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Simultaneous optimization of pH and organic modifier content of the mobile phase for the separation of chlorophenols using a Doehlert design

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ABSTRACT

By optimizing the pH and volume fraction of organic modifier, the complete resolution of an isocratic separation of a mixture of phenol and thirteen of the nineteen chlorophenol isomers and of a mixture of the three tetrachlorophenols and pentachlorophenol was achieved. The effectiveness of the Doehlert design in optimizing both experimental parameters was investigated. A quadratic model was applied. For mixtures of a small number of compounds, a retention boundary map is proposed to determine limits of the concentration of organic modifier so as to elute compounds within a reasonable analysis time. The resulting three-dimensional graph of the minimum resolution as a function of the experimental parameters allows the direct visual evaluation of the ruggedness of the optimum conditions that are attainable in the selected parameter space and with a given stationary phase.

INTRODUCTION

Chlorophenols are used extensively as fungicides, herbicides, algicides, insecticides, ovicides, pharmaceuticals, dyes, as preservatives for wood, glue, paint, vegetable fibres and leather and as intermediates in chemical syntheses [1]. About 200 000 tons of chlorophenols are manufactured annually, while some additional chlorophenols are formed by the reaction of chlorinated water supplies with phenol in the environment or through the degradation of chlorinated pesticides [2].

Procedures for the separation and determination of the chlorophenols, as such or after derivatization, include gas chromatography–mass spectrometry, thin-layer chromatography, gas–liquid chromatography and high-performance liquid chromatography (HPLC). A great number of HPLC methods have been reported, which for the greater part do not allow quantification as the peaks are incompletely resolved. Most often the pH of the mobile phase and/or concentration(s) of organic modifier are applied as the experimental parameters. Only a few representative examples are mentioned to give an idea of what has already been achieved. Nair *et*

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al. [3] and Sha and Stanley [4] used isocratic elution for the separation of thirteen and nineteen isomers, respectively, of phenol. Paleologou *et al.* [2] reported an isocratic separation of the members of each category of chlorophenols (*e.g.*, mono-, di-) and a gradient elution method to separate fifteen of the nineteen chlorophenols on a β-cyclodextrin-bonded column. Ugland *et al.* [5] resolved most congeners in a mixture of phenol and eighteen chlorophenols using a linear gradient with a mobile phase of pH 4.

These separations were obtained by trial-and-error, which involves several well known disadvantages. The most evident disadvantage is the often long development time that is required to select experimental conditions that are not necessarily the optimum ones. The first application of a systematic approach to the optimization of the mobile phase composition for the separation of phenols was described by Ong *et al.* [6]. He applied a simplex method combined with overlapping resolution mapping and successfully improved the separation of eleven substituted phenols with a quaternary mobile phase (methanol–acetonitrile–tetrahydrofuran–water).

In this paper the systematic optimization of the isocratic separation of a mixture of phenol and fourteen of the nineteen chlorophenol isomers and of a mixture of three tetrachlorophenols and pentachlorophenol by applying a Doehlert design is described. The former mixture contains phenol and mono-, di- and trichlorophenols, with the exception of 3,4,5-trichlorophenol. As far as we know, the complete resolution of a reversed-phase isocratic separation of such a complex mixture of chlorophenols has not been reported previously. Lores *et al.* [7] resolved a mixture of ten mono- and dichlorophenols by applying a ternary mobile phase in the isocratic mode. We achieved the complete resolution of an isocratic separation of a mixture of phenol and thirteen of the nineteen chlorophenol isomers.

EXPERIMENTAL APPROACH

It was decided to optimize the pH of the mobile phase and the volume fraction of organic modifier. Preliminary experiments indicated that volume fractions of methanol higher than 75% were required to elute tetra- and pentachlorophenols within a reasonable analysis time. Such a volume fraction of methanol is already very close to the solubility limit of the buffer, which in practice imposes the highest possible limit of organic modifier content, and a few percent more of methanol would certainly cause precipitation problems. For that reason, it was decided to apply a modifier with a higher solvent strength, namely acetonitrile. With volume fractions of modifier of at most 61% this allows the same solvent strength to be obtained as with 75% of methanol without problems of buffer solubility.

The chlorophenols are more acidic than phenol and the acidity of the phenol function increases with increasing chlorination of the benzene ring. Therefore, the pH of the mobile phase is a powerful parameter for optimizing selectivity [2,3,8]. The concentration of modifier also has an effect on the selectivity, although a smaller effect than pH. Both parameters should be optimized simultaneously in view of the dependence of both pH [9] and pK_a values [10] on the modifier content of the mobile phase. Because of this dependence, the optimum pH depends on the modifier concentrations (and *vice versa*).

In interpretive optimization methods, a model is needed to describe the retention as a function of the parameters to be optimized. The relationship between retention ($\log k'$) and volume fraction of organic modifier has been shown to be linear over a limited range of capacity factors (*e.g.*, $1 < k' < 10$) and can be described by the following equation [11,12]:

$$\ln k' = \ln k'_0 - S\,\varphi \qquad (1)$$

where k'_0 is the extrapolated retention of the solute in the aqueous phase, S is the slope and φ is the volume fraction of organic modifier. Over larger ranges of mobile phase composition, the following quadratic equation, proposed by Schoenmakers [13], provides a better approximation:

$$\log k' = A\,\varphi^2 + B\,\varphi + \log k'_0 \qquad (2)$$

The relationship between retention and pH is sigmoid, as has already been shown for the chlorophenols in several studies [14,15]. Two very different approaches to pH optimization have been described. That proposed by Dolan *et al.* [16] uses linear models in a restricted pH range whereas the other [17] uses non-linear models (in both the statis-

tical and chemical sense). Both approaches are very interesting and allow useful results, but may have some disadvantages. The former has the advantage of being very simple, but works in a restricted range. Often this will be sufficient or even necessary, but occasionally it would be preferable to be able to work in a larger range. This approach was successfully applied in the systematic optimization of a mixture of acids with, however, a pH range of 1 [16] or 2 pH units [17]. Even in the latter range one risks considerable deviations from linearity. Consider, for instance, Fig. 1. In regions A and C a linear relationship perhaps may be assumed. When selecting a larger region of pH , such as A + B (see further), the model of Horvath *et al.* [18] was shown to be appropriate in the optimization of pH in a reversed-phase liquid chromatographic separation of weak organic acids by a window diagram method [19]. This model has also been applied in combination with eqn. 1 [20] and computer simulation software (Drylab I/mp) was described for predicting HPLC separation as a function of both pH and solvent strength [21]. However, to obtain reliable predictions it is recommended that the pH region is restricted.

The second approach has the advantages and disadvantages of requiring a physical model with nine coefficients. Such non-linear models were recently proposed by Schoenmakers and co-workers [22,23] and require a considerable amount of experimental data. Moreover, a non-linear physical model is not easy to construct and is limited to the particular situation(s) for which it was constructed. We wondered whether a mid-way approach between the two would be useful.

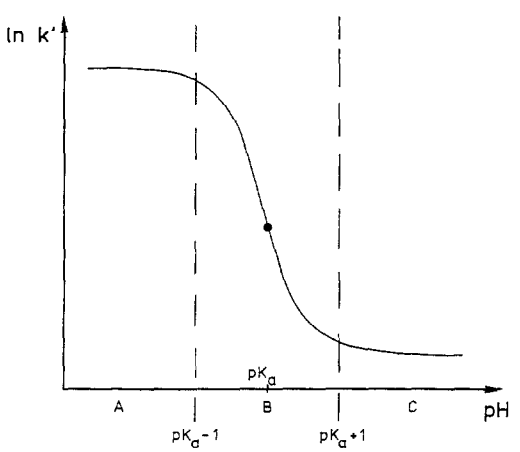

Fig. 1. Evolution of retention (ln k') as a function of pH for an acidic compound.

In experimental design, it is usual to apply quadratic models to optimization. This does not necessarily mean that one assumes the actual relationship to be quadratic, but rather that it is a relatively smooth curved (hyper)plane, which can be approached by a quadratic plane. In the present instance, it is known that the model is not quadratic, but one can hope that in many instances the quadratic curve approaches reality sufficiently closely to allow good prediction. It is hoped that by making the equation quadratic instead of linear, a larger pH range would be feasible. The utility of the following model for the simultaneous optimization of the two experimental parameters will be established:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2 \quad (3)$$

where x_1 and x_2 are the experimental parameters.

It is expected that over moderately broad pH ranges, such as regions A+B or B+C in Fig. 1, eqn. [3] may be an improvement over linear approximations when only a limited number of experiments are performed. The strategy we proposed to investigate can be considered as an extension of that described by Dolan *et al.* [16]. Eqn. 3 is fitted by the Doehlert design, which was introduced into the optimization of HPLC methods by Hu and Massart [24]. They showed this design to have a higher efficiency (expressed by number of coefficients/number of experiments to be carried out) than other designs. A Doehlert design with two factors forms a centred hexagon (Fig. 2) where each point represents an experiment. Seven experiments are to be carried out for a model with two factors.

