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Finding the best separation in situations of extremely low chromatographic resolution

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ABSTRACT

Samples with a large number of compounds or similarities in their structure and polarity may yield insufficient chromatographic resolution. In such cases, however, finding conditions where the largest number of compounds appears sufficiently resolved can be still worthwhile. A strategy is here reported that optimises the resolution level of chromatograms in cases where conventional global criteria, such as the worst resolved peak pair or the product of elementary resolutions, are not able to detect any separation, even when most peaks are baseline resolved. The strategy applies a function based on the number of "well resolved" peaks, which are those that exceed a given threshold of peak purity. It is, therefore, oriented to quantify the success in the separation, and not the failure, as the conventional criteria do. The conditions that resolve the same amount of peaks are discriminated by either quantifying the partial resolution of those peaks that exceed the established threshold, or by improving the separation of peaks below it. The proposed approach is illustrated by the reversed-phase liquid chromatographic separation of a mixture of 30 ionisable and neutral compounds, using the acetonitrile content and pH as factors.

1. Introduction

When an analytical sample contains a large number of compounds, the application of trial and error assays to get complete resolution can be very expensive, slow, or even unfeasible. The best separation conditions are most efficiently found by means of computer-assisted strategies that are based on the global measurement of the separation quality, using the so-called chromatographic objective functions (COFs) [1–5]. With this aim, the chromatographic signals should be described and further predicted through adequate retention and peak profile models, in a selected experimental region. These models allow the detailed examination of the resolution behaviour in a wide range of conditions, without the requirement of more experimental effort, and the final selection of the most appropriate conditions (those optimal according to different criteria).

The quality of a chromatogram is usually evaluated according to two main strategies: the resolution of the worst resolved peak pair (or single peak), and the combination (according to different criteria) of the resolution of all peaks (or groups of peaks) in a chromatogram. These strategies offer satisfactory results when full (or almost full) resolution can be reached in the separation, or at least, there is an experimental condition where each compound in the sample can be clearly observed. In many instances, however, this is not possible; in such situations, conventional COFs are scarcely informative. An extreme case happens when at least one compound appears severely overlapped at any experimental condition: the global resolution will be null, even when practically all other compounds were baseline resolved. In general, the scores of conventional COFs tend to be dominated by the separation level reached by the poorly resolved peaks.

Several approaches have been proposed to appraise the resolution level of chromatograms in the cases where the conventional COFs are not able to detect any resolution (even existing baseline-resolved peaks), which are based on the counting of apparent peaks [6–8]. These approaches focus on the well resolved peaks, in contrast to conventional resolution assessments that attend mainly to (are affected by) the least resolved peaks. In other words, they are oriented to quantify the success in the separation, and not the failure.

The most known COF of this type was proposed by Berridge [6]. This author defined an objective function, which combines in a weighted summation, the number of detected peaks (N), a term that accounts for the resolution (the Snyder resolution between adjacent peak pairs, R_S), and the total time for the elution of the

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whole sample.

$$\mathrm{COF_{Berridge}} = N^x + \sum_{i=1}^{N-1} R_{S,i} - a|t_{max} - t_{R,last}| - b|t_{min} - t_{R,first}| \quad (1)$$

where t_{max}, t_{min}, $t_{R,last}$ and $t_{R,first}$ are the maximal acceptable time, the minimal retention time for the first peak, and the retention times for the last and first peaks, respectively; x, a and b are arbitrary weights selected by the operator (usually set between 0 and 3). The two last terms are applied only when the first and/or last peaks are beyond the window of acceptable times.

We proposed another COF exclusively based on the number of resolved peaks, which were qualified according to the peak purity criterion [7]. Recently, a COF of the type of the Berridge function has been reported by Duarte and Duarte, where the peak resolution is measured using the valley-to-peak ratio for adjacent peaks (θ_i) [8].

$$\mathrm{COF_{Duarte}} = N + \sum_{i=1}^{N-1} \theta_i - \frac{t_{R,last} - t_0}{t_{R,last}} \quad (2)$$

where t_0 is the dead time. Eq. (2) was applied to mixtures of unknown compounds, being well suited for describing the separation of peak pairs of highly unequal area, and also for overlapped and asymmetrical peaks.

In this work, a new COF based on the peak count, measured as the number of peaks exceeding a certain peak purity threshold, is discussed. This function is not only oriented to get good chromatograms for qualitative purposes, but to guarantee that a high number of compounds in the mixture can be quantified. In contrast to the Berridge [6], and Duarte and Duarte [8] functions, the proposed approach is applied to simulated chromatograms, based on the information obtained from standards of the analysed compounds.

The evaluation of the new COF, which is suitable for situations of extremely low resolution, is illustrated by the reversed-phase liquid chromatographic analysis of a mixture of 30 compounds (most of them ionisable with an acidic or basic character), using the acetonitrile content and pH as factors. This is a particularly problematic mixture under the point of view of the resolution, due to the diversity in acid-base behaviour, which is translated into sudden drops in retention at specific pH values for each compound, giving rise to multiple peak crossings. Therefore, finding common high resolution regions was especially difficult. The study was performed with the data obtained in a former work to develop and validate different retention models [7]. For this reason, it involved measurements in a large variety of experimental conditions in a two-dimensional space. It should be noted, however, that the discussion and conclusions are of general application beyond the particular example itself. The case of study is just a good example to evaluate the performance of approaches oriented to problems of low resolution.

2. Theory

2.1. Description of the retention behaviour

The retention of neutral compounds at varying organic solvent content in the mobile phase can be described using simple models, as the following [9,10]:

$$\log\ k = q + pP_m^N \quad (3)$$

where P_m^N is the normalised solvent polarity, which depends on the concentration and nature of the organic solvent. For acetonitrile:

$$P_m^N = 1 - \frac{2.13\varphi}{1 + 1.42\varphi} \quad (4)$$

φ being the organic solvent content. Eq. (3) provides better predictions of the retention than the direct quadratic relationship with φ [10].

