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Experimental design for the rapid selection of separation conditions for methyl and propyl parahydroxybenzoate, phenylephrine hydrochloride and chlorphenamine maleate by ion-pair liquid chromatography

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Abstract: Methyl and propyl parahydroxybenzoate (MPHB, PPHB), phenylephrine hydrochloride (PE) and chlorphenamine maleate (CPM) are often combined as ingredients in cough-syrups. Due to distinct chemical structures, pK_a values among other chemical properties are different. This may result in a particular chromatographic behaviour on ion-pair reversed-phase liquid chromatographic (LC) systems. A face-centred central composite design was applied to study the impact of four LC mobile phase parameters and parameter interactions on the retention of these four compounds. The mobile phase parameters studied were the concentration of methanol as organic modifier, the concentration of sodium dioctylsulphosuccinate (SDSS) as counter-ion, the concentration of dimethyloctylamine (DMOA) as competitive base and the pH. By means of the proposed design, mathematical regression models and response surface plots were calculated, which could predict the compounds' retention times with good statistical reliability. Adequate combination of the most relevant of these mobile phase parameters enabled complete chromatographic separations within short times of analysis.

Keywords: *Ion-pair reversed-phase liquid chromatography; methyl and propyl parahydroxybenzoate; phenylephrine hydrochloride; chlorphenamine maleate; face-centred central composite design; regression models; response surface plots.*

Introduction

Some cough-syrup formulations contain 0.1–0.2% of methyl and propyl parahydroxybenzoate (MPHB, PPHB) as preservatives in a ratio of 7:3 parts, phenylephrine hydrochloride (PE) as vasoconstrictor and chlorphenamine maleate (CPM) as H_1-antihistamine. From the pharmaceutical-industrial point of view, a fast isocratic liquid chromatographic (LC) method for simultaneous analysis of these ingredients would be most welcome for quality control purposes.

The four compounds have rather distinct chemical properties: MPHB and PPHB are hydrophobic phenolic compounds with a pK_a value of 8.4; PE has a phenylethylamine structure and a phenolic function with pK_a values of 8.8 and 9.8, whereas CPM has pK_a values of 4.0 and 9.2 [1]. Their individual chromatographic behaviour on a classical isocratic ion-pair reversed-phase LC system is therefore influenced by mobile phase parameters such as organic modifier concentration, counter-ion concentration, competitive base concentration and pH. Apparently the chromatographic behaviour of CPM with a pK_a value of 4.0, will dramatically be influenced by mobile phase pH fluctuations between 3.0 and 5.0. The chromatographic behaviour of the other three compounds (with pK_a values exceeding 8) will be less affected by these pH fluctuations. On the other hand, a competitive base in the mobile phase can interact with the retention of PE and CPM, while MPHB and PPHB will be unaffected.

In this study the relative importance of four mobile phase parameters and their interactions, governing the chromatographic behaviour of MPHB, PPHB, PE and CPM, was

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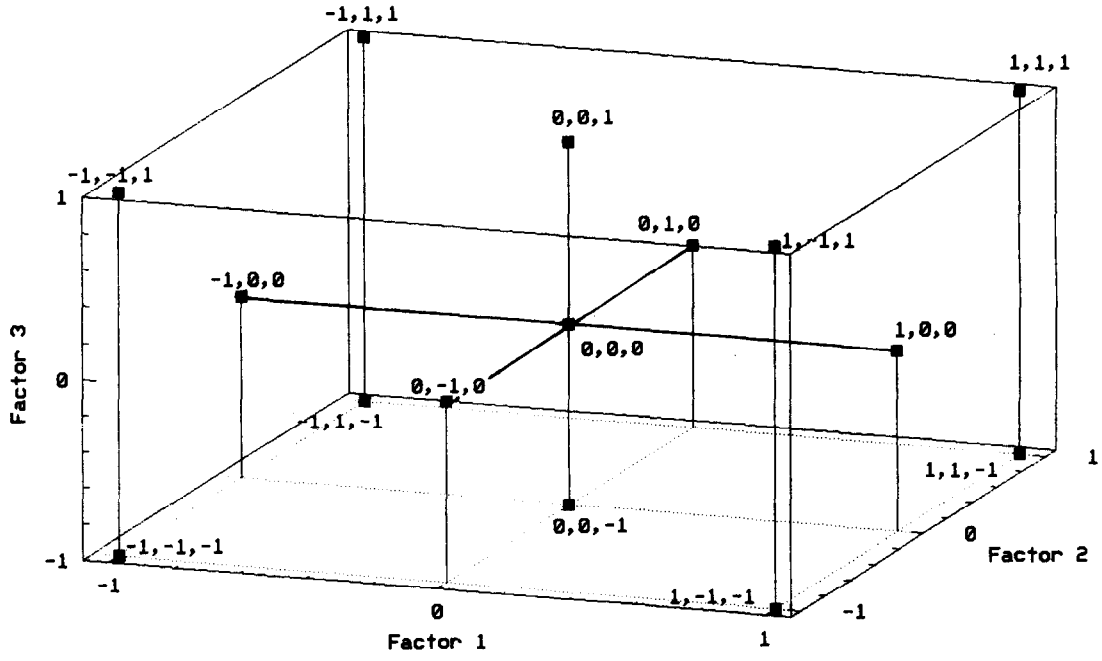

Figure 1
Cube diagram, representing a three factor face-centred central composite design.

examined by application of a face-centred central composite design (Fig. 1). This involved at least (2^k + 2k + 1) different experimental measurements, combining k (= 4) chromatographic parameters at a high extreme, a low extreme and a central level for each examined parameter [2, 3].

The four examined mobile phase variables were:

(1) the concentration of methanol (MeOH) as organic modifier;

(2) the concentration of sodium dioctylsulphosuccinate (SDSS) as ion-pairing reagent;

(3) the concentration of dimethyloctylamine (DMOA) as competitive base; and

(4) the pH.

The experimentally measured response variable was the retention time for each of MPHB, PPHB, PE and CPM.

Multiple regression modelling and response surface plots helped to find those adjustments of mobile phase parameter combinations, which resulted in a complete, short-duration chromatographic separation of the four examined substances. An experimental design approach is more and more being applied to expedite chromatographic analysis. It is classified as a simultaneous optimization method. Factorial and simplex lattice designs, sometimes referred to as mixture designs, are well-known examples of these. The first theoretical studies, however, answering problems of determining optimum conditions in chemical investigations, date from more than 40 years ago. Plackett and Burman [4], Box and Wilson [2] and Box and Behnken [5] among others, developed the mathematical insights in the theory of factorial experimentation. More mathematical and chemometric insights are presented by Deming and Morgan [6] and by Box, Hunter and Hunter [7]. A comprehensive tutorial article on theory and application of experimental design is written by Morgan *et al.* [3]. Chromatographic theory was integrated with experimentation by Snyder to predict satisfactory separation conditions [8, 9]. Morgan and Deming reviewed the experimental optimization of chromatographic systems by means of factorial designs [10]. Later, Kong *et al.* [11] and Sachok *et al.* [12] used experimental designs in multifactor optimization of reversed-phase LC separations. Several factorial designs have previously been used by Lindberg *et al.* [13], by Cotton and Down [14], by Wester *et al.* [15] and by Yuzhu Hu and Massart [16] to investigate and optimize LC systems. In a review article Glajch and Kirkland described method development in LC using retention mapping and mixture design techniques [17].

Experimental

Apparatus and column

The LC equipment was constructed with a Model 600 multisolvent delivery system (Millipore–Waters, Milford, MA, USA), allowing mobile phase composition from four separate reservoirs, continuously degassed with helium. The pump system was provided with a built-in "silk" feature, which is a pump-transducer feedback loop, allowing pump flow to be constantly monitored and adjusted for pulse-free flow. Samples were injected with a Marathon autosampler (Spark Holland, Emmen, The Netherlands) equipped with a loop of about 20 μl. The Waters 990 photodiode array detector was linked to a Nec Powermate 386/33i data station. Two-dimensional chromatograms were recorded at 273 nm. Peek tubing was used at all connections. A flow rate of 0.9 ml min^{-1} was used throughout the study. The 15 × 0.39 cm i.d. LC column was a C_{18} Novapak column (Millipore–Waters). Its silica is spheric and has a particle size of 4 μm.

Samples, chemicals and solvents

The reference samples of two substances (PE and CPM) were supplied by Laboratoria Qualiphar (Bornem, Belgium). MPHB and PPHB were own-laboratory reference samples. Water was prepared with a Milli Q system (Millipore, Milford, MA, USA). MeOH was of LC quality (Lab-Scan Ltd, Dublin, Ireland). Phosphate buffer solutions were prepared with *pro analysi* potassium dihydrogen phosphate (Merck, Darmstadt, Germany) and adjusted to a suitable pH by means of phosphoric acid (Merck) or a 1 M sodium hydroxide solution. Their pH values were controlled with a Φ 43 Beckman pH-meter (Beckman, San Ramon, CA, USA), equipped with a combined glass electrode (Beckman). DMOA 95% was from Janssen Chimica (Beerse, Belgium) and SDSS from Aldrich Chemie (Brussels, Belgium).

