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Journal of Chromatography A, 886 (2000) 31–46

JOURNAL OF CHROMATOGRAPHY A

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Interpretive optimisation strategy applied to the isocratic separation of phenols by reversed-phase liquid chromatography with acetonitrile–water and methanol–water mobile phases

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Received 17 January 2000; received in revised form 14 April 2000; accepted 25 April 2000

Abstract

An optimisation protocol is presented for the resolution of complex mixtures in isocratic RPLC with binary mobile phases of organic solvent and water, which is based on the prediction of peak position and shape of the individual compounds. A good description of the retention was achieved through the application of statistical weights to the widely used linear or quadratic relationships between the logarithm of the retention factor (log k) and the organic solvent concentration in the mobile phase. The maximisation of the product of peak purities for each compound is shown as a competitive resolution strategy versus the worst value of a selectivity parameter. Peak purities allow one to associate a single resolution value to each compound, which is not affected by the identity of the interfering peaks. It is shown how when full resolution is not achieved with a single mobile phase, the same experimental data set (retention factors, asymmetries and efficiencies) can be used for finding two or three optimal complementary mobile phases (CMPs). Each CMP resolves fully some compounds in the mixture, while the remaining compounds can overlap among them. The elementary limiting resolutions, which measure the maximal separation degree for each compound, are also given as a useful guide in the selection of the elution conditions. A mixture of 13 phenols (phenol, chloro-, bromo-, nitro- and methyl-derivatives), eluted with acetonitrile–water or methanol–water mobile phases, is used to show the proposed methodology.

Keywords: Optimization; Mobile phase composition; Peak purity; Phenols

1. Introduction

The chromatographer is often concerned with the separation of complex mixtures with a variable behaviour of their components, which makes good resolution sometimes extremely difficult. Several optimisation strategies have been proposed to solve this problem. The most reliable and less time-consuming strategies apply resolution criteria based on empirical or mechanistic models to describe the retention of solutes [1,2]. In reversed-phase liquid chromatography (RPLC), the elution is governed by the strength of solute–stationary phase and solute–mobile phase interactions. In binary mobile phases, numerous studies have established that an exponential decay yields a satisfactory description of the

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0021-9673/00/$ – see front matter
PII: S0021-9673(00)00507-0

retention. This can be linearised to give the following empirical relationship [3]:

$$\log k = a_0 + a_1\varphi \quad (1)$$

where k is the retention factor for a given mobile phase composition, φ the volume fraction of organic solvent in the organic–water mixture, and a_0 and a_1 are constants related to the nature of organic solvent and solute. The intercept of the fitted straight-line (Eq. (1)) refers to the extrapolated log k value for neat water as mobile phase, and the slope indicates the sensitivity of the retention of a given compound to the change of organic solvent concentration in the mobile phase. This equation is however only valid in limited ranges of organic solvent concentration. The quadratic relationship:

$$\log k = a_0 + a_1\varphi + a_{11}\varphi^2 \quad (2)$$

diminishes the deviations from linearity, especially important at the highest and lowest concentrations [4].

Although the accuracy of the predictions of k values severely affects the reliability of the optimisation procedure [5], other secondary factors related to the response function used for describing the separation should be considered, such as the prediction of peak shape. The response function itself introduces some bias. For characterising the separation degree achieved in a chromatogram with a single numerical value (i.e., global resolution), a separation descriptor (i.e., elementary resolution) is first computed for each peak or pair of peaks. Very often, the worst resolution value is straightforwardly taken as a representative measurement of the separation achieved in the whole chromatogram [6–9]. Alternatively, other combination (or reduction) methods can be used such as the product of peak resolutions, which has demonstrated to be also a good descriptor [10–12].

Different measurements of diverse complexity which depict the separation performance have been proposed as indexes of elementary resolution. Criteria based on peak position, such as the separation factor and selectivity, are suitable for comparing chromatograms if all peaks are symmetrical and show similar efficiencies [13,14]. Other criteria allow different efficiencies for each peak in the chromatogram [15,16], or/and consider individual widths, asymmetries and peak heights [6–9]. This is the case of peak purities, which in contrast to most elementary resolution criteria, associate a numerical value to each individual peak instead to each pair of peaks.

In this work, an optimisation strategy for isocratic RPLC is presented for the resolution of complex mixtures with binary mobile phases. The methodology is based on the calculation of combined peak purities for the compounds present in the mixture. Empirical equations are used for the prediction of the retention. It is shown how when full resolution with a single mobile phase fails, the same experimental data set (k values, asymmetries and efficiencies) obtained to model the system can be used for finding two or three complementary mobile phases (CMPs) [17]. Each optimal CMP concerns the resolution of only some compounds in the mixture, whereas the other compounds [resolved by other mobile phase(s)] are not considered, and can thus overlap among them. The increased separation space achieved for the compounds of interest in each CMP enhances the possibilities of good resolution.

A mixture of 13 phenols (phenol, chloro-, bromo-, nitro- and methyl-derivatives), eluted with acetonitrile–water or methanol–water mobile phases, has been chosen as a good probe sample to show the proposed methodology. Several RPLC procedures have been reported for the analysis of mixtures of phenols, which are priority pollutants in natural, drinking and wastewater [18–23]. Other samples where phenol analysis is concerned are urine [24,25], cigarette smoke condensate [26], coal-derived products [27], and shale oil [28]. Isocratic elution from C_{18} columns with binary eluents of methanol–water [21,22,25]or acetonitrile–water [27,28], and ternary eluents of methanol–acetonitrile–water [18,20] are commonly used. Gradient elution procedures have been employed to a lesser extent for these analyses [19,23].

2. Experimental

2.1. Reagents

The following phenols were studied: phenol, 2-chlorophenol and 4-chlorophenol (Carlo Erba, Milan,

Italy), 3-bromophenol (Aldrich, Steinheim, Germany), 2,4-dimethylphenol, 2-nitrophenol, 4-nitrophenol and 2,4-dinitrophenol (Scharlau, Barcelona, Spain), 3-nitrophenol and 2,6-dichlorophenol (Fluka, Buchs, Switzerland), 3-chlorophenol and 4-bromophenol (Merck, Darmstadt, Germany), and 2-methylphenol (Doesder, Barcelona, Spain). An amount of 20 to 200 mg/l of each phenol was dissolved in pure methanol. The acid–base constants (pK_a) and octanol–water partition coefficients (log $P_{o/w}$) are given in Table 1. Methanol and acetonitrile (Merck, for chromatography) mobile phases were prepared. The aqueous phase was 0.1 *M* in acetic acid (Merck) to avoid ionisation of the eluted compounds. Triply distilled water was used throughout.

2.2. Apparatus

The chromatographic equipment was a dual-pump system from ISCO (Lincoln, NE, USA) Model 2350, provided with an ISCO variable-wavelength V^4 absorbance detector, which was set at 282 nm. Data acquisition was made by a Chemresearch chromatographic data managing system controller from ISCO. A Merck LiChrospher 100 RP-18 column (250 mm×4.0 mm I.D., 5 μm particle size), and a precolumn of similar characteristics were used. The injection volume was 10 μl and the flow-rate was 1 ml/min. The chromatographic column was thermostated at 25.0±0.2°C. A 0.01% potassium bromide solution measured at 200 nm was used as dead volume marker. The mean dead times were 1.34 and 1.31 min for acetonitrile–water and methanol–water mobile phases, respectively.

