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Journal of Chromatography A, 1078 (2005) 42–50
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Simultaneous optimization of the resolution and analysis time in micellar liquid chromatography of phenyl thiohydantoin amino acids using Derringer's desirability function

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Received 6 October 2004; received in revised form 24 April 2005; accepted 27 April 2005

Abstract

The chemometrics approach was applied for simultaneous optimization of resolution and analysis time of nine phenyl thiohydantoin amino acids in micellar liquid chromatography. Derringer's desirability function, a multi-criteria decision making method, was tested for the evaluation of the two different chromatographic performance goals. The effect of five experimental parameters on a chromatographic response function formed using two sigmoidal desirability functions was investigated. The sigmoidal functions were used to transform the optimization criteria, resolution and analysis time, into the desirability values. The factors studied were the concentration of sodium dodecyl sulfate, alkyl chain length of the alcohol used as the organic modifier, organic modifier content, mobile phase pH and temperature. The experiments were performed according to a face-centred cube response surface experimental design to map the chromatographic response surface. Then, calculated chromatographic response functions were fitted to a polynomial model. The obtained regression model was characterized by both descriptive and predictive ability ($R^2 = 0.988$ and $R^2_{cv} = 0.973$). The model was verified, as good agreement was observed between the predicted and experimental values of the chromatographic response function in the optimal condition. Based on the results of the study, combination of response surface mapping with Derringer's desirability function allows to predict the best operating condition in micellar liquid chromatography of phenyl thiohydantoin amino acids with respect to resolution and analysis time.

Keywords: Phenyl thiohydantoin amino acids; Micellar liquid chromatography; Optimization; Derringer's desirability function; Modeling

1. Introduction

Micellar liquid chromatography (MLC) is a mode of reversed phase liquid chromatography which employs aqueous micellar solutions as the mobile phases [1]. The use of micellar mobile phases in RPLC leads to some advantages such as low cost, nontoxicity, unique separation selectivity, detection sensitivity enhancement, possibility of direct injection of biological fluids, application in quantitative structure-activity relationship studies, etc. [2–7].

Due to the dependence on a large number of factors including the type and concentration of surfactant and organic modifier, solute nature, temperature and mobile phase pH, optimization of the experimental conditions is a complicated process in MLC. The systematic approach to the optimization of chromatographic separations is more expedient for such complicated method since the effect of the factors on retention can be interdependent and nonlinear [8–10]. However, chromatographic optimization requires to select a suitable criterion for the evaluation of the results and to choose the optimum conditions. Furthermore, it is usually necessary to judge the very different quality aspects of a chromatogram and to find a compromise between conflicting goals such as maximizing the separation while minimizing the analysis time. Different approaches from multi-criteria decision making (MCDM) have been used for simultaneous optimization of the criteria in RPLC methods [11–14]. However, adequate

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doi:10.1016/j.chroma.2005.04.081

attention has not been paid to the problem of MCDM in MLC. Our previous study [15] investigated the possibility of using the Pareto-optimality method, an approach from MCDM, in simultaneous optimization of separation quality and analysis time of micellar liquid chromatographic separation of chlorophenols.

In the present work, the feasibility of the Derringer's desirability function, other approach from MCDM, in MLC is demonstrated. In order to simultaneous optimization of resolution and analysis time in micellar liquid chromatographic separation of a group of nine phenyl thiohydantoin amino acids (PTH-amino acids), chemometric protocols of experimental design, response surface mapping and multi-criteria decision making (Derringer's desirability function) were employed. PTH-amino acids were selected as test solutes because their separation is of prime importance in determination of the amino acid sequencing of peptides and proteins [16]. The experiments for the optimization were performed according to the face-centred cube central composite design that is one of the most known response surface experimental designs for the purpose of modeling and optimization. The experimental factors considered were: concentration of sodium dodecyl sulfate, alkyl chain length of the alcohol used as the organic modifier, volume percentage of the organic modifier, mobile phase pH and temperature. For evaluation of the chromatograms using a chromatographic response function (CRF), an approach similar to the method proposed by Divjak et al. [17] was used. The method of stepwise multiple linear regression was employed to select the most important effects and to calculate the coefficients relating the effects to the chromatographic response functions. The experiment performed at the optimal condition predicted by the model actually produced the chromatogram of high quality. To the best of our knowledge, no chemometric treatment has been already reported concerning the simultaneous optimization of resolution and analysis time in MLC using Derringer's desirability function.

2. Theory

Simultaneous optimization of resolution and analysis time is the most important aspect of method development in liquid chromatography. Response surface mapping methods are effective optimization tools because the global optimum can be found [18]. Response surface mapping describes the relationship between the criteria and the experimental variables. Multi-criteria decision making, a branch of operations research, is a useful method that is applied when more than one optimization criterion has to be taken into account. The essence of MCDM is to judge the different quality aspects of a chromatogram individually and quantitatively [18,19].