In the case of ionisable compounds, the effect of both organic solvent and pH on retention should be considered. It should be also taken into account that the changes in the solvent concentration affect the acid-base equilibria, which should be described by conditional constants. Therefore, the interaction of the retention with the pH requires more complex models [7,11,12].

Since the intrinsic retention of the acidic and basic species for ionisable compounds is different, a sudden drop in retention will happen at pH values close to the logarithm of the conditional protonation constant. The retention of the neutral species will be higher due to the stronger interaction with the stationary phase. Thus, for acidic compounds, where the basic species is anionic: $k_{HA} > k_{A^-}$, whereas for basic compounds where the acidic species is cationic: $k_B > k_{HB^+}$. This gives rise to a decrease or an increase in the retention with pH, respectively. The amphoteric compounds present a particular behaviour: low retention at low pH (where the cationic species dominates), increased retention at intermediate pH (since the neutral species becomes the most abundant), and a further decrease in the retention at higher pH (due to the shift of the equilibrium toward the anionic species).

For acidic and basic compounds, the following model was used [7]:

$$\log\ k = q + pP_m^N + \log\left(\frac{1}{1 + 10^{\log K_0 + m\varphi h}}\right) \quad (5)$$

where h is the molar concentration of the hydrogen ion, K_0 the protonation constant in water, and m quantifies the dependence of the logarithm of the protonation constant with φ (i.e. slope). For amphoteric compounds [7]:

$$\log\ k = q + pP_m^N + \log\left(\frac{10^{\log\ \beta_{1,0} + m_1\varphi h}}{1 + 10^{\log\ \beta_{1,0} + m_1\varphi h} + 10^{\log\ \beta_{2,0} + m_2\varphi h^2}}\right) \quad (6)$$

where $\beta_{1,0}$ and $\beta_{2,0}$ are the global protonation constants in water, and m_1 and m_2 the slopes of the dependence of the logarithm of the global protonation constants with φ.

The coefficients of these models can be calculated from the experimental retention times through non-linear regression [13].

2.2. Measurement of the resolution

The peak purity (or free peak area fraction) is the ideal measurement to quantify the interference level for a given peak in a chromatogram [5,14]:

$$p_s = 1 - \frac{a'_s}{a_s} \quad (7)$$

a'_s being the area under the peak overlapped by the chromatogram that would be obtained for the set of accompanying compounds (the possible interferents), and a_s, the total area of the peak of interest. The calculation of peak purities requires the prediction not only of the peak location but also of its profile, that is, the width and asymmetry for each peak in a chromatogram (see Section 2.3).

The peak purity is a normalised measurement that ranges between zero for complete overlapping to one for full resolution. In practice, a peak with $p = 0.97$ can be considered as completely resolved (close to the baseline). The most important features of the peak purity criterion are its clear meaning and its capability for evaluating the separation without considering the identity of the interferents, since the effects of all of them are considered altogether. The latter feature was fundamental for its use in the COF developed for this work.

A representative value of the global resolution in a chromatogram can be achieved according to different approaches, such

as the worst elementary purity or different mean values or products [5,15]. In situations of low resolution, which are frequent with complex mixtures, the peak purity for one or more compounds will be likely null or at least very low. In such cases, the use of a measurement of the separation quality not penalised by the non-resolved compounds may be more appropriate. One of such measurements is the number of resolved peaks (peak count, PC), which in this work will be those that exceed a pre-established threshold of peak purity [7].

2.3. Simulation of chromatograms

A reliable optimisation of the resolution in a chromatogram requires realistic simulations. In order to quantify the peak overlapping, an idealised description based on the retention times is not sufficient. Information on the width, or even more, on the complete profile for all peaks in the chromatogram should be included. In the case of mixtures, the simulation of a complete chromatogram is built by adding the signals for all compounds.

A variety of functions has been proposed for the simulation of peak signals [16]. Practically any chromatographic signal departs from the ideal Gaussian behaviour, presenting skewness (the peaks are mostly tailing). The peak model used in signal simulation should include parameters routinely monitored: retention time, efficiency, asymmetry (measured as the right-to-left peak half-width ratio), and area (or height). We have used a function that fulfills this condition, which is a modification of the Gaussian equation, where the standard deviation varies linearly with the distance to the peak maximum (a polynomially modified Gaussian, PMG) [17,18]. This function was combined with exponential decays out of the peak region (where the peak height is below 10% of the maximal value) [19]. This allowed an appropriate modelling of the peaks and baseline, which is important to accurately quantify the peak overlapping in a chromatogram, minimising cummulative errors.

We have considered peaks with normalised areas. Nevertheless, the optimisation approach allows the inclusion of real areas to find optimal conditions taking into account the signal size (i.e. solute concentration and the relative detection sensitivities for each compound). Optimisations based on normalised areas provide, however, more general solutions and are, frequently, valid for most problems.

As commented, the simulation of a peak for each compound at each experimental condition (whose separation performance is being evaluated) requires predictions of the efficiencies and peak asymmetries. In contrast to what happens with retention, the changes in efficiency and asymmetry are often too large and irregular to be predicted, with enough accuracy, by means of models. In this study, we applied local interpolations using the experimental values of efficiency and asymmetry in the three mobile phases closest to the mobile phase that is being simulated.

3. Experimental

3.1. Reagents, column and apparatus

The probe compounds are detailed in Table 1. Aqueous mobile phases containing acetonitrile (HPLC grade, Merck, Darmstadt, Germany) at several pH values in the range 2–13 were used. The pH was adjusted with 0.01 M buffers prepared with the phosphoric, citric, boric, and butylammonium systems [20]. A polymeric C18 column (Polymer Labs, model PLRP-S, 150 mm × 4.6 mm I.D., 15–20 μm particle size) was used. This column allowed mobile phases in the extended pH range.

The chromatograph was equipped with a dual pump (Jasco, model 2350, Lincoln, NE, USA), an injection loop of 20 μL, and a UV–visible detector (Shimadzu, model SPD-10Avp, Kyoto, Japan) set at 254 nm, except for phenols, which were detected at 282 nm. Three-fold injections were made, processing the mean values. The dead time (1.80 min) was obtained as the average of the signals for KBr at 200 nm, in the set of mobile phases. The flow-rate was 1 mL/min.