Table 1
Acid–base constants and octanol–water partition coefficients of several phenols

Compound	pK_a [a]	Log $P_{o/w}$ [b]
Phenol	9.99	1.50
4-Nitrophenol	7.18	1.91
3-Nitrophenol	8.36	2.00
2-Methylphenol	10.31	1.98
2-Chlorophenol	8.51	2.15
2,4-Dinitrophenol	4.10	1.67
2-Nitrophenol	7.23	1.77
3-Chlorophenol	9.02	2.50
3-Bromophenol	9.01	2.63
4-Bromophenol	9.36	2.59
4-Chlorophenol	9.38	2.39
2,4-Dimethylphenol	10.54	2.35
2,6-Dichlorophenol	6.79	2.64

[a] Refs. [29] and [30].
[b] Ref. [31].

2.3. Software

A software called CHROM, which is an evolution of MICHROM (available through Marcel Dekker) [32], was developed to model the chromatographic behaviour of sets of compounds under isocratic conditions, and optimise their separation. CHROM is able to simulate chromatograms at any mobile phase composition, and used in an interactive way, it is a practical tool for method development in liquid chromatography. MATLAB 4.2c (Mathworks) Laboratory-written routines were used for finding the optimal CMPs.

3. Mathematical treatment

3.1. Description of the retention

The retention behaviour of phenols was modelled according to the usual linear and quadratic relationships with the volume fraction of organic solvent in the mobile phase (Eqs. (1) and (2)). These equations are often linearly fitted, without any consideration of the transformation carried out in the response. The regression process consists of building the best possible relationship between a given response and the predicting variable. This is done by minimising the sum of squared residuals, that is, the square of the differences between actual and predicted responses extended to the whole set of experimental data. However, when the response is transformed to achieve a more convenient relationship (i.e., a linear dependence such as the logarithmic conversion of Eqs. (1) and (2)), the set of optimal (regressed) parameters will minimise the residuals for the transformed response (log *k*) but not for the original response (*k*).

In any least-squares calculation, the residuals for the experimental data of greater magnitude are considered as important as those ones of smaller

magnitude. One thus obtains a homoscedastic error distribution: all the data are predicted with a similar absolute error. When a transformation is done, a homoscedastic distribution is also achieved, but for the transformed response. Therefore, the error distribution for the original response will not be uniform, as desired. This drawback can be compensated through the application of weights. In the case of a logarithmic transformation, the weights can be obtained using the error theory as follows [33]:

$$W = \frac{\left(\frac{\partial F}{\partial P}\right)^d}{\left(\frac{\partial f}{\partial P}\right)^2} = \frac{1}{\left(\frac{\partial \log k}{\partial k}\right)^2} = 2.303k^2 \quad (3)$$

where F and f are the non-linearised and linearised equations, and P any constant in the retention model. A value of d in the 1–2 range is often accepted.

The errors in the predictions made with Eqs. (1) and (2) can be evaluated in several ways. The usual definition of mean relative error is only adequate when the measurements have similar magnitude. However, with measurements of different magnitude as the case of the retention data for a given compound eluted under different conditions, the usual calculation of mean relative error overestimates the importance of the deviations in the prediction of small retention factors. The following expression overcomes this limitation, leading to more reliable error estimation:

$$\mathrm{RE}_i = \frac{\sum_{i=1}^{m} |k_i^{\mathrm{exp}} - k_i^{\mathrm{pred}}|}{\sum_{i=1}^{m} k_i^{\mathrm{exp}}} \quad (4)$$

where k_i^{exp} and k_i^{pred} are the experimental and predicted retention factors, and m is the number of mobile phases included in the experimental design, for the studied compound.

3.2. Measurement of the resolution

Two optimisation methodologies were considered. The first one consists of a modification of the selectivity parameter, $\alpha_{i,i+1}$ [14], which associates one resolution measurement to each pair of neighbouring peaks (i and $i+1$):

$$\beta_{i,i+1} = 1 - \frac{1}{\alpha_{i,i+1}} = \frac{k_{i+1} - k_i}{k_{i+1}} \quad (5)$$

where $k_{i,i+1} > k_i$. The second methodology builds resolution diagrams using an estimation of peak purity as elementary resolution criterion [34]:

$$r_i = 1 - \frac{w_i'}{w_i} \quad (6)$$

w_i being the total area of a given peak, and w_i' the area of the peak overlapped by the chromatogram yielded by the remaining peaks. Peak purities depend on the relative peak areas, that can increase or decrease the peak fraction overlapped by other peaks. In this work, normalised areas have been used, but true areas can be used instead to improve the reliability of the predictions when a difficult separation is expected. Note that, as commented, this criterion associates one resolution measurement to each peak (not to each pair of peaks, as $\beta_{i,i+1}$ does). Also, r_i measurements are not disturbed by peak reversals since they are straightforwardly related to a specific compound, whereas all the remaining peaks are considered as interferents: which of them overlaps with peak i is irrelevant. The individual nature of the peaks is not important as happens in other criteria where knowledge of the identity of neighbouring peaks is required for each simulated mobile phase. Measuring the resolution in this way for each peak avoids problems related to peak reversals and makes some operations easier, such as weighting or exclusion of peaks. Peak purities are normalised measurements, which simplifies their mutual combination into a single final value and, eventually, a further combination with other quality criteria (i.e., to penalise longer retention times or larger asymmetry factors) via multicriteria decision-making functions.

The information about peak resolution provided by the peak purity criterion has a better quality, but depends strongly on the accuracy of peak shape description. In this work, peak shape was modelled using a Gaussian modified function defined as [10]:

$$h(t) = H \exp\left(-\frac{1}{2} \cdot \frac{(t - t_{\mathrm{R}})^2}{[s_0 + s_1(t - t_{\mathrm{R}})]^2} \right) \quad (7)$$

where H is the peak height and t_{R} the retention time.

The coefficient s_0 is a measurement of peak width at the maximum, whereas s_1 quantifies peak distortion. Both parameters are obtained from the values of retention time, efficiency (N), and asymmetry factor (B/A) measured at 10% of peak height:

$$s_0 = 0.466 \cdot \frac{\sqrt{\frac{41.7(1+t_R)^2}{N(1.25+B/A)}}}{1+\frac{1}{B/A}} \cdot \left(1-\frac{B/A-1}{B/A+1}\right) \quad (8)$$

$$s_1 = 0.466 \cdot \frac{B/A-1}{B/A+1} \quad (9)$$

The retention factors were given by Eqs. (1) or (2), and the efficiencies and asymmetry factors required for these estimations were obtained through linear interpolation, using the experimental data corresponding to the closest available experimental mobile phases to the predicted one.

A set of $n-1$ (selectivity parameter) or n (peak purity) measurements of resolution (n being the number of compounds) are thus obtained for each eventual chromatogram simulated for a given mobile phase composition. The resolution values (Eqs. (5) and (6)) should be finally reduced into a single measurement describing the overall separation for all the peaks present in the simulated chromatogram. Usually, the resolution value for the limiting pair (worst elementary value) is taken for depicting the combined resolution in the full chromatogram. The selectivity parameter and other elementary resolution measurements, such as R_s or the separation factor, have been usually treated in this way to obtain the so-called window diagram.

Another way of combining resolution values, which gives good results with normalised elementary measurements, is the product of elementary resolutions [11]:

$$cR = \prod_{i=1}^{n} r_i \quad (10)$$

which varies between 0 (when at least one peak is fully overlapped) and 1 (when all peaks are baseline resolved). This product improves the resolution of all the peaks in the chromatogram instead of only the limiting peak.