A compromise between very different chromatographic goals may be achieved using the utility functions. In this MCDM method, a combined criterion called utility function (U_i) is used for simultaneous optimization of different chromatographic aspects. If there are m criteria Y_j ($Y_1, \ldots, Y_m$) to be optimized, the utility function for experiment i may be formulated as follows:

$$U_i = \sum_{J=1}^{m} W_j Y_{ji} \tag{1}$$

where the importance of the criteria is expressed by weighting factors W_j ($W_1, \ldots, W_m$). The multi-criteria problem is then reduced to the single criterion problem of optimizing U_i [19]. Some utility functions consisting the factors related to different chromatographic aspects have been developed including the chromatographic exponential function [20], chromatographic optimization function [21] and chromatographic response function [22–24]. Although the utility functions have been extensively used in chromatography, the procedure has some important disadvantages. It is difficult to consider the priori weights for all the criteria. Furthermore, it is possible the multi-criteria optimum found leads to an unacceptable value of one or more of the criteria. It can happen that very good solutions are found for one of the criteria with high weight, so that the bad results for some of the other criteria are compensated [19].

Another MCDM method of simultaneously optimizing different criteria, was first presented by Harrington [25]. He proposed that one can multiply the criteria instead of summing them. According to the method, values of the criteria should be scaled between 0 (unacceptable) and 1 (optimal). These values are then called desirabilities. This mathematical model was put into a more general form by Derringer [26]. Derringer's desirability function was introduced in chromatography by Bourguignon and Massart [13]. It is based on the transformation of the measured properties to a dimensionless desirability scale for each criterion, so that values of several properties, obtained from different scales of measurements, may be combined. The values for desirability ranges from zero for the undesirable level of quality to the value of unity which indicates an ultimate level of quality beyond which further improvements would have no value.

The transformation of the individual criteria into desirability values are possible using a one-sided or a two-sided transformation. In the one-sided transformation, the response variables Y_i ($i = 1, 2, \ldots, n$, where n is the number of response variables) are transformed into the desirability functions, d_i, according to the following equations:

$$\begin{aligned} \mathrm{d}_i &= 0 && \text{if } Y_i \le Y_i^{(-)} \\ \mathrm{d}_i &= \left(\frac{Y_i - Y_i^{(-)}}{Y_i^{(+)} - Y_i^{(-)}} \right)^r && \text{if } Y_i^{(-)} < Y_i < Y_i^{(+)} \\ \mathrm{d}_i &= 1 && \text{if } Y_i \ge Y_i^{(+)} \end{aligned} \tag{2}$$

where $Y_i^{(-)}$ is the minimum acceptable value of the criterion Y_i and $Y_i^{(+)}$ is the value beyond which improvements would serve no further benefit. Both values and the parameter r which is in fact a kind of weighting factor should be selected

by the user. It is noteworthy that such transformation is valid for separation criteria. Although this is not the case with the criteria used for evaluation of the analysis time where d-value needs to be minimized. Therefore, $d = 0$ for $Y_i \geq Y_i^{(+)}$, $d = 1$ for $Y_i \leq Y_i^{(-)}$ and a value in between for $Y_i^{(-)} < Y_i < Y_i^{(+)}$ should be considered in such cases.

In a second step, the overall quality D is calculated by multiplying the desirability values obtained for the different criteria [26] or by using the geometric mean of them [27].

The advantage of the Derringer's desirability function is that if one of the criteria has an unacceptable value, then the overall product will also be unacceptable. While, with the utility functions, this is not the case [26]. It is noteworthy that the outcome of the overall quality D depends on the r-value and selection of the suitable r-value offers the user flexibility in the definition of desirability functions.

Divjak et al. [17] showed the usefulness of the sigmoidal functions (instead of exponential functions as in Eq. (2)) for one-sided transformation of different criteria into desirability values. According to their method, transformation of the resolution values between the neighbouring peaks, $R^{P,P+1}$, to desirability values, $S^{P,P+1}$, ranging between 0 and 1 may be performed using the following equation:

$$S^{P,P+1} = \frac{1}{1 + \exp(-b_0 \cdot R^{P,P+1} + b_1)} \tag{3}$$

The $S^{P,P+1}$ value should be high (≈1) for maximum and low (≈0) for minimum acceptable values of resolution. These limiting conditions determine the values of the parameters b_0 and b_1 in the equation.

In the next step, overall desirability value (f) for the evaluation of the chromatograms in regard to the integral resolution of n analytes is calculated using geometrical average of all individual desirability values $S^{P,P+1}$ ($P = 1, 2, \ldots, n-1$).

$$f = \left(\prod_{P=1}^{n-1} S^{P,P+1} \right)^{1/(n-1)} \tag{4}$$

The evaluation of the desirability of the analysis time, g, of the chromatograms may be also performed using a sigmoidal transformation:

$$g = \frac{1}{1 + \exp(\, b_2 \cdot t + b_3)} \tag{5}$$

where t is a criteria used as a measure of the analysis time. The g value should be high (≈1) for very short and low (≈0) for long analysis time. Calculation of the parameters b_2 and b_3 is done by employing these limiting conditions.

