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Half-fraction and full factorial designs versus central composite design for retention modelling in reversed-phase ion-pair liquid chromatography

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Abstract

In a previous paper (J. O. De Beer, C. V. Vandenbroucke and D. L. Massart, J. Pharm. Biomed. Anal., 12, (1994) 1379–1396) liquid chromatographic (LC) retention modelling of the cough-syrup compounds methyl *para*-hydroxybenzoate (MPHB) and propyl *para*-hydroxybenzoate (PPHB), phenylephrine hydrochloride (PE) and chlorphenamine maleate (CPM) was studied using a face-centred central composite design. It is examined whether smaller half-fractional and full factorial designs with fewer experiments tend to reliably predict retention times of the latter compounds as well. Simplified regression modelling, however, neglecting more first-order and interactive effects and disregarding pure second-order effects, has to be set up. These smaller designs finally satisfy the prediction of the retention of MPHB, PPHB and PE also. Retention prediction of CPM is much less accurate. CPM has a pK_a value of 4.0, which is encompassed by the examined mobile phase pH limits 3.0 and 5.0. Since the largest retention shifts occur near the pK_a value, retention prediction in this area becomes more complex. CPM retention modelling from a full factorial design is useful if the mobile phase pH is fixed at 5.0 for methanol as well as for acetonitrile as organic modifers. The full factorial design, applied with acetonitrile as organic modifer, enables the selection of suitable LC parameter combinations for fast and complete separation of the four compounds in cough-syrup analysis.

Keywords: Half-fraction factorial design; Full-factorial design; Ion-pair reversed-phase liquid chromatography; Regression modelling; Response surface plots; Cough-syrup analysis

1. Introduction

Fast isocratic liquid chromatographic (LC) analysis of the cough-syrup ingredients methyl *para*-hydroxybenzoate (MPHB) and propyl *para*-

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hydroxybenzoate (PPHB) as preservatives with concentrations of 0.1–0.2% in the ratio 7:3, phenylephrine hydrochloride (PE) as vasoconstrictor at a concentration of 5 mg per 5 ml and chlorphenamine maleate (CPM) as H_1-antihistamine at a concentration of 2 mg per 5 ml is most satisfactory for quality control purposes.

The efficiency of a face-centred central composite design for modelling the retention times of four cough-syrup ingredients in an ion-pair reversed phase LC system with photodiode-array detection at 273 nm was proved in a previous paper [1]. Like a full factorial design at three levels, a central composite design enables the modelling of retention with full second-order regression equations with fewer experiments [2]. The four LC variables were the pH of the mobile phase, and the concentrations of methanol (MeOH) as organic modifier, sodium dioctyl sulfosuccinate (SDSS) as ion-pair reagent and of dimethyloctylamine (DMOA) as competitive base.

Multivariate regression models could predict the retention times of MPHB, PPHB, PE and CPM with good statistical reliability. Response surfaces helped to select those LC parameter combinations that led to chromatograms with well-resolved peaks within a reasonable time of analysis. Nevertheless, a set of 26 different LC runs had to be executed.

In this study, it is examined to what extent a half-fraction and a full factorial design with fewer experiments (10–18 runs) may lead to similar estimates of the main and interaction effects of the same LC variables on the retention of the same compounds. Therefore, it is surveyed whether simplified regression models can predict the retention times of MPHB, PPHB, PE and CPM with the same statistical reliability as the more complex regression models derived for the face-centred central composite design.

Further, it is examined whether the retention times of the same compounds can be reliably predicted with full factorial designs (9–11 runs), the mobile phase pH being fixed at 5.0. MeOH as well as acetonitrile (AcN) as organic modifiers are compared. By means of new experiments it is verified whether the factorial design, applied to the LC system with AcN as organic modifier and with a fixed pH of 5.0, can model the retention times of MPHB, PPHB, PE and CPM and can reveal suitable LC parameter combinations for rapid analysis of the four compounds in the cough syrup. By holding the mobile phase pH constant, three factorial designs are created. As a consequence, the retention modelling of, for example, CPM, of which the retention is considerably influenced by mobile phase pH fluctuations from 3.0 to 5.0 as one of its pK_a values is 4.0 [3], is simplified. Moreover, important interactions of pH with other variables are eliminated. The utility of several factorial designs in predicting chromatographic retention has been shown by Lindberg et al. [4], Cotton and Down [5], Wester et al. [6], Hu and Massart [7], Mulholland and Waterhouse [8,9], Otto and Wegscheider [10] and Mulholland et al. [11]. Van Leeuwen et al. [12,13] describe a ruggedness test in LC method validation based on factorial designs.

2. Experimental

2.1. Apparatus, chemicals, mobile phase composition and sample preparation

The LC runs in the designs were performed by means of the model 600 multisolvent delivery system of the LC apparatus (Millipore-Waters, Milford, MA, USA), combined with a Waters 990 photodiode-array detector, which was linked to a Nec Powermate 386/33i data station. Four separate solutions, each containing a component of the mobile phase, were prepared and stored in the four solvent reservoirs A, B, C and D of the LC equipment. The preparation of each of the four solutions was as follows.

Reservoir A contained a mixture of 80% (v/v) organic modifier, which was MeOH or AcN (Lab-Scan Ltd, Dublin, Ireland), according to the design applied, and 20% (v/v) of a 50 mM solution of p.a. potassium dihydrogen phosphate (Merck, Darmstadt, Germany).

Reservoir B contained a 50 mM solution of SDSS (Aldrich Chemie, Brussels, Belgium) in a mixture of 80% (v/v) organic modifier and 20% (v/v) water.

Reservoir C contained a 50 mM solution of DMOA (Janssen Chimica, Beerse, Belgium) in a mixture of 80% (v/v) organic modifier and 20% (v/v) water.

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

The apparent pH* of the solutions in each reservoir was previously adjusted to 3.0, 4.0 or 5.0 with p.a. phosphoric acid (Merck) or a 1 M p.a. sodium hydroxide solution (Merck), according to the prescribed pH value for an individual run of the design.

The volumes taken from each reservoir by the multisolvent delivery system of the LC equipment were chosen to fulfill the different mobile phase parameter combinations in each run of the proposed factorial design. Reservoir D was only used to top up the total volume to 100% (v/v) [1]. The 15×0.39 cm^2 i.d. LC column was a C_{18} Novapak column (Millipore–Waters) with 4 μm spherical silica. A flow rate of 0.9 ml min^{-1} was kept constant.

Reference solutions of MPHB and PPHB at a concentration of 0.05 mg ml^{-1}, and PE·HCl at a concentration of 0.5 mg ml^{-1} and CPM at a concentration of 0.25 mg ml^{-1} were prepared in a 50:50 (v/v) mixture of the organic modifier used in the design and phosphate buffer (pH* 3.0, 4.0, 5.0). With three to four consecutive injections of each solution it was verified whether stable retention times were obtained for each of the four compounds. Stable retention times confirmed a good column equilibration each time the mobile phase composition had been changed by a new combination of the four solvent reservoirs for each individual run. This does not mean that the mentioned retention times should be interpreted as average retention times from replicates. The cough syrup, composed of sugar syrup, fluid extract of *Papaver rhoeas*, vermouth extract and alcohol, was diluted 1:10 with the selected mobile phase before LC analysis.