The pH was measured with a Crison potentiometer (model MicropH 2002, Barcelona, Spain), with a precision of ±0.002 pH units. The electrode was calibrated with the usual aqueous buffers, and the pH in the mobile phase measured after mixing the aqueous buffer with the organic modifier, therefore, the $^{s}_{w}$pH scale was used [21]. All measurements were carried out at 25 °C. Other experimental details are given in Ref. [7].

3.2. Software

Several routines were developed in MATLAB 2010b (The MathWorks, Inc., Natick, MA, USA) for the modelling and simulation of chromatograms under isocratic conditions, the calculation of the matrices of peak purities, and the evaluation of the quality of the separation in the selected domain of conditions. Another series of routines was developed specifically for the optimisation of the mobile phase, according to different resolution requirements.

4. Results and discussion

4.1. Retention behaviour of the probe compounds

The most important parameter that should be accurately modelled in the studies of chromatographic resolution is, obviously, the retention. For this purpose, the retention factors of each compound under different experimental conditions should be fitted to appropriate models. The mixture examined in this work contained 24 ionisable compounds (15 acids, 8 bases and one amphoter) and 6 neutral compounds (Table 1). The retention behaviour of these compounds was described according to different equations: Eq. (3) for the neutral compounds, Eq. (5) for the acidic and basic, and Eq. (6) for the amphoteric one. The reliability of these equations was checked previously [7,11,12].

The experimental design consisted of three levels of organic modifier content (20, 40 and 60% acetonitrile, v/v). At each concentration, the retention was measured at 10 pH levels, covering approximately the 2–13 range. The wide pH range of the analytical column allowed, therefore, a detailed examination of the complete acid–base transition experienced by the ionisable compounds, when the pH of the mobile phase was varied.

The model obtained for each compound was used to predict its retention inside an experimental domain constituted by a regular grid of 101 × 111 (solvent content × pH) conditions. Therefore, the grid step was 0.1 units for the pH and 0.2 units for the solvent content, expressed as v/v percentage. The inspection in the pH dimension was more exhaustive, since this is the factor that offered more possibilities to modify the selectivity. The detail level in the organic solvent dimension was maybe excessive considering the changes in selectivity associated to this factor (as was further checked). In this way, 11,211 possible synthetic conditions were evaluated throughout the optimisation. The practical unfeasibility of carrying out experimentally this evaluation is evident.

Fig. 1 depicts the retention time ranges for the 30 probe compounds, considering the whole set of experimental conditions. The central line in the "box and whiskers plots" is the median and the lower and upper ends in the boxes indicate the first and third quartiles. The values outside the whiskers are those out of the confidence interval of the median. This diagram evidences

Table 1
Probe compounds and protonation constants.[a]

	Compound	Character	Acetonitrile (v/v)		
			20%	40%	60%
1	Naphthoic acid	Acid	4.41	5.09	5.80
2	2-Nitrobenzoic acid	Acid	2.93	3.59	4.26
3	3-Nitrobenzoic acid	Acid	3.91	4.38	5.00
4	4-Nitrobenzoic acid	Acid	3.79	4.29	4.92
5	Benzoic acid	Acid	4.73	5.28	5.77
6	Resorcinol	Acid	10.47	11.18	11.88
7	Phenol	Acid	10.77	11.61	11.98
8	2,4-Dichlorophenol	Acid	8.15	8.86	9.69
9	2,4-Dinitrophenol	Acid	4.04	4.36	4.79
10	β-Naphthol	Acid	10.24	11.23	11.61
11	2-Nitrophenol	Acid	7.37	7.91	8.74
12	3,5-Dichlorophenol	Acid	8.68	9.34	9.83
13	3-Aminophenol	Amphoteric (K_1)	10.83	11.60	12.38
		Amphoteric (K_2)	4.18	3.79	3.40
14	3-Bromophenol	Acid	9.60	10.43	10.79
15	*p*-Chlorophenol	Acid	10.05	10.85	11.20
16	*m*-Cresol	Acid	11.03	11.70	12.36
17	*N*-Ethylaniline	Basic	4.95	4.57	3.87
18	*N*,*N*-Dimethylbenzylamine	Basic	8.51	8.11	7.68
19	2,6-Dimethylaniline	Basic	3.57	3.22	2.78
20	Benzene	Neutral			
21	Acetophenone	Neutral			
22	Benzaldehyde	Neutral			
23	Nitrobenzene	Neutral			
24	Methylphenylether	Neutral			
25	Benzonitrile	Neutral			
26	2,4,6-Trimethylpyridine	Basic	7.03	6.59	6.10
27	4-Chloroaniline	Basic	3.55	3.11	2.92
28	Aniline	Basic	4.35	3.90	3.45
29	*p*-Toluidine	Basic	4.83	4.48	4.13
30	Pyiridine	Basic	4.92	4.61	4.04

[a] Ref. [20].

those compounds with extreme behaviour, which allows a first evaluation of the chromatographic system. Thus, compounds 6 (resorcinol, acidic), 13 (3-aminophenol, amphoteric) and 30 (pyridine, basic) were poorly retained in the whole domain. Also, some compounds were excessively retained in some conditions, with retention times exceeding 100 min, and even 200 min (case of 3,5-dichlorophenol). The retention time medians indicate that most poorly retained compounds are acidic, and the neutral compounds are largely retained. On the other hand, the amphoteric compound elutes close to the void volume at both low and high pH, and it only reaches $k \approx 1$ at intermediate pH values, interfering both acidic and basic compounds. Therefore, in a mixture of the 30 probe compounds, the changes in retention times with the nature of the mobile phase will give rise to multiple peak crossings, and frequently, long analysis times.