4. Results and discussion

4.1. Description of the retention

The 13 phenols were eluted with 11 regularly distributed mobile phases in the 0–100% (v/v) range for acetonitrile–water and methanol–water mixtures (separated in 10% intervals from each other) [35]. At concentrations of organic solvent below 20%, some phenols were strongly retained, exceeding 200 min. Chromatographic data in the 20–100% range were, therefore, only considered. For acetonitrile–water mixtures, the retention factors ranged between $k=$ 0.75 and 53 for the least retained compound eluted with the strongest mobile phase, and the most retained compound eluted with the weakest mobile phase, respectively. For methanol–water mixtures, k values ranged between 0.69 and 131. The median of the efficiencies and asymmetry factors were 5550 and 1.14 for acetonitrile, and 4430 and 1.17 for methanol, respectively.

Any resolution strategy requires a description of the retention behaviour as good as possible. For RPLC, two equations are traditionally used (Eqs. (1) and (2)). Figs. 1 and 2 illustrate the performance of both equations when ordinary (a, b) and weighted linear least-squares fittings (c, d) are performed, for the two organic solvent–water mixtures. In the plots, the abscissas are logarithmic to avoid clumping of the data in the region of lower retention. It is evident that an ordinary fitting is unacceptably inaccurate. For the studied compounds, the retention covers more than two-orders of magnitude. For this reason, and also because a logarithmic transformation is made, the deviations between experimental and predicted data ($k_i^{exp}-k_i^{pred}$) exceeded largely 10 units for the most retained compounds (the points having larger deviations are not plotted in Figs. 1 and 2). For narrower retention ranges, a heteroscedastic error distribution still exists, but in a lesser extent. Weighted linear fitting partially corrected the results.

As expected, the best results were achieved using a weighted quadratic fitting (Eq. (2), Figs. 1d and 2d), which yielded very low errors independently from the compound and organic solvent concentration. This equation needs at least three experiments to be fitted. Table 2 summarises the errors obtained for each phenol when all available retention

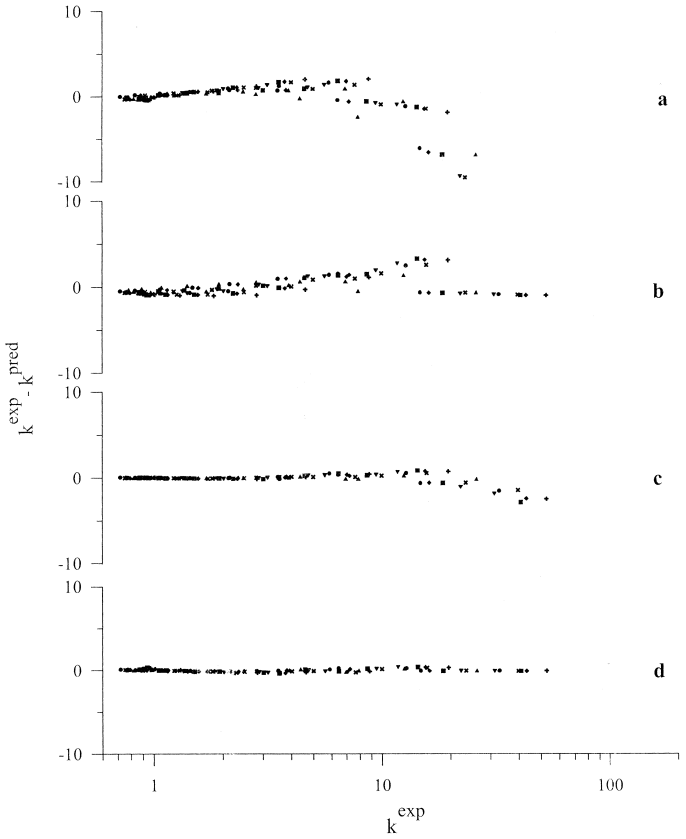

Fig. 1. Accuracy in the prediction of retention factors for the set of 13 phenols, according to Eq. (1) (a, c) and Eq. (2) (b, d), after non-weighted (a, b) and weighted (c, d) fitting of the experimental retention factors obtained in nine mobile phases containing 20–100% acetonitrile.

data are used in the fitting, or only the data from four mobile phases (20%, 40%, 60% and 100%), that is, the three mobile phases needed to fit the quadratic model plus an extra one to check whether a minimised experimental work could still lead to acceptable predictions. It is noteworthy that the errors for the nine- and four-experiment designs are almost coincident, and usually below 0.5 *k* units for the most retained compounds. The aim of covering a very wide organic solvent range has not been translated into dramatic uncertainties in the prediction of retention as certainly happens when correcting weights are not applied.

The predictions were more accurate for acetonitrile–water mixtures. This fact stands out clearly in Figs. 1 and 2. The mean relative error for the 13 compounds was 2.1% for acetonitrile and 2.6% for methanol. Fig. 3 compares predicted and experimental chromatograms for mixtures of four (4-nitrophenol, 2,4-dinitrophenol, 4-chlorophenol and 2,6-dichlorophenol), and five compounds (phenol, 3-nitrophenol, 2-chlorophenol, 3-bromophenol and 4-chlorophenol), eluted with 40% acetonitrile and 20% methanol, respectively. True areas were considered for the predicted chromatograms.

4.2. Resolution with a single mobile phase

For 50% acetonitrile and 60% methanol, the largest *k* values were only 4.6 and 5.6, respectively. For higher modifier contents, the compounds co-eluted in groups near the dead volume. For this reason, the useful region of concentrations for finding the optimal separation was limited to 20–50%. In

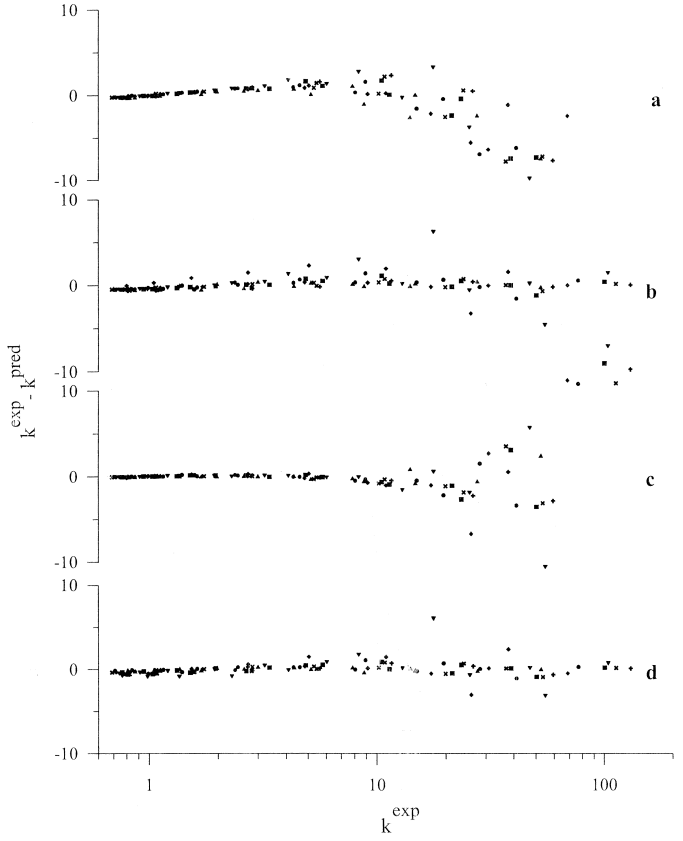

Fig. 2. Accuracy in the prediction of retention factors for the set of 13 phenols, according to Eq. (1) (a, c) and Eq. (2) (b, d), after non-weighted (a, b) and weighted (c, d) fitting of the experimental retention factors obtained in nine mobile phases containing 20–100% methanol.

this region, the changes in resolution behaviour were very complex. Not only the retention exhibited extreme variations when the contents of organic solvent increased in the mobile phase, multiple peak reversals took place also. Baseline separation was not found at any mobile phase composition.