The reference mixtures and diluted cough syrup samples were injected with a Marathon autosampler (Spark Holland, Emmen, The Netherlands) equipped with a loop of about 20 μl. Two-dimensional chromatograms were recorded by the photodiode-array detector at 273 nm. The drafts of all designs, as well as the complete statistical and regression analysis for each compound per design applied, were supported by the softwere package STATGRAPHICS version 5.0 (STSC Inc., Rockville, MD, USA).

2.2. Half-fraction and full factorial design with four LC mobile phase parameters

The two-level half-fractional and full factorial designs studied are composed of distinct LC parameter combinations (blocks) of the face-centred central composite design described elsewhere [1]. In both designs the central level LC parameter combinations are included. Tables 1 and 2 represent this half-fraction and full factorial design together with the measured retention times of MPHB, PPHM, PE and CPM as response variables corresponding to each run. The coded values -1, 0 and $+1$ correspond respectively to 60% (v/v), 70% (v/v) and 80% (v/v) for MeOH, to 3.0 mM, 9.0 mM and 15.0 mM for SDSS and DMOA and to 3.0, 4.0 and 5.0 for the pH as mobile phase variable parameters. For practical reasons, related to the composition of the mobile phase provided by the multisolvent delivery system of the LC set-up, retention times of the four solutes for each different run in these designs were measured per pH level starting with pH 3.0. This explains why no real randomization of the runs in these desigms was applied. Estimated main and interactive effects, ANOVA tables, regression models with residuals, predicted response variables, etc. for MPHB, PPHB, PE and CPM can be calculated with known retention times as response variables.

2.3. Full factorial designs with three LC mobile phase parameters (pH fixed at 5.0)*

In the three-factor full factorial design in Table 3 only three of the four LC parameters are investigated (MeOH, SDSS and DMOA concentration in the mobile phase), the mobile phase pH* being fixed at 5.0. The coded parameter values and the measured retention times of MPHB, PPHB, PE and CPM as response variables in the corresponding runs originate from the earlier studied face-centred central composite design.

Table 1
Four-factor half-fraction factorial design with retention times (in minutes) of MPHB, PPHB, PE and CPM as the corresponding response variables.

Run[a]	MeOH (Vol.%)	SDSS (mM)	DMOA (mM)	pH*	Retention times (min)			
					MPHB	PPHB	PE	CPM
1 (1)	0	0	0	0	1.45	1.94	1.89	6.67
2 (2)	−1	−1	−1	−1	1.69	2.90	2.42	–
3 (11)	1	−1	−1	1	1.34	1.55	1.41	2.42
4 (12)	−1	1	−1	1	1.51	2.32	2.79	15.50
5 (5)	1	1	−1	−1	1.36	1.62	1.83	6.80
6 (14)	−1	−1	1	1	1.63	2.63	1.65	5.27
7 (7)	1	−1	1	−1	1.38	1.67	1.30	1.93
8 (8)	−1	1	1	−1	1.64	2.67	1.93	17.12
9 (17)	1	1	1	1	1.36	1.59	1.57	2.88
10 (26)	0	0	0	0	1.45	1.99	1.94	6.68

[a] Run numbers in parentheses correspond to run numbers of the face-centred central composite design [1].

Another three-factor full factorial design with AcN as organic modifier and a constant mobile phase pH* of 5.0 was introduced and applied experimentally also. Actually, this design is a full factorial design with three variables, expanded with two supplementary variable combinations (11 runs). This factorial design with its coded values for the LC parameters and the measured retention times for MPHB, PPHB, PE and CPM for each different run of the design is reproduced in Table 4. The mobile phase parameter boundaries in this design, with their nominal values corresponding to −1, 0 and +1 are given in Table 5.

Randomization was accomplished by mixing up the order in which the individual runs of this design for retention time measurements of MPHB, PPHB, PE and CPM were carried out. Randomization offers some assurance that uncontrolled variation of all factors, other than the ones under study, will not systematically influence the results.

3. Results

3.1. Half-fraction and full factorial design with four LC mobile phase parameters

For the calculation methods of the main effects of the parameter and their interactions, we refer to the literature [14–17]. An estimated effect is considered as significant when its calculated value is greater than twice its standard error [18]. Calculation of standard errors for effects, using replicated runs as well as higher-order interactions if there are no replicates, is explained by Box et al. [16]. Twice the standard error of an estimated effect means that a confidence interval about each estimated effect, including or not the value zero, may be constructed at the 95% confidence level ($\alpha = 0.05$; Student's $t \simeq 2$). If this confidence interval contains zero, the estimated effect is not considered as significant. The results with an experimental design are further examined using the ANOVA F-test [19–21]. Parameter interactions in the half-fraction factorial design are confounded. This is not a real restriction for further evaluation, if interactions between insignigicant parameters are considered as insignificant too. For MPHB, PPHB, PE and CPM, factor and factor interaction ANOVA F (Fisher variance) ratios are expressed by their corresponding P-level values. These P-level values are reproduced in Tables 6 and 7 for the half-fraction and the full factorial design. Significant factors and interactions have P-level values less than 0.05. This means that the probability for a factor or an interaction to be significant is greater that 95%. For simple models, Student's t-test with the confidence interval for the significance of an effect is

Table 2
Four-factor full factorial design with retention times (in minutes) of MPHB, PPHB, PE and CPM as corresponding response variables.

Run[a]	MeOH	SDSS	DMOA	pH*	Retention time (min)			
					MPHB	PPHB	PE	CPM
1 (1)	0	0	0	0	1.45	1.94	1.89	6.67
2 (2)	−1	−1	−1	−1	1.69	2.90	2.42	-
3 (3)	1	−1	−1	−1	1.38	1.64	1.44	2.82
4 (4)	−1	1	−1	−1	1.55	2.42	2.86	-
5 (5)	1	1	−1	−1	1.36	1.62	1.83	6.80
6 (6)	−1	−1	1	−1	1.73	3.03	1.70	8.99
7 (7)	1	−1	1	−1	1.38	1.67	1.30	1.93
8 (8)	−1	1	1	−1	1.64	2.67	1.93	17.12
9 (9)	1	1	1	−1	1.36	1.60	1.59	3.96
10 (10)	−1	−1	−1	1	1.66	2.81	2.37	13.06
11 (11)	1	−1	−1	1	1.34	1.55	1.41	2.42
12 (12)	−1	1	−1	1	1.51	2.32	2.79	15.50
13 (13)	1	1	−1	1	1.33	1.54	1.75	3.63
14 (14)	−1	−1	1	1	1.63	2.63	1.65	5.27
15 (15)	1	−1	1	1	1.35	1.59	1.28	1.89
16 (16)	−1	1	1	1	1.53	2.31	1.93	6.61
17 (17)	1	1	1	1	1.36	1.59	1.57	2.88
18 (26)	0	0	0	0	1.45	1.99	1.94	6.68

[a] Run numbers between brackets correspond to run numbers of the face-centred central composite design [1].

equivalent to the ANOVA F-test for the adequacy of the model. However, for more complex models, the two tests are not always equivalent. The t-test can be used to test the significance of a single parameter. The ANOVA F-test is generally useful as a means of testing the significance of a set of parameters or testing the lack of fit of a multi-parameter model [15].