4.2. Resolution capability for the mixture of 30 probe compounds

Aiming at obtaining the best separation conditions, an expert analyst can decide to assay different mobile phases, based on his/her knowledge of the problem, and then evaluate their respective success. For example, considering the nature of the mixture of 30 probe compounds, it seems logical to examine the behaviour of an acidic mobile phase, on the one hand, and of a basic mobile phase, on the other, as those in Fig. 2. In this figure, it can be checked that only 12 and 8 compounds are resolved at 10% interference level,

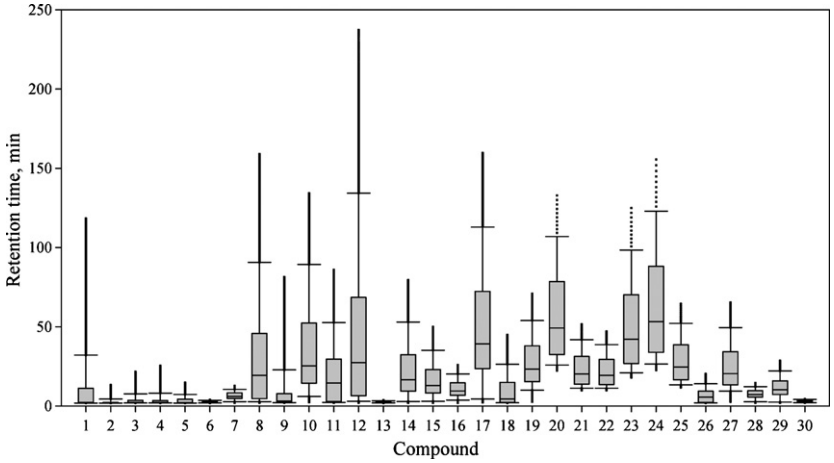

Fig. 1. Statistical distribution (box and whisker plots) of the retention times for each compound in the mixture of 30 ionisable and neutral compounds, in the whole domain of mobile phases in the synthetic design (11,211 experimental conditions). Compound identity is given in Table 1.

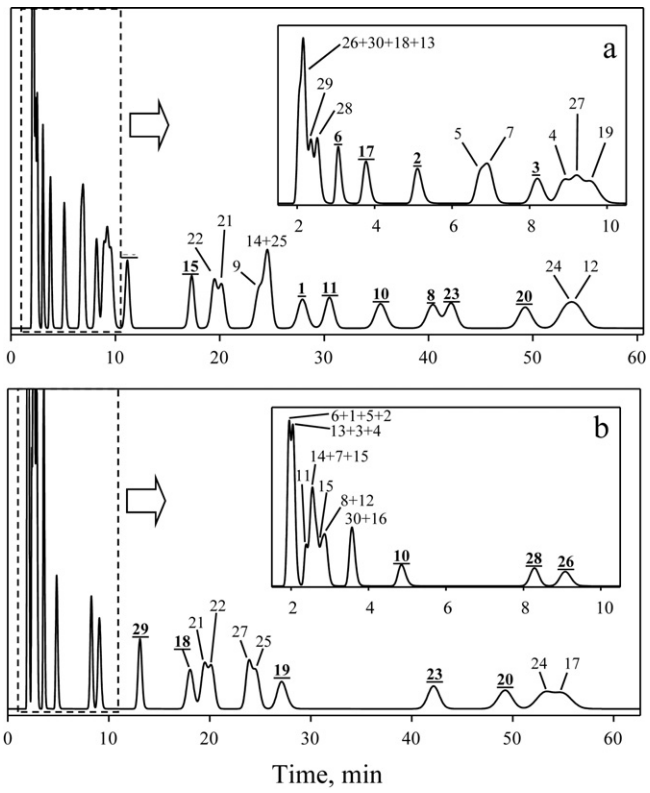

Fig. 2. Simulated chromatograms for the mixture of the 30 compounds included in Table 1, corresponding to mobile phases containing 30% acetonitrile at pH: (a) 3, and (b) 12. The compounds with $p \geq 0.90$ are indicated in underlined bold characters.

at acidic and basic pH, respectively. Other conditions selected on a trial and error basis have few chances of improving the situation.

The simple inspection of the chromatograms in Fig. 2 evidences the complexity of the problem, with multiple coelutions as well as an accumulation of peaks for poorly retained compounds, whose identity is strongly affected by the pH of the mobile phase. The solution for finding the experimental conditions for the best possible separation is performing a computer-assisted optimisation, based on previously established models describing the compound behaviour, followed by the simulation of the separation with mobile phases in the pre-established solvent content and pH ranges.

Once the system has been modelled, the elementary peak purities for the whole set of compounds can be calculated for each mobile phase inside the selected experimental domain. With this aim, for each compound and each condition, two simulated chromatograms are built: the first one includes only the peak of interest, and the second one, the peaks of the other compounds in the mixture, considered as potential interferents. The comparison of both chromatograms indicates the area free of interference (or peak purity p, Eq. (7)).

This process is repeated for each experimental condition in the design. A data matrix of peak purities describing the behaviour of the chromatographic system is finally built, with as many rows as chromatographic conditions, and as many columns as compounds (for the studied example: 11,211 and 30, respectively). The data in the matrix for each compound can be plotted as a contour map to visualise the changes in peak purity, as a function of the two optimisation factors: solvent content and pH. The maximal value of the resolution map corresponds to the "limiting peak purity", which is the maximal value that can be obtained for the considered compound in the chromatographic system [14]. These data are listed in Table 2 for the studied example.

An even simpler way of visualising the resolution behaviour for each compound is to represent maps showing only the