Finally, the chromatographic response function is calculated by multiplying the two desirability values f and g:

$$\mathrm{CRF}(f, g) = f \times g \tag{6}$$

As can be easily found, no priori decisions about the weighting factors have to be made in the procedure. It should be also noted that the optimization criterion (CRF) is not sensitive to possible changes in the elution order of the components.

3. Experimental

3.1. Apparatus

The HPLC system consisted of a model 515 solvent delivery system equipped with model U6K injector fitted with a 20 μl loop, all from Waters (Milford, MA, USA) and a Perkin-Elmer LC-95 UV detector (Norwalk, CT, USA). A Jenway model 3030 digital pH meter (Jenway, UK) equipped with a combined glass-calomel electrode was employed for pH measurements.

3.2. Chemicals

The surfactant, sodium dodecyl sulfate (SDS), and HPLC grade methanol, ethanol, propanol and butanol were purchased from Fluka (Buchs, Switzerland). The test solutes including PTH-aspargine (PTH-Asp), PTH-glutamine (PTH-Glu), PTH-glycine (PTH-Gly), PTH-alanine (PTH-Ala), PTH-methionine (PTH-Met), PTH-valine (PTH-Val), PTH-tryptophane (PTH-Trp), PTH-leucine (PTH-Leu) and PTH-phenylalanine (PTH-Phe) were used as received from Fluka. Phosphoric acid, disodium hydrogenphosphate and sodium dihydrogenphosphate were Fluka analytical grade chemicals.

3.3. Chromatographic conditions

A Spherisorb C18 column (250 mm × 4.6 mm, 5 μm particle size) from Waters was used for all the separations. The column was thermostated at the different temperatures by a water circulator bath. Stock solutions of PTH-aminoacids (0.5–1.0 mg/ml) were prepared in methanol and were stored at −20 °C. The micellar solutions were prepared in double distilled, deionized water and were filtered through a 0.45 μm Millipore solvent filter. The mobile phase pH was adjusted at 3–7 using phosphate buffer.

The experiments were performed according to the experimental design using a number of eluents prepared with different combinations of the values of the five variables. The sequence of experiments was randomized. The mobile phase flow rate was maintained at 1.2 ml/min and spectrophotometric detection at 254 nm was employed.

The isocratic chromatographic system was conditioned by passing the eluent through the column until a stable base line was observed. Then, repeatable retention times were obtained for three subsequent injections. Dead time value was measured from the time of injection of methanol to the first deviation of the base line.

All statistical analyses of the multiple regression were performed on range scaled factor values of [−1, +1] with SPSS/PC software [28].

4. Results and discussion

4.1. Chromatographic response function

In order to study the application of the desirability function in MLC, it was applied to locate the optimum condition for separation of PTH-amino acids with regard to resolution as well as analysis time. To evaluate the quality of the chromatograms using a chromatographic response function, an approach similar to that proposed by Divjak et al. [17] was followed. The resolution between peaks and the retention time of the last peak in the chromatogram were used as the measures of separation and analysis time, respectively.

The individual resolution ($R^{P,P+1}$) between the neighbouring peaks P and P + 1 for n analytes ($P = 1, 2, \ldots, n-1$) was calculated by the following expression:

$$R^{P,P+1} = \sqrt{N}\frac{(k_{p+1} - k_p)}{2(k_{p+1} + k_p + 2)} \quad (7)$$

where k and N are the retention factor and plate number, respectively. Transformation of the resolution values to desirability values ranging between 0 and 1 was performed using Eq. (3), Where minimum acceptable value of the response variable ($R^{P,P+1}$) was set at 0.5 because peaks can not be recognized as being separated until $R^{P,P+1} = 0.5$. On the other hand, maximum acceptable value of $R^{P,P+1}$ was set at 2.5, since higher resolutions resulting in increasing analysis time are of no further benefit. Determination of the parameters b_0 and b_1 in the Eq. (3) was done by employing the limiting conditions for values $S^{P,P+1} = 0.95$ and 0.10 for $R^{P,P+1} = 2.5$ and 0.5, respectively. The values obtained for b_0 and b_1 were 2.567 and 3.481, respectively. Then, overall desirability value (f) was calculated using geometrical mean of all individual desirability values $S^{P,P+1}$ (Eq. (4)).

The desirability values of the analysis time (g) of the chromatograms was also evaluated using the sigmoidal transformation (Eq. (5)). Calculation of the parameters b_2 and b_3 was done by employing the limiting conditions for values $g = 0.9$ and 0.1 for $t = 10$ and 45 min, respectively. The values obtained for b_2 and b_3 were 0.126 and −3.458, respectively.