Mobile phase composition

From the experimental point of view, the four examined mobile phase parameters (concentrations of MeOH, SDSS and DMOA, and the pH) had to be independently adjusted to their central, high and low extreme value levels in 25 different combinations, as determined within the proposed central composite design. By means of a multisolvent delivery system these 25 different mobile phase parameter combinations could be easily established. Four separate solutions, each containing a component of the mobile phase, were prepared at each pH level and stored in the four available solvent reservoirs A, B, C and D of the LC equipment. These four buffered solutions were then properly mixed in the different required proportions, using the multi-solvent delivery system.

Preliminary experiments revealed that the mobile phase should contain at least 60% (v/v) of MeOH, so that it could be composed with the prescribed high DMOA and SDSS concentration boundaries (15 mM) as prescribed for some runs of the design, without turbidity or precipitation of both ingredients occurring. The final preparation of each of the four solutions for the composition of the 25 different mobile phase combinations, was as follows:

Reservoir A. This contained a mixture of 80% (v/v) of MeOH and 20% (v/v) of a 0.05 M solution of potassium dihydrogen phosphate.

Reservoir B. This contained a 50 mM solution of SDSS in a mixture of 80% (v/v) of MeOH and 20% (v/v) of water.

Reservoir C. This contained a 50 mM solution of DMOA in a mixture of 80% (v/v) of MeOH and 20% (v/v) of water.

Reservoir D. This contained a 50 mM solution of potassium dihydrogen phosphate.

In each reservoir the pH had been previously adjusted to the required pH level (3.0, 4.0 or 5.0) with phosphoric acid or a 1 M sodium hydroxide solution.

The amounts (%, v/v) taken from reservoirs A, B and C were chosen to fulfil the different mobile phase combinations in the design per pH level, and reservoir D was only used to top-up the total volume of 100% (v/v).

Sample preparation

Reference solutions of the four compounds together, as well as of separate mixtures of MPHB and PPHB and of PE and CPM were prepared in a 60/40 (v/v, %) mixture of MeOH and phosphate buffer (pH 3.0, 4.0 or 5.0). Each time the concentrations were 0.05 mg ml^{-1} for MPHB and PPHB, 0.5 mg ml^{-1} for PE and 0.25 mg ml^{-1} for CPM.

Face-centred factorial design and analysis of results

To establish with statistical reliability the influence of the four mobile phase parameters and their mutual interactions on the retention times of MPHB, PPHB, PE and CPM, a face-centred central composite design was applied. This design also allowed the estimation of second-order effects. The design was developed by Box and Wilson [2], who added a star design to a full factorial design at two levels. Figure 1 represents such a central composite design for three independent variables in a "cube diagram". For practical reasons a "face-centred" design rather than an "orthogonal" design was applied, as a minimal final MeOH concentration of 60% (v/v) in the mobile phase had always to be maintained. To examine the influence of four independent variables on one or more response variables, at least 25 different experiments were needed with this design. During these experiments, the measurements at the central level parameter combination were repeated once.

The implementation of the design, the printout of the worksheet as well as the statistical analysis of the measured response variables were supported by the graphic software 'STATGRAPHICS' version 5.0 (STSC Inc., Rockville, MD, USA). It enabled the calculation of the estimated parameters for main and second-order effects, the analysis of variance (ANOVA) tables, the standardized Pareto charts, the residuals, the predicted response variables and the response surface plots.

Practical performance of the applied central composite design

The central levels of the mobile phase parameters in the applied design were fixed at 70% (v/v) for MeOH, 9.0 mM for SDSS, 9.0 mM for DMOA and 4.0 for the pH. Similar SDSS (13 mM) and MeOH (68%, v/v) concentrations were already described by Halstead for the preparation of a mobile phase with pH 3.8 to separate phenylpropanolamine, dextromethorphan and CPM by LC [18].

To overcome solubility problems during solvent mixing, SDSS in reservoir B and DMOA in reservoir C had to be dissolved in 80% (v/v) of MeOH solutions. This had to be taken into account when each examined mobile phase combination was composed with the Multisolvent Delivery System. For instance, to prepare the central level combination, 52 volumes of MeOH solution in reservoir A were mixed with 18 volumes of SDSS solution in reservoir B, 18 volumes of DMOA solution in reservoir C and 12 volumes of buffer solution (pH 4.0) in reservoir D.

The final mobile phase parameter settings in the design are given in Table 1.

The worksheet of the design, with the coded values −1, 0 and +1, is reproduced in Table 2.

Results

For practical reasons, related to the preparation of the mobile phase by the multisolvent delivery system, the experimental measurements had to be performed per pH level starting with pH 3.0. This explains why no randomization of the experiments was applied. The response variables were the measured retention times of MPHB, PPHB, PE and CPM, recorded for each of the 25 + 1 chromatographic runs. In Table 3 their measured retention times are compiled. Consecutive measurements (n) were only performed to verify if stable retention times were recovered and to confirm that the column was well equilibrated after tuning to each run's new chromatographic conditions. This does not mean that each mentioned retention time in Table 3 should be interpreted as an average retention time of n measurements. The retention times of CPM in runs 2 and 4 (cf. Table 2) were 38.05 min and 75.5 min, respectively. These rather extreme and unpractical values

Table 1
Nominal values corresponding to −1, 0 and +1

Chromatographic parameter	Low value (−1)	Central value (0)	High value (+1)
MeOH % (v/v) (reservoir A + B + C)	60	70	80
SDSS mM (reservoir B)	3.0	9.0	15.0
DMOA mM (reservoir C)	3.0	9.0	15.0
pH	3.0	4.0	5.0

Table 2
Applied "face-centred central composite design" (coded units)

Run	MeOH (Vol. %)	SDSS (mM)	DMOA (mM)	pH
1	0	0	0	0
2	−1	−1	−1	−1
3	+1	−1	−1	−1
4	−1	+1	−1	−1
5	+1	+1	−1	−1
6	−1	−1	+1	−1
7	+1	−1	+1	−1
8	−1	+1	+1	−1
9	+1	+1	+1	−1
10	−1	−1	−1	+1
11	+1	−1	−1	+1
12	−1	+1	−1	+1
13	+1	+1	−1	+1
14	−1	−1	+1	+1
15	+1	−1	+1	+1
16	−1	+1	+1	+1
17	+1	+1	+1	+1
18	−1	0	0	0
19	+1	0	0	0
20	0	−1	0	0
21	0	+1	0	0
22	0	0	−1	0
23	0	0	+1	0
24	0	0	0	−1
25	0	0	0	+1
26	0	0	0	0

seemed unsuitable to fit in the proposed second-order regression model and were omitted in further calculations. However, if both response values are maintained in the design, the influence of the mobile phase parameter SDSS on the retention time of CPM is considered as insignificant in the calculted ANOVA table and standardized Pareto chart. This finding seems illogical from chromatographic point of view. Another anomaly which is established if both response values of CPM are included, is the poor agreement between the observed and the estimated CPM retention times, using a linear quadratic regression model. This poor agreement dramatically improves if both values are left out. A more complex regression model is probably needed to fit well both out-lying retention times of runs 2 and 4 together with the other measured retention times of CPM. We are aware, however, that simply omitting these results destroys the balance of the face-centred design, leading to a more arbitrary design.

Estimation of the individual mobile phase parameter effects

The effect of an individual mobile phase parameter on the response variable is the mean

Table 3
Measured response variables: retention times in min

Run	MPHB	PPHB	PE	CPM
1	1.45 (n = 3)	1.94 (n = 3)	1.89 (n = 4)	6.67 (n = 4)
2	1.69 (n = 2)	2.90 (n = 2)	2.42 (n = 1)	38.05* (n = 1)
3	1.38 (n = 2)	1.64 (n = 2)	1.44 (n = 2)	2.82 (n = 2)
4	1.55 (n = 3)	2.42 (n = 3)	2.86 (n = 2)	75.50* (n = 1)
5	1.36 (n = 3)	1.62 (n = 3)	1.83 (n = 3)	6.80 (n = 2)
6	1.73 (n = 2)	3.03 (n = 2)	1.70 (n = 3)	8.99 (n = 3)
7	1.38 (n = 3)	1.67 (n = 3)	1.30 (n = 3)	1.93 (n = 3)
8	1.64 (n = 2)	2.67 (n = 2)	1.93 (n = 2)	17.12 (n = 2)
9	1.36 (n = 2)	1.60 (n = 2)	1.59 (n = 4)	3.96 (n = 4)
10	1.66 (n = 3)	2.81 (n = 3)	2.37 (n = 3)	13.06 (n = 3)
11	1.34 (n = 1)	1.55 (n = 3)	1.41 (n = 1)	2.42 (n = 3)
12	1.51 (n = 2)	2.32 (n = 2)	2.79 (n = 2)	15.50 (n = 3)
13	1.33 (n = 3)	1.54 (n = 3)	1.75 (n = 3)	3.63 (n = 3)
14	1.63 (n = 3)	2.63 (n = 3)	1.65 (n = 3)	5.27 (n = 3)
15	1.35 (n = 2)	1.59 (n = 2)	1.28 (n = 3)	1.89 (n = 2)
16	1.53 (n = 2)	2.31 (n = 2)	1.93 (n = 2)	6.61 (n = 2)
17	1.36 (n = 3)	1.59 (n = 1)	1.57 (n = 1)	2.88 (n = 3)
18	1.63 (n = 2)	2.66 (n = 2)	2.24 (n = 2)	16.15 (n = 2)
19	1.34 (n = 3)	1.58 (n = 3)	1.51 (n = 3)	2.90 (n = 3)
20	1.50 (n = 2)	2.10 (n = 2)	1.62 (n = 3)	4.60 (n = 3)
21	1.42 (n = 3)	1.89 (n = 3)	2.00 (n = 3)	7.66 (n = 3)
22	1.46 (n = 2)	2.00 (n = 2)	2.28 (n = 2)	11.30 (n = 2)
23	1.43 (n = 3)	1.91 (n = 3)	1.68 (n = 3)	4.91 (n = 3)
24	1.44 (n = 2)	1.99 (n = 2)	1.74 (n = 2)	8.41 (n = 2)
25	1.40 (n = 4)	1.84 (n = 1)	1.79 (n = 1)	4.68 (n = 3)
26	1.45 (n = 1)	1.99 (n = 1)	1.94 (n = 1)	6.68 (n = 1)