3.2. Full factorial designs with three LC mobile phase parameters (pH fixed at 5.0)*

Analogously, the ANOVA tables for MPHB, PPHB, PE and CPM may be calculated from both the full factorial designs with three mobile phase parameters as independent variables, with MeOH or AcN as organic modifier and the mobile phase pH* fixed at 5.0. The corresponding P-level values for the factor and factor interaction effect F-ratios are represented in Tables 8 and 9. The reason for applying these three-factor full factorial designs at a mobile phase pH* of 5.0 involves CPM, of which the pK_a values are 4.0 and 9.2 [3]. A compound's largest retention shifts occur near its pK_a value(s). Below a mobile phase pH of 4.0, both CPM nitrogen atoms are protonated. As a consequence, both nitrogen atoms may form an ion pair with SDSS, leading to a significant rise in the retention time of CPM. Increasing the mobile phase pH to 5.0 results in the protonation of only one nitrogen atom that may form an ion pair with SDSS. Therefore retention times of CPM are much shorter and are more practical for LC analysis.

4. Discussion

4.1. The half-fraction factorial design with four LC mobile phase variables.

The major advantage of a fractional factorial design is that the main effect and two-parameter interactions of a large number of independent variables can be examined with a minimum of experimental runs. The fractional factorial design

Table 3
Three-factor full factorial design with retention times (in minutes) of MPHB, PPHB, PE and CPM as corresponding response variables (pH* 5.0; organic modifier is MeOH)

Run[a]	MeOH (vol.%)	SDSS (mM)	DMOA (mM)	Retention times (min)			
				MPHB	PPHB	PE	CPM
1 (10)	−1	−1	−1	1.66	2.81	2.37	13.06
2 (11)	1	−1	−1	1.34	1.55	1.41	2.42
3 (12)	−1	1	−1	1.51	2.32	2.79	15.50
4 (13)	1	1	−1	1.33	1.54	1.75	3.63
5 (25)	0	0	0	1.40	1.84	1.79	4.68
6 (14)	−1	−1	1	1.63	2.63	1.65	5.27
7 (15)	1	−1	1	1.35	1.59	1.28	1.89
8 (16)	−1	1	1	1.53	2.31	1.93	6.61
9 (17)	1	1	1	1.36	1.59	1.57	2.88

[a] Run numbers between brackets correspond to run numbers of the face-centred central composite design [1].

applied is a half-fraction 2^4 − 1 design with resolution R IV. This means that the number of variable levels is 2 and the number of variables is 4; −1 indicates that a half-fraction design is concerned. The resolution R reflects the confounding pattern in the design. A R code IV confounds two-level interactions with two-level interactions [16].

Another advantage of a fractional factorial design is that a full factorial design can be obtained in every set of $R-1$ variables. This is especially useful when in a large set of variables only $R-1$ of them may have a significant effect on the response. The use of such a design of resolution R gives then a full factorial design plus replicates for the significant variables.

The half-fraction factorial design applied needs at least eight different retention time measurements for each of the four compounds examined. Two additional measurements with central parameter conditions were included to increase the number of degrees of freedom (d.f.) for calculation of the total error mean square. The high retention time value for CPM in the second run,

Table 4
Three-factor full factorial design with retention times (in minutes) of MPHB, PPHB, PE and CPM as corresponding response variables (pH* 5.0; organic modifer is AcN)

Run[a]	AcN (vol.%)	SDSS (mM)	DMOA (mM)	Retention time (min)			
				MPHB	PPHB	PE	CPM
1	3	0	0	1.27	1.58	1.24	1.93
2	−1	−1	−1	1.67	2.67	1.43	4.12
3	1	−1	−1	1.43	1.96	1.22	2.25
4	−1	1	−1	1.49	2.26	2.26	7.82
5	1	1	−1	1.38	1.84	1.73	4.11
6	−1	−1	1	1.60	2.51	1.31	2.62
7	1	−1	1	1.40	1.92	1.16	1.72
8	−1	1	1	1.45	2.12	1.65	3.67
9	1	1	1	1.38	1.81	1.54	2.80
10	−1	0	0	1.52	2.32	1.66	4.33
11	3	0	0	1.27	1.58	1.24	1.93

Table 5
Mobile phase parameter boundaries in the three-factor full factorial design with AcN as organic modifier and a constant mobile phase pH* of 5.0

LC parameter	Low value, −1	High value, +1	Supplementary combinations	
			1 deg	2 deg
AcN (%v/v)[a]	50	60	70 (+3)	50 (−1)
SDSS (mM)[b]	3.0	15.0	9.0 (0)	9.0 (0)
DMOA (mM)[c]	3.0	15.0	9.0 (0)	9.0 (0)

[a] Reservoir A+B+C.
[b] Reservoir B.
[c] Reservoir C.

38.05 min, was omitted as it did not even fit in a pure second-order regression model, as demonstrated earlier [1]. From Table 6, the MeOH concentration in the mobile phase is the most important LC parameter that influences the retention times of MPHB, PPHB and PE. Moreover, no other factors within the parameter space, affect the LC retention behavior of MPHB and PPHB. This is entirely what may be expected. As there is no nitrogen function in their chemical structure, no ion-pair formation with SDSS and no competition with DMOA occurs during the chromatography. Their retention as phenolic compounds is mainly determined by the solvent strength of the mobile phase. The retention of PE is influenced furthermore by competition with DMOA and by the ion-pair forming reagent SDSS in the mobile phase. Both variables, however, exhibit opposite effects: ion-pair formation enhances retention, competition lowers retention. Mobile phase pH* variations between 3.0 and 5.0 have no effect on the retention of PE with pK_as of 8.77 and 9.84 [22]. Any interaction between pH and other LC variables as, for example with SDSS is considered insignificant. As a consequence, only the parameter interaction between MeOH and DMOA is considered significant. The retention of CPM is definitely affected by each of the four mobile phase parameters and their interactions. As these interactions are conpounded, selection of the significant interactions is hampered. Mixed second-order regression modelling is therefore not possible without prior knowledge of the statistical significance of these interactions.

Table 6
ANOVA on retention times of MPHB, PPHB, PE and CPM in the four-factor half-fraction factorial design: P-values of factor and factor interaction effects

Effect	P-value			
	MPHB	PPHB	PE	CPM
A: MeOH	0.0089[a]	0.0092[a]	0.0031[a]	0.0002[a]
B: SDSS	0.2250	0.2987	0.0124[a]	0.0010[a]
C: DMOA	0.3784	0.7132	0.0056[a]	0.0307[a]
D: pH	0.1436	0.1908	0.7324	0.0244[a]
AB+CD	0.2250	0.3120	0.8178	0.0015[a]
AC+BD	0.7914	0.9824	0.014[a]	0.0031[a]
AD+BC	0.2656	0.3565	0.2518	0.0055[a]

[a] $P<0.05$; indicates significant effect.