A feature of the Doehlert design is that the number of levels for each experimental parameter is not the same. A Doehlert design with two parameters uses three levels for one parameter and five levels for the other. One should use the design so that the parameter with the most complex relationship is modelled with the largest number of levels [24]. Therefore, the highest number of levels (= 5) is used to model the pH. Once the experimental parameters

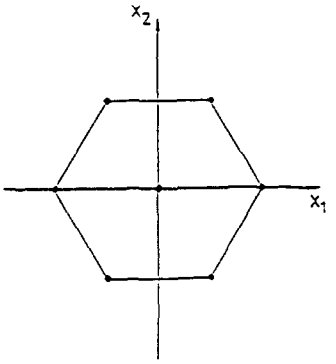

Fig. 2. The Doehlert design.

have been selected, the parameter space has to be defined by fixing boundary values. For the volume fraction of acetonitrile, the highest concentration should be selected so that the peak of the first eluted compound is still separated from the solvent peak. The lower limit should be selected so that the analysis time will not exceed a certain time.

Different approaches have been proposed in the literature, such as selected isocratic experiments or, most often, gradient runs. Berridge [25] carried out an initial gradient separation in order to estimate the likely range of solvent strength required in the simplex optimization of a reversed-phase separation. A similar approach was followed by De Smet *et al.* [26] who combined an initial gradient separation with a small number of isocratic experiments in a normal-phase separation of sulphonamides. Preliminary isocratic separations combined with low-wavelength detection termed "sequential isocratic step" (SIS) chromatography have been applied successfully in ion-pair separations [27].

A retention boundary map is proposed to establish in a reasonable time an optimum region of concentrations of organic modifier. Conventionally with binary mobile phases the organic modifier content is selected to give capacity factors in the range $1 < k' < 10$ for all compounds, as only a very small gain in resolution is obtained with k' values > 10. By applying eqn. 1 and capacity factors measured at two different solvent strengths, percentages of acetonitrile that lead to capacity factors between 1 and 10 are calculated. In this way, a region of acceptable concentrations of organic modifier is defined for each compound at the upper and lower limit of pH. Both regions are connected to constrain an area in the plane of pH *versus* organic modifier content of experimental conditions that should lead to the desired retention times for one compound. This is, of course, an approximation but it allows an area to be established with sufficient accuracy and with a minimum of experimental effort. By overlapping of the areas of each compound one can select one or more areas where for all compounds of interest or at least for most of them the postulated requirements are fulfilled (Fig. 3).

One should not confuse the retention boundary map with overlapping mapping techniques as described by Glajch and Kirkland [28]. The aim of constructing a retention boundary map is to select a region of concentrations of organic modifier so that the capacity factors of all compounds are within a certain range. When optimizing selectivity it is recommended to select pH limits so that for all compounds both ionized and non-ionized forms are chromatographed.

As it is known that the lifetime of silica-based stationary phases may be seriously reduced in the presence of aqueous buffer–mobile phase systems with a pH that is outside the range 2–7.5, the upper limit of pH is set at 7 as imposed by the stability of the stationary phase. The lower limit of pH is set at

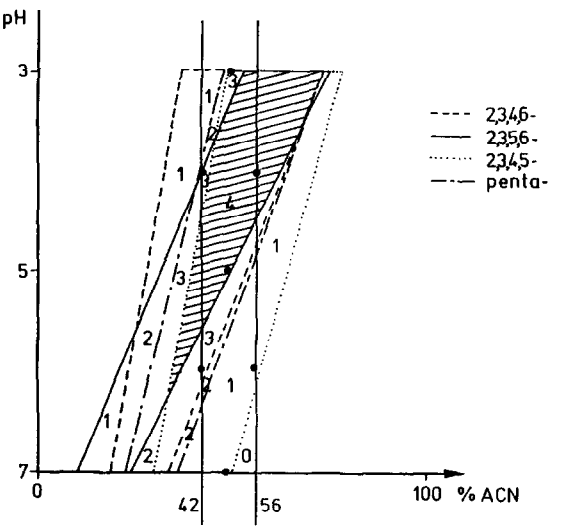

Fig. 3. Retention boundary map of the mixture of tetra- and pentachlorophenols. The numbers refer to the number of compounds for which $1 < k' < 10$. ACN = Acetonitrile.

3 because all compounds remain uncharged at lower pH. This pH region encompasses all pH values that range from $pK_a - 1$ to $pK_a + 1$ for the mixture of tetrachlorophenols and pentachlorophenol. The pK_a values of the fifteen chlorophenols range from 5.80 for 2,3,6-trichlorophenol to 9.92 for phenol [14] (see Table V). One can argue that in view of the lowest pK_a value of the sample set it is not reasonable to include a mobile phase pH as low as 3.0. However, it was decided to do so as several published RPLC separations of smaller mixtures of chlorophenols, obtained by trial-and-error, were achieved at pH values of 4.0 and lower [5,29,30]. In the low-pH region, there will be no pH effect. However, it is not necessary that this should be so: if a good separation is found that is not influenced by pH, this will be preferable as it leads to increased robustness.

The effective buffer range for a weak acid or base is approximately from $pH = pK_a - 1$ to $pH = pK_a + 1$. This implies that a buffer system should be selected with successive pK_a values that differ by about 2 pH units to ensure adequate buffering ability at each pH within the selected region. When applying only one type of buffer, the buffer capacity will not be sufficient over one or more pH ranges in the selected pH region [31]. For phosphoric acid, for instance, the good buffering ranges between pH 3 and 7 are pH 3–3.2 and 6.2–7. For this reason, stoichiometric mixtures of citrate and phosphate buffers (1:1) were used.

EXPERIMENTAL CONDITIONS

Chromatographic equipment and parameters

All measurements were carried out with a Varian Model 5000 liquid chromatograph equipped with a Rheodyne injection valve (5-μl sample loop) and coupled with a Shimadzu SPD-2A UV detector. Detection was performed at 260 nm. The attenuation was set at 0.04 a.u.f.s. The chromatograms were recorded with a Spectra-Physics SP 4290 integrator. The column used was LiChrospher RP 18 (250 x 4 mm I.D.) from Merck with a particle size of 5 μm because octadecyl-modified stationary phases were found to be superior for the analysis of chlorinated phenols in reversed-phase chromatography [1,4]. The flow-rate was maintained at 1 ml/min. During chromatography, the column temperature was maintained at 30°C with a Prolabo Sup-Rs stabitherm column thermostat. pH measurements of buffer solutions were carried out with a Corning Model 240 pH meter.

Standards and reagents

All chlorophenols were of reference grade and were obtained from Aldrich. Standard solutions in the mobile phase with concentrations of ca. 300 mg/l were daily prepared by dilution of stock solutions in acetonitrile. Acetonitrile of HPLC gradient quality was purchased from Distrilab. The water used was purified in a Milli-Q system (Millipore). Sodium dihydrogenphosphate, phosphoric acid, citric acid, trisodium citrate and sodium hydroxide of analytical-reagent grade were obtained from Prolabo.

Citrate and phosphate buffers of pH 3, 4, 5, 6 and 7 and ionic strength 0.05 mol/l were prepared with purified water. At each pH value, the electrode was calibrated with standard solutions of pH 4 and 7. After controlling the pH, the buffers were filtered through a 0.45-μm Millipore filter under vacuum.

Procedures

Retention times were obtained from the peak maximum by the integrator. Peak widths at half-height were measured manually. The pH of the mobile phase was assumed to be the pH of the aqueous fraction. To avoid increasing the number of experiments too much, several standards with large enough differences in retention were chromatographed in one run.

TABLE I

EXPERIMENTAL CONDITIONS FOR THE SEVEN EXPERIMENTS OF THE TWO DESIGNS

Experiment No.	pH	Acetonitrile (%)	
		15 phenols	4 phenols
1	7	38	49
2	5	38	49
3	3	38	49
4	6	45	56
5	4	45	56
6	6	31	42
7	4	31	42

The experimental conditions for the seven experiments of the two designs are given in Table I. The central experiment was carried out twice, on different days, to obtain an idea of the experimental error (see further). The seven experiments were performed in random order and, to avoid a memory effect from previous eluents, the column each time was equilibrated for 2 h with the next mobile phase at a flow-rate of 1 ml/ min.

Description of the software

A program for a Doehlert matrix design of experiments was developed by Hu and Massart [24]. As their program is limited to the use of at most ten compounds, a new version in Excell and Quickbasic was written. The program calculates the coefficients in eqn. 3 for y = retention time, t_R, and y = peak width at half-height, $w_{1/2}$, for each compound so that it can predict the values of both variables for a large set of experimental conditions (= different mobile phases). Subsequently the resolution of the worst separated peak pair, $R_{s\min}$, is calculated as a chromatographic response. All values of $R_{s\min}$, t_R and $w_{1/2}$ are saved in spreadsheets in Excell which allows the user to construct a three-dimensional graph of the optimization criterion or of each of the two variables as a function of the experimental parameters.