For acetonitrile–water, some compounds were at least partially overlapped at any mobile phase composition. This is the case of 2-chlorophenol and 2,4-dinitrophenol. In contrast, phenol and 2,6-dichlorophenol (the first and last eluting compounds) were always fully resolved. At increasing concentrations of acetonitrile, two compounds (2-nitrophenol and 2,4-dimethylphenol) increased progressively their retention with respect to other phenols, suffering overlapping and reversing their elution order, whereas other compounds became closer (4-nitrophenol and 3-nitrophenol, or 2-chlorophenol and 2,4-dinitrophenol).

For methanol, the first and last eluting compounds were also phenol and 2,6-dichlorophenol. Owing to the lower elution strength, the working concentration range of methanol in which the peaks were not clumped was larger, reaching 60–70%. The least retained compounds were also well resolved in a wider range. Phenol, 4-nitrophenol and 2-nitrophenol were always completely resolved, whereas a small overlapping existed between 2-methylphenol and 2,4-dinitrophenol. 4-Bromophenol, 4-chlorophenol and 2,4-dimethylphenol were in contrast extremely overlapped, giving rise to a wide band. Also, 3-bromophenol and 3-chlorophenol, on the one hand, and 4-bromophenol and 4-chlorophenol, on the other, changed their elution order.

Table 2
Relative errors (REs), mean deviations (MDs) and correlation coefficients obtained in the prediction of retention factors with the weighted quadratic model (Eq. (2)), using all the available (nine) or only four mobile phases in the 20–100% modifier range for the learning test, and the nine mobile phases for the test set

Compound	Nine mobile phases			Four mobile phases		
	RE (%)	MD	Correlation coefficient	RE (%)	MD	Correlation coefficient
Acetonitrile						
Phenol	1.7	0.04	0.9997	1.5	0.04	0.9996
4-Nitrophenol	2.9	0.10	0.9996	2.6	0.09	0.9994
3-Nitrophenol	2.0	0.08	0.9998	1.9	0.07	0.9998
2-Methylphenol	2.4	0.11	0.9997	2.3	0.10	0.9997
2-Chlorophenol	2.2	0.11	0.9998	2.2	0.11	0.9998
2,4-Dinitrophenol	1.3	0.07	0.9999	1.2	0.06	0.9999
2-Nitrophenol	1.0	0.06	0.9999	1.1	0.07	0.9998
3-Chlorophenol	2.4	0.16	0.9998	2.3	0.16	0.9998
3-Bromophenol	2.3	0.20	0.9998	2.2	0.18	0.9997
4-Bromophenol	2.7	0.21	0.9998	2.6	0.21	0.9997
4-Chlorophenol	2.8	0.18	0.9997	2.6	0.17	0.9996
2,4-Dimethylphenol	1.7	0.14	0.9999	1.5	0.12	0.9998
2,6-Dichlorophenol	1.7	0.18	0.9999	1.6	0.17	0.9999
Methanol						
Phenol	3.8	0.16	0.9990	3.4	0.14	0.9988
4-Nitrophenol	2.0	0.14	0.9998	2.1	0.14	0.9998
3-Nitrophenol	3.1	0.24	0.9996	3.1	0.24	0.9993
2-Methylphenol	2.6	0.24	0.9997	2.7	0.26	0.9996
2-Chlorophenol	3.4	0.37	0.9995	3.6	0.40	0.9993
2,4-Dinitrophenol	2.7	0.24	0.9997	2.9	0.25	0.9995
2-Nitrophenol	1.2	0.15	0.9999	1.2	0.15	0.9999
3-Chlorophenol	3.1	0.55	0.9997	3.4	0.59	0.9992
3-Bromophenol	5.3	0.78	0.9990	5.3	0.78	0.9987
4-Bromophenol	2.4	0.52	0.9998	2.5	0.55	0.9996
4-Chlorophenol	0.3	0.06	1.0000	0.4	0.06	1.0000
2,4-Dimethylphenol	2.0	0.46	0.9999	2.1	0.50	0.9997
2,6-Dichlorophenol	1.4	0.37	0.9999	1.6	0.42	0.9999

Not only the elution strength was different for both acetonitrile and methanol, also the selectivity changed significantly. This was especially outstanding for 2-nitrophenol, which successively reversed its elution order with other phenols when was eluted with acetonitrile, but was completely resolved with methanol in a wide range of concentrations.

The resolution behaviour of the 13 phenols is described in Fig. 4 for acetonitrile–water mixtures. The global resolution for a single mobile phase is plotted in Fig. 4a and b for the worst elementary modified selectivity and the product of peak purities, respectively. There is a rough agreement between the location of the maximums in both diagrams, but the relative importance is not the same. Thus, for the first criterion several maximums are observed, the main maximum of which corresponds to 34.3% acetonitrile. Meanwhile, for the second criterion this maximum is smaller, and the two main maximums are observed for 20.0 and 24.8% acetonitrile, with combined resolution values: cR=0.476 and 0.389, respectively.

Fig. 4a shows a window diagram where each minimum corresponds to a peak reversal. In contrast, Fig. 4b is simpler, and less clear in indicating the position of peak reversals. Co-elution can be revealed however easily making a log-transformation of the resolution axis (Fig. 4c). As can be seen, the resolution behaviour of phenols is very complex, showing numerous changes in elution order, which are described by the minimums in the plot. At increasing acetonitrile contents, the resolution be-

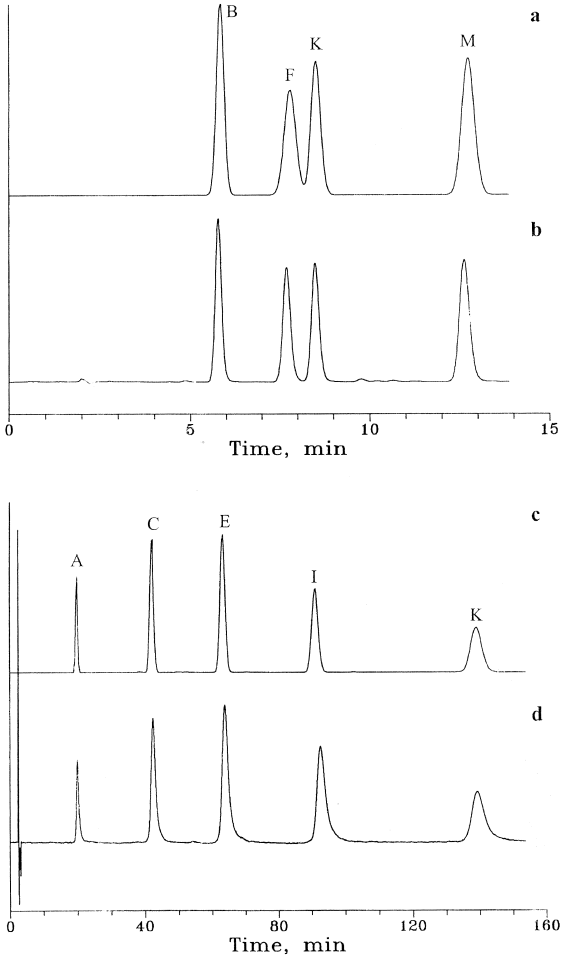

Fig. 3. Predicted (a, c) and experimental (b, d) chromatograms for mixtures of 4-nitrophenol (B), 2,4-dinitrophenol (F), 4-chlorophenol (K) and 2,6-dichlorophenol (M), eluted with 40.0% acetonitrile (a, b), and phenol (A), 3-nitrophenol (C), 2-chlorophenol (E), 3-bromophenol (I) and 4-chlorophenol (K), eluted with 20.0% methanol (c, d).

comes worse because the greater elution strength pushes the compounds towards the beginning of the chromatogram.