Table 7
ANOVA on retention times of MPHB, PPHB, PE and CPM in the four-factor full factorial design: P-values of factor and factor interaction effects

Effect	P-value			
	MPHB	PPHB	PE	CPM
A: MeOH	0[a]	0[a]	0[a]	0.0002[a]
B: SDSS	0.002[a]	0.0042[a]	0[a]	0.0097[a]
C: DMOA	0.1857	0.5190	0[a]	0.0058[a]
D: pH	0.0102[a]	0.0236[a]	0.0692	0.0106[a]
AB	0.005[a]	0.0077[a]	0.7038	0.1745
AC	0.4945	0.8385	0[a]	0.0195[a]
AD	0.1427	0.1442	0.8985	0.0368[a]
BC	0.3074	0.5763	0.0122[a]	0.7865
BD	0.8615	0.8035	0.8985	0.0738
CD	0.3990	0.2812	0.3891	0.8394

[a] $P<0.05$: indicates significant effect.

Table 8
ANOVA on retention times of MPHB, PPHB, PE and CPM in the three-factor full factorial design (MeOH as organic modifier; mobile phase pH* 5.0): *P*-values of factor and factor interaction effects

Effect	*P*-value			
	MPHB	PPHB	PE	CPM
A: MeOH	0.0161[a]	0.0108[a]	0.0019[a]	0.0123[a]
B: SDSS	0.1771	0.1757	0.008[a]	0.2131
C: DMOA	0.8311	0.8276	0.004[a]	0.0324[a]
AB	0.1771	0.1823	0.6233	0.6853
AC	0.7257	0.5615	0.0088[a]	0.0434[a]
BC	0.6297	0.6999	0.2533	0.7328

[a] $P<0.05$; indicates significant effect.

Table 9
ANOVA on retention times of MPHB, PPHB, PE and CPM in the three-factor full factorial design (AcN as organic modifier; mobile phase pH* 5.0): *P*-values of factor and factor interaction effects

Effect	*P*-value			
	MPHB	PPHB	PE	CPM
A: AcN	0[a]	0.0002[a]	0.0015[a]	0.0027[a]
B: SDSS	0.001[a]	0.0103[a]	0.0005[a]	0.0063[a]
C: DMOA	0.0408[a]	0.176	0.0078[a]	0.0069[a]
AB	0.0052[a]	0.0647	0.2311	0.2835
AC	0.1639	0.3651	0.0729	0.0597
BC	0.2708	0.9019	0.0354[a]	0.0789

[a] $P<0.05$; indicates significant effect.

4.2. The full factorial design with four LC mobile phase parameters

Full factorial designs determine and estimate whether the effects of independent variables are important, and detect and quantify their interactions. From Table 7, there is statistical evidence that other parameters and interactions, with respect to the half-fraction factorial design, affect the retention times of some of the four compounds. Apart from the MeOH concentration, determining the solvent strength of the mobile phase, the SDSS concentration and the pH have an impact on the phenolic MPHB and PPHB retention. An increase of the latter three parameters, within the examined parameter space, lowers their retention. A significant interaction between MeOH and SDSS is revealed: the competitive effect of SDSS in the mobile phase on the retention times of MPHB and PPHB is more pronounced at a lower MeOH level than at a higher one. Compared to the previous half-fraction factorial design, no more information concerning the significance of other LC parameter or interactive effects on the retention of PE is supplied. A significant interaction between SDSS and DMOA, however, is discovered whereas the significant interaction between MeOH and DMOA is confirmed. The competitive effect of DMOA with PE is more pronounced at a lower MeOH level than at a higher one and is less at a lower SDSS level than at a higher one. It is worth reporting that the order of significance of the examined LC parameters on the retention of PE is similarly expressed by the half-fraction, the full factorial and the earlier examined face-centred central composite design. The two impractical retention times of CPM in this design (38.05 and 75.50 mins) were deleted. The significance of each individual LC parameter on the retention of CPM, which is revealed in the half-fraction factorial design, is confirmed. The size order of the effects, however, is altered and approximates to that calculated with the earlier studied face-centred central composite design. Two significant parameter interactions are shown, concerning the interactions between MeOH and DMOA, and between MeOH and the pH. The effects of both the pH and the DMOA concentration on the retention time of CPM are stronger at a lower MeOH level than at a higher one.

4.3. Full factorial designs with three LC mobile phase parameters (MeOH as organic modifier; mobile phase pH 5.0)*

From Table 8, acquired from the three-factor design with MeOH as organic modifier, only the significance of the MeOH concentration on the retention time of both MPHB and PPHB is evident. Analogously to the findings with the former designs, estimating four LC parameter effects, the

Table 10
Four-factor half-fraction factorial design: regression results for MPHB, PPHB and PE from significant parameters and parameter interactions

	Regression coefficients of significant variables		
	MPHB	PPHB	PE
Constant	1.481	2.088	1.873
A:MeOH	−0.1287	−0.5112	−0.335
B: SDSS	–	–	0.1675
C:DMOA	–	–	−0.25
AC	–	–	0.1575
d.f.	8	8	5

Run	Fitted values with standardized residuals					
	MPHB		PPHB		PE	
1	1.48	−0.62	2.09	−0.94	1.87	0.31
2	1.61	2.17	2.60	2.85	2.45	−0.82
3	1.35	−0.26	1.58	−0.17	1.46	−2.08
4	1.61[a]	−3.39	2.60	−2.46	2.78	0.19
5	1.35	0.16	1.58	0.28	1.80	0.97
6	1.61	0.43	2.60	0.20	1.63	0.48
7	1.35	0.59	1.58	0.62	1.28	0.63
8	1.61	0.65	2.60	0.46	1.97	−1.21
9	1.35	0.16	1.58	0.09	1.61	−1.44
10	1.48	−0.62	2.09	−0.60	1.87	1.53

[a] Result beyond 3σ.

effects of MeOH, DMOA and SDSS in the mobile phase on the retention of PE are confirmed. The significant interaction between MeOH and DMOA is demonstrated again. CPM is predominantly influenced by the MeOH and DMOA concentrations in the mobile phase. A significant interaction between both parameters is revealed. The lack of any significant effect of SDSS on the retention time of CPM within the examined parameter boundaries is curious. Its effect seems to apply only at mobile phase pH fluctuations between 3.0 and 5.0.

