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Optimization of phenylthiohydantoinamino acid separation by micellar electrokinetic capillary chromatography

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ABSTRACT

Optimization of phenylthiohydantion (PTH)-amino acid separation by micellar electrokinetic capillary chromatography was achieved by the use of a weighted variable-size simplex algorithm. The optimization procedure concerned the pH of the aqueous buffer, the sodium dodecyl sulphate concentration and the percentage of organic solvent; the organic solvent used was either methanol or acetonitrile. In both instances the optimization procedure led to very similar final experimental conditions and migration order and times of the PTH-amino acids, showing that the organic solvent probably provides a better polydispersity of the micellar phase. The elution pattern observed in the two instances suggests that ionic interactions and polar repartition play a role in the separation mechanism, but other types of interaction cannot be excluded.

INTRODUCTION

Micellar electrokinetic capillary chromatography (MECC) is a separation technique, deriving from capillary electrophoresis (CE), that allows very sensitive analysis [1,2]. The use of sodium dodecyl sulphate (SDS) in the separation buffer at a concentration higher than its critical micellar concentration (CMC) generates a biphasic system: the aqueous phase migrates towards the cathode because of the electro-osmotic flow and can be considered as the chromatographic mobile phase; the micellar phase, moving towards the anode owing to its negative charge, can be regarded as a pseudo-stationary phase. Solutes are separated as a function of their different thermodynamic interactions with the two pseudo-phases, such as an electro-driven chromatographic separation.

The molecular mechanism is complex, as many forces such as ionic or polar interactions play an important role in the separation [1,2]. Further, the improvement of the separation and the selectivity also depend on the presence of an organic solvent and on the pH of the aqueous phase, all these experimental parameters being connected with each other. In spite of this complex experimental picture, the separation capacity of MECC is interesting, because in a few minutes it allows the detection of analytes at a sensitivity in the femtomole (10^{-15} mol) range.

This study was devoted to the MECC separation of phenylthiohydantoin (PTH)-amino acids. The reliable detection of these derivatives in the low femtomole range could represent a real improvement in manual and automatic sequencing strategies (of almost three orders of magnitude), as LC methods show a sensitivity at the low picomole level. Some workers [3,4] have demonstrated that a sensitive separation of PTH-amino acids is obtainable by MECC.

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The possibility of varying several experimental parameters permits a useful manipulation of the selectivity of the separation, but this is accompanied by long and tedious attempts to find the optimum separation parameters. As the theory of MECC separation is complex and a prediction on the basis of the thermodynamics of the system is impossible, we adopted for the optimization of the separation of PTH-amino acids a weighted variable-size simplex algorithm, which explores the parameter range without requiring information on the physics of the separation system or the behaviour of any individual solute. The simplex optimization was applied to three experimental parameters: the pH of the aqueous buffer, the percentage of organic solvent in the separation buffer and the concentration of SDS.

EXPERIMENTAL

Materials

All common reagents were of analytical-reagent grade purchased from Carlo Erba (Milan, Italy) or Merck (Darmstadt, Germany). SDS was obtained from Merck, the PTH-amino acid standards used were the PTH-amino acids standard kit from Pierce (Rockford, IL, USA) or the PTH-amino acids standard, complete, from Sigma (St. Louis, MO, USA). Methanol and acetonitrile were of HPLC grade from Baker (Deventer, Netherlands) and were used as received. All aqueous buffers, prior to the CE separation, were filtered through 0.2-μm flowpore FN filters obtained from Flow Labs. (McLean, VA, USA).