RESULTS AND DISCUSSION

Separation of a mixture of tetra- and pentachlorophenols

For the Doehlert design with tetra- and pentachlorophenols, the upper and lower limit are fixed at 56 and 42% of acetonitrile, respectively, using a retention boundary map (Fig. 3). The area where $1 < k' < 10$ for all compounds is large at pH 3 but becomes smaller with increasing pH and at pH 7 it is no longer possible to obtain $1 < k' < 10$ for 2,3,4,5-tetrachlorophenol and pentachlorophenol at the same time. Therefore, this area is combined with other areas each with as large a number of compounds as possible that fulfil the postulated requirements. As a result of combining areas where not all compounds have $1 < k' < 10$, the capacity factors of all solutes except 2,3,4,5-tetrachlorophenol will be smaller than 1 in the experiment at pH 7. In only two of the seven chromatograms of the experiments of the design (Table I) are four peaks observed. These chromatograms are obtained under experimental conditions 2 and 6.

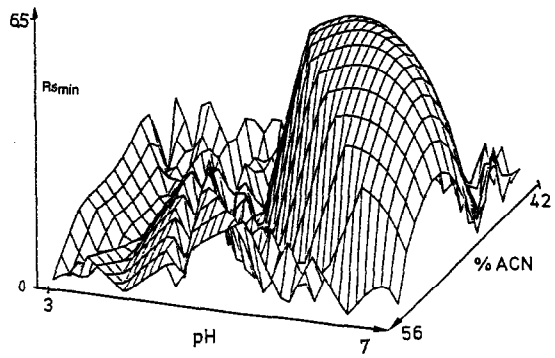

Fig. 4. Three-dimensional graph of $R_{s\min}$ as a function of pH and concentration of acetonitrile for the mixture of tetra- and pentachlorophenols.

The resulting three-dimensional graph of $R_{s\min}$ (Fig. 4) as a function of pH and concentration of acetonitrile and the graph in two dimensions representing areas of equal $R_{s\min}$ (Fig. 5) show a wide optimum which allows the development of a rugged method. Both graphical presentations allow an evaluation of the ruggedness of the optimum. A maximum value of $R_{s\min}$ of 6.63 is predicted at pH 6 and with a mobile phase that contains 49% of acetonitrile.

Two experiments were carried out with a mobile phase containing 49% of acetonitrile at pH 5.5 and 6.0. The predicted minimum resolution for the for-

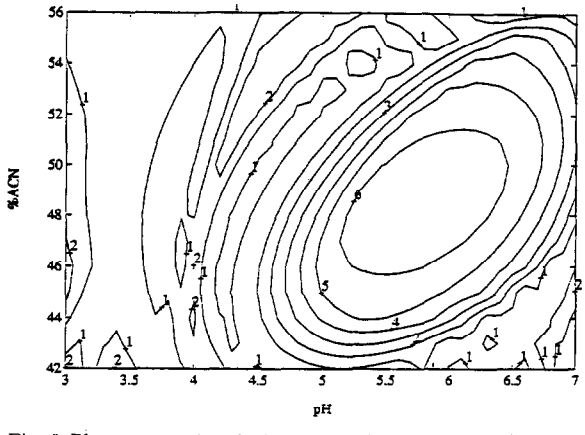

Fig. 5. Plot representing the interaction between pH and volume percentage of acetonitrile. The lines delimit areas of equal $R_{s\min}$.

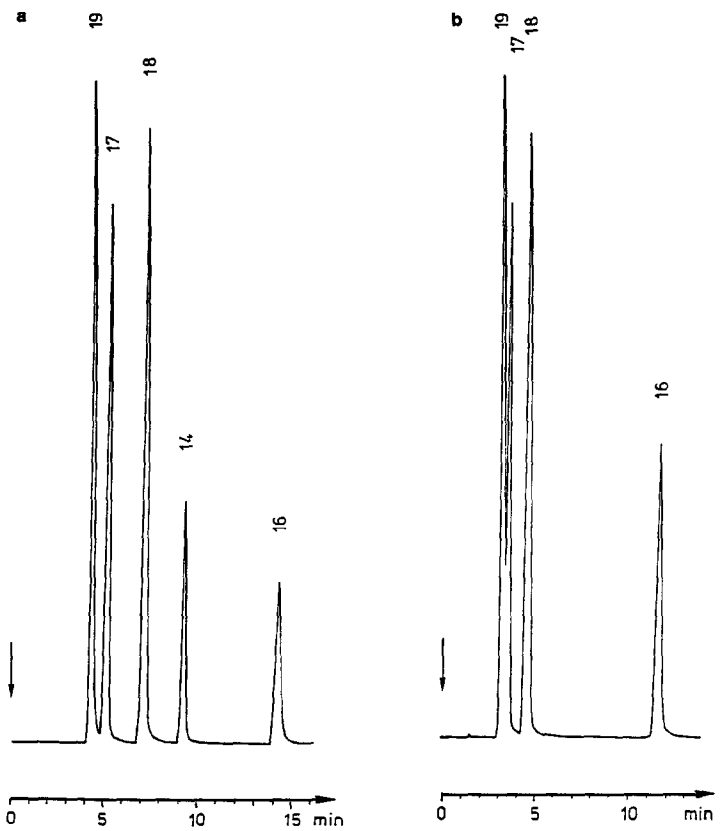

Fig. 6. Chromatogram of the mixture of tetra- and pentachlorophenols obtained with a volume fraction of acetonitrile of 49% at pH (a) 5.5 and (b) 6.0. Numbers of the solutes refer to Table II.

mer experimental conditions is 6.35. Baseline resolution was obtained by applying the first experimental conditions (Fig. 6a). Compared with reversed-phase isocratic separations of the same mixture that have been published [2,5], the resolution in Fig. 6a is superior, although the analysis time is about 4 min longer. The second experiment led to partial overlap of penta- and 2,3,5,6-tetrachlorophenol

TABLE II

COMPARISON BETWEEN PREDICTED RETENTION TIMES AND EXPERIMENTAL VALUES FOR THE MIXTURE OF TETRA- AND PENTACHLOROPHENOLS

Compound No.	Name[a]	pK_a	pH 5.5			pH 6		
			t_R (pred.)	t_R (exp.)	deviation (%)	t_R (pred.)	t_R (exp.)	deviation (%)
16	2,3,4,5-	5.64	13.7	14.3	4.3	11.1	11.6	3.78
18	2,3,4,6-	5.22	8.75	7.29	20	5.99	4.57	31.1
17	2,3,5,6-	5.02	6.62	5.29	25.1	4.17	3.48	19.8
19	Penta-	4.74	4.26	4.42	3.62	1.51	3.12	107

[a] Tetra- and pentachlorophenols.

(Fig. 6b). The pH of the mobile phase in the two experiments differed only by 0.5, which demonstrated that the separation of this, at first sight simple, mixture of four compounds requires the use of an optimization procedure in which pH is a very important parameter. At pH 6 also a complete separation of all compounds was predicted. The deviations of the predicted $R_{s_{min}}$ must be due to deviations between the predicted values of retention and peak width and the results obtained by experiment. This might indicate a lack of fit of the experiments to the model. To investigate this the retention times of the compounds obtained experimentally are compared with the predicted values in Table II. The precision of predicted values of the peak width was investigated in the separation of the mixture of fifteen phenols. The deviations between predicted values of t_R and experimental results are larger in the experiment at pH 6, which indicates that the accuracy of the predictions is higher in experiments that are closer to the central experiment. For all solutes except 2,3,4,5-tetrachlorophenol the model does not provide accurate predictions. The pK_a values of the former compounds are nearly in the middle of the investigated pH range, so that an almost complete sigmoidal curve of retention *versus* pH is assumed in the selected parameter space. This was confirmed in later experiments performed with a larger set of pH-values for the computer-assisted determination of pK_a values of chlorophenols by means of HPLC [32]. For 2,3,4,5-tetrachlorophenol with a pK_a value of 5.64 there is only one "leg" of this curve that is better fitted by the second-order polynomial than a complete curve. This is illustrated in Fig. 7, which presents predicted and experimental values of t_R *versus* pH for 2,3,4,5-tetrachlorophenol and pentachlorophenol.