4.4. Full factorial designs with three LC mobile phase parameters (AcN as organic modifier; mobile phase pH 5.0)*

Table 9 demonstrates the significance of the effects of the mobile phase parameters of this particular LC system. Within the parameter space, the retention times of MPHB and PPHB are predominantly affected by the AcN (solvent strength) and SDSS concentrations of the mobile phase. An increase of the SDSS concentration lowers the retention time of both compounds, which indicates a competitive effect. For MPHB a significant interaction exists between both parameters. The retention time of PE in this LC system is mainly determined by the concentration of the ion-pairing SDSS in the mobile phase. A higher SDSS concentration results in an increase in the PE retention time. Higher concentrations of AcN and the competitive base DMOA in the mobile phase decrease the retention time of PE. A significant interactive effect is seen between SDSS and DMOA. Competition between DMOA and PE is more pronounced at a higher SDSS concentration in the mobile phase than at a lower one. The retention time of CPM is influenced by each of the three mobile phase variables, without sig-

Table 11
Four factor full factorial design: regression results for MPHB, PPHB, PE and CPM from significant parameters and parameter interactions

	Regression coefficients of significant variables			
	MPHB	PPHB	PE	CPM
Constant	1.4833	2.1011	1.8694	8.1945
A: MeOH	−0.13	−0.5181	−0.3425	−5.0932
B: SDSS	−0.0325	−0.1094	0.1675	1.4371
C: DMOA	–	–	−0.245	−2.3032
D: pH	−0.0237	−0.0756	–	−1.9769
AB	0.0275	0.0969	–	–
AC	–	–	0.1587	1.6769
AD	–	–	–	1.3907
BC	–	–	−0.0312	–
d.f.	13	13	12	9

Run	Fitted values with standardized residuals							
	MPHB		PPHB		PE		CPM	
1	1.48	−1.21	2.10	−1.77	1.87	0.53	8.19	−0.94
2	1.70	−0.28	2.90	0	2.42	0	–	–
3	1.38	0	1.67	−0.36	1.41	0.83	2.87	−0.04
4	1.58	−1.14	2.49	−0.81	2.81	1.59	–	–
5	1.37	−0.49	1.65	−0.30	1.81	0.58	5.75	0.82
6	1.70	1.43	2.90	1.63	1.67	0.91	11.24	−2.49
7	1.38	0	1.67	0	1.3	0	1.62	0.23
8	1.58[a]	3.67	2.49	2.59	1.94	−0.46	14.11[a]	5.35
9	1.37	−0.49	1.65	−0.53	1.58	0.41	4.50	−0.41
10	1.65	0.42	2.75	0.70	2.42	−1.65	12.46	0.50
11	1.33	0.22	1.52	0.35	1.41	−0.14	1.70	0.55
12	1.53	−0.81	2.34	−0.20	2.81	−0.78	15.34	0.14
13	1.32	0.22	1.49	0.52	1.81	−2.40	4.58	−0.73
14	1.65	−0.81	2.75	−1.49	1.67	−0.70	4.50	0.68
15	1.33	0.63	1.52	0.82	1.3	−0.79	0.45	1.17
16	1.53	0	2.34	−0.31	1.94	−0.46	7.38	−0.68
17	1.32	1.55	1.49	1.15	1.58	−0.22	3.33	−0.34
18	1.48	−1.21	2.10	−1.15	1.87	2.16	8.19	−0.93

[a] Result beyond 3σ

nificant interactive effects. An increase of the AcN concentration and competition with DMOA decreases the retention time of CPM. In contrast to the previous three-factor design with MeOH as organic modifier, an increase of the ion-pairing SDSS concentration enhances its retention.

4.5. Regression modelling and retention time predictions

For both the four-factor half-fraction and full factorial design, with the mobile phase pH as a variable, first-order regression equations are calculated for MPHB, PPHB and PE, relating their retention times to significant parameters and parameter interactions. Regression modelling of the retention times of CPM is performed with significant parameters and parameter interactions, established with the full factorial design. Tables 10 and 11 give the calculated regression equation characteristics, including the intercept and the regression coefficients of the significant parame-

Table 12
Four-factor half-fraction factorial design: measured and predicted retention times of MPHB, PPHB and PE for parameter combinations belonging to the other half-fraction factorial and to the star design as blocks of the face-centred composite design

Run	Parameter combinations[a]				Measured retention times			Predicted retention times		
	MeOH	SDSS	DMOA	pH*	MPHB	PPHB	PE	MPHB	PPHB	PE
3[b]	1	−1	−1	−1	1.38	1.64	1.44	1.35	1.58	1.46
4	−1	1	−1	−1	1.55	2.42	2.86	1.61	2.60	2.78
6	−1	−1	1	−1	1.73	3.03	1.70	1.61	2.60	1.63
9	1	1	1	−1	1.36	1.60	1.59	1.35	1.58	1.61
10	−1	−1	−1	1	1.66	2.81	2.37	1.61	2.60	2.44
13	1	1	−1	1	1.33	1.54	1.75	1.35	1.58	1.80
15	1	−1	1	1	1.35	1.59	1.28	1.35	1.58	1.28
16	−1	1	1	1	1.53	2.31	1.93	1.61	2.60	1.97
18	−1	0	0	0	1.63	2.66	2.24	1.61	2.60	2.21
19	1	0	0	0	1.34	1.58	1.51	1.35	1.58	1.54
20	0	−1	0	0	1.50	2.10	1.62	1.48	2.08	1.71
21	0	1	0	0	1.42	1.89	2.00	1.48	2.08	2.04
22	0	0	−1	0	1.46	2.00	2.28	1.48	2.08	2.12
23	0	0	1	0	1.43	1.91	1.68	1.48	2.08	1.62
24	0	0	0	−1	1.44	1.99	1.74	1.48	2.08	1.87
25	0	0	0	1	1.40	1.84	1.79	1.48	2.08	1.87

ARD: MPHB 2.8%; PPHB 5.8%; PE 3.2%.
[a] Coded values.
[b] Run number in the face-centred central composite design [1].

ters and interactions, the degrees of freedom (d.f.) left for standard error calculation and fitted retention times with their standardized residuals at each run of the design. The number of d.f. is calculated by subtracting the number of significant parameters and interactions from the total number of runs minus one in the design.

The agreement between observed and fitted retention times for each compound may be expressed by the average relative deviation (ARD) between both as a percentage. For MPHB, PPHB and PE, ARD values are 2.3%, 4.6% and 1.9% in the half-fraction factorial design and 1.2%, 3.2% and 1.3% in the full factorial design, respectively. For CPM, however, an ARD value of 19.6% is calculated. This value expresses an obvious inadequacy of this regression model, compared with the regression equation, derived from the face-centred central composite design, yielding an ARD value for CPM of 12.9%

Predicted retention times of MPHB, PPHB, PE and CPM for LC parameter that belong to the other half of the full factorial design and/or to the star design as blocks of the face-centred central composite design are presented in Table 12 for the half-fraction and Table 13 for the full factorial design. The low ARD values confirm the reliability of the derived regression equations within the parameter space. Table 13 indicates that accurate retention time prediction for CPM fails using this full factorial design (ARD value of 22.3%). This inaccurate CPM retention prediction is visualized in Fig. 1, plotting 24 measured retention times for CPM versus 24 fitted retention times, calculated by the regression models derived from the face-centred central composite and from the full factorial design.

For the three-factor full factorial design with MeOH as organic modifier and a fixed mobile phase pH* of 5.0, the calculated regression equation characteristics for each compound using their significant variables are given in Table 14. ARD values of 2.6% for MPHB, 5.3% for PPHB, 1.4% for PE and 16.8% for CPM are found.

For the three-factor full factorial design with AcN as organic modifier and a fixed mobile phase

Table 13
Four-factor full factorial design: measured and predicted retention times of MPHB, PPHB, PE and CPM for parameter combinations belonging to the star design as a block of the face-centred central composite design.