*Measured values not used for calculations; n = number of consecutive measurements.

response value at its high levels (+) minus the mean response value at its low levels (−). The effects of parameter interactions are calculated by subtracting the mean response values at their positive products and the mean response values at their negative products. An important question is whether or not the calculated estimated effects are significantly different from the experimental measurement errors. An estimated effect may be considered as significant if its value is greater than twice its standard error. A detailed discussion of how this standard error may be calculated is given by Massart *et al.* [19]. The same algorithm is applied by *STATGRAPHICS*. An equivalent mathematical approach, using least-squares, is discussed by Box, Hunter and Hunter [20]. To obtain the estimates of the main parameter effects, of their interaction effects and even of their second order effects, the least-squares estimates of the regression coefficients in the multiple regression model are multiplied by 2. This is because the regression coefficients of the variables in the regression model measure the change in response, as each variable changes by one unit from 0 to +1 and not from −1 to +1. In this regression calculation the nominal values in the design matrix are substituted by the coded values −1, 0 and +1.

For each of the mobile phase parameters, a complete summary of their estimated effects on the retention times of each of the four compounds, and their standard errors are given in Tables 4, 5. The mentioned *t*-values at the foot of these tables are used for construction of the vertical line in the standardized Pareto charts, indicating the significant limit for the parameters.

Table 4
Estimated effects with their standard errors on the retention times of MPHB and PPHB

Parameter	MPHB	PPHB
A: MeOH	$-0.263 \pm 9.48 \times 10^{-3}$*	-1.041 ± 0.0296*
B: SDSS	$-0.067 \pm 9.48 \times 10^{-3}$	-0.218 ± 0.0296
C: DMOA	$0.014 \pm 9.48 \times 10^{-3}$	0.022 ± 0.0296
D: pH	$-0.047 \pm 9.48 \times 10^{-3}$	-0.151 ± 0.0296
AB	0.055 ± 0.0101	0.194 ± 0.0314
AC	-0.01 ± 0.0101	-0.011 ± 0.0314
AD	0.023 ± 0.0101	0.086 ± 0.0314
BC	0.015 ± 0.0101	0.031 ± 0.0314
BD	$2.5 \times 10^{-3} \pm 0.0101$	0.014 ± 0.0314
CD	-0.013 ± 0.0101	-0.061 ± 0.0314
AA	0.087 ± 0.0251	0.327 ± 0.0786
BB	0.037 ± 0.0251	0.077 ± 0.0786
CC	$6.76 \times 10^{-3} \pm 0.0251$	$-2.86 \times 10^{-3} \pm 0.0786$
DD	-0.043 ± 0.0251	-0.083 ± 0.0786

* "Standard error" estimated from "total error" with 11 d.f. ($t = 2.20156$).

Table 5
Estimated effects with their standard erors on the retention times of PE and CPM

Parameter	PE	CPM
A: MeOH	-0.69 ± 0.0205*	-11.219 ± 0.600†
B: SDSS	0.34 ± 0.0205	3.322 ± 0.513
C: DMOA	-0.502 ± 0.0205	-5.497 ± 0.600
D: pH	-0.03 ± 0.0205	-4.621 ± 0.600
AB	$-7.5 \times 10^{-3} \pm 0.0217$	-1.302 ± 0.55
AC	0.318 ± 0.0217	4.133 ± 0.652
AD	$2.5 \times 10^{-3} \pm 0.0217$	3.560 ± 0.652
BC	-0.063 ± 0.0217	-0.232 ± 0.55
BD	$-2.5 \times 10^{-3} \pm 0.0217$	-1.859 ± 0.55
CD	0.018 ± 0.0217	0.895 ± 0.652
AA	0.029 ± 0.0542	4.782 ± 1.24
BB	-0.101 ± 0.0542	-2.008 ± 1.24
CC	0.239 ± 0.0542	1.942 ± 1.24
DD	-0.191 ± 0.0542	-1.178 ± 1.24

* "Standard error" estimated from "total error" with 11 d.f. ($t = 2.20156$).
† "Standard error" estimated from "total error" with 9 d.f. ($t = 2.26277$).

ANOVA tables

Estimating the effects of the mobile phase parameters and their standard errors enables the parameter effects on the response variables to be distinguished as being significant or not. ANOVA, however, separates the total variation of the experimental measurements into sections. One represents experimental error and estimates the variance it introduces, while the others can be associated with the separate parameters studied and can be presented as variance estimates, which are compared with the error variance. The variance ratios or *F*-ratios are compared with critical *F*-values. For each parameter or parameter interaction, the ANOVA tables include the calculation of the "sum of squares", the "degrees of freedom" (d.f.) and the "mean square". As a central composite design was applied, the total experimental error could be estimated and *F*-ratios and significance levels, expressed as *P*-values could be calculated.

The ANOVA tables for MPHB, PPHB, PE and CPM are reproduced in Tables 6–9.

Standardized Pareto charts

A standardized Pareto chart consists of bars, of which each length is proportional to the absolute value of the estimated parameter (or parameter interaction) effect, divided by its standard error. The bars are displayed in size order of the effects. The chart includes a vertical line at a critical *t*-value, mentioned in Tables 4, 5, for $\alpha = 0.05$.

Parameter effects for which the bars are smaller than this critical *t*-value line are considered as not significant and not affecting the response variables. Standardized Pareto charts for MPHB, PPHB, PE and CPM are depicted in Figs 2–5.

Table 6
ANOVA table for retention times of MPHB

Effect	Sum of squares	d.f.	Mean square	*F*-ratio	*P*-value
A: MeOH	0.312	1	0.312	771.13	0.0000
B: SDSS	0.02	1	0.02	49.42	0.0000
C: DMOA	0.0009	1	0.0009	2.32	0.156
D: pH	0.0098	1	0.0098	24.22	0.0005
AB	0.0121	1	0.0121	29.90	0.0002
AC	0.0004	1	0.0004	0.99	0.352
AD	0.002	1	0.002	5.00	0.047
BC	0.0009	1	0.0009	2.22	0.164
BD	0.000025	1	0.000025	0.06	0.811
CD	0.0006	1	0.0006	1.54	0.24
AA	0.0048	1	0.0048	11.91	0.0054
BB	0.0009	1	0.0009	2.14	0.172
CC	0.00003	1	0.00003	0.07	0.796
DD	0.0012	1	0.0012	2.96	0.113
Total error	0.00445	11	0.0004	—	—

Total (corr.) 0.3754, 25 d.f.

Table 7
ANOVA table for retention times of PPHB

Effect	Sum of squares	d.f.	Mean square	*F*-ratio	*P*-value
A: MeOH	4.8776	1	4.8776	1234.23	0.0000
B: SDSS	0.2134	1	0.2134	54.00	0.0000
C: DMOA	0.0022	1	0.0022	0.56	0.477
D: pH	0.1027	1	0.1027	26.00	0.0003
AB	0.1501	1	0.1501	38.00	0.0001
AC	0.0005	1	0.0005	0.13	0.731
AD	0.0297	1	0.0297	7.53	0.019
BC	0.0039	1	0.0039	0.99	0.352
BD	0.0007	1	0.0007	0.19	0.675
CD	0.015	1	0.015	3.80	0.077
AA	0.0685	1	0.0685	17.34	0.0016
BB	0.0038	1	0.0038	0.96	0.358
CC	0.000005	1	0.000005	0.00	0.972
DD	0.0044	1	0.0044	1.11	0.314
Total error	0.0435	11	0.0039	—	—

Total (corr.) 5.5893, 25 d.f.