Methods

The CE experiments were performed using a Beckman (Palo Alto, CA, USA). P/ACE 2000 system. The PTH-amino acids were dissolved in methanol or acetonitrile so as to give a 0.2 mmol/l concentration of each PTH-amino acid; a small increase in the concentration of a single PTH-amino acid in the complete mixture was used for the final identification of PTH-amino acids. Prior to injection, the mixture of PTH-amino acids was diluted 1:1 (v/v) with the separation buffer. The capillary used was the standard capillary provided by Beckman in the cartridge; its dimensions were 56.5 cm total length, 50.0 cm to the detection window and 75 μm I.D. The sample injection, unless stated otherwise, was always performed by pressure, with a time ranging from 1 to 5 s. The wavelength of detection was 254 nm. The compositions of the solutions are described in the Results section; the aqueous buffer used was sodium phosphate (40 mmol/l)–sodium tetraborate (10 mmol/l) adjusted to the desired pH value in the experiment. The SDS concentration and the percentage of organic solvent used are described in the Results section. Unless stated otherwise the MECC separation was performed at 35°C and at a constant voltage of 18 kV.

Weighted variable-size simplex algorithm

As described in the Introduction, the parameters (or coordinates or factors) studied in the optimization algorithm were the pH of the aqueous buffer, the percentage of organic solvent and the concentration of SDS. The choice of these three factors was made on the basis of preliminary experiments (not reported), which demonstrated that these are the major factors responsible for modulation of the selectivity of the separation of PTH-amino acids. The efficiency of the MECC separation was analysed, as usual, in terms of the mean resolution, R_{s_m}; the resolution R_s between two peaks, as is well known, is defined as

$$R_s = 2(t_2 - t_1)/(w_2 + w_1)$$

where t_2 and t_1 are the elution times measured at the maximum peak height and w_1 and w_2 are the peak widths measured at the peak base. If the separation allows the detection of n peaks, the mean resolution, R_{s_m} can be calculated as:

$$R_{s_m} = \frac{\sum_{z=2}^{z=n} 2(t_z - t_{z-1})/(w_z + w_{z-1})}{L - 1}$$

where L corresponds to the total number of substances under analysis (in this instance the 20 PTH-amino acids). In general, care must be taken in the use of this parameter as a general representation of the efficiency of separation as (hypothetically in some instances) a better R_{s_m} can be obtained in a chromatogram with a small number of peaks, n, less than the number of

substances, L, under analysis. As the final optimization procedure should offer a chromatogram in which each substance is resolved from any other, accurate control of the calculated R_{s_m} should be exercized in any step of the simplex algorithm to avoid erroneous interpretations of the response of a factorial point.

The simplex optimization strategy starts from a minimum number of initial experiments established as $k+1$, where k represents the number of experimental factors that are explored [5]. Here, as the experimental factors under study were the pH of aqueous buffer, the SDS concentration and the percentage of organic solvent (three factors), a minimum of four starting experiments were required, organized, in the space defined by each parameter, as the four vertices (corners) of a tetrahedron. After a measure of the response of the experiments, in the fixed-size simplex algorithm, the worst vertex W was eliminated and a new vertex is generated by a reflection R:

$$R = P + (P - W)$$

obtained by extending the line segment WP beyond P by the operation where P is the centroid of the face defined by the three vertices of the tetrahedron remaining when W is eliminated from the starting simplex [5]. The response of the new vertex R of the tetrahedron is established and the elimination of the new worst vertex is the second step of the algorithm, and so on through further new steps until an optimum response or a complete separation is obtained. This strategy presents some disadvantages that can be summarized as follows: (i) it is impossible to reduce the dimensions of the tetrahedron from the starting ones; (ii) a local optimum may be the final result; (iii) little insight into the response surface is obtained; and (iv) the centroid P is obtained by considering the three vertices of the face with the same experimental statistical weight.