Run	Parameter combinations[a]				Measured retention times				Predicted retention times			
	MeOH	SDSS	DNOA	pH*	MPHB	PPHB	PE	CPM	MPHB	PPHB	PE	CPM
18[b]	−1	0	0	0	1.63	2.66	2.24	16.15	1.61	2.62	2.21	13.29
19	1	0	0	0	1.34	1.58	1.51	2.90	1.35	1.58	1.53	3.10
20	0	−1	0	0	1.50	2.10	1.62	4.60	1.52	2.21	1.70	6.76
21	0	1	0	0	1.42	1.89	2.00	7.66	1.45	1.99	2.04	9.63
22	0	0	−1	0	1.46	2.00	2.28	11.30	1.48	2.10	2.11	10.50
23	0	0	1	0	1.43	1.91	1.68	4.91	1.48	2.10	1.62	5.89
24	0	0	0	−1	1.44	1.99	1.74	8.41	1.51	2.18	1.87	10.17
25	0	0	0	1	1.40	1.84	1.79	4.68	1.46	2.03	1.87	6.22

ARD: MPHB, 2.4%; PPHB, 5.9%; PE, 4.1%; CPM, 22.3%.
[a] Coded values.
[b] Run number in the face-centred central composite design [1].

pH* of 5.0, the same regression characteristics are given in Table 15. ARD values of 1.1% for MPHB, 3.4% for PPHB, 3.2% for PE and 16.2% for CPM are recovered. The rather high ARD values found for CPM, ranging from 19.6 and 22.3% to 16.2 and 16.8%, are due to the complex pH-dependent chromatographic retention behaviour of CPM on the examined ion-pair LC

Table 14
Full factorial design (MeOH as organic modifier; mobile phase pH 5.0): regression results for MPHB, PPHB, PE and CPM from significant parameters and parameter effects

	Regression coefficients of significant variables			
	MPHB	PPHB	PE	CPM
Constant	1.4567	2.02	1.8378	6.2155
A: MeOH	−0.1187	−0.475	−0.3412	−3.7025
B: SDSS	–	–	0.1662	–
C: DMOA	–	–	−0.2362	−2.245
AC	–		0.1587	1.925
d.f.	7	7	4	5

Run	Fitted values with standardized residuals							
	MPHB		PPHB		PE		CPM	
1	1.58	2.26	2.5	2.96	2.41	−1.48	14.09	−1.20
2	1.34	0	1.55	0.03	1.41	0.07	2.83	−0.42
3	1.58	−1.51	2.5	−1.16	2.74	2.85	14.09	2.00
4	1.34	−0.16	1.55	−0.03	1.74	0.29	2.83	0.87
5	1.46	−1.14	2.02	−1.09	1.84	−1.12	6.22	−1.44
6	1.58	1.19	2.5	0.86	1.62	1.15	5.75	−0.49
7	1.34	0.24	1.55	0.27	1.25	0.92	2.19	−0.31
8	1.58	−0.96	2.5	−1.24	1.95	−0.64	5.75	0.96
9	1.34	0.44	1.55	0.27	1.59	−0.47	2.19	0.73

