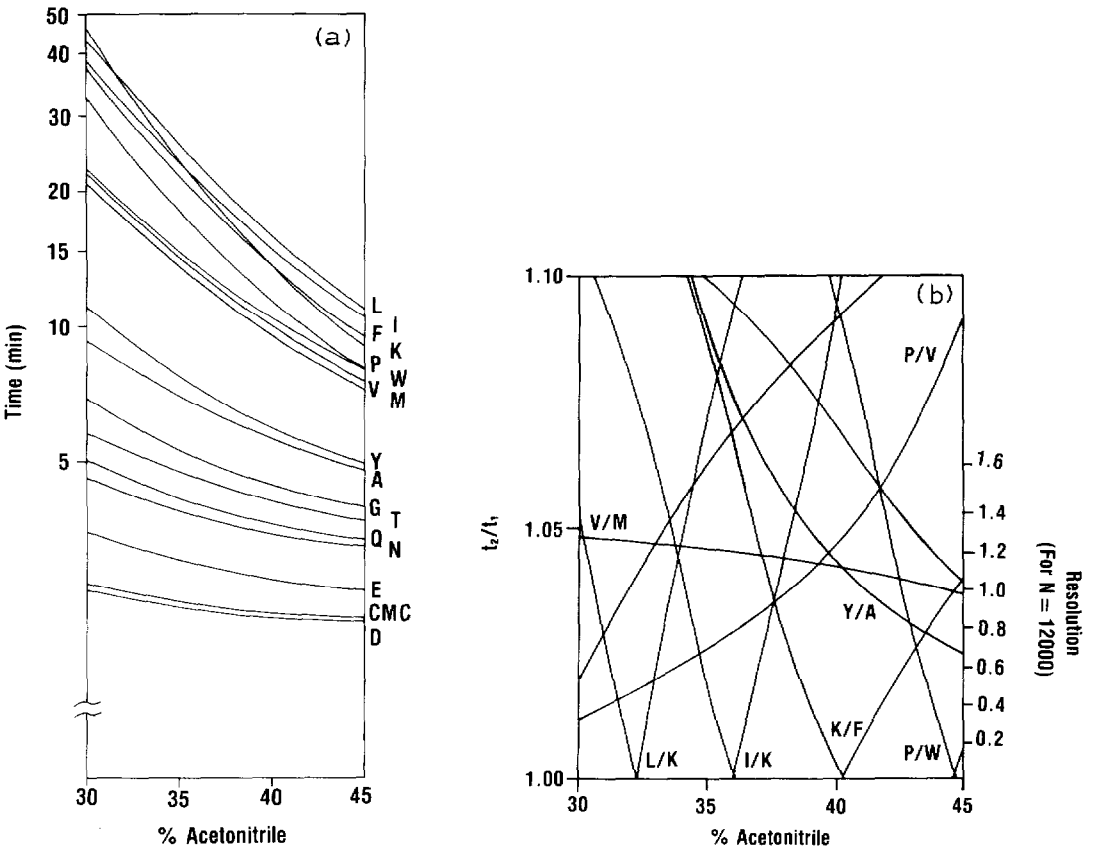

Fig. 3. (a) Plot of log retention time *vs.* percent acetonitrile on Ultrasphere-ODS, 25 cm × 4.6 mm I.D. Mobile phase, aqueous component 0.01 *M* sodium acetate, pH 4.9; flow-rate, 1.0 ml/min; $T = 55°C$. (b) Window diagram, t_2/t_1 *vs.* percent acetonitrile, for data shown in (a).

columns used, one of the plots led directly to an adequate analysis. If examination of intermediate values of temperature and solvent strength had been necessary, retention times for those conditions could have been calculated by fitting a model to the data already collected[11,12]. Empirical models of the form $\log k' = b_0 + b_1\varphi + b_2/T + b_3\varphi/T$ (where b_0, b_1, b_2 and b_3 are the parameters of the experimental model) fit the Partisil ODS-2 data well, but the Ultrasphere-ODS data would probably require higher-order models. Such models could also be used in conjunction with a computer to generate a two-factor window diagram[3].

The log t plots and t_2/t_1 window diagrams have the further advantage of not requiring measurement of t_0, the dead volume elution time, whereas calculated values of k' are highly dependent on t_0. Exact evaluation of t_0 is not trivial; McCormick and Karger[10], for example, discuss the problem and recommend measurement of the retention time of deuterium oxide. However, in PTH-amino acid chromatography PTH-Asp and -S-carboxymethyl-Cys were eluted earlier than deuterium oxide at the pH values used.