Table 8
ANOVA table for retention times of PE

Effect	Sum of squares	d.f.	Mean square	*F*-ratio	*P*-value
A: MeOH	2.1424	1	2.1424	1137.31	0.0000
B: SDSS	0.5202	1	0.5202	276.15	0.0000
C: DMOA	1.135	1	1.135	602.52	0.0000
D: pH	0.00405	1	0.00405	2.15	0.171
AB	0.00022	1	0.00022	0.12	0.74
AC	0.4032	1	0.4032	214.05	0.0000
AD	0.000025	1	0.000025	0.01	0.912
BC	0.0156	1	0.0156	8.29	0.015
BD	0.000025	1	0.000025	0.01	0.912
CD	0.0012	1	0.0012	0.65	0.45
AA	0.00054	1	0.00054	0.28	0.61
BB	0.0065	1	0.0065	3.47	0.09
CC	0.0365	1	0.0365	19.41	0.0011
DD	0.0234	1	0.0234	12.41	0.005
Total error	0.0207	11	0.0019	—	—

Total (corr.) 4.2956, 25 d.f.

Table 9
ANOVA table for retention times of CPM

Effect	Sum of squares	d.f.	Mean square	*F*-ratio	*P*-value
A: MeOH	340.8125	1	340.8125	350.15	0.0000
B: SDSS	40.8227	1	40.8227	41.94	0.0001
C: DMOA	81.8151	1	81.8151	84.06	0.0000
D: pH	57.8275	1	57.8275	59.41	0.0000
AB	5.4527	1	5.4527	5.60	0.042
AC	39.1585	1	39.1585	40.23	0.0001
AD	29.0609	1	29.0609	29.86	0.0004
BC	0.1729	1	0.1729	0.18	0.688
BD	11.1228	1	11.1228	11.43	0.008
CD	1.8376	1	1.8376	1.89	0.203
AA	14.4804	1	14.4804	14.88	0.004
BB	2.5533	1	2.5533	2.62	0.14
CC	2.3881	1	2.3881	2.45	0.152
DD	0.8787	1	0.8787	0.90	0.377
Total error	8.76	9	0.973	—	—

Total (corr.) 483.4837, 23 d.f.

Discussion

From Tables 3–8 and Figs 2–5, it can be established which mobile phase parameters and parameter interactions significantly influence the retention behaviour of MPHB, PPHB, PE and CPM. As could be expected, the retention times of all four of the compounds are dramatically modified by the MeOH concentration in the mobile phase. Increase of the modifier concentration always decreases their retention times. However, the estimated effects of the three other mobile phase parameters on the retention behaviour of each of the four compounds, are dissimilar and even opposite.

MPHB and PPHB are both equally but rather weakly influenced by other mobile phase parameter modifications. An increase of the SDSS concentration and of the pH slightly lowers their retention times. One significant parameter interaction (between MeOH and SDSS) is established. As there is no basic nitrogen function in their chemical structures (cf. PE and CPM), there will be no competition with DMOA during the chromatographic process. Indeed, no significant effect of DMOA on the retention times of MPHB and PPHB can be estimated.

On the contrary, PE competes with DMOA. An increase of the DMOA concentration clearly lowers the retention of PE. SDSS on the other hand forms an ion-pair with PE; an increase of the SDSS concentration enhances its retention. Obviously, DMOA and SDSS have an opposite effect here. There is no effect of the pH of the mobile phase on the retention of PE. Indeed, its pK_a values largely exceeded the examined mobile phase pH interval. There seems to be a clear interaction between MeOH

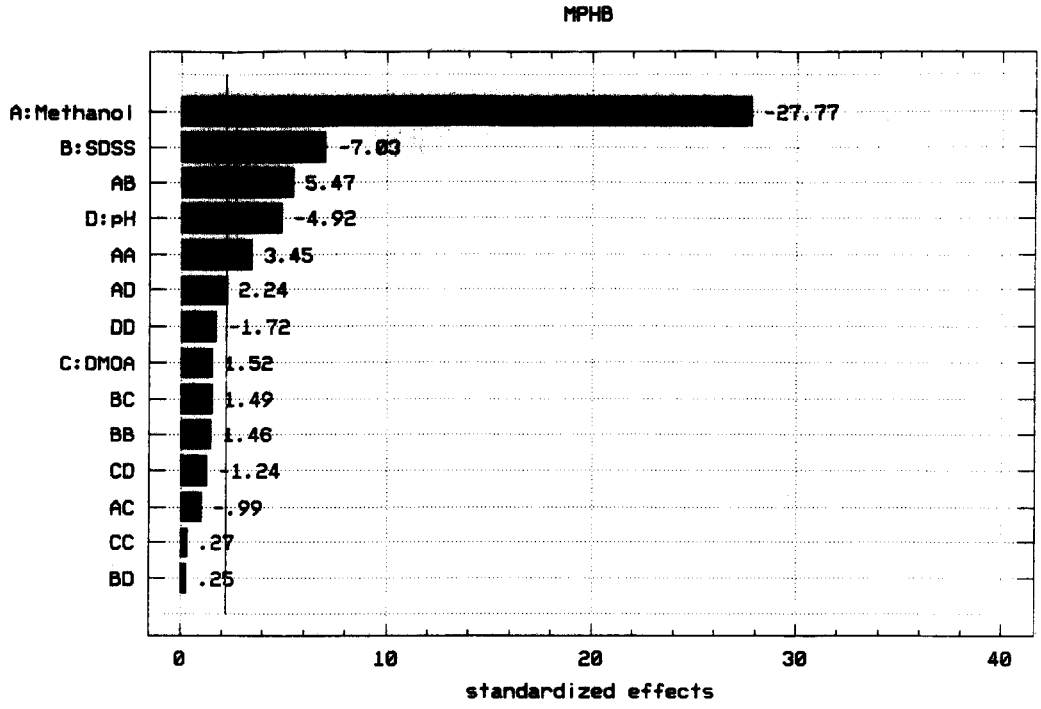

Figure 2
Standardized Pareto chart, representing the estimated effects of mobile phase parameters and parameter interactions on the retention times of MPHB.

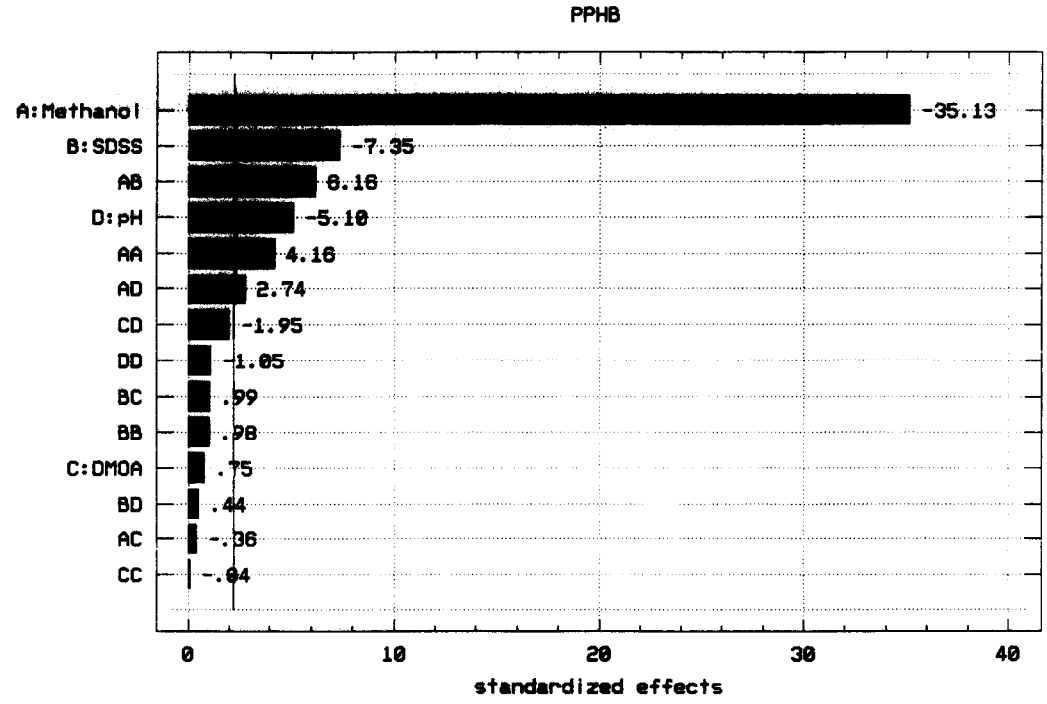

Figure 3
Standardized Pareto chart, representing the estimated effects of mobile phase parameters and parameter interactions on the retention times of PPHB.