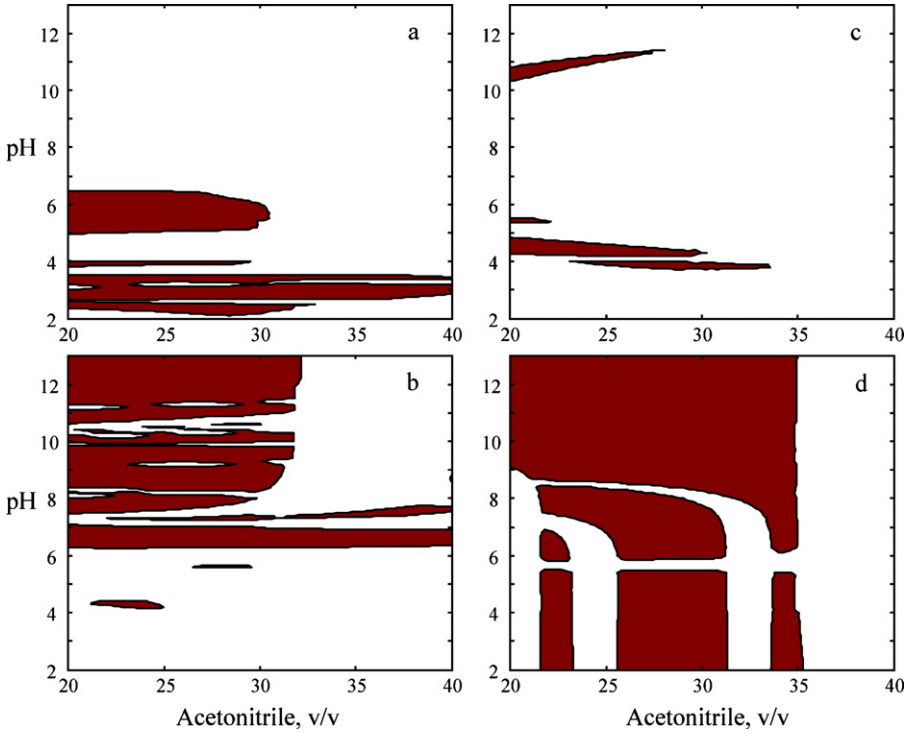

Fig. 3. Contour maps depicting the regions of peak purity above $p = 0.90$ (in black) for four representative compounds of different character: (a) 2-nitrobenzoic acid, (b) 2,4,6-trimethylpyridine (basic), (c) 3-aminophenol (amphoteric), and (d) benzene (neutral).

Table 2
Elementary limiting peak purities in a mixture of the 30 probe compounds, considering the full domain, or restricted analysis time and pH domains.[a]

Compound	Full domain	<45 min	pH 3–7<45 min
Naphthoic acid	1.0000	1.0000	1.0000
2-Nitrobenzoic acid	1.0000	1.0000	1.0000
3-Nitrobenzoic acid	1.0000	**0.9806**	**0.9199**
4-Nitrobenzoic acid	1.0000	**0.9806**	**0.9089**
Benzoic acid	1.0000	1.0000	1.0000
Resorcinol	1.0000	0.9989	0.9989
Phenol	1.0000	1.0000	1.0000
2,4-Dichlorophenol	1.0000	1.0000	1.0000
2,4-Dinitrophenol	1.0000	1.0000	1.0000
β-Naphthol	1.0000	1.0000	1.0000
2-Nitrophenol	1.0000	1.0000	1.0000
3,5-Dichlorophenol	1.0000	1.0000	0.9999
3-Aminophenol	0.9949	**0.9226**	**0.9226**
3-Bromophenol	1.0000	1.0000	**0.9364**
p-Chlorophenol	1.0000	1.0000	1.0000
m-Cresol	1.0000	1.0000	1.0000
N-Ethylaniline	1.0000	1.0000	1.0000
N,*N*-Dimethylbenzylamine	1.0000	1.0000	**0.8828**
2,6-Dimethylaniline	0.9999	0.9999	0.9987
Benzene	1.0000	**0.9822**	**0.9376**
Acetophenone	0.9977	**0.6905**	**0.6905**
Benzaldehyde	0.9972	**0.6900**	**0.6900**
Nitrobenzene	1.0000	1.0000	1.0000
Methylphenylether	1.0000	**0.9800**	**0.9789**
Benzonitrile	1.0000	1.0000	1.0000
2,4,6-Trimethylpyridine	1.0000	1.0000	1.0000
4-Chloroaniline	0.9984	**0.9861**	**0.9861**
Aniline	1.0000	1.0000	1.0000
p-Toluidine	1.0000	1.0000	1.0000
Pyridine	1.0000	1.0000	**0.9873**

[a] Numbers in bold indicate incomplete resolution.

regions offering good resolution. Fig. 3 shows the regions with an elementary peak purity $p > 0.90$ (i.e. 90% of the peak free of interference) for four representative compounds of different character. It can be observed that 2-nitrobenzoic acid shows good resolution for mobile phases at an acidic pH (Fig. 3a), whereas for 2,4,6-trimethylpyridine, a basic compound, the regions of good resolution correspond to basic pH (Fig. 3b).

The resolution contour maps for ionisable compounds (acidic, basic or amphoteric) offer, generally, structures parallel to the solvent content axis (which is a factor with a much smaller influence on the resolution than pH), together with rapid changes in the pH direction (the fundamental factor that governs the resolution of ionisable compounds). In contrast, for benzene (a neutral compound), several regions with discontinuities are observed in both directions, indicating peak crossings or coelution (the resolution drops to zero) (Fig. 3d).

The data in Table 2 indicate that there is at least one mobile phase where each compound appears totally (or virtually) resolved (i.e. with $p = 1$ or $p \approx 1$). However, in other cases (e.g. another column, organic modifier, range of conditions, or compounds), the situation can be more adverse, and one or more compounds will remain unresolved at any condition. To illustrate this, the limiting peak purities in restricted analysis time and pH domains are also given in Table 2. In both cases, the poorly retained compounds did not reach full resolution (i.e. p was well below 1). Therefore, there are more chances of coelution. Two neutral compounds (acetophenone and benzaldehyde) show, in the best case, peak purities below $p = 0.7$, which probably will not be enough for the final purpose of the analysis.

4.3. Global resolution assessments penalised by the worst resolved peaks

An interpretive optimisation process is based on the simulation of a large number of experimental conditions, and the evaluation of the global resolution through a COF. Finally, the conditions that offer the highest resolution or those most convenient (e.g. in terms of analysis time or organic solvent consumption) are selected. In situations of extremely low resolution, conventional COFs (based on the worst resolved peak or a combination of the resolution of all peaks in the chromatogram) are not informative, since they are dominated by the poorly resolved compounds. This means that they are highly affected by the failure in the separation.