Disadvantage (i) obliges one to choose a small starting tetrahedron with a subsequent large number of experiments required for the optimization. An effective solution to this problem was first described by Nelder and Mead [6] and makes use of other operations besides the reflections, such as contractions or expansions. This is called a variable-size simplex algorithm and these movements in the parameter space were adopted in our optimization strategy. It is difficult to eliminate disadvantage (ii); a possibility for finding the global optimum instead of a local optimum is to restart the simplex procedure from different sets of initial factor points and to verify if the same final result is obtained; however, this is in conflict with the large number of experiments required for the total optimization process; in any event, it is impossible, at the end of the optimization procedure by the simplex algorithm, to have the assurance that the global optimum is the final result. Disadvantage (iii) was in part avoided by starting the experiments with an initial scouting factorial design that allowed a partial exploration of the parameter space (see Results). Finally, disadvantage (iv) can increase the number of experiments necessary to reach the optimum; it was partly corrected for by calculating the centroid P not as the simple mean of the values of each factor under study, but by considering in the mean each point with a statistical weight proportional to its response. In fact, the coordinates (factors) of a vertex in the parameter space can be defined as $a_v, b_v, c_v, \ldots, k_v$. The coordinates of the $k+1$ vertices chosen in the starting set of experiments can be organized in the array

$$(V) = \begin{matrix} a_1 & b_1 & c_1 & \cdots & k_1 \\ a_2 & b_2 & c_2 & \cdots & k_2 \\ a_3 & b_3 & c_3 & \cdots & k_3 \\ \vdots & \vdots & \vdots & & \vdots \\ a_k & b_k & c_k & \cdots & k_k \\ a_{k+1} & b_{k+1} & c_{k+1} & \cdots & k_{k+1} \end{matrix}$$

which can be ordered in the rows from the best to the worst response; in this instance the vertex that must be eliminated during the simplex strategy is that corresponding to the $(k+1)$th row. Then, in normal simplex algorithm, the coordinate i_p of the centroid P for a general factor (array column) can be obtained by the mean

$$i_p = \frac{\sum_{z=1}^{z=k} i_z}{k}$$

In this mean, any vertex used to calculate the

coordinates of the centroid P has the same statistical weight. We preferred to define a weighted centroid P_w obtained as follows after calculation of R_{s_m}, a statistical weight for any row j of the matrix was established as

$$sw(j) = \frac{R_{s_m}(j)}{\sum_{z=1}^{z=k} R_{s_m}(z)}$$

and the weighted coordinates of the centroid P_w were established as

$$i_p = \sum_{z=1}^{z=k} i(z)sw(z)$$

The centroid P_w is then used in substitution of P for the common operations suggested by Nelder and Mead [6] in the variable-size simplex algorithm, which are:

(Reflection) $R = P_w + (P_w - W)$

(Expansion) $E = R + (P_w - W)$

(Contraction towards reflection) $C_r = P_w + [(P_w - W)/2]$

(Contraction towards worst res.) $C_w = P_w - [P_w - W)/2]$

RESULTS

The initial set of experiments chosen to explore the parameter space is reported in Table I. It was initially established that reasonable ranges for the three parameters under optimization were as follows:

(a) A pH range of the aqueous buffer ranging from 7.00 to pH 9.50. The lowest limit was established to ensure always an acceptable electroosmotic flow and the highest value was fixed so as to avoid instability of PTH-amino acids at high pH values.

(b) The limits of SDS concentration were established as 30 and 60 mmol/l, respectively: the lowest limit to ensure a sufficient total concentration of the micellar phase and the highest to avoid too high an ionic strength and a consequent too high current during the separation, for the purpose of maintaining a constant voltage of 18 kV. A constant voltage is essential when using R_{s_m} as an index of the separation performance; in fact, R_{s_m} is directly linked to the applied voltage. Further, too high an SDS concentration can easily generate capillary obstructions.

(c) The limits of the percentage of organic solvent were established as 10 and 40% (v/v), respectively. Less than 10% of organic solvent strongly reduces the resolution, whereas greater than 40% strongly reduces the electroosmotic flow.