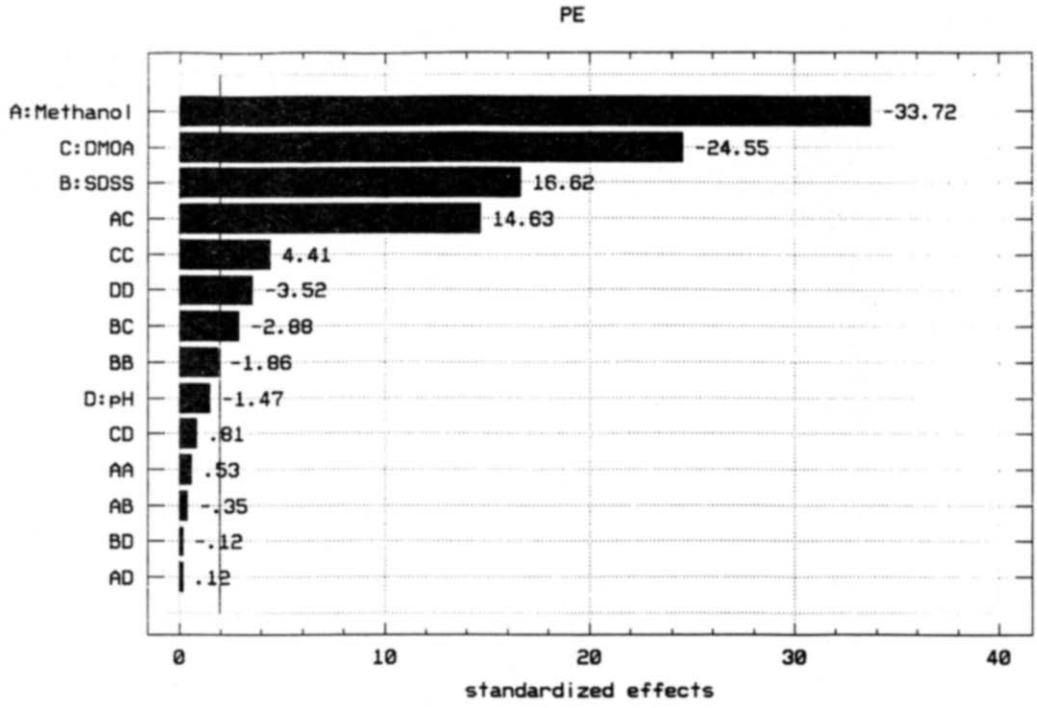

Figure 4
Standardized Pareto chart, representing the estimated effects of mobile phase parameters and parameter interactions on the retention times of PE.

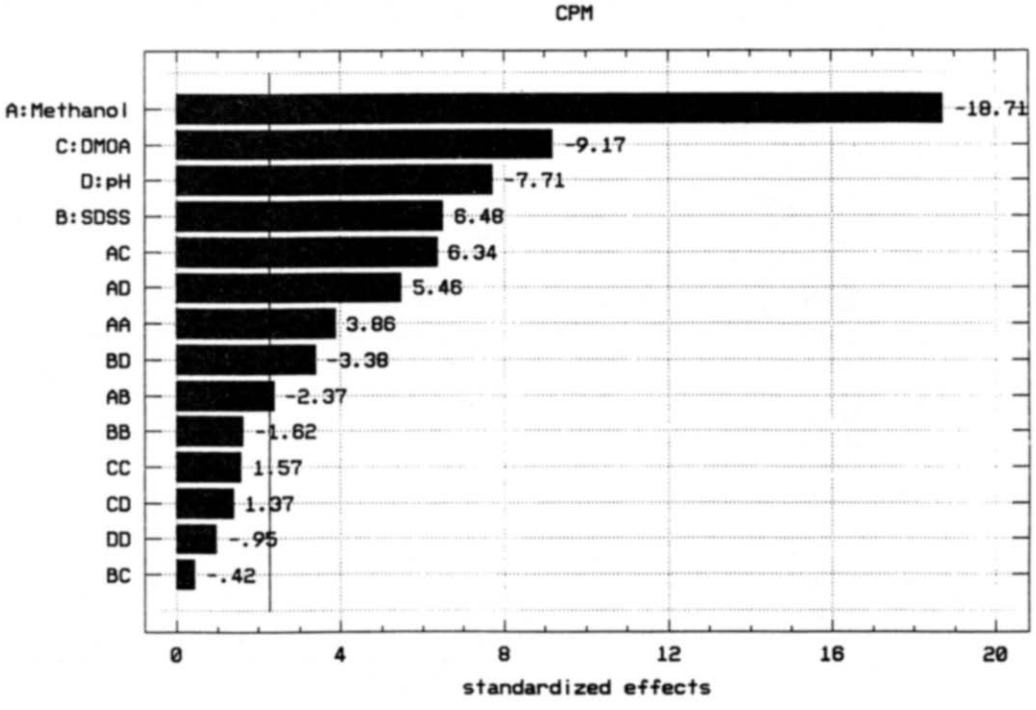

Figure 5
Standardized Pareto chart, representing the estimated effects of mobile phase parameters and parameter interactions on the retention times of CPM.

and DMOA. The competitive effect of DMOA with PE is stronger at a lower MeOH concentration than at the higher one.

The retention of CPM is also influenced by competition with the DMOA in the mobile phase. An increase of the DMOA concentration also reduces its retention time. The effect of SDSS is the same as for PE, due to ion-pairing. Unlike PE, the pH of the mobile phase considerably influences the chromatographic behaviour of CPM. At the high pH level of the mobile phase (pH 5.0), the retention time of CPM may be less than half the one measured at the low pH level (pH 3.0), the other parameters kept equal (compare runs 8 and 16).

A plausible explanation may be that below a pH of 4.0 (= pk_{a1} of CPM) of the mobile phase, both nitrogen atoms of CPM are protonated. This implies that they are both able to form an ion-pair with SDSS. Consequently much longer retention times are measured. This hypothesis seems to be confirmed by the interaction between the pH of the mobile phase and its SDSS concentration. At a lower pH-level, SDSS affects much more the retention of CPM than at the higher pH-level.

Other important interactions, such as between MeOH and DMOA (cf. PE) and between MeOH and the pH, also seem to influence the chromatographic behaviour of CPM. The effect of pH modification is more important at lower than at higher MeOH concentrations.

Summarizing we can argue that in a reversed-phase LC system with a mobile phase, composed by MeOH as modifier, DMOA as competitive base, SDSS as ion-pairing reagent, and having a pH between 3.0 and 5.0:

(1) the retention of MPHB and PPHB is predominantly determined by the MeOH concentration in the mobile phase.

(2) the retention of PE is also determined by the DMOA and/or SDSS concentration and not by the pH of the mobile phase.

(3) the retention of CPM is the only one which is influenced by each of the four mobile phase parameters.

(4) several specific mobile phase parameter interactions are concerned in the LC separation of these four compounds.

Regression modelling

Multiple regression models express the mathematical relationship between the experimentally measured response variables and the independent system variables or system parameters. Linear quadratic regression equations can be computed, if a full factorial design at three levels is applied. However, a central composite design, which is a combined design of a full factorial design at two levels and a star design with a central level, also enables to form second-order regression equations with less experiments. At least 25 experiments are needed for a central composite design, when four different parameters are concerned in an optimization procedure. In such a design, the response variables are modelled by the general equation:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_4X_4 + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{14}X_1X_4 + B_{23}X_2X_3 + B_{24}X_2X_4 + B_{34}X_3X_4 + B_{11}X_1^2 + B_{22}X_2^2 + B_{33}X_3^2 + B_{44}X_4^2 + \text{error}$$

where Y is the measured retention time for each compound, B_0 is the intercept, B_{1-4} are the slopes in the directions X_{1-4}, $B_{12} \ldots B_{34}$ are the interaction coefficients, $B_{11} \ldots B_{44}$ are the curvature coefficients, X_{1-4} are the values of the independent chromatographic variables and "error" is the difference between the experimental and the estimated (or predicted) retention time. There may be a concern that this full quadratic regression model has 15 parameters to be estimated, while it is fitted to only 26 experimental runs. However, the residual errors in the ANOVA tables (Tables 6–9) have sufficient d.f. to allay this concern. Parameters or parameter interactions without significant effect on the response variables, as estimated in the ANOVA tables or in the Pareto charts, are excluded from the regression equation.