When a complete sigmoidal curve of retention *versus* pH is observed in the selected parameter space, other approaches are required. One such approach is to use non-linear regression using a model such as that by Horváth *et al.* [18]. The use of this method was described by us for pK_a determination in an optimization context [32]. The other approach is to transform the data to obtain a response surface that is more amenable to the usual experimental design approaches. We are currently investigating such transforms. Two of them are well known,

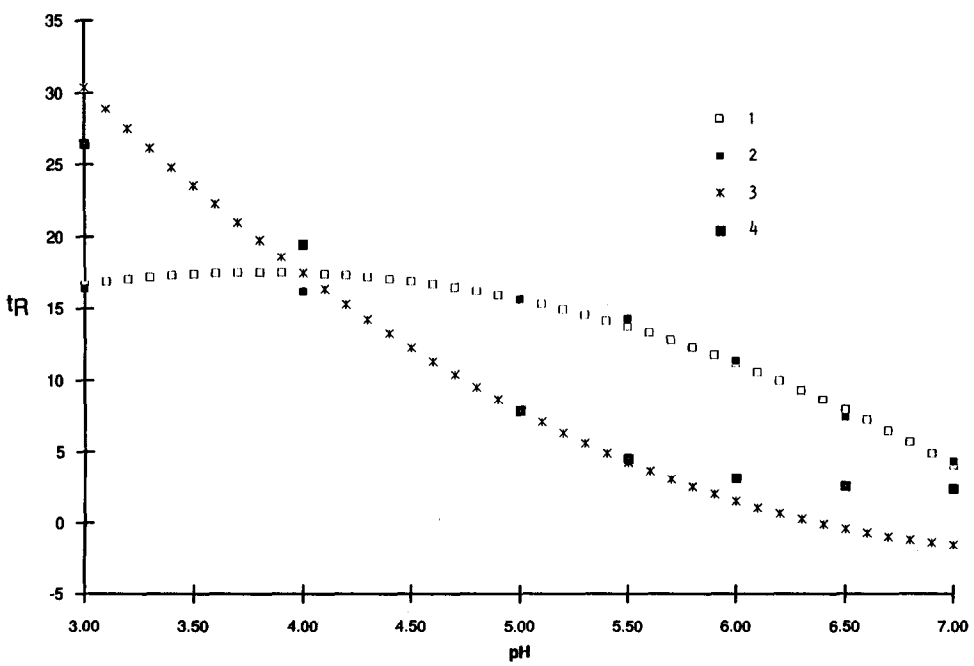

Fig. 7. Predicted and experimental retention times *versus* pH at 49% of acetonitrile. 2,3,4,5-Pentachlorophenol: 1 = predicted; 2 = experimental. Pentachlorophenol: 3 = predicted; 4 = experimental.

namely the probit transform and the logit transform. We chose to study the latter. In the present case it is carried out as follows:

$$z = \log [k'^*/(1 - k'^*)] \quad (4)$$

where k'^* values are standardized k' values, obtained by transformation to a scale between 0 and 1 by applying the following equation:

$$k'^* = [Y_i^{(+)} - k']/[Y_i^{(+)} - Y_i^{(-)}] \quad (5)$$

where $Y_i^{(+)}$ and $Y_i^{(-)}$ are the largest and smallest values of measured k' values which are respectively increased and decreased by 1% to avoid losing information of the experiments at the smallest and largest pH by k'^* equaling 0 or 1. A first- or second-order model should allow z to be accurately described as a function of the independent variable (here pH). We have verified this for a few examples, *e.g.*, on the data of Schoenmakers *et al.* [22] and for

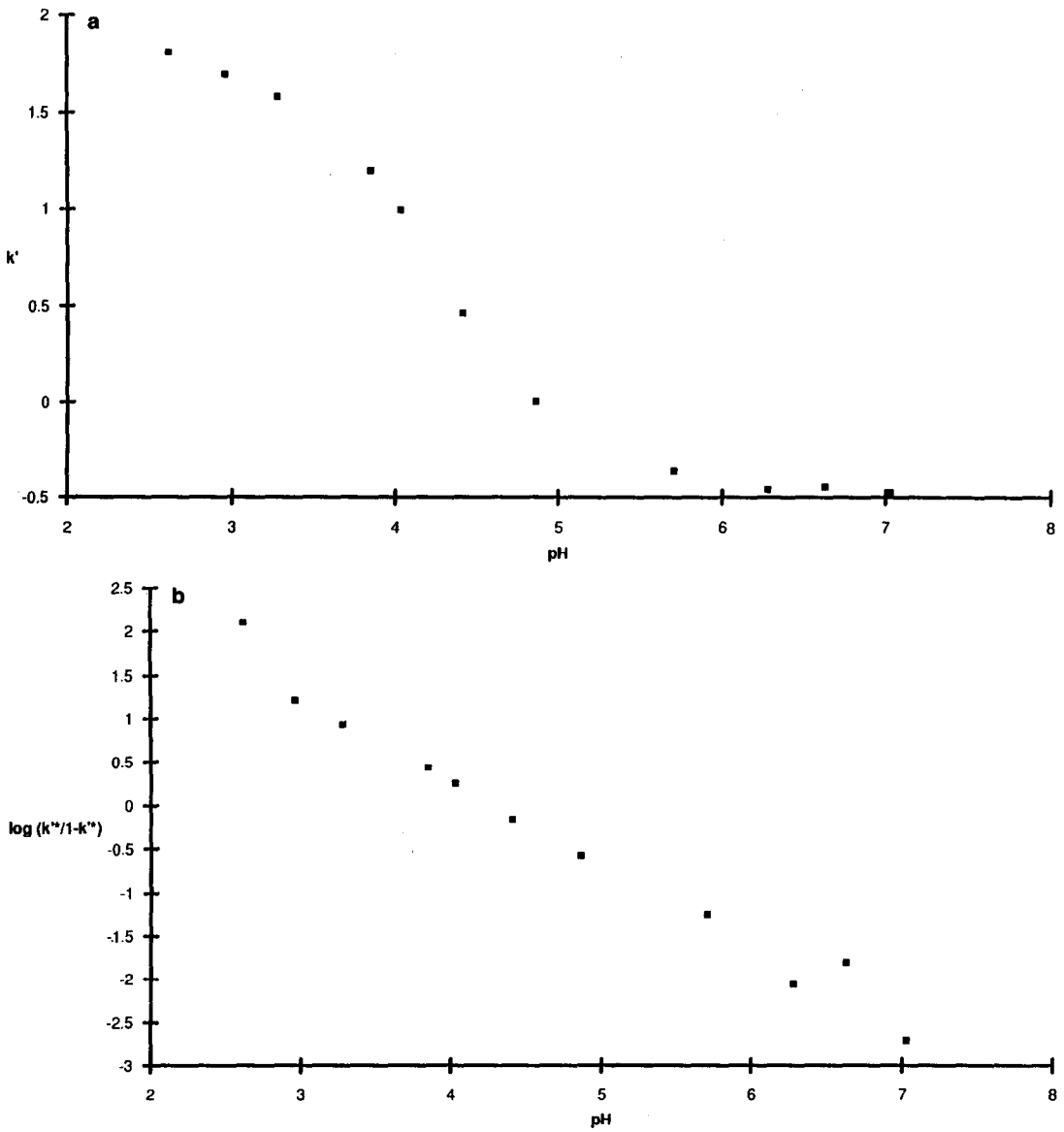

Fig. 8.

(Continued on p. 180)

2,3,4,5-tetrachlorophenol and pentachlorophenol. For these three solutes the curves of k' and z as a function of pH are represented in Fig. 8a–f. Both a first- and a second-order equation were applied to model z and the precision of the predictions was compared with that obtained by modelling k' *versus* pH with a quadratic equation. The precision was significantly better with the logit transform for benzoic acid and pentachlorophenol but not for 2,3,4,5-tetrachlorophenol (Table III, Fig. 9a and b). For the former solutes no difference in precision was obtained on applying a first- or second-order equation to model z *versus* pH (Table III). We are now verifying whether this transformation can be used in general and how to apply it to the bivariate optimization of pH and solvent strength.