Fig. 5 shows chromatograms at the optimal compositions for the three main maximums observed in Fig. 4a and b. Baseline resolution could not be achieved at any mobile phase composition. The best separation was obtained for 20.0% acetonitrile (Fig. 5a), but an important overlapping still existed between 2,4-dimethylphenol (L) and 4-bromophenol (J). The elution time was also rather long. The use of

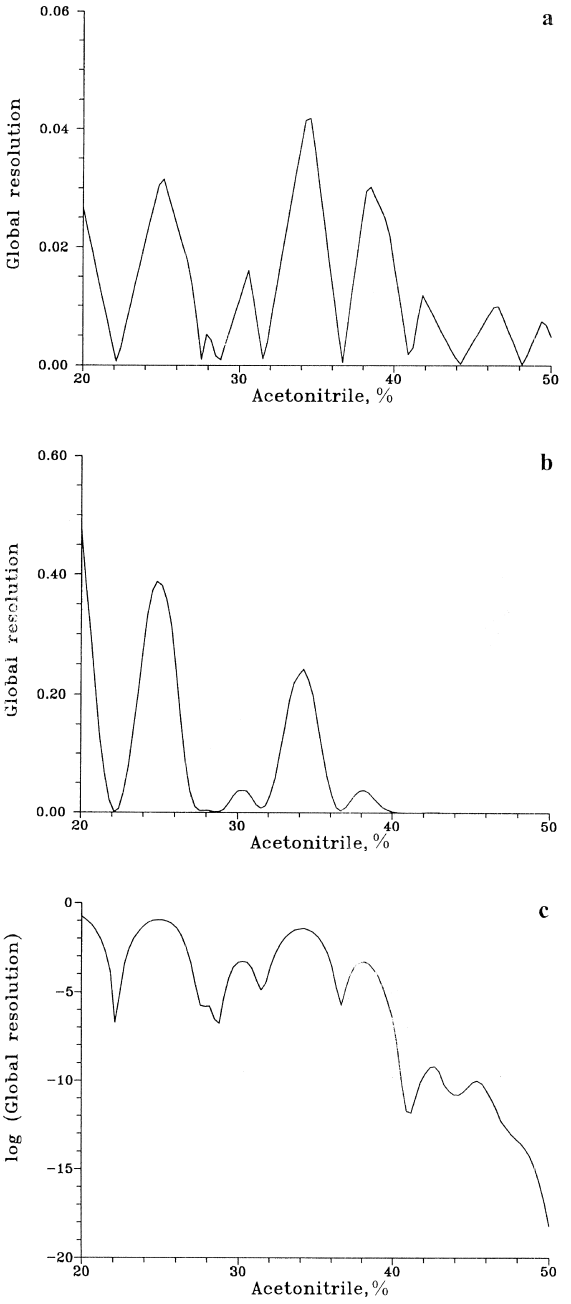

Fig. 4. Global resolution for mixtures of the 13 phenols eluted with a single mobile phase of acetonitrile–water, according to the worst elementary modified selectivity (a), and the product of peak purities (b, c).

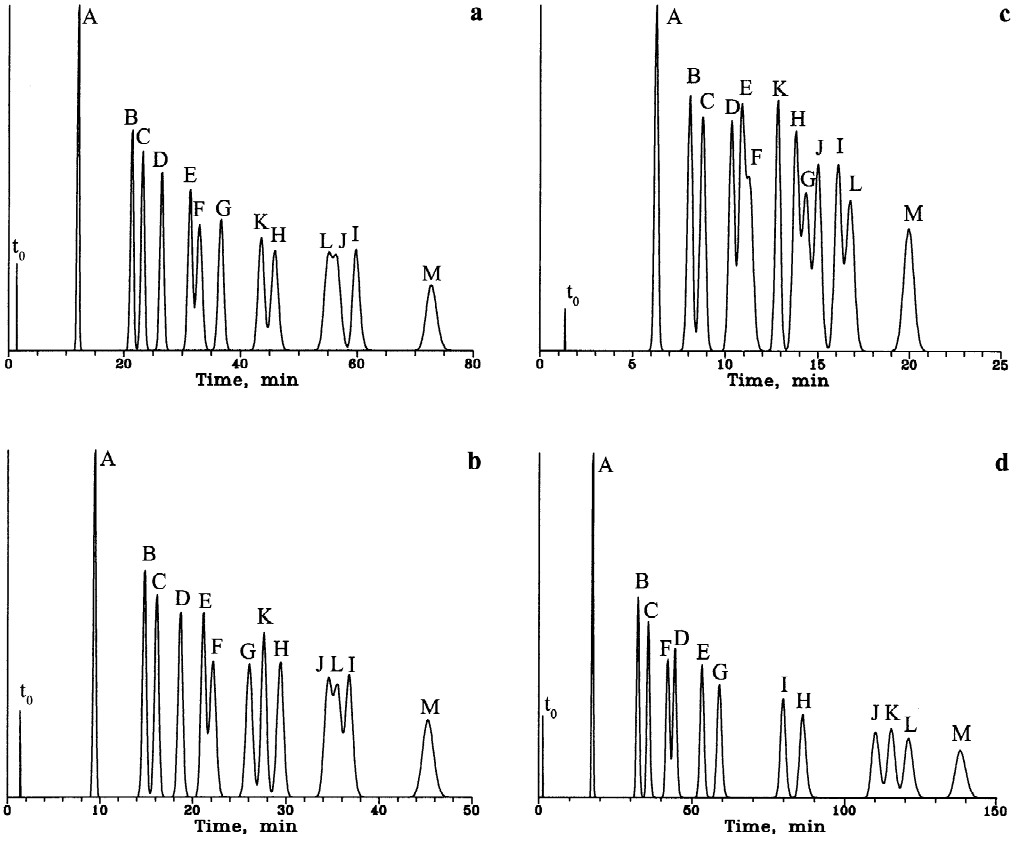

Fig. 5. Chromatograms for the three main maximums shown in Fig. 4a and b for acetonitrile: 20.0% (a), 24.8% (b), and 34.3% (c), and main maximum obtained for methanol: 23.2% (d). Compounds: phenol (A), 4-nitrophenol (B), 3-nitrophenol (C), 2-methylphenol (D), 2-chlorophenol (E), 2,4-dinitrophenol (F), 2-nitrophenol (G), 3-chlorophenol (H), 3-bromophenol (I), 4-bromophenol (J), 4-chlorophenol (K), 2,4-dimethylphenol (L), and 2,6-dichlorophenol (M).

24.8% acetonitrile (Fig. 5b) decreased the analysis time, but at the cost of an increased overlapping of 3-bromophenol (I) with 2,4-dimethylphenol and 4-bromophenol. The third maximum at 34.3% acetonitrile was very unsatisfactory (Fig. 5c). According to these considerations, it can be concluded that the product of peak purities indicates the optimal resolution better.