For each compound, a summary of its regression equation characteristics, including the significant regression coefficients, their standard errors, t-values and significance levels (P values), is given in Table 10. Their fitted retention times at each run of the design, the residuals and the standardized residuals are compiled in Table 11. In this table, runs with deviating residuals of more than 3σ, are indicated by an asterisk. The fair agreement between observed and predicted retention times for each compound is illustrated in Figs 6–9, and may be expressed by the average relative deviation between predicted and

Table 10
Regression equation characteristics

Mobile phase parameter	Regression coeff.	Standard error	*t*-value	*P*-value
MPHB				
Intercept	5.2426	0.4766	10.9993	0.0000
A: MeOH	−0.0827	0.0131	−6.2878	0.0000
B: SDSS	−0.0376	0.0064	−5.8849	0.0000
D: pH	−0.1021	0.0384	−2.6601	0.015
AB	0.0005	0.00009	5.0616	0.0001
AD	0.0011	0.0005	2.0706	0.052
AA	0.0004	0.00009	4.7076	0.0002
PPHB				
Intercept	16.1725	1.353	11.9534	0.0000
A: MeOH	−0.309	0.0373	−8.2815	0.0000
B: SDSS	−0.1312	0.0182	−7.2250	0.0000
D: pH	−0.3774	0.109	−3.4649	0.003
AB	0.0016	0.00026	6.2816	0.0000
AD	0.0043	0.0015	2.7963	0.012
AA	0.0016	0.00026	6.1357	0.0000
PE				
Intercept	5.999	0.201	29.8397	0.0000
A: MeOH	−0.0583	0.0028	−20.9328	0.0000
B: SDSS	0.0361	0.0046	7.7853	0.0000
C: DMOA	−0.2192	0.0189	−11.5867	0.0000
AC	0.0026	0.0003	10.0964	0.0000
BC	−0.0009	0.0004	−1.9875	0.06
CPM				
Intercept	199.7966	28.8668	6.9213	0.0000
A: MeOH	−3.9881	0.7302	−5.4616	0.0001
B: SDSS	1.611	0.4347	3.7059	0.002
C: DMOA	−2.6653	0.3902	−6.8313	0.0000
D: pH	−12.1895	2.3823	−5.1166	0.0002
AB	−0.0105	0.0049	−2.1304	0.05
AC	0.0318	0.0053	5.9712	0.0000
AD	0.1625	0.032	5.0770	0.0002
BD	−0.1512	0.0491	−3.0757	0.008
AA	0.0186	0.0048	3.8399	0.002

experimental retention times (ARD in %). This is 0.98% for MPHB, 1.82% for PPHB, 2.09% for PE and 12.95% for CPM. It can also be demonstrated using the Kolmogorov–Smirnov test, that the model residuals are normally distributed. This is a basic assumption for the validity of the models.

Response surface plots

The object of an experimental design is to discover those parameter combinations, leading to an appropriate value for a response variable or a combination of response variables. This has also been illustrated in the design of enantiomer separations using CCD with chiral HPLC [21]. In the present work, an appropriate value for each response variable results in a chromatogram, characterized by complete peak resolution, within a short time of analysis. By means of the calculated regression models, retention times for each compound can be estimated for each combination of significant mobile phase parameters. Response surface plots are three-dimensional plots, visualizing how response variables change, if two independent variables are modified within the previously fixed boundaries. Another advantage is that by means of combined response surface plots, parameter combinations can be selected, which lead to a complete chromatographic resolution. In Fig. 10 a response surface plot shows how the retention times of MPHB, PPHB and PE vary as a function of the MeOH and DMOA concentrations in the mobile phase. A pH of 5.0 and an SDSS concentration of 15 mM were kept constant. This plot shows that PE may elute as well before as after PPHB, depending on the DMOA concentration in the mobile phase. Similar plots are recovered at pH values

Table 11
Compilation of regression results

MPHB (Methylparahydroxybenzoate)				PPHB (Propylparahydroxybenzoate)			
Run	Fitted retention time	Residuals	Standardized residuals	Run	Fitted retention time	Residuals	Standardized residuals
1	1.44	0.01	0.30	1	1.96	−0.02	−0.30
2	1.71	−0.02	−1.42	2	2.96	−0.06	−1.29
3	1.37	0.01	0.36	3	1.64	0.00	0
4	1.59	−0.04	−2.85	4*	2.55	−0.13	−3.24
5	1.36	0.00	0	5	1.62	0.00	0
6	1.71	0.02	0.90	6	2.96	0.07	1.37
7	1.37	0.01	0.36	7	1.64	0.03	0.55
8	1.59	0.05	3.34	8	2.55	0.12	2.75
9	1.36	0.00	0	9	1.62	−0.02	−0.36
10	1.64	0.02	0.84	10	2.73	0.08	1.78
11	1.35	−0.01	−0.52	11	1.58	−0.03	−0.54
12	1.52	−0.01	−0.76	12	2.31	0.01	0.11
13	1.34	−0.01	−0.43	13	1.55	−0.01	−0.26
14	1.64	−0.01	−0.86	14	2.73	−0.1	−0.29
15	1.35	0.00	0	15	1.58	0.01	0.25
16	1.52	0.01	0.36	16	2.31	0.00	0
17	1.34	0.02	1.30	17	1.55	0.04	0.73
18	1.62	0.01	0.53	18	2.64	0.02	0.35
19	1.36	−0.02	−0.75	19	1.60	−0.02	−0.30
20	1.48	0.02	1.18	20	2.07	0.03	0.60
21	1.41	0.01	0.48	21	1.85	0.04	0.73
22	1.44	0.02	0.79	22	1.96	0.04	0.73
23	1.44	−0.01	−0.67	23	1.96	−0.05	−0.82
24	1.47	−0.03	−1.41	24	2.03	−0.04	−0.76
25	1.42	−0.02	−1.04	25	1.88	−0.04	−0.74
26	1.44	0.01	0.30	26	1.96	0.03	0.55

PE (Phenylephrine hydrochloride)				CPM (Chlorphenamine maleate)			
Run	Fitted retention time	Residuals	Standardized residuals	Run	Fitted retention time	Residuals	Standardized residuals
1	1.87	0.02	0.38	1	6.86	−0.19	−0.19
2	2.42	0.00	0	2	—	—	—
3	1.41	0.03	0.54	3	2.58	0.24	0.32
4	2.82	0.04	0.73	4	—	—	—
5	1.81	0.02	0.30	5	6.41	0.39	0.51
6	1.66	0.04	0.73	6	10.29	−1.30	−2.70
7	1.29	0.01	0.20	7	1.18	0.75	1.03
8	1.94	−0.01	−0.18	8	16.64	0.48	0.81
9	1.57	0.02	0.43	9	5.02	−1.06	−1.50
10	2.42	−0.05	−0.96	10	13.55	−0.49	−0.73
11	1.41	0.00	0	11	3.29	−0.87	−1.22
12	2.82	−0.03	−0.61	12	16.28	−0.78	−1.18
13	1.81	−0.06	−1.27	13	3.50	0.13	0.17
14	1.66	−0.01	−0.23	14	4.51	0.76	1.19
15	1.29	−0.01	−0.18	15	1.90	−0.01	−0.01
16	1.94	−0.01	−0.18	16	7.23	−0.62	−0.96
17	1.57	0.00	0	17	2.11	0.77	1.07
18	2.21	0.03	0.48	18	14.19	1.96	2.31
19	1.52	−0.01	−0.18	19	3.25	−0.35	−0.33
20	1.70	−0.08	−1.29	20	5.22	−0.62	−0.63
21	2.04	−0.04	−0.59	21	8.50	−0.84	−0.86
22*	2.12	0.16	3.35	22	9.47	1.83	2.11
23	1.61	0.07	1.10	23	4.25	0.66	0.67
24	1.87	−0.13	−2.23	24	9.04	−0.63	−0.64
25	1.87	−0.08	−1.25	25	4.69	−0.01	−0.01
26	1.87	0.07	1.22	26	6.86	−0.18	−0.18

*Denotes "residuals" greater than 3σ.

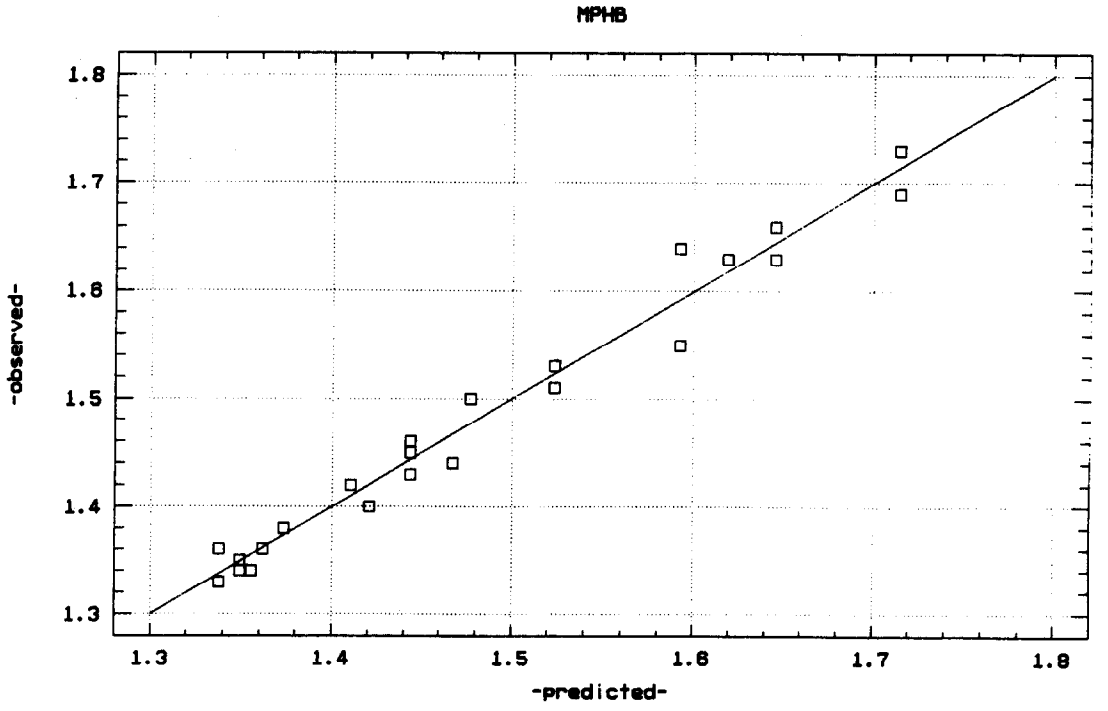

Figure 6
Diagnostic plot, displaying the observed retention times of MPHB vs the retention times, predicted from its regression model.