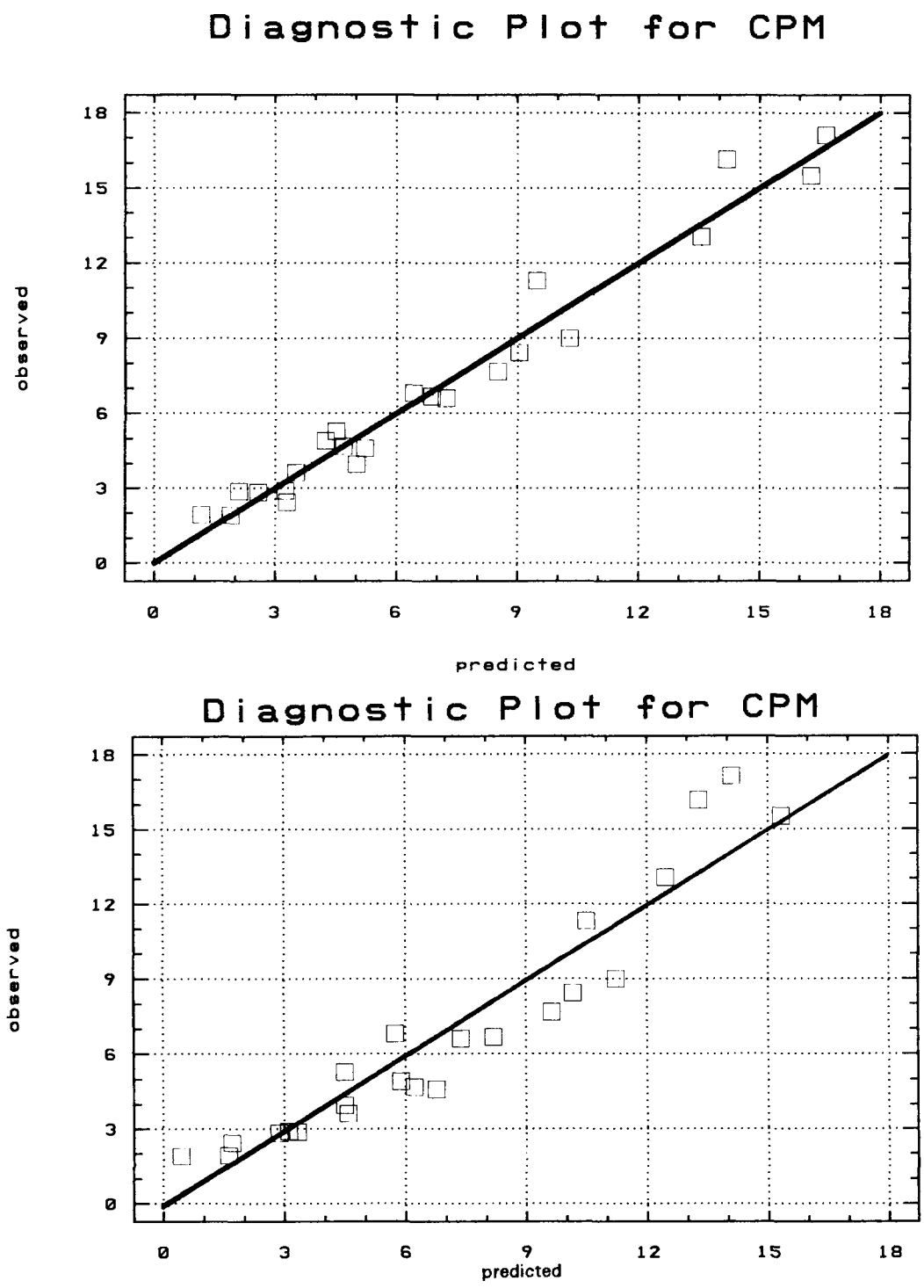
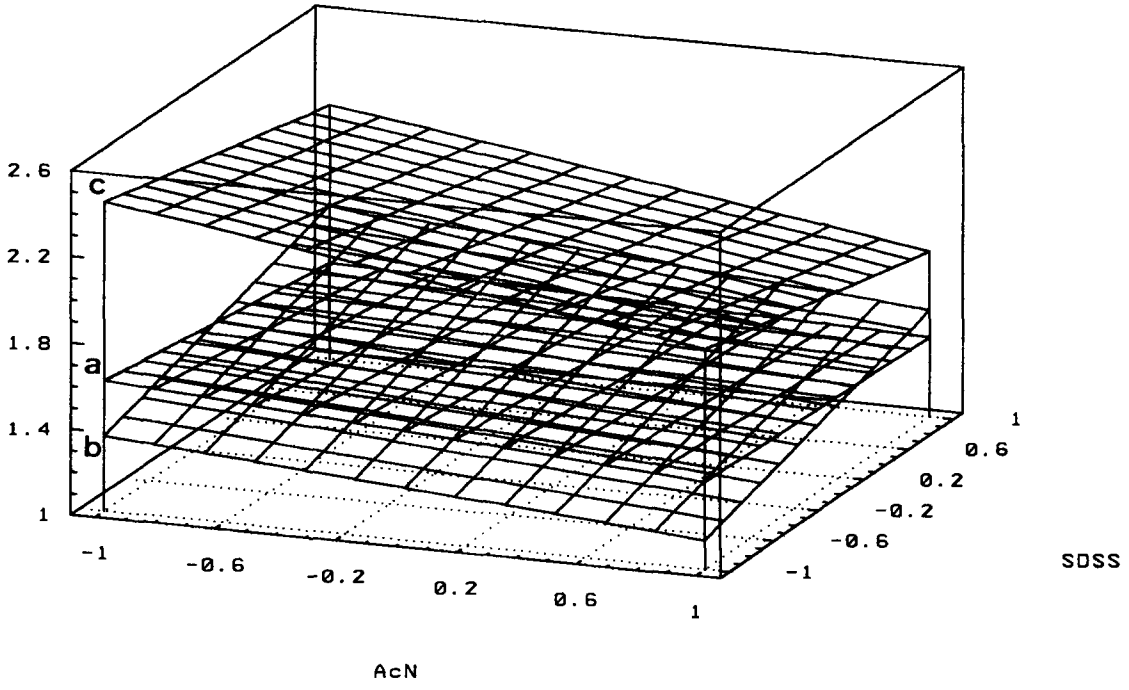
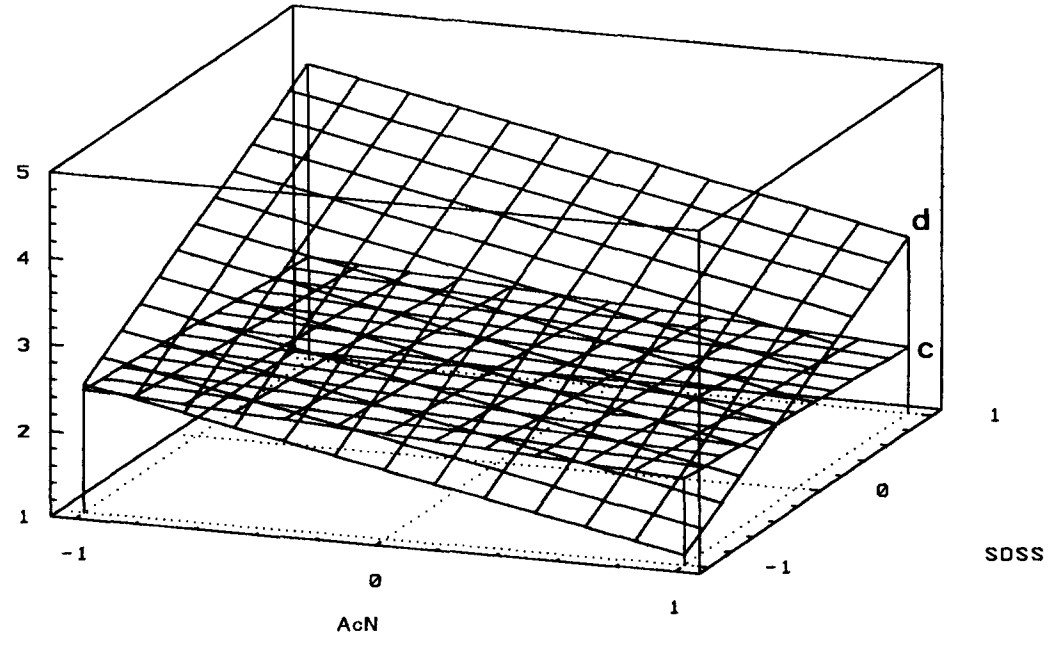


Fig. 1. Diagnostic plots displaying the 24 measured retention times of CPM versus their predicted values, fitted using the face-centred central composite design and using the full factorial design.



(a)



(b)

Fig. 2. (a) Response surface plot representing the retention times of MPHB (surface a), PPHB (surface c), and PE (surface b), as a function of acetonitrile % (v/v) and SDSS (mM) concentrations in the mobile phase (DMOA concentration 15 mM. (b) Response surface plot representing the retention times of PPHB (surface c) and CPM (surface d) as a function of acetonitrile %(v/v) and SDSS (mM) concentrations in the mobile phase (DMOA concentration 15 mM).

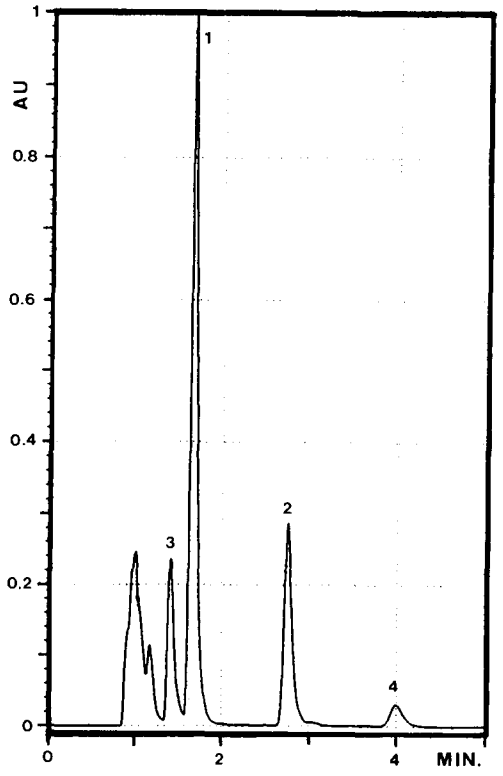


Fig. 3. Liquid chromatogram recorded for cough-syrup analysis. Mobile phase (pH* 5.0) 47 vol. % acetonitrile, 2.5 mmol $^{-1}$ SDSS; 4.5 mmol $^{-1}$ DMOA. Peak 1, MPHB; peak 2, PPHB; peak 3, PE; peak 4, CPM.