The resolution obtained with methanol is similar to that of acetonitrile, but considering that the retention times are much greater, the former modifier should be discarded for the analysis of the studied mixture. The chromatogram for the optimal composition (23.2% methanol) showed a combined resolution value of cR=0.798 (Fig. 5d). Only a partial overlapping was observed between 2,4-dinitrophenol (F) and 2-methylphenol (D), on the one hand, and 4-bromophenol (J), 4-chlorophenol (K) and 2,4-dimethylphenol (L), on the other.

4.3. Limiting resolution

Using a single mobile phase, the separation degree of the mixture of 13 phenols was not enough with any modifier at any concentration. The limiting resolutions (r_L) measured as peak purities that can be obtained for each compound are given in the last column of Table 3. Each limiting resolution is associated to a given compound, and corresponds to the maximal peak purity that can be achieved when that compound is resolved with the mobile phase that separates it from the others better [17]. A similar

Table 3
Maximal elementary resolution values obtained using a single mobile phase (20.0% acetonitrile or 23.2% methanol), or two (22.4% and 40.3% acetonitrile, or 21.8% and 29.7% methanol), and three (21.4%, 32.1% and 40.0% acetonitrile, or 20.0%, 29.1% and 46.4% methanol) complementary mobile phases to resolve the mixture of the 13 phenols

Compound	Maximal resolution			Limiting
	Single phase	Two CMPs	Three CMPs	resolution
Acetonitrile				
Phenol	1.000	1.000	1.000	1.000
4-Nitrophenol	0.999	0.999	0.999	0.999
3-Nitrophenol	0.999	0.999	0.999	0.999
2-Methylphenol	1.000	1.000	1.000	1.000
2-Chlorophenol	0.950	0.940	0.945	0.950
2,4-Dinitrophenol	0.945	0.941	0.945	0.946
2-Nitrophenol	1.000	1.000	1.000	1.000
3-Chlorophenol	0.966	0.988	0.982	0.998
3-Bromophenol	0.995	0.995	0.996	0.996
4-Bromophenol	0.751	0.889	0.997	0.998
4-Chlorophenol	0.966	0.988	0.999	0.999
2,4-Dimethylphenol	0.761	0.988	0.988	0.988
2,6-Dichlorophenol	1.000	1.000	1.000	1.000
Methanol				
Phenol	1.000	1.000	1.000	1.000
4-Nitrophenol	1.000	1.000	1.000	1.000
3-Nitrophenol	1.000	1.000	1.000	1.000
2-Methylphenol	0.952	0.974	0.984	0.986
2-Chlorophenol	1.000	1.000	1.000	1.000
2,4-Dinitrophenol	0.950	0.973	0.971	0.980
2-Nitrophenol	1.000	1.000	1.000	1.000
3-Chlorophenol	0.999	1.000	1.000	1.000
3-Bromophenol	1.000	1.000	1.000	1.000
4-Bromophenol	0.964	0.975	0.977	0.980
4-Chlorophenol	0.940	0.940	0.946	0.946
2,4-Dimethylphenol	0.975	0.992	0.999	0.999
2,6-Dichlorophenol	1.000	1.000	1.000	1.000

parameter has been described using the R_s values for peak pairs [36]. If limiting resolutions are compared with the elementary resolution values (r) for each compound at the optimal mobile phase found in the previous section, it can be concluded that:

(i) The separation of some compounds can be improved. For acetonitrile–water mixtures: 3-chlorophenol (from $r=0.966$ to $r_L=0.998$), 4-bromophenol (from $r=0.751$ to $r_L=0.998$), 4-chlorophenol (from $r=0.966$ to $r_L=0.999$), and 2,4-dimethylphenol (from $r=0.761$ to $r_L=0.988$). For methanol–water mixtures: 2-methylphenol (from $r=0.952$ to $r_L=0.986$), 2,4-dinitrophenol (from $r=0.950$ to $r_L=0.980$), and 2,4-dimethylphenol (from $r=0.975$ to $r_L=0.999$).

(ii) Some compounds will not be completely resolved at any mobile phase composition. With acetonitrile–water mixtures, the limiting values are: $r_L=0.950$ for 2-chlorophenol and 0.946 for 2,4-dinitrophenol; and with methanol–water mixtures: $r_L=0.946$ for 4-chlorophenol. Other compounds will not reach baseline resolution, but the separation can be quite satisfactory.

The product of limiting resolutions for the mixture of 13 phenols is $cR_L=0.879$ and 0.894 for acetonitrile and methanol, respectively. Since the combined resolutions obtained with these solvents for the optimal single mobile phase were $cR=0.476$ and 0.798, respectively, and selectivity changes are produced when the modifier contents is varied, an alternative approach is to use two or more CMPs, instead of a single mobile phase. Each compound

should be well resolved in at least one of these mobile phases.

4.4. Use of optimal complementary mobile phases

Complete separation of a mixture is not always possible using a single mobile phase. In this case, the chromatographer may decide to change the nature of the separation system (organic modifier, column or even chromatographic technique). However, the separation can succeed without the need of such drastic changes, finding mobile phases with complementary behaviour. This strategy is applied here to improve the separation of the mixture of 13 phenols.

The methodology is based, as before, on the calculation of peak purities for the n compounds in the mixture at varying mobile phase composition. This allows building a set of n vectors, each one associated with a specific compound. The elements of these vectors store the resolution at different mobile phase compositions for the considered compound. In the examined example, 13 vectors (one by phenol) were calculated for a set of pre-defined regularly spaced mobile phase compositions. The set of vectors can be used for finding either the optimal composition to resolve each compound, a subset of compounds or, eventually, all the compounds in the sample.

In the CMP approach, two or three mobile phase compositions giving complementary resolutions are found. This is done measuring the resolution values for all possible arrangements that can be defined distributing the n compounds in m (i.e., 2 or 3) subsets, and then finding for each distribution the optimal mobile phase [17]. For any arbitrary distribution of the compounds, the resolution is calculated in two consecutive steps.

In the first step, intermediate combined resolutions are obtained according to Eq. (10) including only the compounds assigned to the considered subset. The mobile phase giving maximal resolution for each subset is found as in a conventional optimisation. These maximal values [$R_m(1)$, $R_m(2)$,...$R_m(m)$ for the m CMPs] are then multiplied to obtain a value representing the resolution for that compound distribution (cR_m). One obtains thus a global combined resolution value, and m (two or more) linked complementary mobile phases. This process is carried out for all possible distributions that can be made with the considered compounds. The maximal resolution value points out the best combination of mobile phases that will resolve the mixture in a complementary way.

In order to apply this approach to the mixtures of phenols, peak purities were calculated for each phenol in one hundred simulated mobile phases evenly distributed in the 20–50% modifier range. The global combined resolution for the different distributions of the 13 phenols in two subsets, each of them resolved with a different mobile phase, was then calculated. The number of different distributions that should be examined was relatively low: 4095. The computer time needed to find the optimal CMPs in a 266 MHz Pentium personal computer was less than 30 s. The results obtained after applying this strategy for two optimal CMPs are given next. For acetonitrile (Fig. 6a and 6b), the first mobile phase [phase a, 22.4% acetonitrile, $R_2(1)=0.857$] resolves optimally 4-bromophenol (J), 2,4-dimethylphenol (L) and 2,6-dichlorophenol (M), and the second one [phase b, 40.3% acetonitrile, $R_2(2)=0.878$] the other 10 phenols. Note however, that phenol (A) and 2,6-dichlorophenol are well resolved with both phases a and b. The two $R_2(i)$ values should not be compared each other because the first one involves the product of three elementary resolution values, while the second one includes 10 values. Finally, the global combined resolution for the two optimal CMPs is the product of both values: $cR_2=0.857\times0.878=0.752$, appreciably greater than that obtained with a single mobile phase (20.0% acetonitrile, $cR=0.476$). As observed, in phase a, 2-methylphenol (D), 2-chlorophenol (E) and 2,4-dinitrophenol (F), on the one hand, and 2-nitrophenol (G) and 3-bromophenol (I), on the other, co-elute. These compounds are nevertheless resolved in phase b (three of them fully). In phase b, 4-bromophenol (J) and 2,4-dimethylphenol (L) co-elute, but these compounds are partially resolved in phase a. These two compounds also overlap for 20.0% acetonitrile.