In the last step, the chromatographic response function was calculated by multiplying the two desirability values f and g (Eq. (6)).

4.2. Experimental design

To locate the optimum condition for separation of PTH-amino acids in MLC, a simultaneous optimization strategy was adopted. In this strategy, a face-centred cube response surface experimental design was used to map the chromatographic response surface. This design is one of the experimental designs suitable for modeling and optimization which results from the addition of a factorial design and of a star design [17,29].

Table 1
The five chromatographic factors and corresponding three level settings[a]

Level	[SDS]	N	V_m	pH	T
−	0.030	2	3.0	3	30
0	0.065	3	6.5	5	35
+	0.100	4	10.0	7	40

[a] [SDS], sodium dodecyl sulfate concentration (M); N, alkyl chain length of the organic modifier; V_m, organic modifier content (v/v %); pH, mobile phase pH; T, temperature (°C).

The effect of five experimental factors on the quality of the chromatograms was studied using the multivariate analysis. The factors studied were SDS concentration, alkyl chain length of the alcohol used as the organic modifier (N), volume percentage of the organic modifier (V_m), mobile phase pH and temperature (T). Table 1 shows the feasible region of the selected factors in which experimental optimization could be carried out. The experimental range of the factors was selected on the basis of chromatographic insight and physical limitations.

Table 2
Experimental conditions according to the face-centred cube response surface experimental design for five factors studied

Experiment	[SDS] (M)	N	V_m (v/v %)	pH	T (°C)
Fractional factorial design					
1	−1	−1	−1	−1	+1
2	+1	−1	−1	−1	−1
3	−1	+1	−1	−1	−1
4	+1	+1	−1	−1	+1
5	−1	−1	+1	−1	−1
6	+1	−1	+1	−1	+1
7	−1	+1	+1	−1	+1
8	+1	+1	+1	−1	−1
9	−1	−1	−1	+1	−1
10	+1	−1	−1	+1	+1
11	−1	+1	−1	+1	+1
12	+1	+1	−1	+1	−1
13	−1	−1	+1	+1	+1
14	+1	−1	+1	+1	−1
15	−1	+1	+1	+1	−1
16	+1	+1	+1	+1	+1
Central points					
17	0	0	0	0	0
18	0	0	0	0	0
19	0	0	0	0	0
20	0	0	0	0	0
21	0	0	0	0	0
22	0	0	0	0	0
Star design					
23	−1	0	0	0	0
24	+1	0	0	0	0
25	0	−1	0	0	0
26	0	+1	0	0	0
27	0	0	−1	0	0
28	0	0	+1	0	0
29	0	0	0	−1	0
30	0	0	0	+1	0
31	0	0	0	0	−1
32	0	0	0	0	+1

Table 3
Experimental retention time (min) and CRF values obtained by the face-centred cube response surface experimental design

Experiment	PTH-Asp	PTH-Glu	PTH-Gly	PTH-Ala	PTH-Met	PTH-Val	PTH-Trp	PTH-Leu	PTH-Phe	CRF
1	6.47	9.99	10.97	16.94	41.88	42.59	53.54	70.18	72.03	0.05
2	5.11	6.89	8.24	11.01	19.29	19.71	19.76	25.73	25.72	0.23
3	4.36	5.93	6.36	8.78	16.86	17.85	24.17	27.71	29.99	0.30
4	3.63	4.46	4.97	6.22	9.45	9.96	10.71	12.99	12.96	0.48
5	5.04	7.28	8.23	11.45	25.93	27.35	36.16	43.59	48.99	0.08
6	3.92	4.87	5.66	7.17	12.11	12.59	13.60	17.51	17.58	0.42
7	3.45	3.77	4.28	5.77	9.79	11.22	14.21	17.97	15.68	0.68
8	3.07	3.38	3.73	4.38	6.14	6.51	7.15	8.65	8.25	0.58
9	6.98	11.10	12.55	19.86	47.48	50.38	60.12	77.39	80.10	0.07
10	4.86	6.39	7.59	10.22	17.84	18.52	18.63	24.51	24.37	0.26
11	4.28	5.52	5.91	8.18	15.56	16.77	22.00	26.27	27.80	0.34
12	3.92	4.74	5.35	6.60	9.91	10.36	11.36	13.47	13.56	0.48
13	4.70	6.63	7.22	10.11	22.98	23.58	31.47	40.57	43.79	0.07
14	4.10	5.24	6.15	7.71	13.15	13.40	14.42	18.48	18.56	0.38
15	3.37	3.76	4.21	5.71	9.72	11.14	14.74	18.35	16.00	0.66
16	2.91	3.12	3.36	3.90	5.08	5.50	5.76	7.03	6.55	0.52
17	3.81	4.78	5.33	6.78	11.58	12.14	14.21	17.32	17.89	0.47
18	3.80	4.76	5.32	6.76	11.55	12.12	14.16	17.18	17.84	0.48
19	3.79	4.74	5.28	6.69	11.37	11.91	13.91	16.80	17.47	0.49
20	3.89	4.84	5.37	6.76	11.36	11.90	13.92	16.68	17.38	0.49
21	3.72	4.63	5.13	6.47	10.84	11.37	13.20	15.94	16.60	0.50
22	3.81	4.75	5.29	6.70	11.37	11.92	13.91	16.82	17.49	0.49
23	4.00	5.27	5.74	7.54	14.72	15.48	20.89	24.88	27.50	0.37
24	3.61	4.39	4.96	6.10	9.53	9.96	10.96	13.38	13.45	0.49
25	4.56	6.14	7.06	9.41	17.47	17.88	20.35	25.35	26.07	0.30
26	3.29	3.79	4.08	5.02	7.24	8.06	9.31	11.05	10.94	0.58
27	4.61	6.18	7.01	9.38	16.68	17.27	19.57	23.85	24.58	0.34
28	3.41	4.04	4.40	5.39	8.64	9.14	10.72	13.06	13.40	0.51
29	3.75	4.66	5.20	6.53	10.96	11.46	13.44	16.24	16.85	0.49
30	3.74	4.66	5.15	6.49	10.84	11.33	13.21	15.94	16.64	0.50
31	3.89	4.98	5.56	7.07	12.18	12.65	14.92	17.95	18.75	0.46
32	3.71	4.58	5.10	6.49	11.03	11.68	13.52	16.70	17.09	0.48