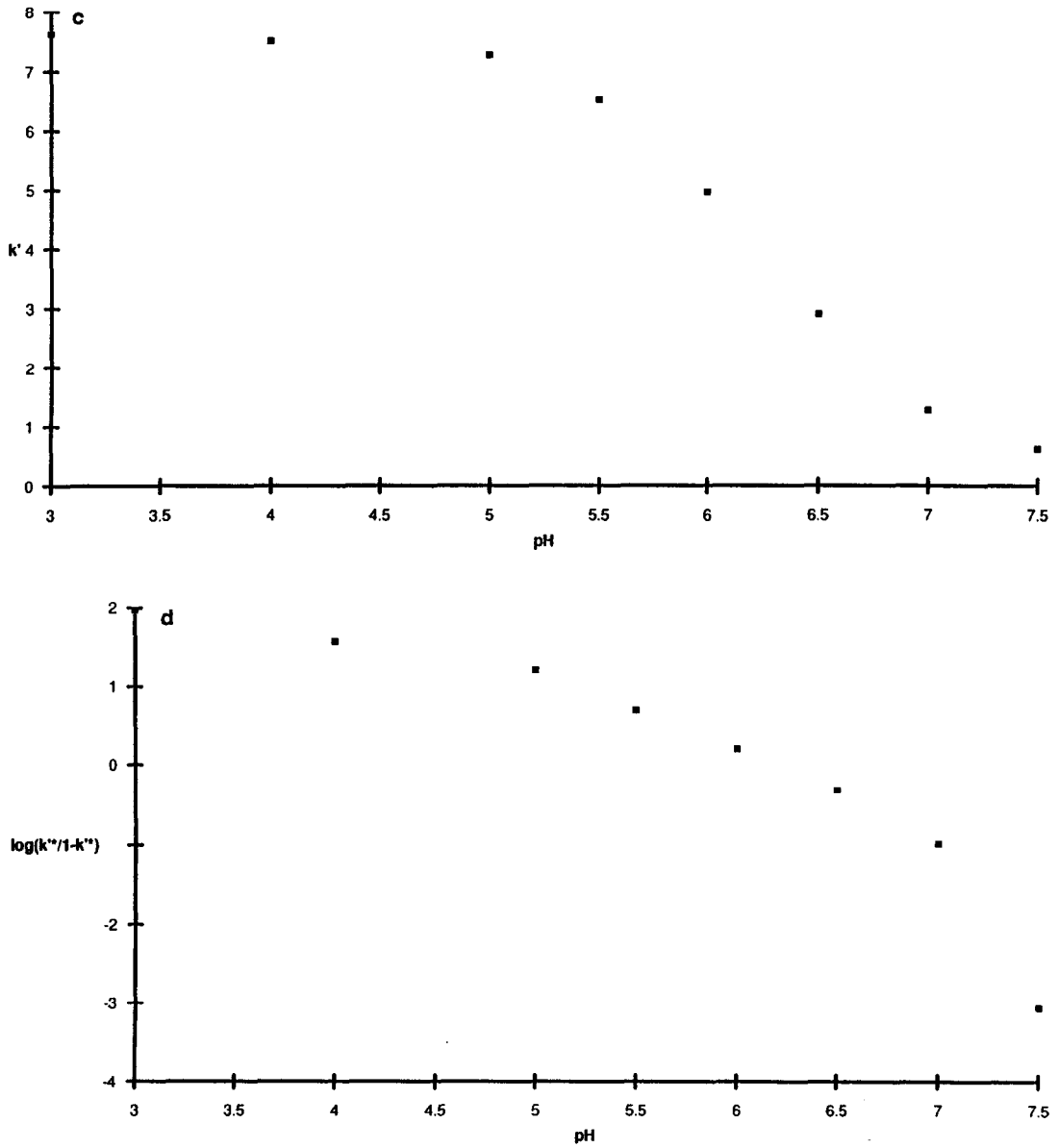

Fig. 8.

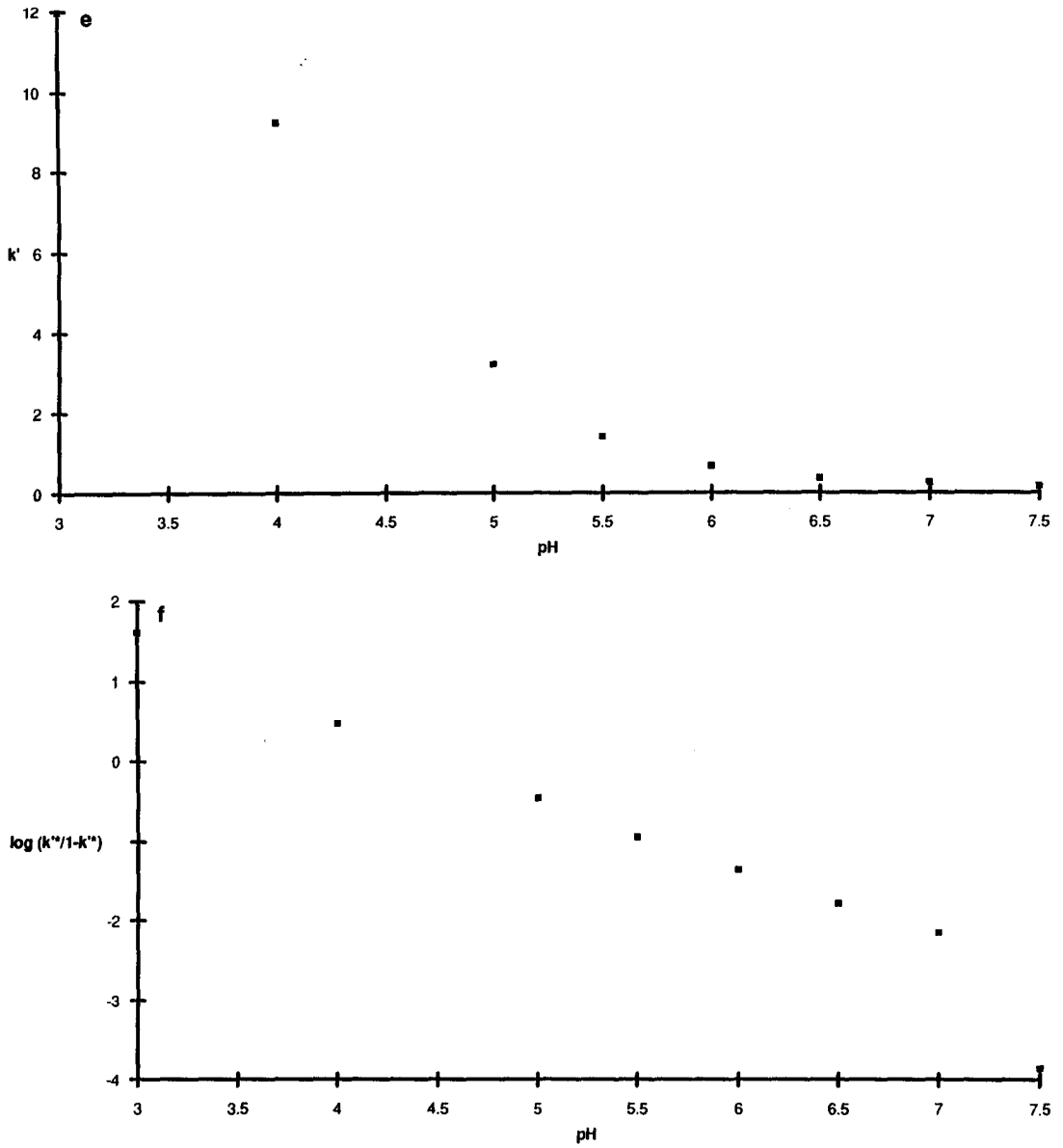

Fig. 8. Evolution of capacity factors and z values as a function of pH for (a, b) benzoic acid, (c,d) 2,3,4,5-tetrachlorophenol and (e,f) pentachlorophenol.

Separation of a mixture of phenol and fourteen chlorophenols

The requirement of $1 < k' < 10$ as imposed by applying eqn. 1 may be fulfilled if the optimization problem concerns a small set of compounds with similar polarities. However, this was not the case for the mixture of tetra- and pentachlorophenols. It is therefore not surprising that this requirement also was not fulfilled for the much more complex mixture of fifteen phenols. By performing two isocratic experiments at two different concentrations of acetonitrile at pH 3 and 7, an upper limit was set at 45% of acetonitrile so that phenol and 2,3,6-trichlorophenol, which have the shortest retention times at

TABLE III

COMPARISON BETWEEN EXPERIMENTAL VALUES OF CAPACITY FACTORS [k'(exp.)] AND VALUES PREDICTED BY FITTING A LINEAR MODEL [k'(a)] AND A SECOND-ORDER MODEL [k'(b)] TO z *VERSUS* pH, AND BY MODELLING THE EXPERIMENTAL DATA WITH A SECOND-ORDER EQUATION [k'(c)]

Compound	k'(exp.)	k'(a)	Deviation (%)	k'(b)	Deviation (%)	k'(c)	Deviation (%)
Pentachlorophenol	12.0	12.0	0.00	11.8	1.67	12.8	6.67
	9.25	9.89	6.86	10.1	9.19	7.44	19.6
	3.21	3.28	2.18	4.20	30.7	3.52	9.78
	1.39	1.27	8.35	1.70	22.6	2.11	51.4
	0.66	0.51	22.6	0.62	6.08	1.04	58.3
	0.36	0.27	25.0	0.28	21.8	0.34	5.14
	0.25	0.20	20.8	0.19	22.5	0.00	101
	0.17	0.18	4.06	0.17	2.06	0.01	94.5
2,3,4,5-Tetrachlorophenol	7.62	7.61	0.20	7.50	1.52	7.63	0.08
	7.51	7.48	0.36	7.52	0.10	7.80	3.80
	7.28	6.51	10.5	7.29	0.09	6.89	5.29
	6.52	5.06	22.4	6.70	2.74	6.04	7.31
	4.96	3.15	36.5	4.88	1.65	4.92	0.71
	2.89	1.71	41.0	2.07	28.5	3.54	22.4
	1.27	1.01	20.5	0.82	35.1	1.88	48.4
	0.61	0.75	22.1	0.63	3.11	−0.04	106
Benzoic acid[a]	1.81	1.78	1.16	1.79	0.78	2.06	14.3
	1.69	1.73	2.07	1.73	2.49	1.67	1.24
	1.58	1.63	3.42	1.63	3.23	1.39	11.8
	1.20	1.20	0.08	1.15	4.33	0.90	25.3
	1.00	1.09	9.45	1.03	3.02	0.82	17.6
	0.46	0.57	23.3	0.49	6.09	0.53	15.9
	−0.36	−0.39	9.10	−0.39	7.78	−0.18	48.7
	−0.46	−0.46	0.43	−0.45	1.98	−0.41	11.6
	−0.45	−0.47	5.39	−0.46	4.04	−0.49	10.6
	−0.48	−0.48	0.00	−0.47	0.63	−0.58	21.9

[a] Experimental data from ref. 22.

pH 3 and 7, respectively, should be eluted with a capacity factor of at least 0.5. The lower limit was relaxed and was set at 31% of acetonitrile, which should correspond to a capacity factor of about 40. This value, however, can only be used as a first approximation because extrapolations have been made outside the region $1 < k' < 10$. Once boundary values of the experimental parameters had been fixed, the seven experiments of the design were performed (Table I).