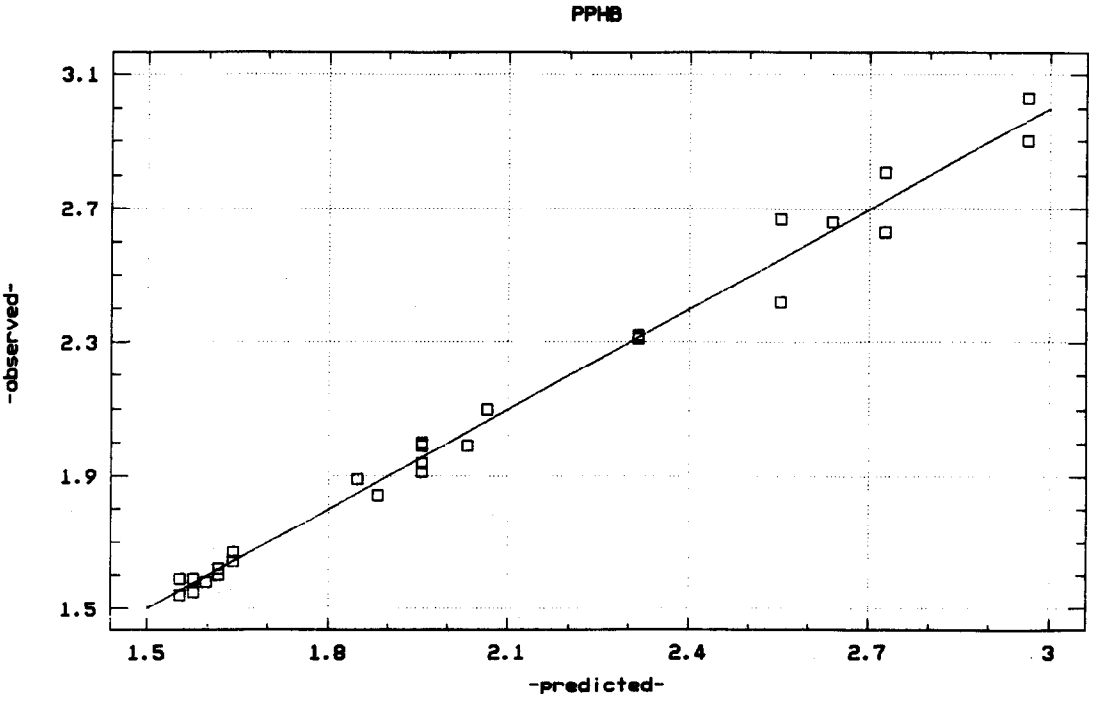

Figure 7
Diagnostic plot, displaying the observed retention times of PPHB vs the retention times, predicted from its regression model.

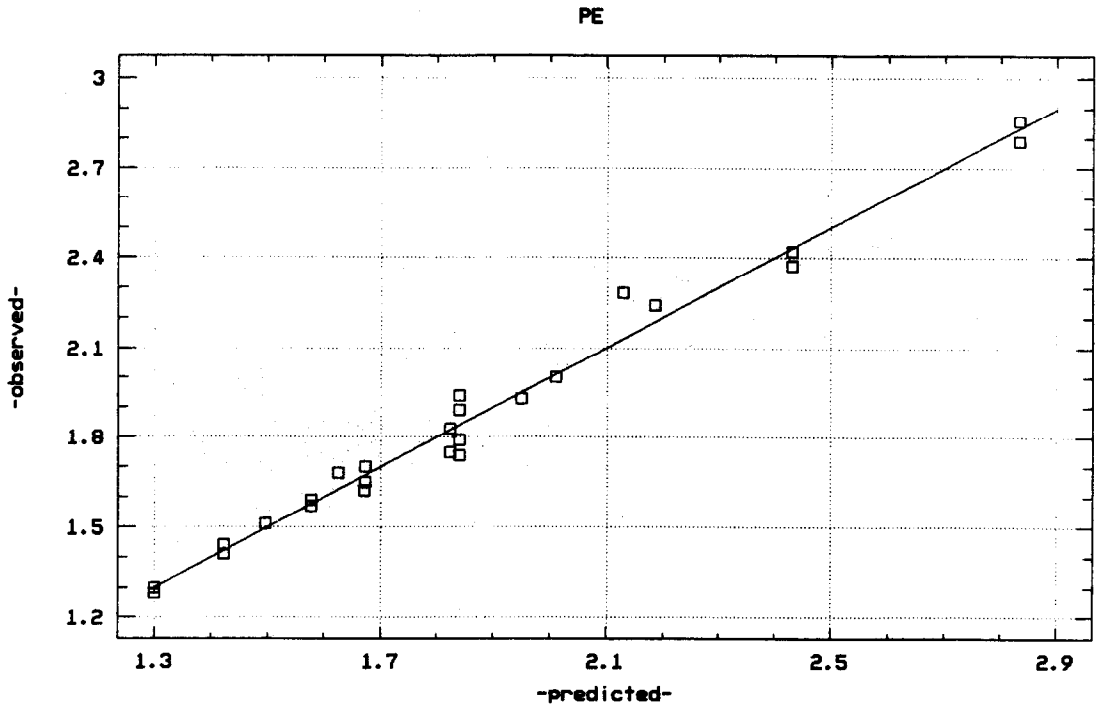

Figure 8
Diagnostic plot, displaying the observed retention times of PE vs the retention times, predicted from its regression model.

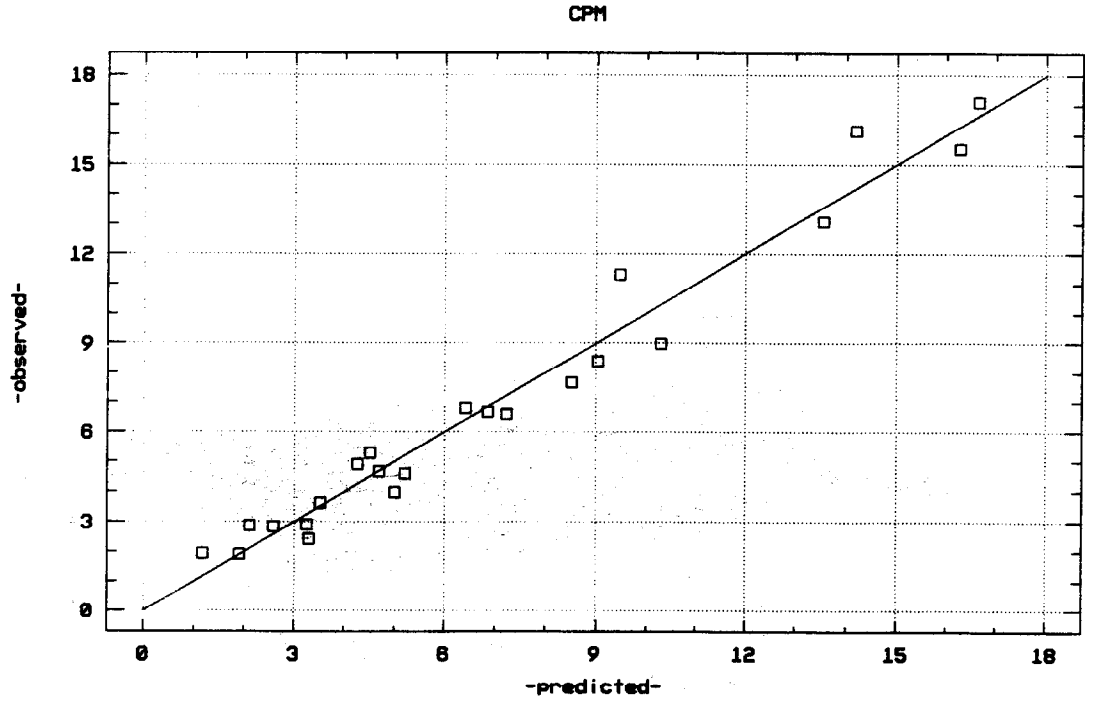

Figure 9
Diagnostic plot, displaying the observed retention times of CPM vs the retention times, predicted from its regression model.