The exploration of the experimental domain was started with a factorial design. A full factorial design for five factors and two levels would require 32 experiments. To reduce the number of experiments, a two-level half fractional factorial design consisting 2^{5-1} experiments was used. The experiments 1–16 in Table 2 show the fractional factorial design (fFD). The values of retention times and calculated CRF for the experiments were reported in Table 3. The reduced design allows the first estimation of the effects of the main factors and of their second order interactions, that are presented in Table 4. It can be observed that the most important effect on retention time (t_R) values of the analytes was due to the alkyl chain length of the organic modifier. As expected, an increase in N leads to a decrease in retention time. The effect of the

Table 4
The effects of the factors and of their interactions calculated for PTH-amino acids from the fractional factorial design (experiments 1–16 in Table 2)

Factors	PTH-Asp	PTH-Glu	PTH-Gly	PTH-Ala	PTH-Met	PTH-Val	PTH-Trp	PTH-Leu	PTH-Phe	CRF
[SDS]	−0.89	−1.86	−1.84	−3.70	−12.15	−13.04	−15.83	−24.21	−25.85	0.14
N	−1.52	−2.96	−3.56	−5.62	−14.77	−14.85	−17.20	−23.19	−25.04	0.31
V_m	−1.13	−2.12	−2.39	−3.95	−9.17	−9.36	−10.35	−10.39	−13.89	0.15
pH	0.01	−0.01	−0.01	0.07	0.03	0.23	−0.10	0.22	−0.05	0.00
T	−0.22	−0.45	−0.61	−0.87	−1.72	−2.00	−2.25	3.41	−2.55	0.01
[SDS] × N	0.41	1.04	1.00	1.86	6.82	6.88	9.34	12.17	13.82	−0.12
[SDS] × V_m	0.25	0.65	0.58	1.23	4.17	4.22	5.47	7.00	7.47	−0.04
[SDS] × pH	0.01	−0.02	−0.03	−0.16	0.29	−0.48	−0.16	−0.57	−0.31	−0.01
[SDS] × T	0.00	0.09	0.14	0.33	0.72	1.14	1.25	0.97	1.39	0.00
N × V_m	0.28	0.47	0.64	1.45	3.91	4.21	3.75	6.15	4.43	0.06
N × pH	−0.02	−0.09	−0.12	−0.26	−0.53	−0.68	−2.20	−0.77	−0.68	−0.03
N × T	0.10	0.21	0.33	0.52	1.04	1.39	1.06	1.06	1.35	−0.01
V_m × pH	−0.11	−0.13	−0.23	−0.41	−0.79	−1.25	−1.08	−1.04	−1.34	−0.04
V_m × T	0.07	0.13	0.16	0.30	0.48	0.62	0.39	0.55	0.52	−0.01
pH × T	−0.19	−0.35	−0.44	−0.99	−2.98	−3.23	−3.45	−5.29	−3.88	−0.11

factor N on t_R values is more significant for PTH-leucine and PTH-phenylalanine. This result can be correlated to the predominantly hydrophobic properties of these solutes with respect to the other solutes. In agreement with previous reports [30,31], the results emphasize that hydrophobicity of the solute and organic modifier are important factors in controlling their interactions with the micelles which affect the solute retention in MLC. The retention time value for the analytes is also largely affected by the main factors [SDS] and V_m. It should be noted that the effects of these factors on retention are in expected direction. Retention time was found to decrease as SDS concentration or modifier content was increased. The other main factors, pH and T, showed the minor effects on the t_R values.