The product of peak purities is easily obtained from the matrix of peak purities, as the product of the values in a row. The worst resolution corresponds to the minimal purity in a row. In both cases, practically all conditions yielded null global resolution for the mixture of 30 probe compounds, without significant variations throughout the studied domain. Fig. 4 illustrates this limitation with the contour maps depicting the worst elementary value and the product of elementary resolutions for the case of study. The elementary resolution function used to draw the maps was the peak purity, but the situation is analogous for other resolution criteria. As observed, the contour maps do not assist the chromatographer in the selection of the best separation conditions. This happens because both functions fall to zero when at least one peak is overlapped, even when the other compounds in the mixture were well resolved.

4.4. Global resolution measurements that attend preferably to well resolved peaks

4.4.1. Peak count

In the analysis of complex mixtures, frequently, one or more peaks remain unresolved in the whole experimental domain. However, there is still an interest in finding conditions where the other peaks in the chromatogram are satisfactorily resolved. As we have seen in Section 4.3, the resolution measurements oriented to quantify the failure level (e.g. worst elementary value and product of the resolution of all peaks in the chromatogram) do not offer use-

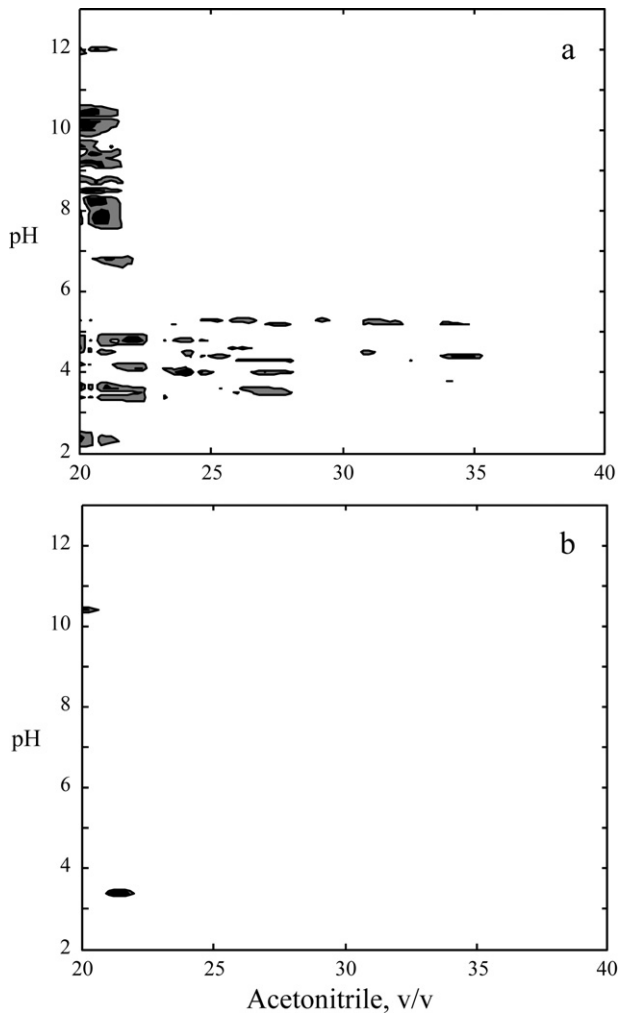

Fig. 4. Global resolution contour maps for: (a) the worst elementary peak purity and (b) the product of elementary peak purities of all peaks, in the domain of mobile phases considered in the optimisation. The maximal global purity was: (a) 0.3449 and (b) 4.7×10^{-4}.

ful information in situations of extremely low resolution. In such cases, these measurements do not discriminate among different conditions. However, finding conditions where the largest number of compounds appears sufficiently resolved can be still worthwhile. In order to get this information, the global resolution measurement must be based on the success in the separation and not on the failure.

We will consider, first, an optimisation strategy oriented to the success, which simply counts the number of "well resolved" peaks (peak count, PC). This is a practice that intuitively a chromatographer makes when he/she examines visually a chromatogram. The elementary peak purity will be used to determine whether a compound is "well resolved" [7]. For "well resolved", we understand that the elementary purity exceeds a certain arbitrary threshold (for instance, $p = 0.95$ or 0.85). One would expect that the threshold value for practical purposes should be fixed according to the chromatographer requirements (e.g. safe quantitation or spectra acquisition).

Fig. 5 shows a contour map depicting the number of peaks that exceed the threshold of elementary peak purities $p = 0.85$, throughout the complete domain of experimental conditions without attending to the analysis time. In this map, the regions with a maximal peak count are more intensely shaded. Several conditions appear with 20–22 compounds resolved exceeding the threshold $p = 0.85$. Most of them correspond to four regions in the contour map (indicated with Roman numbers): regions I (20.0–20.7% acetonitrile, pH 10.35–10.45), II (25.4–26.6% acetonitrile, pH 4.0–4.4), III (25.6–26.6% acetonitrile, pH 3.3–3.5), and IV (25.6–26.6% acetonitrile, pH 2.2–2.5). It is possible to resolve up to 20 peaks in regions III and IV, 21 peaks in region I, and 22 peaks in region II. Of course, there is a larger number of conditions where only 20 compounds are resolved.

Other contour maps as that in Fig. 5 can be drawn for different thresholds, depending on the required separation quality. To understand the separation capability of the system, it is also interesting to build a plot of the maximal number of resolved peaks (PC), as the peak purity threshold is changed (a threshold scan plot, Fig. 6). It is observed that all 30 compounds reach a threshold of $p = 0.34$, indicating that all the peaks can be resolved with at least that purity level in at least one condition. However, this resolution is absolutely insufficient. In the same way, 29 compounds would reach a threshold of $p = 0.52$, simultaneously. Increasing the p threshold level, a more intense decrease in the number of peaks is found, followed by a "plateau" for 23–24 peaks (23 compounds would reach $p = 0.83$). It could be interesting to check if this minimal resolution quality is enough for the purpose of the analysis, through the simulation of chromatograms at the corresponding conditions. Beyond this point, the rate of reduction in the number of compounds that can be simultaneously resolved becomes larger, as the demand in the resolution quality increases. In the mixture of 30 compounds, only five can be resolved exploiting the whole capacity of the system simultaneously, since PC = 5 is the maximum for a threshold of $p = 1$.