Organic solvent methanol

As shown in Table I, the best response among the four scouting vertices was observed for the point B, where an R_{s_m} about twice than at any other point was obtained. The low resolution of the points A, C, D suggests that their conditions are probably far from a local or a global optimum. For this reason, the simplex strategy was started from the conditions of point B. The initial experimental design was prepared by

TABLE I

SCOUTING EXPERIMENTS FOR THE OPTIMIZATION

Organic solvent: methanol.

Vertex	pH	SDS (m*M*)	Methanol (%)	*n* (No. of peaks)	R_{s_m}
A	9.50	30	10	12	2.36
B	7.00	30	10	14	6.10
C	7.00	30	40	9	3.63
D	7.00	60	10	12	3.74

considering a variation of 0.5 pH unit in the aqueous buffer, a 5% variation in the concentration of methanol and a 5 mmol/l variation in the SDS concentration.

The starting tetrahedron (or factorial design) was obtained by fractional replication and is shown in Table II. From this starting tetrahedron, a series of simplex movements in the parameter space were performed and they are also reported in Table II. The optimization continued to the eleventh step, which must be considered as the final step as the next movement is very close to the previous conditions.

In Fig. 1 the MECC separation of PTH-amino acids obtained at the eleventh step of the optimization is shown, in Fig. 2 the performance obtained at any step is reported and in Fig. 3 the conditions used in the steps of the optimization are reported. The conditions of the eleventh step are pH 7.27, 9% methanol and 34.8 mmol/l SDS. The number of resolved peaks is 18, as the pairs PTH-gln–PTH-gly and PTH-met–PTH-val migrate as a unique peak.

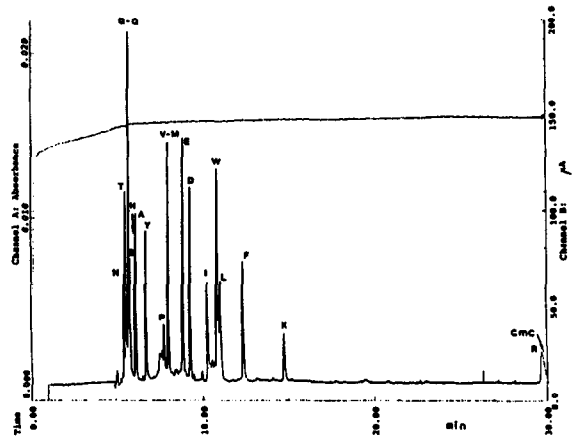

Fig. 1. MECC separation of the PTH-amino acids (the one-letter code is utilized; CmC = carboxymethyl cysteine) obtained at the eleventh step of the optimization using methanol as organic solvent.

Organic solvent acetonitrile

The optimization of the separation (of PTH-amino acids using acetonitrile instead of methanol is reported in Table III. In this instance the good resolution observed using the conditions of the starting four scouting vertices suggested performing the optimization starting from these conditions. The separation of the PTH-amino acids obtained at the tenth step of the optimization is shown in Fig. 4.

The performance of the optimization, represented in Fig. 5, showed a serious decrease in the performance at the fifth step, the first after the scouting experiments. The separation was so bad that a definition of R_{s_m} and of the number of the peaks was impossible. The next step restored acceptable separation conditions. The optimiza-

TABLE II

VARIABLE-SIZED WEIGHTED SIMPLEX OPTIMIZATION

Organic solvent: methanol.

Vertex	pH	Methanol (%)	SDS (m*M*)	*n* (No. of peaks)	R_{s_m}	Rejected vertex	Simplex operation[a]
1	7.00	10.0	30.0	14	6.10	–	Start 1
2	7.50	10.0	35.0	13	10.98	–	Start 2
3	7.00	15.0	35.0	10	2.67	–	Start 3
4	7.50	15.0	30.0	13	7.43	–	Start 4
5	7.76	8.0	29.4	14	6.46	3	*R*
6	8.14	12.0	34.0	15	6.29	1	*R*
7	7.85	11.5	33.0	14	6.05	1	C_r
8	7.28	10.5	31.0	14	9.98	1	C_w
9	7.05	14.9	35.0	13	5.93	5	*R*
10	7.22	13.2	33.6	15	7.01	9	C_w
11	7.27	9.0	34.8	18	11.76	4	C_r