Further analysis of the results of the experiments of the fFD showed that the most significant effect on CRF values is due to the main factor N, followed by the effect of V_m and of [SDS] (last column in Table 4). Surprisingly, these factors have positive effects on the CRF values, although they showed negative effects on the values of t_R. On the basis of the results, no evidence for significant effects of pH and T was found. It must be underlined immediately that the second order interaction between pH and T is significant and characterized by a negative value. It is noteworthy that existence of significant two-factor interaction term pHξT (coefficient value = −0.11) emphasises that the effect of the two main factors needs to be looked at further.

To estimate the pure experimental error and to check system reproducibility, the experiment in the central point was replicated (experiments 17–22 in Table 2). Subsequently, existence of quadratic (or higher) significant effects was tested by means of F-test that compares the difference between the responses in the central point and factorial design with the

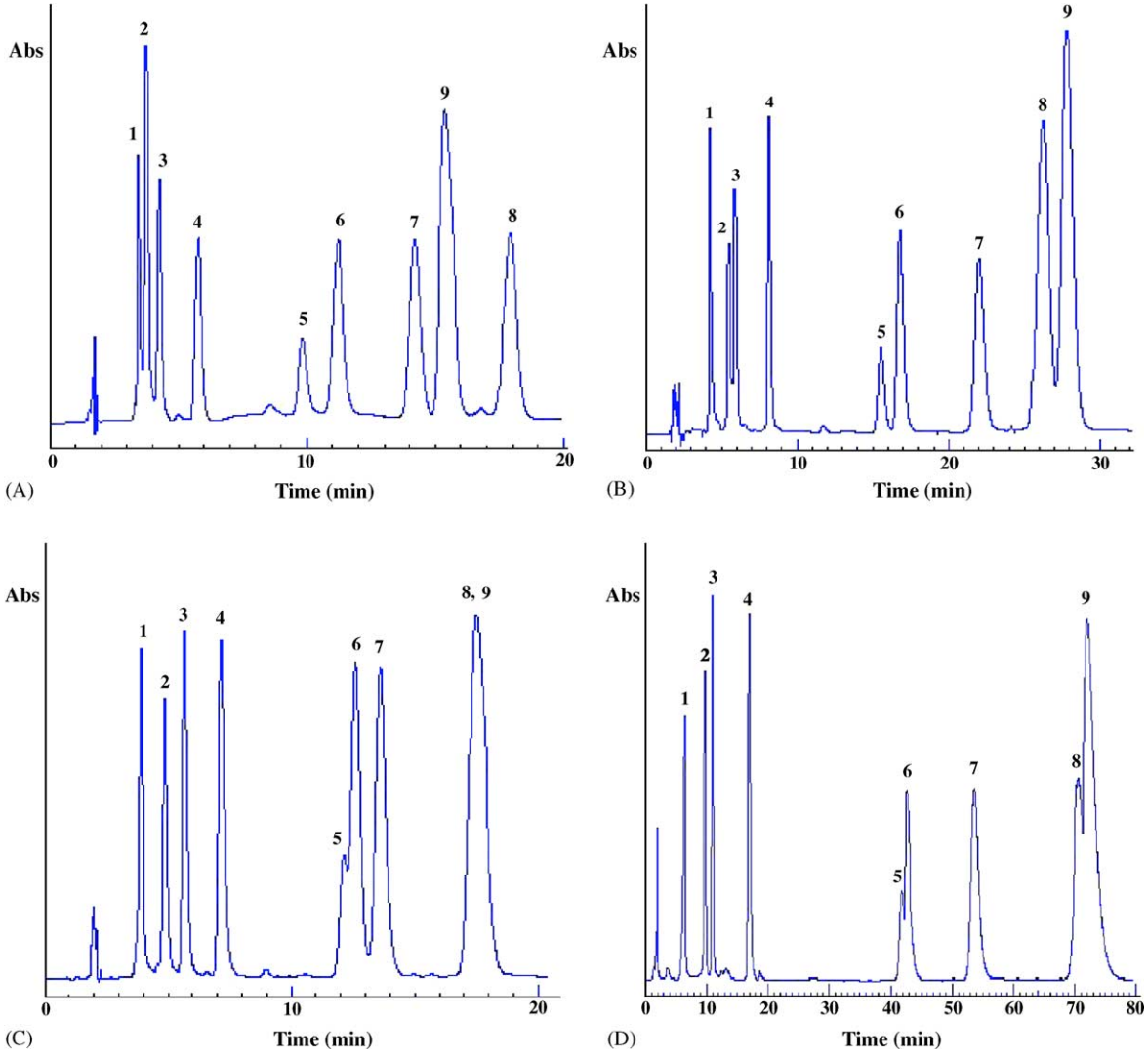

Fig. 1. The chromatograms giving CRF values of 0.68 (A), 0.34 (B), 0.42 (C) and 0.05 (D). Conditions are as those of the experiments 7, 11, 6 and 1 (Table 2), respectively. Peaks identification: 1, PTH-aspargine; 2, PTH-glutamine; 3, PTH-glycine; 4, PTH-alanine; 5, PTH-methionine; 6, PTH-valine; 7, PTH-tryptophane; 8, PTH-leucine; 9, PTH-phenylalanine.