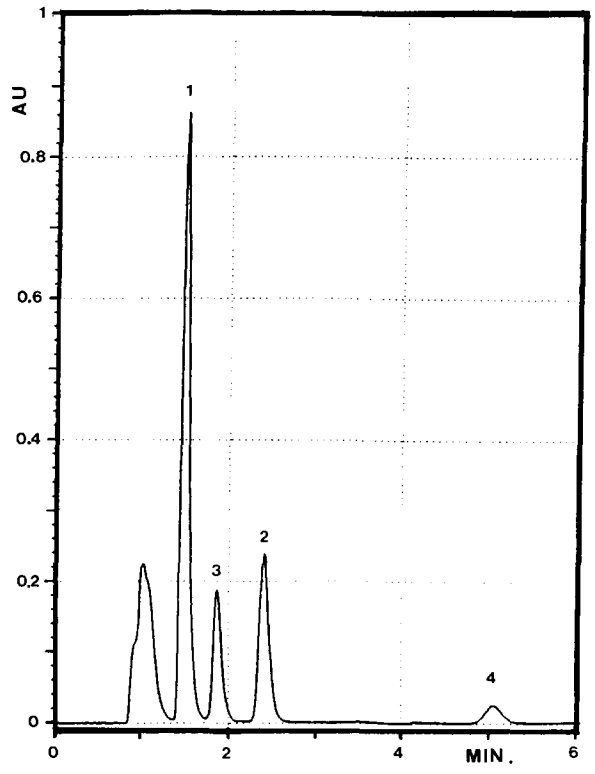


Fig. 4. Liquid chromatogram recorded for cough-syrup analysis. Mobile phase (pH* 5.0) 43 vol.% acetonitrile, 16.0 mmol $^{-1}$ SDSS; 11.0 mmol $^{-1}$ DMOA. Peak 1, MPHB; peak 2, PPHB; peak 3, PE; peak 4, CPM.

system and to the limitation of simplified linear regression models for its accurate retention prediction. The calculated regression models at pH 5.0, however, satisfactorily indicate its elution order on the chromatogram with respect to the other eluting compounds and find suitable LC parameter combinations for complete separation.

4.6. Selection of LC mobile phase parameter combinations (AcN as organic modifier; mobile phase pH 5.0) for cough-syrup analysis*

Three-dimensional response surface plots visualize how retention times change if two mobile phase parameters vary within their proposed boundaries. Combined response surface plots can help to select those parameter combinations which may lead to a fast and complete LC separation of the compounds of interest. If the DMOA concentration in the mobile phase is kept constant at 15.0 mmol l^{-1}, PE can elute before or after MPHB, depending on the SDSS concentration as shown in Table 4, run 6 (3.0 mmol l^{-1}) or run 8 (15 mmol l^{-1}). The separation between PPHB and CPM is improved with increasing SDSS concentration in the mobile phase. However, if the SDSS concentration in the mobile phase is low (3.0 mmol l^{-1}) and the DMOA concentration is high (15.0 mmol l^{-1}), CPM can even elute before PPHB as measured in run 7. A general survey of these changing separations between the examined compounds is illustrated by the response surface plots in Figs. 2a and 2b. Chromatograms recorded with two different LC parameter combinations, which ensure fast and complete compound separation in cough-syrup

Table 15
Full factorial design (AcN as organic modifier; mobile phase pH 5.0): regression results for MPHB, PPHB, PE and CPM from significant parameters and parameter effects

	Regression coefficients of significant variables			
	MPHB	PPHB	PE	CPM
Constant	1.4736	2.1461	1.5440	3.7044
A: AcN	−0.0699	−0.2074	−0.1089	−0.6897
B: SDSS	−0.05	−0.1287	0.2575	0.9612
C: DMOA	–	–	−0.1225	−0.9362
AB	0.0325	–	–	–
BC	–	–	−0.0775	–
d.f.	7	8	6	7

Run	Fitted values with standardized residuals							
	MPHB		PPHB		PE		CPM	
1	1.26	0.27	1.52	0.63	1.22	0.26	1.64	0.42
2	1.63[a]	3.76	2.48	2.99	1.44	−0.14	4.37	−0.37
3	1.42	0.39	2.07	−1.18	1.22	−0.03	2.99	−1.14
4	1.46	1.62	2.22	0.38	2.11[a]	5.44	6.29[a]	5.95
5	1.39	−0.27	1.81	0.30	1.89[a]	−5.77	4.91	−1.26
6	1.63	−1.40	2.48	0.29	1.35	−0.58	2.5	0.18
7	1.42	−1.01	2.07	−1.79	1.13	0.35	1.12	0.90
8	1.46	−0.53	2.22	−1.22	1.71	−0.90	4.42	−1.25
9	1.39	−0.27	1.81	0	1.49	0.63	3.04	−0.34
10	1.54	−0.99	2.35	−0.33	1.65	0.07	4.39	−0.08
11	1.26	0.27	1.52	0.63	1.22	0.26	1.64	0.42

[a] Result beyond 3σ.

analysis, are shown in Figs. 3 and 4. In the first combination, consisting of 47 vol.% AcN, 2.5 mmol l^{-1} SDSS and 4.5 mmol l^{-1} DMOA, PE elutes before MPHB. In the second combination, composed of 43 vol.% AcN, 16.0 mmol l^{-1} 16.0 mmol l^{-1} SDSS and 11.0 mmol l^{-1} DMOA, PE elutes between MPHB and PPHB. Under both sets of chromatographic conditions, LC analysis is performed within 6 min. With the first mobile phase combination, experimental retention times of 1.43 min for PE, 1.65 min for MPHB, 2.75 min for PPHB and 4.0 min for CPM were measured. Their predicted retention times are respectively, 1.54 min, 1.70 min, 2.62 min and 4.47 min. With the second mobile phase combination, experimental retention times of 1.50 min for MPHB, 1.90 min for PE, 2.40 min for PPHB and 5.10 min for CPM were measured. Their predicted retention times are, respectively, 1.49 min, 1.99 min, 2.49 min and 6.17 min. Although the latter retention time prediction for CPM differs more than 1 min from the measured one, important practical information about the elution order of the compounds and the expected duration of the chromatographic run is provided.

5. Conclusion

A possible relevant criticism on the application of a face-centred central composite design, developed for retention time prediction, might be that the derived regression models are often overdetermined. It is demonstrated that a half-fraction factorial design of type 2^4 − 1 with *R* IV (10 runs), as a block of the face-centred central composite design (26 runs), might be suitable also for selecting significant main and interactive parame-

ter effects of an ion-pair LC system with four mobile phase variables. Within the parameter space, the selected LC parameters may serve as independent variables of simplified regression models that fit well the retention times of the compounds MPHB, PPHB and PE. Their statistical reliability is equivalent to that performed by the more complex regression models derived from the central composite design. It should be emphasized, however, that rather narrow retention time areas for the latter three compounds were scanned. This is not the case with a compound like CPM, which shows a more complex pH-dependent retention behavior over a much wider retention time area, as one of its pK_a values is encompassed by the examined mobile phase pH boundaries. The half-fraction factorial design only indicates the significance of most of the parameter interactions but fails for further CPM retention time modelling. The full factorial design, selecting significant parameter interactions, overcomes to some extent the mentioned restrictions inherent in the fractional factorial design. Retention time modelling of CPM, however, using the full factorial design, provides fitted retention times which are inferior to these obtained using the face-centred central composite design. The ability of a full factorial design to predict the CPM retention in the ion-pair LC system, however, is improved if the pH* of the mobile phase is kept constant at 5.0. This is illustrated by the fast and complete LC separation of MPHB, PPHB, PE and CPM in cough-syrup analysis.

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