For methanol (Fig. 6c and 6d), the first mobile phase [phase c, 21.8% methanol, $R_2(1)=0.933$] resolves 2-chlorophenol (E), 2-nitrophenol (G), 3-bromophenol (I), 3-chlorophenol (H), 4-chlorophenol (K) (this one only partially), and 2,4-di-

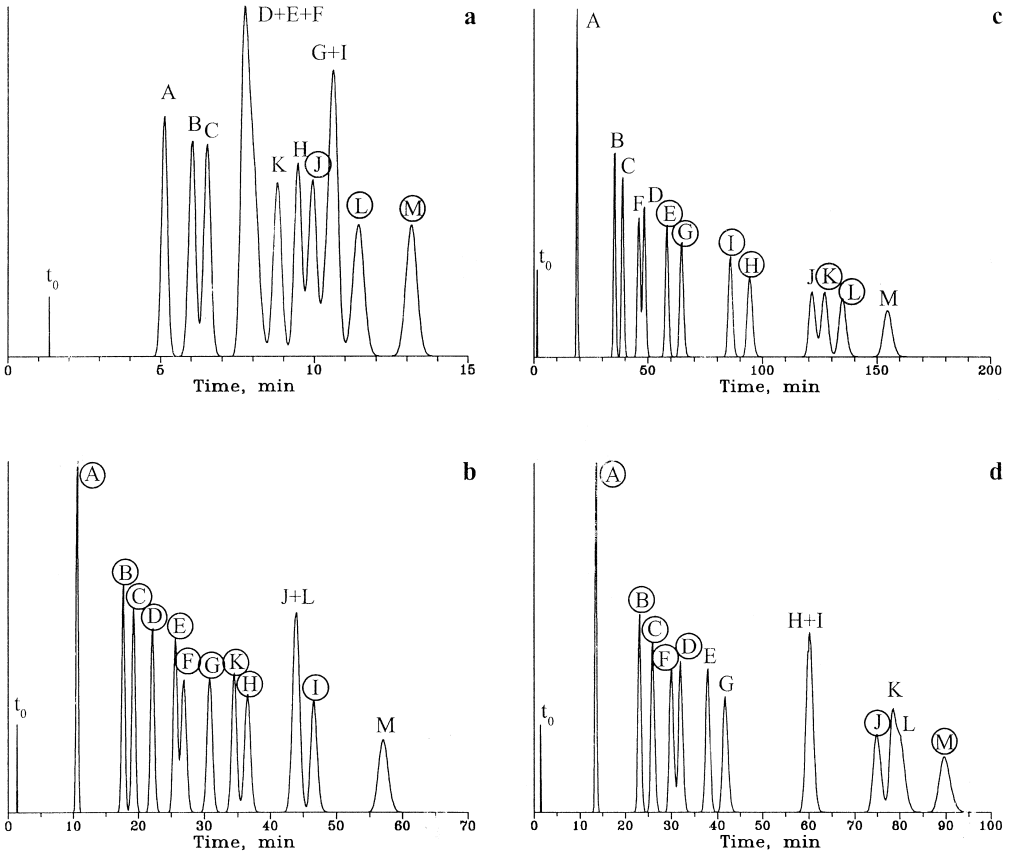

Fig. 6. Chromatograms of mixtures of the 13 phenols eluted with two optimal complementary mobile phases of acetonitrile–water (a, b), and methanol–water (c, d), according to the product of peak purities. Mobile phase composition for acetonitrile: 22.4% (a) and 40.3% (b); for methanol: 21.8% (c) and 29.7% (d). The circles indicate the compounds resolved optimally with each CMP. See Fig. 5 for peak identity.

methylphenol (L), and the second one the remaining seven phenols [phase d, 29.7% methanol, $R_2(2)=0.924$]. The global combined resolution is $cR_2=0.862$, only somewhat greater than the value obtained for a single mobile phase (23.2% methanol, $cR=0.798$), and close to the limiting combined value, $cR_L=0.894$. The retention times are again much longer than those obtained with acetonitrile. It is interesting to note that 3-chlorophenol and 3-bromophenol co-elute in phase d, but are baseline resolved in phase c. Phenol (A) and 2,6-dichlorophenol (M) are again equally resolved with both phases c and d. However, for methanol, the use of two optimal CMPs is not so advantageous as for acetonitrile, since the mixture is quite well resolved with a single eluent containing 23.2% methanol, and the retention times are too high.

Table 3 compares the elementary resolutions of each compound, for both modifiers in the optimum found using a single mobile phase, and for two optimal CMPs. For acetonitrile, 2,4-dimethylphenol reaches the limiting resolution using two CMPs, whereas 3-chlorophenol, 4-bromophenol and 4-chlorophenol although better resolved, can still be improved. The resolution of 2,4-dinitrophenol with a single mobile phase is already the maximal that can be achieved in this system. As commented, with methanol, the improvement achieved with two CMPs is rather moderate, since all the compounds reach practically the limiting resolution with a single mobile phase.

The limiting values given in Table 3 indicate that the resolution achieved with two optimal CMPs can be enhanced further in some extension for acetoni-

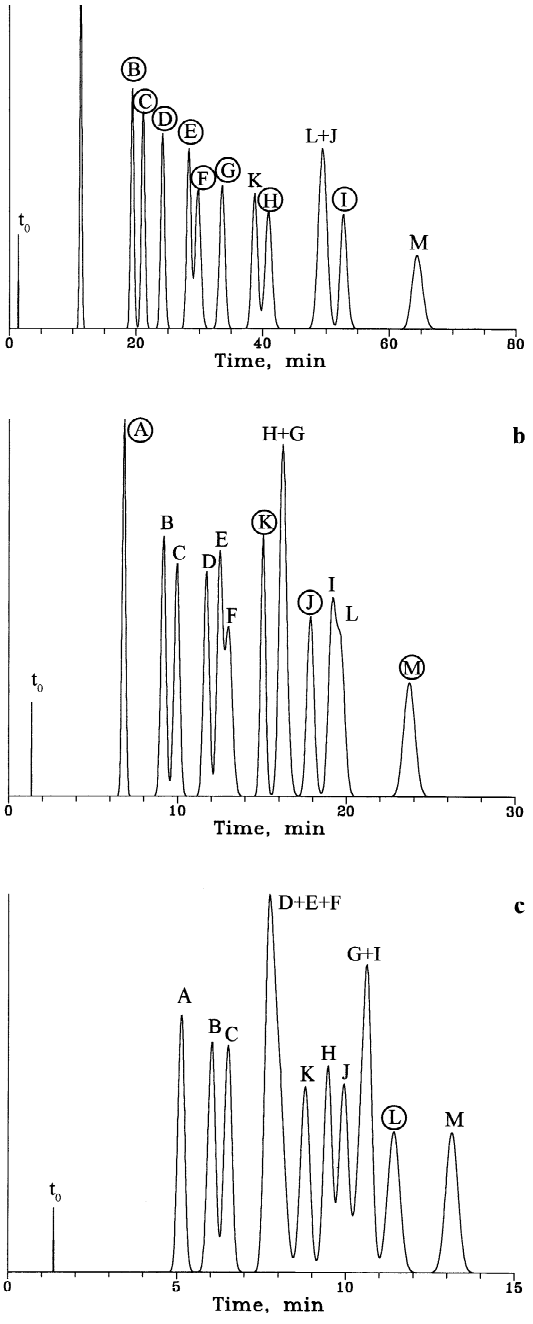

Fig. 7. Chromatograms of mixtures of the 13 phenols eluted with three optimal complementary mobile phases of acetonitrile–water, according to the product of peak purities. Mobile phase composition: 21.4% (a), 32.1% (b), and 40.0% (c). The circles indicate the compounds resolved optimally with each CMP. See Fig. 5 for peak identity.