Only the experimental conditions of experiment 7 led to a chromatogram with 15 peaks but, as can be seen in Fig. 10, the peaks of 2,4- and 2,5-dichlorophenol show considerable overlap while the very large capacity factors cause peak broadening. Compared with the performance obtained in the chromatograms with tetra- and pentachlorophenols, the peaks show much less tailing. This is assumed to be due to reduced stereochemical effects and reduced interaction with residual silanol functions because of a smaller degree of chlorination.

Peak cross-overs occur as a function of pH (Fig. 11) but less when varying the volume fraction of acetonitrile at constant pH (Figs. 12 and 13). Most curves in Figs. 12 and 13 are convergent, as is expected when varying only the organic modifier content. The retention of compounds such as phenol and 2-chlorophenol as a function of pH remains almost constant as they are very weak acids, while the largest decreases in retention are observed for more acidic compounds, *e.g.*, trichlorophenols.

Three-dimensional graphs of $R_{s\min}$ as a function

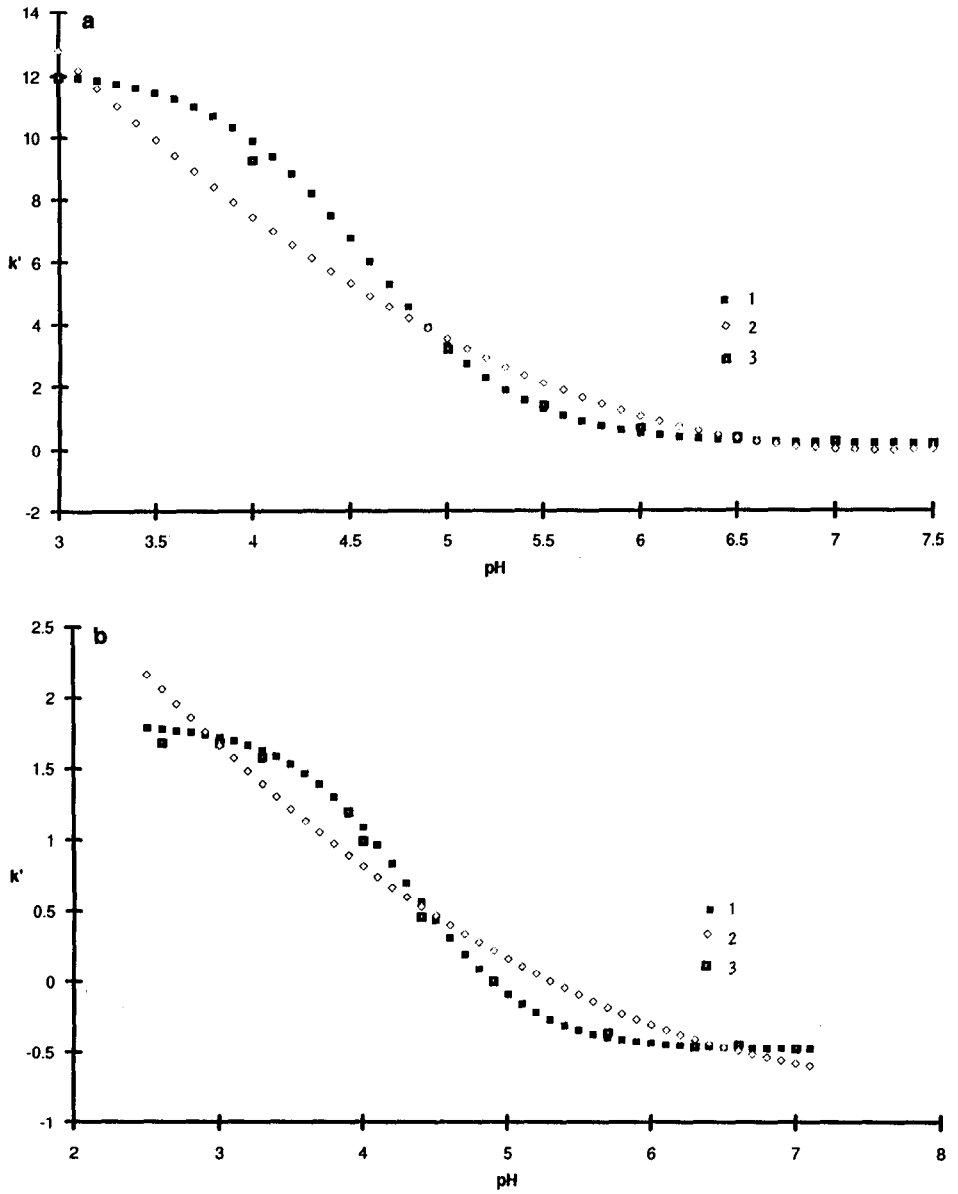

Fig. 9. Evolution of predicted capacity factors as a function of pH. 1 = capacity factors predicted by applying a linear model to z as a function of pH; 2 = capacity factors obtained by fitting a second-order model to the experimental data, represented by symbols 3. (a) Pentachlorophenol; (b) benzoic acid.

of pH and concentration of acetonitrile are represented in Fig. 14. The highest $R_{s\min}$ value of 0.98 is predicted at pH 3.8 and 36% acetonitrile. Considering in more detail the spreadsheet of all $R_{s\min}$ values (part of which is represented in Table IV) and Fig. 13, it can be seen that this optimum is not robust but should be very sensitive to small variations in mobile phase composition; when the volume fraction of acetonitrile is varied by only 1% the value of $R_{s\min}$ is reduced to 0.4. An HPLC method under these conditions certainly will not be rugged.

An experiment was carried out at pH 3.9 and

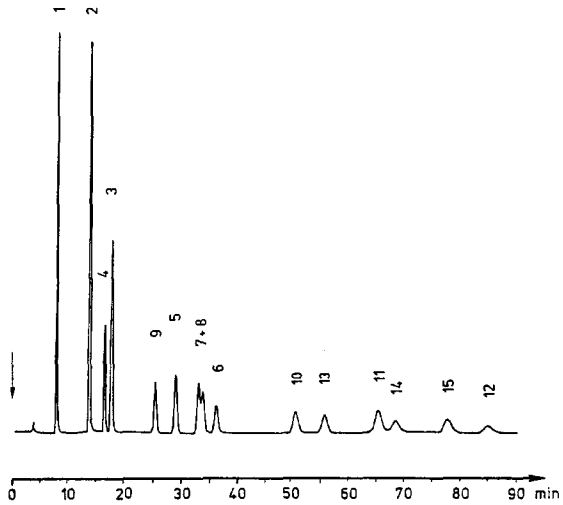

Fig. 10. Chromatogram of the mixture of fifteen chlorophenols, obtained in experiment 7 of the Doehlert design. For experimental conditions, see Table I.

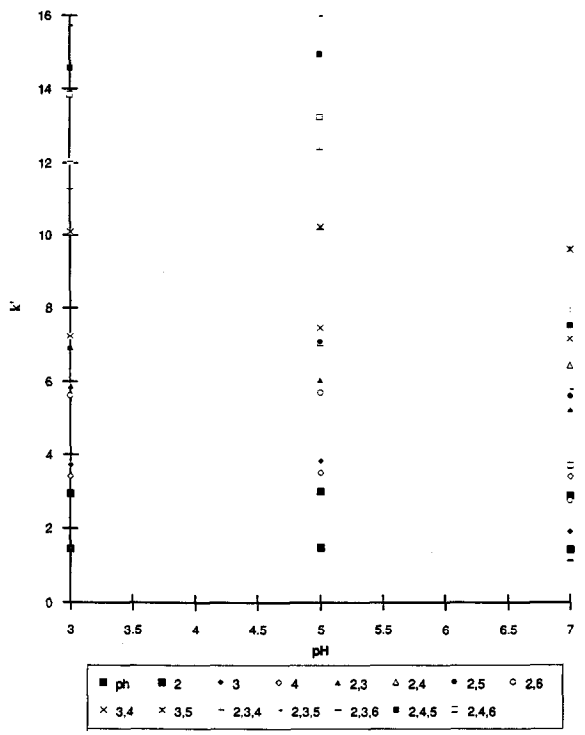

Fig. 11. Evolution of capacity factors as a function of pH for the mixture of fifteen chlorophenols.