Jones and Wellington[5] have recently proposed a similar method, defining a

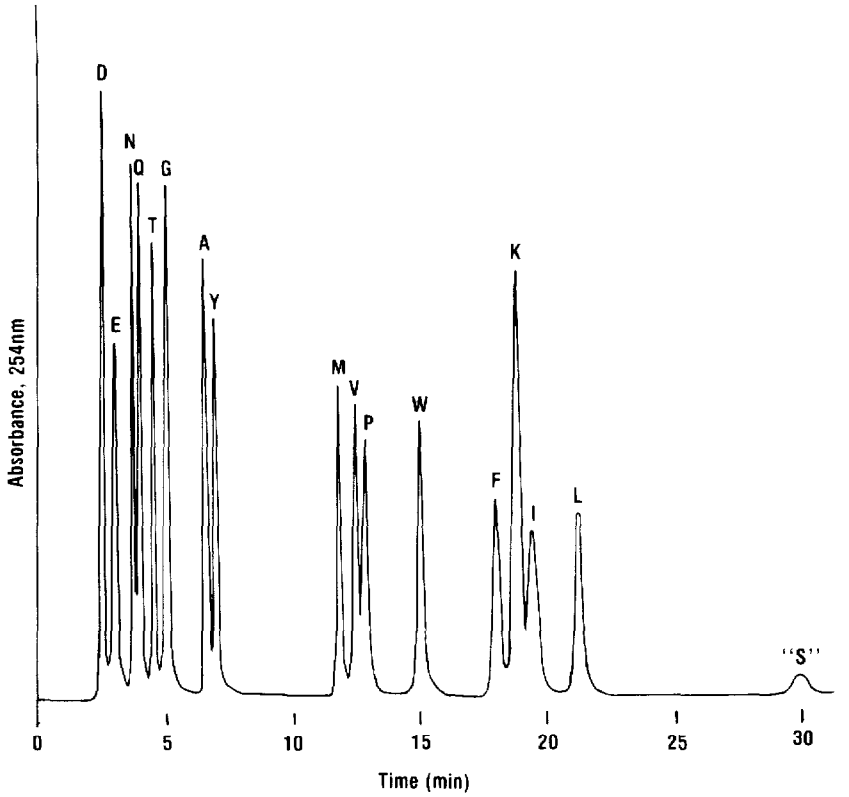

Fig. 4. Isocratic separation of PTH-amino acids on Ultrasphere-ODS, 25 cm × 4.6 mm I.D. Mobile phase, 0.01 *M* sodium acetate (pH 4.9)– acetonitrile (62.2:37.8); flow-rate, 1.0 ml/min; $T = 55°C$.

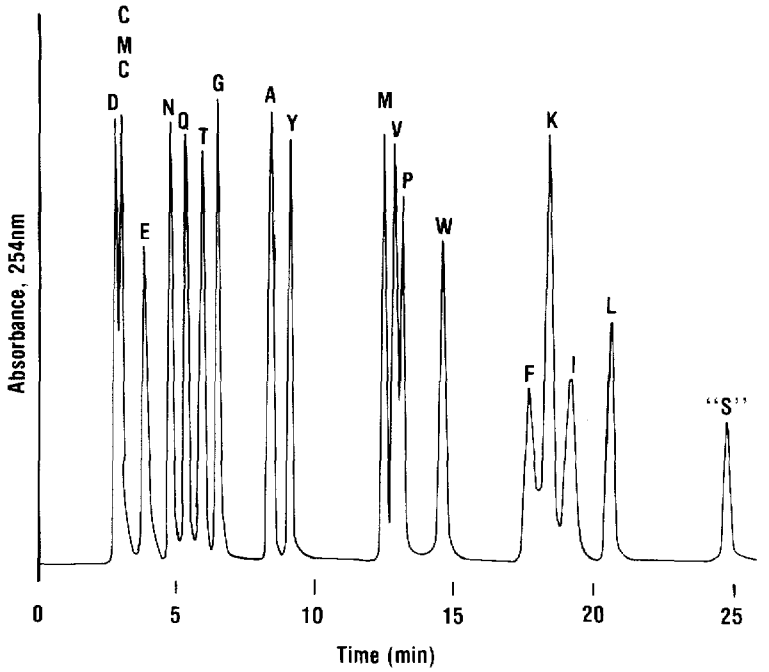

Fig. 5. Gradient separation of PTH-amino acids on Ultrasphere-ODS, 25 cm × 4.6 mm I.D. Solvent A, 0.01 *M* sodium acetate, pH 4.9; solvent B, acetonitrile; flow-rate, 1.0 ml/min; $T = 55°C$. Solvent program (all changes linear): 27% B to 48.6% B in 12 min, then immediately to 30%, held at 30% for 1.0 min, then to 49.8% in 10 min, held at 49.8% for 2 min, then returned to 27%. The sample was injected 3.8 min after the start of the solvent program to compensate for the volume of the mixing chamber.

factor $S = (t_2 - t_1)/(t_2 + t_1) = 2R_s/\sqrt{N}$, which also leads directly to optimization of resolution without requiring determination of t_0.

Retention plots such as Figs. 1c and 3a are helpful in modifying conditions to maintain separation as a column ages. The Ultrasphere-ODS column used in the present experiments required periodic minor adjustments in the gradient program to compensate for a slow decrease in sample retention.

A suggested order of experiments for optimizing the HPLC separation of PTH amino acids or similar complex mixtures is: (1) adjust solvent strength, (2) adjust pH to separate acidic or basic from neutral components, (3) adjust temperature, and (4) add other solvents, if necessary.

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