[a] The symbols used for the simplex operations are explained under *Weighted variable-size simplex algorithm.*

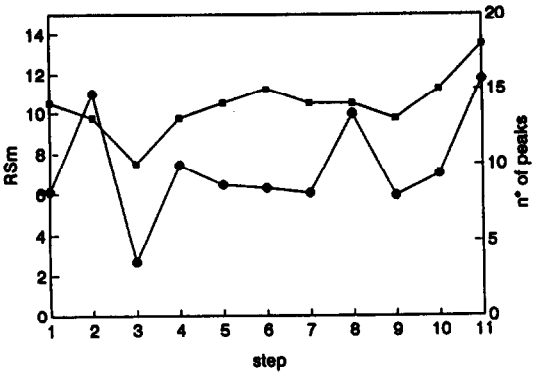

Fig. 2. (●) Mean resolution and (■) number of peaks obtained through the optimization of the separation of PTH-amino acids using methanol as organic solvent.

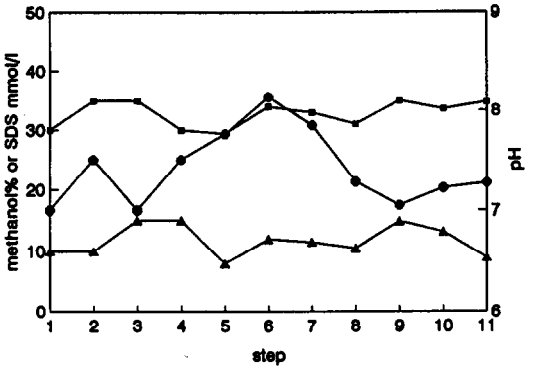

Fig. 3. Experimental conditions utilized through the optimization of the separation of PTH-amino acids using methanol as organic solvent. ▲ = Methanol (%); ■ = SDS (mmol/l); ● = pH.

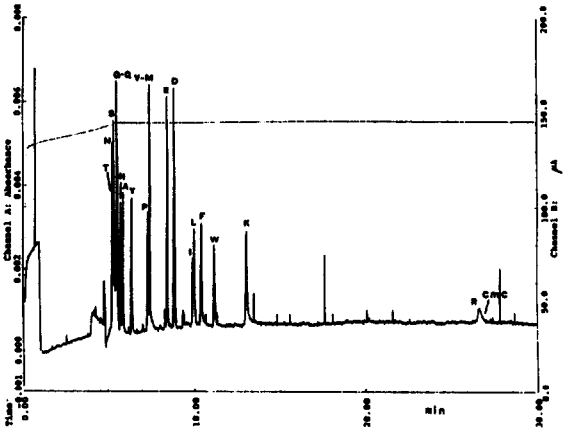

Fig. 4. MECC separation obtained at the tenth step of the optimization using acetonitrile as organic solvent.

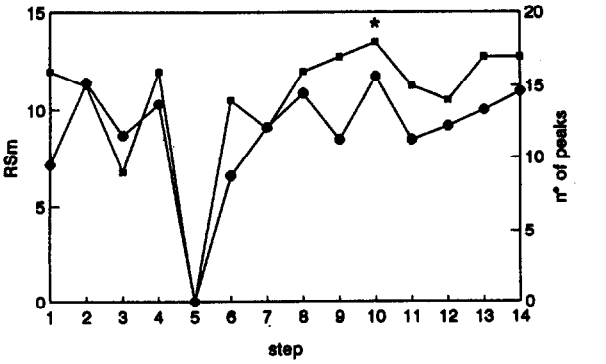

Fig. 5. (●) Mean resolution and (■) number of peaks obtained through the optimization of the separation of PTH-amino acids using acetonitrile as organic solvent. The asterisk indicates the best step.