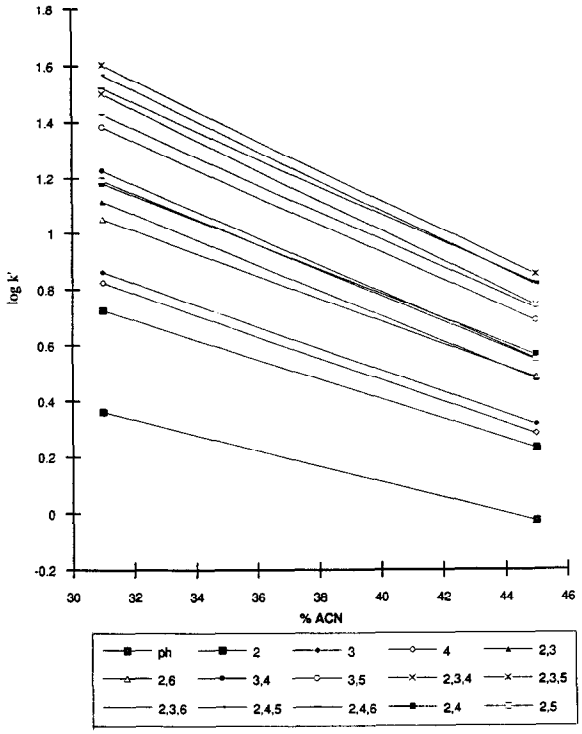

Fig. 12. Evolution of retention (log k') as a function of organic modifier concentration at pH 4.

with a mobile phase containing 36% of acetonitrile. These experimental conditions should lead to almost the same $R_{s\min}$ as predicted at pH 3.8 but are slightly more robust (Table IV). In the resulting chromatogram (Fig. 15), fourteen compounds are well separated in an analysis time of 44 min. The peaks of 2,4- and 2,5-dichlorophenol completely overlap, which was not predicted. The retention times of the compounds obtained experimentally are compared with the predicted values in Table V. The predicted retention times agree with the actual retention times to within 5% for all but two compounds, *i.e.*, 2,4,6- and 2,3,6-trichlorophenol. For each compound the means of the percentage deviations between predicted values of t_R and $w_{1/2}$ and the experimental results for the seven experiments are given in Table V. Compared with the precision of predictions of retention times, a poorer precision is obtained when predicting peak widths. This may be explained by the larger inaccuracy of the measurements of the peak width, which is also included

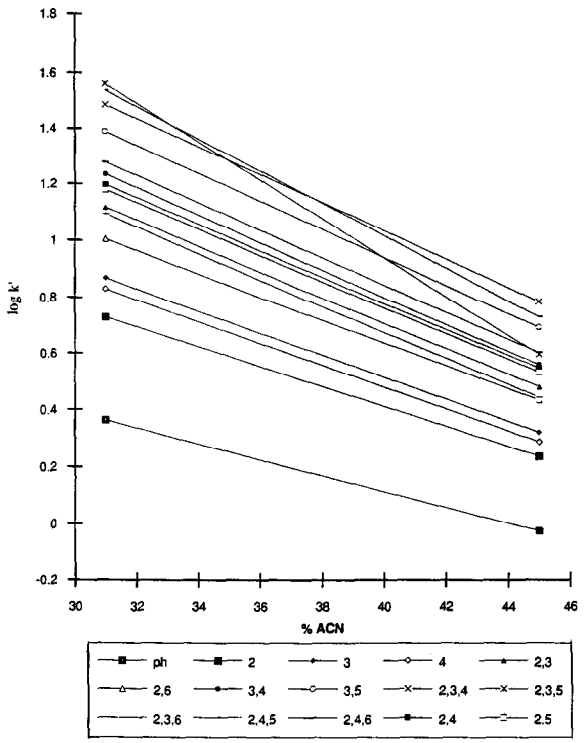

Fig. 13. Evolution of retention (log k') as a function of organic modifier concentration at pH 6.

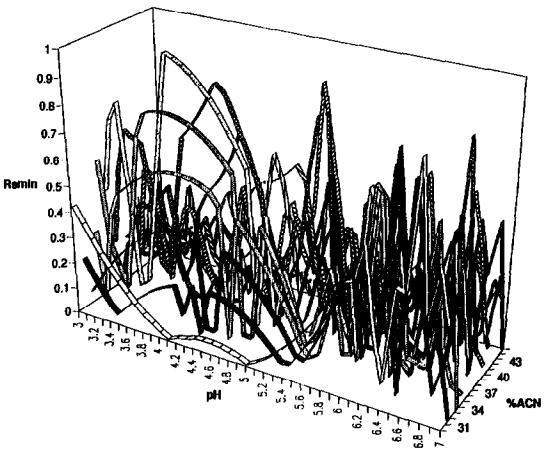

Fig. 14. Three-dimensional graphs of $R_{s_{min}}$ as a function of pH and concentration of acetonitrile for the mixture of 15 chlorophenols.

in the mean percentage deviations in Table V, besides, of course, a lack of fit of the model. The quality of fit of the model depends mainly on the pK_a values of the compounds, but also on the order of elution (magnitude of retention times). In fact, both parameters are interdependent. The model is least accurate for 2,3,6- and 2,4,6-trichlorophenol. These compounds have the smallest pK_a values of the mixture and an almost complete sigmoidal curve of retention *versus* pH was observed in later experiments, as was the case for the tetra- and pentachlorophenols. Consequently, changes in retention times as a function of pH are pronounced for these trichlorophenols. This is illustrated in Fig. 16a and Fig. 16b, which represent the surface of retention times as a function of pH and concentration of acetonitrile for phenol (pK_a = 9.92) and 2,4,6-trichlorophenol (pK_a = 5.99), respectively. The pK_a value

TABLE IV

$R_{s_{min}}$ VALUES FOR THE MIXTURE OF FIFTEEN CHLOROPHENOLS AS A FUNCTION OF pH AND VOLUME FRACTION OF ACETONITRILE

pH	Acetonitrile (%)									
	3.5	3.6	3.7	3.8	3.9	4	4.1	4.2	4.3	4.4
34	0.5072	0.5325	0.5526	0.5675	0.5773	0.5818	0.5811	0.5751	0.5638	0.5471
35	0.6683	0.7632	0.7748	0.7806	0.7805	0.7747	0.7629	0.7453	0.7217	0.6920
36	0.0840	0.3804	0.6826	0.9809	0.9681	0.9488	0.9229	0.8904	0.8512	0.8052
37	0.4260	0.1551	0.1213	0.4038	0.6708	0.7124	0.7637	0.8248	0.8958	0.8682
38	0.1547	0.1774	0.2108	0.0838	0.1556	0.3760	0.4529	0.4966	0.5451	0.6170
39	0.2581	0.2111	0.1115	0.0206	0.0026	0.0116	0.0878	0.0717	0.0304	0.0361
40	0.0245	0.1993	0.3410	0.3251	0.2081	0.0773	0.0256	0.1360	0.3059	0.4782

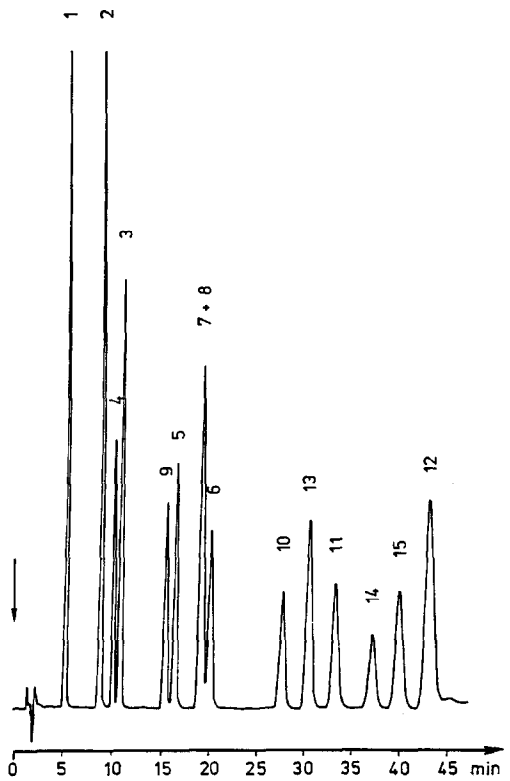

Fig. 15. Chromatogram of the mixture of fifteen chlorophenols obtained under the optimum experimental conditions: pH = 3.9; concentration of acetonitrile = 36%. For identification of the solutes, see Table V.

of phenol is more than two pH units greater than the upper limit of pH and small fluctuations of pH hardly change the retention times.

The experimental error was estimated by repeating the central experiment. Variations of retention times between different days were determined to be of the order of 0.7%, so that results seem to be fairly reproducible.

For the two overlapping compounds, 2,4- and 2,5-dichlorophenol, the deviations between predicted retention times and experimental results are less than 2.5%. Therefore, the difference between observed and predicted $R_{s\min}$ values is probably due to the inaccuracy of the measurements of the peak width and also to small fluctuations in the experimental conditions, especially in the organic modifier content of the mobile phase (see Table V). The latter cause is illustrated in Figs. 12 and 13. At constant organic modifier concentration, differences in the retentions of 2,4- and 2,5-dichlorophenol are very small at pH 4 and 6. However, at pH 4 this difference depends on the concentration of acetonitrile.