purely experimental variance (S^2_{pe}) [32]:

$$F_{(1,\upsilon,\alpha)} = \frac{(\bar{y}_o - \bar{y}_f)^2}{S^2_{pe} \times ((1/n_o) + (1/n_f))} \quad (8)$$

where $\bar{y}_o$ and $\bar{y}_f$, are average response of the replicated central and average response of the factorial design experiments, respectively. The n_o and n_f are number of experiments in the central point and in the factorial design, respectively. From the high F-value ($F = 737.894$), it was concluded that the quadratic (or higher) effects must be used in the regression model to describe the dependence of the chromatographic response function to the experimental factors. Therefore, a star design consisting 10 experiments (experiments 23–32 in Table 2) was added to the factorial design to provide a central composite design that allows to obtain a model containing the main factors plus the interactions and the squared terms.

The results of the study showed that the overall CRF value reasonably represented our evaluation of the obtained chromatograms. They gave high values only for the chromatograms that exhibited good separation in a reasonably short analysis time (CRF = 0.68, Fig. 1A). At the same time, medium values were obtained for the chromatograms with relatively good separation but longer analysis time and for the chromatograms with bad separation regardless of the analysis time (CRF = 0.34 and 0.42, Fig. 1B and C, respectively). The chromatograms that exhibited bad separations in a long analysis time had low CRF values (CRF = 0.05, Fig. 1D).

4.3. Modeling

The overall CRF values for the complete set of 32 experiments were fitted with a polynomial model. An ordinary least square method was used by a variable selection algorithm (stepwise search) to find a model that describes efficiently the dependence of CRF on the experimental parameters. Criteria for the evaluation of the descriptive capability of the models were Fisher-ratio value (F), squared correlation coefficient (R^2) and standard error of estimate (SE). Different polynomials with all possible combinations of the factors were generated. It was found that the simplest polynomial that successfully described the system under study was second order. The best model and the statistics are given in Table 5. The high value of R^2 and F statistics indicates that the model is quite successful in calculating the chromatographic response function. The standard error is 0.022 and greater than 98% of the variance is accounted for by the model. The model obtained showed that the CRF value is influenced by three main factors including alkyl chain length of the alcohol, alcohol content and SDS concentration. Determination of the importance of the factors in the model by the standardized regression coefficient [33] demonstrated that alkyl chain length of the alcohol is the most important factor affecting the CRF values. The value of CRF was found to increase as N was increased. It is noteworthy that modifier content and SDS concentration showed positive contributions to the dependent variable, while the square of these factors showed negative contributions. It can be readily seen that the overall contribution for both factors is positive. Therefore, an increase in CRF should be obtained as a result of an increase of [SDS] or V_m. Mobile phase pH and temperature only appeared in a two-factor interaction term and showed a negative contribution to the CRF value. The existence of two-factor interactions between main factors in conditions of our experiments emphasises once the necessity to carry out active multifactor experiments for optimization of the chromatographic separation process in MLC.

Table 5
Specification of the best polynomial model for prediction of the CRF values

Variable	Coefficient	Standard error	Standardized regression coefficient
Constant	0.478	0.006	–
N	0.154	0.005	0.709
V_m	0.075	0.005	0.346
V_m^2	−0.068	0.012	−0.208
[SDS]	0.066	0.005	0.304
[SDS] × N	−0.058	0.005	−0.252
pH × T	−0.053	0.005	−0.229
$N \times V_m$	0.031	0.005	0.133
[SDS]^2	−0.060	0.012	−0.182
[SDS] × V_m	−0.018	0.005	−0.080
Statistics			
R^2[a]	0.988		
SE[b]	0.022		
F[c]	193.775		
R^2_{cv}[d]	0.973		

[a] Square of correlation coefficient.
[b] Standard error of estimate.
[c] Fisher-ratio value.
[d] Square of the cross-validated correlation coefficient.

In order to test the predictive power and robustness of the model obtained, cross-validation using leave-one-out method [34–36] was employed. In leave-one-out method, a model is constructed after deleting one observation of the data set, then this observation is predicted by a model based on the remaining data and squared difference between the left-out observation and its prediction is calculated. This procedure is repeated for the entire data set and cross-validated R^2 (R^2_{cv}) is calculated. The results showed that the model obtained is quite valid and stable as judged from R^2_{cv} value (Table 5). Fig. 2 shows the plot of cross-validated predicted CRF values according the model reported in Table 5 versus experimental CRF values and related statistics. High R^2 and F values for the plot indicate good stability and predictive ability of the model developed.