The diagram in Fig. 6 can be examined in a reversed way, starting from the threshold limit $p = 1$: five compounds can be resolved simultaneously up to the baseline, to which other nine can be added with $p = 0.99$, three more with $p = 0.98$, and one more with $p = 0.97$, summing up to a total of 18 resolved compounds at that resolution level. The addition of another compound would require to sacrifice the threshold expectancies down to $p = 0.94$.

It should be noted that the threshold is a minimal value. Therefore, for thresholds below $p = 1$, we can affirm with a high probability that some compounds will show better resolution than the threshold. Also, it may happen that for a given threshold (such as $p = 0.94$), no peak will reach full resolution (i.e. $p = 1$). The peak count concept only assures that the compounds will be resolved with peak purities above the threshold, but it does not give information about the specific resolutions, which can range between the threshold and the maximal possible value. This observation is important because, as we have seen in Fig. 5, the condition of maximal PC is not unique. A given PC value can be reached under several experimental conditions, and even in two or more regions of the experimental domain. However, the resolution level, very likely, will change from one condition to another.

4.4.2. Optimisation of the separation of the compounds that exceed the resolution threshold

As commented, a drawback of the peak count concept is that it does not discriminate among situations that resolve the same amount of peaks. Thus, several conditions with the same associated PC value may exist, while the quality of their separation can be clearly different. Also, the identity of the resolved compounds may differ from one region to another. An immediate solution is to classify the compounds in the mixture in two groups for each examined mobile phase, depending on their peak purity with regard to the established resolution threshold (above or below that level), and next, optimise the separation of the compounds that exceed the threshold. In the optimisation process, however, the influence of the non-resolved compounds (those that did not reach the threshold) should be considered. For this purpose, a resolution criterion is

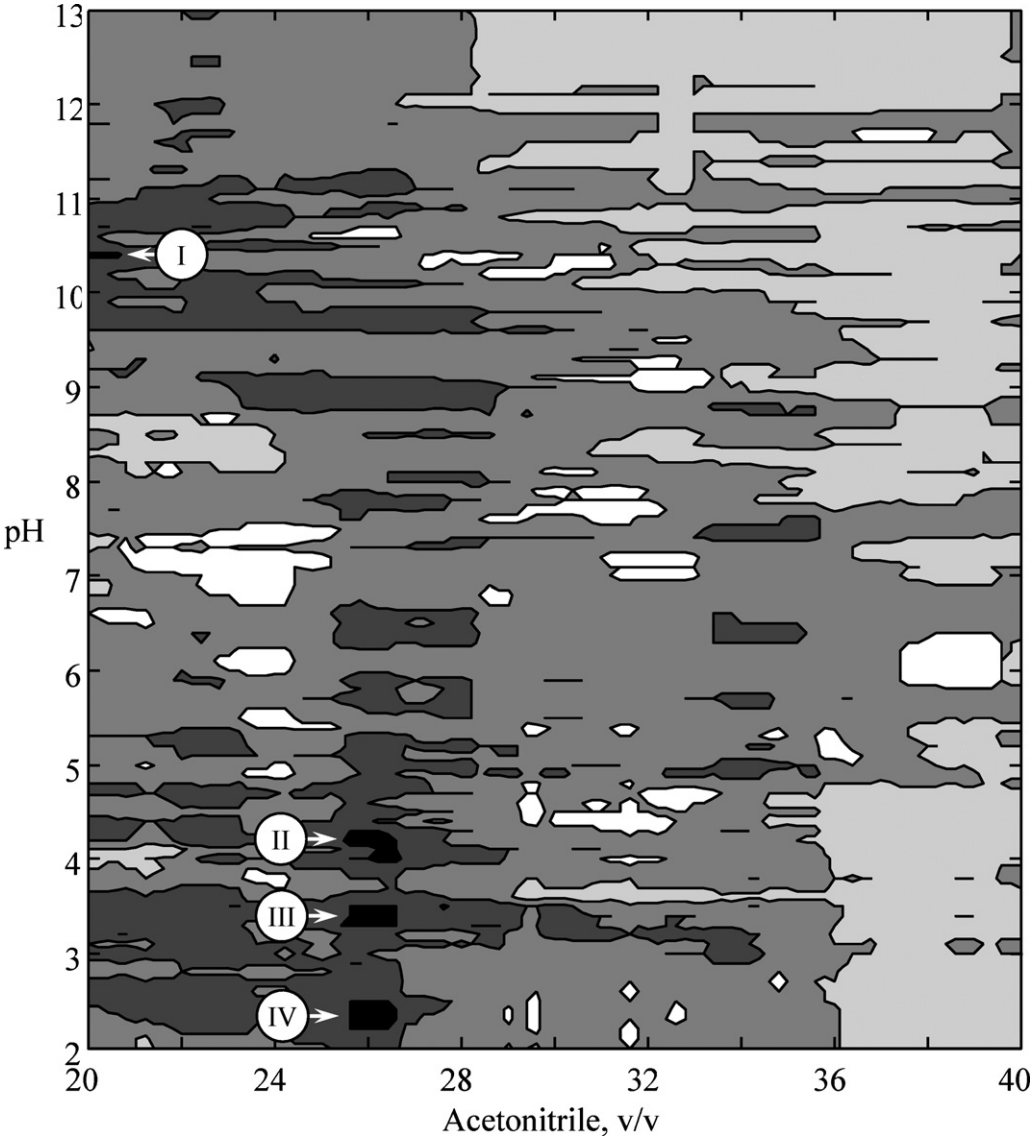

Fig. 5. Peak count (PC) contour map drawn using a peak purity threshold of 0.85, for the mixture of 30 compounds in the full domain of mobile phases considered in the optimisation. The regions for PC ≥0, 5, 10, 15 or 20 appear shaded gradually darker.

needed that qualifies each compound individually, without attending to the nature of the interferents, as is the case of the peak purity. We have used before this criterion to optimise the resolution of selected compounds [4,22].