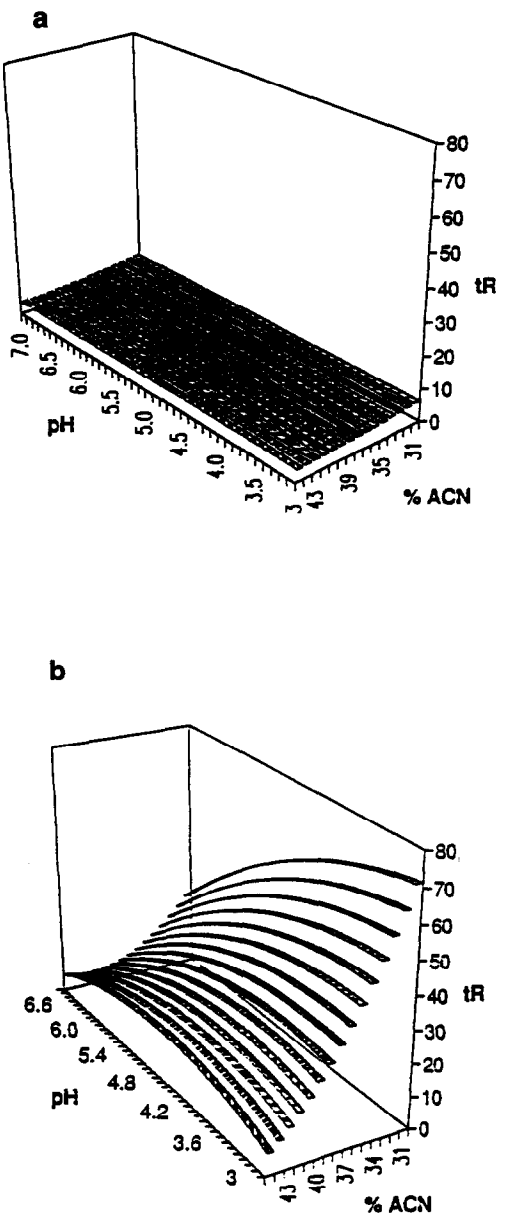

Fig. 16. Retention surfaces as a function of pH and concentration of acetonitrile. (a) Phenol; (b) 2,4,6-trichlorophenol.

After 4 weeks of continuous use, a decrease in

TABLE V

COMPARISON BETWEEN PREDICTED RETENTION TIMES AND EXPERIMENTAL VALUES FOR THE MIXTURE OF FIFTEEN CHLOROPHENOLS, OBTAINED WITH A VOLUME FRACTION OF ACETONITRILE OF 36% AT pH 3.9

In the last two columns the means of the percentage deviations between predicted and experimental t_R and $w_{1/2}$ values for the seven experiments of the design are given.

No.	Compound[a]	pK_a	t_R (pred.)	t_R (exp.)	Deviation (%)	Mean % dev. t_R[b]	Mean % dev. $w_{1/2}$[b]
1	Phenol	9.92	5.38	5.41	0.55	0.35	1.24
2	2-	8.52	9.06	9.12	0.66	0.45	10.9
4	4-	9.37	10.5	10.5	0.48	0.4	4.15
3	3-	8.97	11.4	11.3	0.88	6.07	0
9	2,6-	6.78	16.4	16.1	1.89	3.75	5.38
5	2,3-	7.71	17.3	17.1	0.93	1.36	3.64
7	2,4-	7.9	19.7	19.9	0.81	0.27	3.39
8	2,5-	7.51	20.4	19.9	2.41	2.22	2.77
6	3,4-	8.62	21.0	20.9	0.81	0.72	3
10	3,5-	8.25	28.6	28.6	0	1.23	0.97
13	2,3,6-	5.8	33.5	31.5	6.11	18.3	6.73
11	2,3,4-	6.97	35.5	34.3	3.29	4.7	9.15
14	2,4,6-	5.99	41.0	38.3	6.47	11.9	0.65
15	2,4,5-	6.72	42.6	41.1	3.54	4.1	1.77
12	2,3,5-	6.43	46.4	44.3	4.67	3.91	6.95

[a] Chloro substituent positions.

[b] The mean of the percentage deviations between predicted and experimental t_R and $w_{1/2}$ values for the seven experiments of the design.

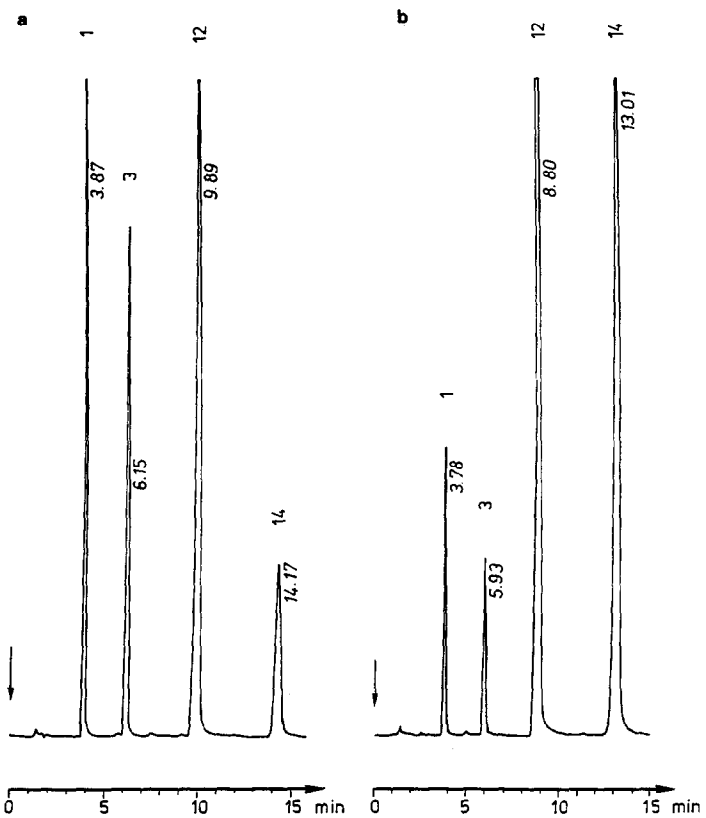

Fig. 17. Chromatograms illustrating the deterioration of the stationary phase. The experimental conditions are the same, *i.e.*, pH = 6 and acetonitrile = 46%, but chromatogram (b) was obtained 4 weeks later than (a). Numbers of the solutes refer to Table II.

retention times of about 10% was observed when repeating experiment 4 of the Doehlert design with fifteen compounds. The peak width remained almost the same and peak tailing also did not increase, as can be seen in Fig. 17. Washing the column with water and methanol, each for 5 h at a flow-rate of 1 ml/min, did not restore the previous retention times; the stationary phase had degraded. Such a decrease in retention is caused by hydrolysis of bonded ligands as solute retention in reversed-phase chromatography as a function of alkyl ligand density follows a linear relationship up to a certain limiting value [33]. Deterioration of the stationary phase was shown to be a cause of bad predictions in the experimental design, so the chromatographer should pay attention when applying conventional silica columns.

CONCLUSIONS

The large difference in retention times between tetra- and pentachlorophenols and the other chlorophenols makes the isocratic separation of all nineteen compounds on the same chromatogram impossible. By optimizing the pH of the mobile phase and the volume fraction of organic modifier using a Doehlert design, two mixtures of fourteen and four chlorophenols could be completely separated. In both separations the systematic approach allowed a short development time of about 2 weeks. One must determine the peak width and retention of each compound in each experiment. For the mixture of tetra- and pentachlorophenols, the deviations that were observed between the predicted values and results obtained by experiment are caused by a lack of fit of the quadratic model.

For the mixture of fifteen compounds, the quadratic model proved to be convenient for the simultaneous optimization of the two experimental parameters. The predictions were found to be accurate. The result obtained, a chromatogram with fourteen well separated peaks, does not correspond to what was predicted (fifteen peaks with an $R_{s\min}$ of 0.96). However, the inaccuracy of the predicted $R_{s\min}$ is not caused by the lack of fit.

In general, the results indicate that a quadratic model is adequate for describing only one leg of the sigmoidal curve of retention *versus* pH. If an (almost) complete curve is observed, a more complex model is required such as the model of Schoenmakers *et al.* [22]. Other approaches might prove worthwhile. One of these (the logit transformation) was described here. Another possibility is to use Drylab to determine an optimum region and to use the quadratic model with a D-optimum design in that region. The quadratic modelling is then an extension of the Drylab strategy. A very different approach based on an optimum mapping of the feasible region is also being investigated [34], but more detailed studies are needed to ascertain the value of such approaches.

The three-dimensional graph of $R_{s\min}$ showed that the development of a rugged HPLC method to separate all the fifteen chlorophenols is not possible in the selected experimental domain and with a LiChrospher RP-18 stationary phase. To resolve this mixture in an isocratic experiment one will require a stationary phase with a higher efficiency and/or other types of organic modifier(s) or HPLC with photodiode-array detection in combination with a chemometric technique such as evolving factor analysis to be able to determine two substances, even when they overlap to a greater extent than usual [35]. It is our intention to explore these possibilities further.

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