tion reached its maximum at the tenth step, where a pH of 7.09, an acetonitrile concentration of 10.2% and an SDS concentration of 33.0 mmol/l were utilized; in further steps the optimization lead to a stabilization of the pH and of the acetonitrile percentage, as shown in Fig. 6, and further increases in the SDS concentration resulted in a decrease in performance. Also, with the use of acetontrile a separation of 18 peaks was the best performance and a close agreement of the migration order of the PTH-amino acids with that obtained using methanol as organic solvent was observed.

With the exception of the fifth step, a better mean resolution was obtained with the use of acetonitrile. For this reason, using the conditions of the tenth point, a change in the applied voltage and the temperature of the capillary was attempted, with the aim of verifying whether a further increase in resolution was possible. As shown in Figs. 7 and 8, a change in either the voltage or the temperature resulted in a decrease in R_{s_m} and did not give a complete separation of all 20 PTH-amino acids.

DISCUSSION

The optimization procedure adopted seems to represent an efficient way to find the optimum conditions for MECC separation. Whereas the usual variable-size simplex algorithm reaches an optimum response after about 20–30 steps, the

TABLE III

VARIABLE-SIZED WEIGHTED SIMPLEX OPTIMIZATION

Organic solvent: acetonitrile.

Vertex	pH	Acctonitrile (%)	SDS (m*M*)	*n* (No. of peaks)	R_{s_m}	Rejected vertex	Simplex operation[a]
1	9.50	10.0	30.0	16	7.13	–	Start 1
2	7.00	10.0	30.0	15	11.42	–	Start 2
3	7.00	40.0	30.0	9	8.63	–	Start 3
4	7.00	10.0	60.0	16	10.30	–	Start 4
5	7.27	25.0	35.0	Not acceptable	Not acceptable	3	C_w
6	7.40	17.5	37.4	14	6.57	5	C_w
7	7.47	13.7	38.5	12	9.05	6	C_w
8	7.50	11.1	39.1	16	10.86	7	C_r
9	6.03	10.2	48.0	17	8.41	1	C_r
10	7.09	10.2	33.0	18	11.75	9	C_w
11	7.26	10.6	20.5	15	8.40	4	C_r
12	7.22	10.5	27.1	14	9.13	11	C_w
13	7.20	10.4	30.4	17	9.98	12	C_w
14	7.19	10.4	32.0	17	10.95	13	C_w

[a] The symbols used for the simplex operations are explained under *Weighted variable-size simplex algorithm.*

weighed procedure adopted seems to reach the same result after about 10–15 steps [7]; the determination of the coordinates of the point *P* is the crucial operation in the performance of the simplex algorithm. When a numerical value of the separation response is available, such as R_{s_m} in this study, a choice of the coordinates of the point *P* proportionally near to the best response should offer a fast approach to optimum conditions [7]. Further studies are in progress on a rigorous comparison between the performances of the different simplex optimization procedures.

Comparison of the two optimization paths (with methanol and acetonitrile) led to surprising results: the paths are very different in the step performance, showing that the behaviour of MECC is sensitively dependent on the solvent. With respect to R_{s_m}, the two solvents show comparable performance, but surprisingly the two optimization procedures, starting from dif-

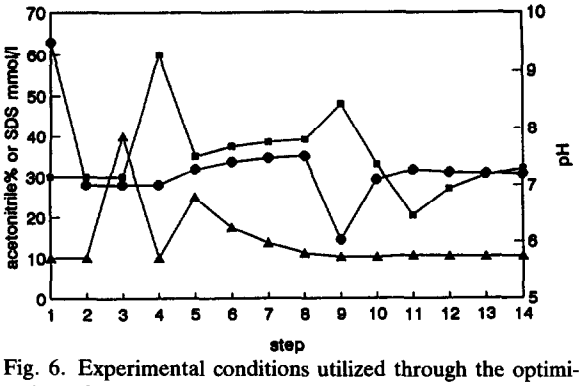

Fig. 6. Experimental conditions utilized through the optimization of the separation of PTH-amino acids using acetonitrile as organic solvent. ▲ = Acetonitrile (%); ■ = SDS (mmol/l); ● = pH.