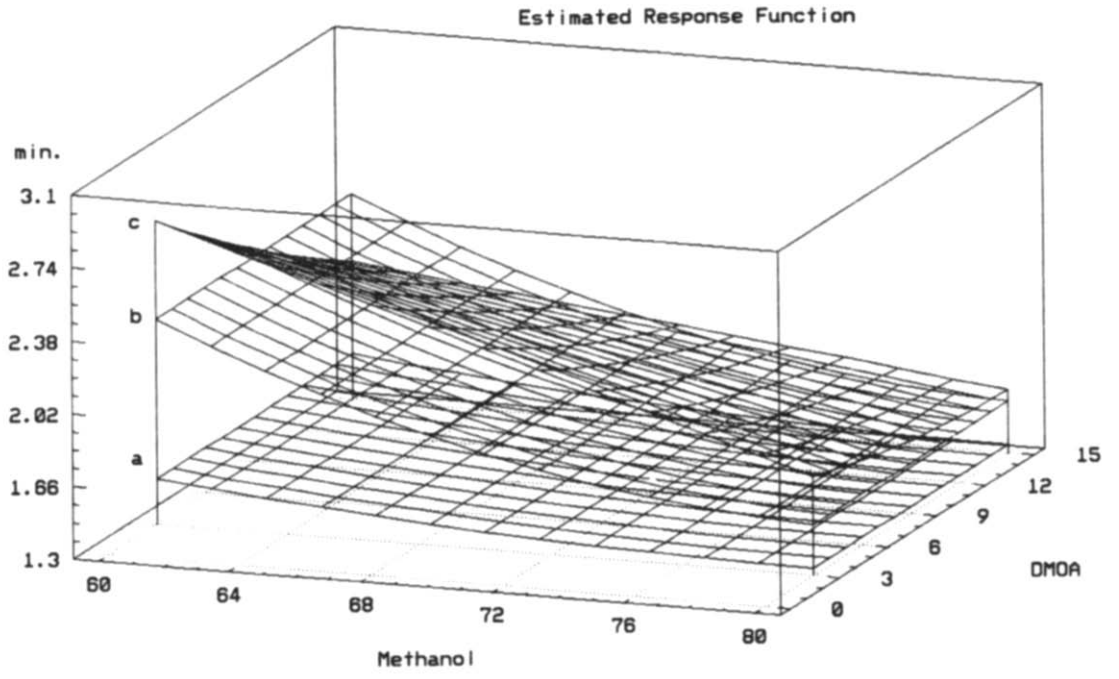

Figure 10
Estimated response surface plots for MPHB[a], PPHB[b] and PE[c], representing their retention times as a function of MeOH (v/v, %) and DMOA (mM) in the mobile phase (pH = 5.0; SDSS = 15 mM).

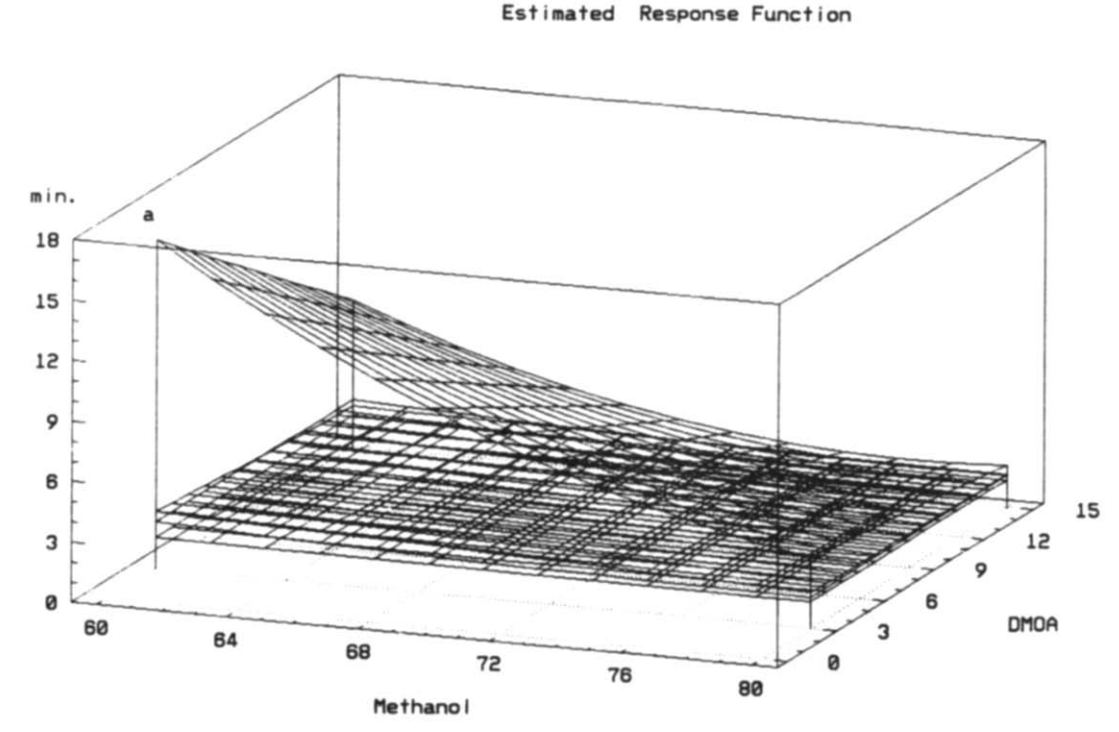

Figure 11
Estimated response surface plots for CPM[a] vs MPHB, PPHB and PE, representing its retention time as a function of MeOH (v/v, %) and DMOA (mM) in the mobile phase (pH = 5.0; SDSS = 15 mM).

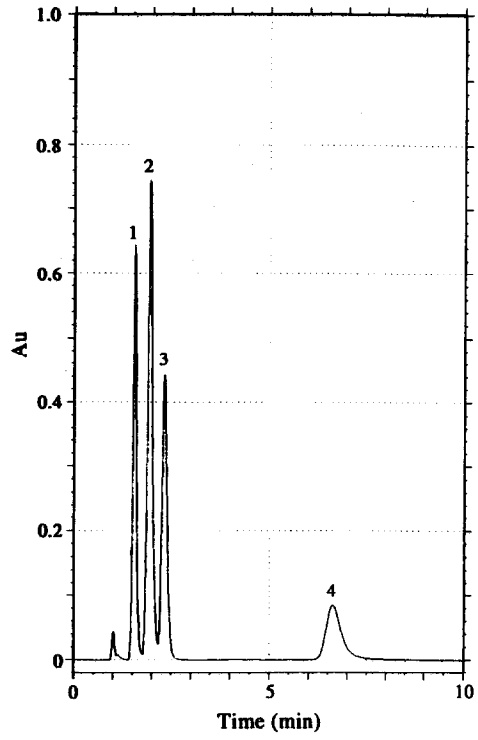

Figure 12
Liquid chromatogram recorded with a mobile phase containing 15 mM DMOA, 15 mM SDSS, 60 (v/v, %) MeOH at a pH = 5.0 (1 = MPHB, 2 = PE, 3 = PPHB, 4 = CPM). Detection at 273 nm.

of 3.0 and 4.0. Figure 11 shows a response surface plot, representing the retention times of CPM in comparison to MPHB, PPHB and PE as a function of the MeOH and DMOA concentrations in the mobile phase. The pH is fixed at 5.0 and the SDSS concentration at 15 mM. In Fig. 12 a chromatogram, corresponding to a location in the response of both surface plots, where the four response surfaces were low (short time) but distinguishable (separated peaks), shows the four peaks separated within eight minutes time of analysis. The mobile phase parameters are 60% (v/v) MeOH, 15 mM SDSS, 15 mM DMOA and pH 5.0. Other mobile phase combinations that also result in chromatograms with well separated peaks, may be selected from both surface plots. This is the case when the mobile phase parameters are 60% (v/v) MeOH, 15 mM SDSS, 3 mM DMOA and pH 5.0, where PE elutes after PPHB on the resulting chromatogram.

Conclusions

By applying a face-centred central composite design, the effects of four mobile phase parameters of an ion-pair reversed-phase LC system, on the individual chromatographic behaviour of MPHB, PPHB, PE and CPM are measured. Using a multisolvent delivery system, the different prescribed mobile phase combinations can easily be composed. It is revealed that MeOH as organic modifier is the most dominant parameter, within its examined concentration interval. Its estimated effect on the retention times is the most important for each compound. The effect of the pH of the mobile phase is highly influential for the retention of CPM. On the contrary, the chromatographic behaviour of MPHB, PPHB and PE is almost insensitive to fluctuations in pH within the range 3.0–5.0. The retention times of PE and CPM are also clearly influenced by the DMOA and SDSS concentrations in the mobile phase. The effects of both parameters, however, are opposite. Some important interactions between mobile phase parameters are discovered. Concerning the chromatographic behaviour of CPM, a remarkable interaction seems to exist between the SDSS concentration and the pH of the mobile phase. The effect of SDSS on the retention time of CPM is stronger at pH 3.0 than at pH 5.0.

Regression models with the significant chromatographic parameters and parameter interactions and the retention times as response variables, enable retention time calculation of the four compounds with good statistical reliability. From these regression models, three-dimensional response surface plots can be constructed, which can help to select those parameter combinations, that ensure chromatograms with well resolved peaks, within a reasonable time of analysis.

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