To find the optimum chromatographic condition in the separation of PTH-amino acids in MLC, a grid search algorithm written in FORTRAN 77 was used. The optimal values of the experimental variables were found to be 0.065 M SDS, 9.3% (v/v) butanol, pH 3.0 and column temperature 40 °C. The efficiency of prediction of the polynomial model was tested by performing the experiment under the predicted optimal condition. Chromatogram obtained under

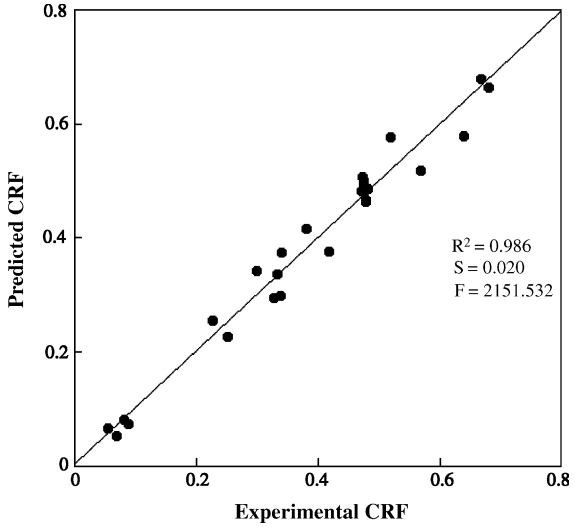

Fig. 2. Plot of cross-validated predicted CRF (according to the regression model reported in Table 5) vs. experimental CRF values.

the predicted condition (Fig. 3) showed complete resolution of all the analytes in a short analysis time. Relative error in prediction of the CRF value for optimal condition was −1.4%. Therefore, suitability of the model developed for interpreting the experimental space and for indicating the optimum experimental condition was confirmed.

The results of the study demonstrated that it is possible to develop the model with descriptive and predictive ability for the chromatographic response function, which allows one to find the optimum conditions in the separation of PTH-amino acids in MLC. Good resolution achieved in the work permits the identification of the PTH-amino acids obtained from sequencing of peptides and proteins.

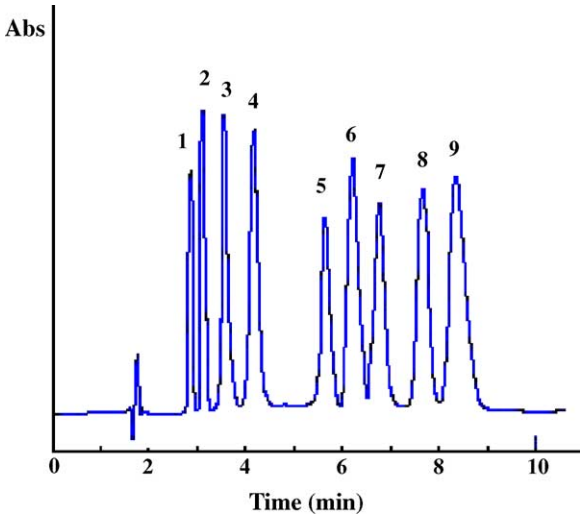

Fig. 3. Chromatogram obtained for PTH-amino acids in optimal condition predicted using polynominal model reported in Table 5. Conditions: 4.6 mm × 250 mm, 5 μm particle size Waters Sphirosorb C18 column, 0.065 M SDS, 9.3% (v/v) butanol, pH 3.0, temperature 40 °C and flow rate 1.2 ml/min. Peak identities are as those in Fig. 1.

5. Conclusions

Derringer's desirability function has been introduced to MLC for optimization of both resolution and the analysis time of PTH-amino acids. Face-centred cube response surface experimental design in conjunction with soft modeling has been shown to be efficient in mapping response surface for changing a chromatographic response function. The results showed that a function composed of two sigmoidal desirability functions can be successfully used to evaluate the chromatograms and to search for an optimum set of experimental conditions in MLC. To find the optimal chromatographic conditions, a second order polynomial equation was generated to model the CRF values as a function of the experimental parameters including the concentration of sodium dodecyl sulfate, alkyl chain length of the alcohol, alcohol content, mobile phase pH and temperature. Robustness of the model obtained was assessed using leave-one-out cross-validation method. The efficiency of the prediction of the model was confirmed by performing the experiment under the optimal condition. The results of the study showed that Derringer's desirability function in combination with response surface mapping can be successfully applied to the micellar liquid chromatographic separation area for modeling and for process optimization. The method offer promising possibilities in MLC because Derringer approach is the only MCDM method for which it is easy to consider simultaneously more than two criteria.

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