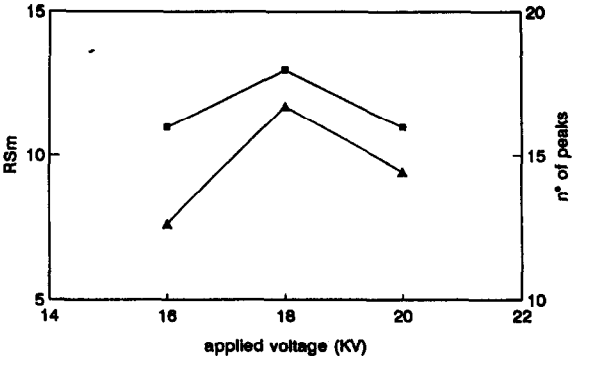

Fig. 7. Mean resolution measured using the experimental conditions of the tenth step of the acetonitrile optimization, changing the applied voltage. ▲ = R_{s_m}; ■ = No. of peaks.

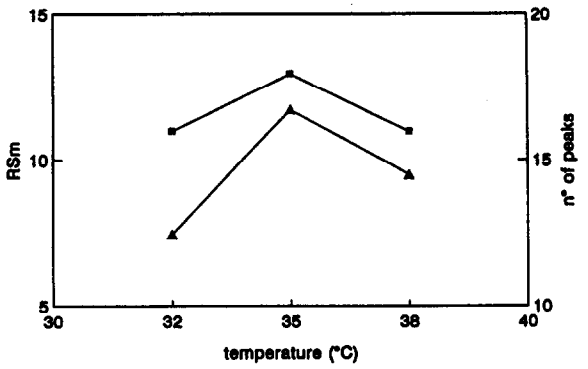

Fig. 8. Mean resolution measured using the experimental conditions of the tenth step of the acetonitrile optimization, changing the temperature of the separation. ▲ = R_{s_m}; ■ = No. of peaks.

ferent initial conditions, arrive at very close final pH values, SDS concentration and percentage of organic solvent. Further, the migration orders of the PTH-amino acids are very similar in the two solvents. This result should not be fortuitous; it probably indicates that the role of the organic solvent is mainly connected with a reduction in the polydispersity of the micellar pseudo-stationary phase. The molecular basis of the separation seems to be connected mainly to a strong ionic interaction between the PTH-amino acids and the micellar phase. This interaction could justify the long migration time of PTH-lys and PTH-arg with respect to other PTH-amino acids. In addition to this ionic interaction, a partition mechanism also plays an important role, as in reversed-phase LC separations. The mixed aqueous–organic mobile phase should represent the polar phase, whereas the micellar pseudo-stationary phase should represent the apolar phase. In fact, generally the non-polar PTH-amino acids are retarded in the separation compared with the polar compounds. However, a strong ionic interaction and a partition mechanism cannot explain the behaviour of some PTH-AAs with respect to others, e.g., the migration time of PTH-thr being shorter than that of PTH-ser, that of PTH-glu shorter than that of PTH-asp, that of PTH-leu shorter than that of PTH-leu and that of PTH-lys noticeably shorter than that of PTH-arg. These migration times are all the reverse of those observed in reversed-phase LC separation, suggesting that some other unknown interactions make important contributions to the separation.

By the use of the optimization procedures reported here, the complete separation of all 20 PTH-amino acids was not achieved; obviously this does not exclude that by using other conditions this goal could be obtainable. Further, under the experimental conditions explored by the optimization, we did not have complete assurance that the global optimum was reached.

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