trile, but not for methanol. The use of three CPMs was therefore considered for acetonitrile. The composition (percentage of acetonitrile) and combined resolution obtained with three optimal CMPs were: phase a [21.4%, $R_3(1)=0.871$], phase b [32.1%, $R_3(2)=0.997$] and phase c [40.0%, $R_3(3)=0.988$], and the global combined resolution: $cR_3=0.858$ (compared with $cR_2=0.752$ with two CMPs, and the limiting value $cR_L=0.879$). Fig. 7 shows the corresponding chromatograms. For methanol, the global combined resolution for three CMPs is $cR_3=0.882$ (against 0.862 for two CMPs).

Fig. 8 depicts the elementary resolution map for each compound at varying mobile phase composition in the 20–50% acetonitrile range. Compounds having compatible maximal resolution have been plotted together. It can be observed that 2,4-dimethylphenol (Fig. 8c) is resolved with phase c, four compounds (phenol, 4-bromophenol, 4-chlorophenol and 2,6-dichlorophenol) are resolved with phase b (Fig. 8b), and the remaining compounds with phase a (Fig. 8a). The compounds resolved optimally in each CMP (40.0%, 32.1% and 21.4% acetonitrile, respectively) have a common composition range of good resolution (not necessarily the optimal for each compound). The elementary contributions are thus balanced in order to obtain the best separation with a reasonable number of mobile phases.

5. Conclusions

In the proposed methodology, the information given by the computed peak purities is combined in order to select chromatographic conditions able to optimally complement each other, and reach the best separation. An interesting parameter that indicates the possibilities of the chromatographic system is the limiting resolution, that is, the maximal elementary resolution that can be obtained for each compound. Limiting resolutions can be used as a guide to know the compounds that can be resolved or will remain unresolved with the selected chromatographic system.

For the success of this strategy, the use of a measurement of the separation quality associating a resolution value to each compound in a mixture, not

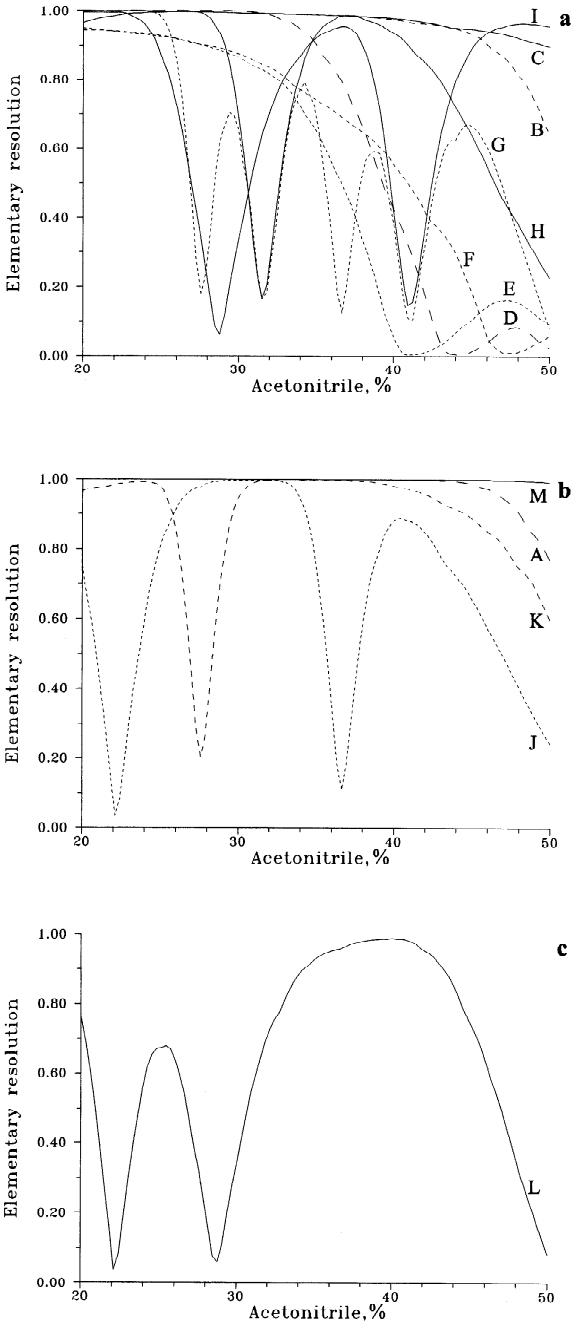

Fig. 8. Elementary resolutions for the 13 phenols at varying acetonitrile–water mobile phase composition. The compounds are distributed in the three diagrams according to the complementary mobile phase where they are assigned in the optimisation process. Compounds I, C, B, G, H, F, E and D are best resolved with 21.4% acetonitrile (a), compounds M, A, K and J are resolved with 32.1% acetonitrile (b), and compound L is resolved with 40.0% acetonitrile (c). See Fig. 5 for peak identity.

affected by the identity of neighbouring peaks, is fundamental together with a good peak description. The criterion of peak purities accomplishes these requirements since it considers peak height, width and asymmetry, and in addition, it isolates the contributions of each component. It has two additional advantages: the resolution values are normalised and have a straightforward meaning, which is useful to understand the information obtained throughout the optimisation process.

In the example considered in this work, the resolution of all the compounds is unfeasible with a single mobile phase. Although the resolution with methanol–water mobile phases was almost complete (except for 4-chlorophenol, $r=0.940$), the retention times were too long to have an analytical interest. The limiting resolutions for acetonitrile indicate that 2-chlorophenol ($r_L=0.950$) and 2,4-dinitrophenol ($r_L=0.946$) cannot be fully resolved at any condition. When a single mobile phase of this modifier is used to resolve the mixture of 13 phenols, some compounds such as 4-bromophenol and 2,4-dimethylphenol are insufficiently resolved. The limiting resolutions for these compounds suggest however that they can be resolved in other conditions, which gives support to the idea of finding complementary mobile phases. Two optimal CMPs were enough to achieve resolutions close to the expected limiting values for all compounds.

The success in obtaining good resolution using CMPs depends on the diversity of retention behaviour of the eluted compounds. The more different the peak distributions that can be yielded by changing the mobile phase composition, the more the probability of finding a set of independent conditions that resolve altogether the compounds in the mixture. These variations in selectivity are restricted with methanol–water and acetonitrile–water mobile phases, since only one experimental factor is available to modify the peak distribution. Therefore, the probability of succeeding is smaller than when two or more variables are used. Analysis time can be also included in the optimisation process via multicriteria decision-making, or just discarding the resolution data in the elementary matrices yielding an undesirable retention. In this work, we focus on isocratic elution. The possibility of using gradient elution remains for further studies.

Acknowledgements

This work was supported by the DGES Projects PB97-0878 and PB97-1384 of Spain.

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