The methodology is similar to that followed to optimise the separation of the whole set of compounds in a mixture, using the product of elementary peak purities as global resolution criterion, restricted to the compounds exceeding the threshold. In the example shown in Fig. 5, 20–22 compounds appeared resolved with $p \geq 0.85$ in different regions of the experimental domain. As commented, the number of conditions able to separate only 20 peaks was larger. We carried out the optimisation, considering the three PC levels individually. In order to make comparable the values of global resolution when changing the number of resolved peaks (20–22 in this example), the geometric mean of elementary purities for the resolved peaks was calculated for each mobile phase in the grid of synthetic conditions. Finally, that mobile phase offering the maximal value (the best resolution) was selected.

It should be kept in mind that the peaks considered as resolved exceeded the chosen threshold, therefore, their elementary purities were in the $0.85 \leq p \leq 1$ range. For this reason, the global resolution calculated as the geometric mean of elementary purities should have a minimal value of $p = 0.85$. The maximal value of global peak purities (P) for the conditions that allowed the resolution of 20, 21 and 22 peaks, with a threshold of $p \geq 0.85$, were (acetonitrile content and pH of the mobile phase): $P = 0.972$ (26.0%, 2.4), 0.961 (26.2%, 4.0), and 0.953 (26.0%, 4.2), respectively. Fig. 7 depicts the respective chromatograms.

4.4.3. Fractional peak count

The definition of peak count can be modified to discriminate among conditions that resolve the same amount of peaks, by adding a fractional term (f) that quantifies the global resolution of the peaks that exceed the established threshold:

$$\mathrm{fPC} = \mathrm{PC} + \mathrm{f} \tag{8}$$

It is also possible to define f to attend the peaks that remain below the threshold, instead.

We have called the combined resolution function in Eq. (8) (fPC) "fractional peak count" or in short, "fractional PC". The integer part of this function indicates the number of compounds that exceeded the threshold, and the fractional part qualifies the peak resolution. The f function discriminates the resolution capability of the mobile phases that resolve the same amount of compounds. It should adopt

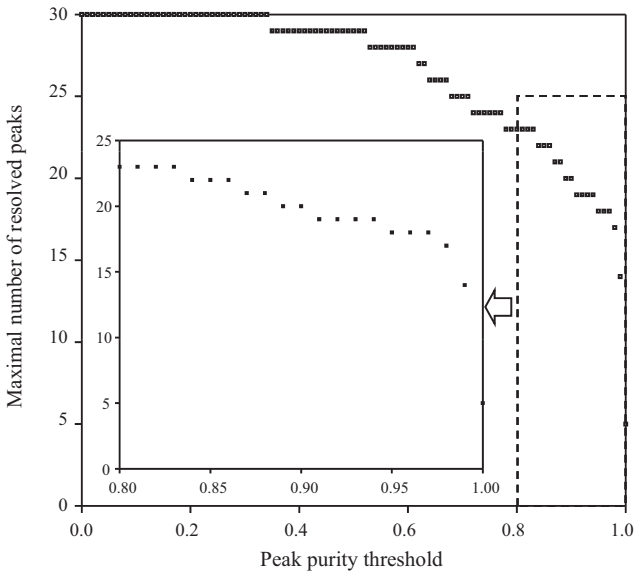

Fig. 6. Change in the maximal PC (number of resolved peaks) as a function of the quality of the separation. The same diagram is presented in a more reduced threshold interval, close to $p = 1$, where in practice the resolution can be appraised as acceptable.

the limits $0 \leq f < 1$, so that fPC is never increased to the next integer value, which could lead to confusion in the number of resolved peaks. Once the desired purity threshold is established, the fPC value can be obtained for each condition in the whole experimental domain. Using this approach, when time restrictions are needed, it is possible to limit the calculation to those mobile phases yielding an acceptable analysis time. In our opinion, this is a preferable option with respect to the inclusion in the fPC function of a term that restricts the analysis time, since this would make the interpretation of the results more difficult: if we added a term accounting for the analysis time penalisation, the summation will adopt also a value with an integer part and a fractional part, but from this summation, we could not derive the number of resolved peaks, neither the achieved resolution.

The fPC function discriminates among the mobile phases providing the same number of resolved peaks. Therefore, an optimisation based on this COF will point to a unique solution. We will consider the two alternatives: that the f function attends to the resolution of the compounds that exceed the threshold (as the approach described in Section 4.4.2), or to insufficiently resolved peaks. In the first alternative, f will be the global purity of the resolved peaks, calculated as the geometric mean of elementary peak purities as in Section 4.4.2. In our example, the maximal value reached in the whole domain was fPC = 22.953, corresponding to a mobile phase of 26% acetonitrile and pH 4.2 (Fig. 7c). If the search is restricted to the mobile phases that resolve only 20 peaks: fPC = 20.972 (26% acetonitrile and pH 2.4, Fig. 7a). This alternative can be interesting when the separation of the compounds in a complex mixture is carried out with quantitative purposes. In this case, we will be interested in determining properly the largest number of compounds, improving the resolution of some compounds at the expense of others with higher resolution.

In contrast with the conventional measurements, the peak count concept is based on the establishment of thresholds delimiting when a practical full separation is reached. In principle, the compounds that have exceeded the threshold are already resolved for practical purposes. If the aim is qualitative, the effort should be better invested in improving the separation of peaks that did not reach the threshold (i.e. the second alternative). The advantage of this approach is that it attends to problematic compounds, indicating the condition where they are best resolved. It has, however, two drawbacks: it does not discriminate the resolution level of the compounds that are resolved (which is not a real problem), and the product of elementary purities can fall to zero if at least one peak is poorly resolved under a given condition (with p close to zero). This is the same problem found in the approaches oriented to the failure (Section 4.3). Consequently, the product of peak purities for all the peaks that did not exceed the threshold cannot be taken as the f function, since probably some peaks will offer null resolution.

The solution is to restrict the calculation of f to the best peaks among those that did not exceed the threshold. On the other hand, it should be noticed that the magnitude of the global resolution will depend on the selected threshold. For instance, the global resolution will be necessarily smaller for a threshold of $p = 0.85$ with regard to $p = 0.95$. In order to make the global resolution comparable for different thresholds, the elementary peak purities of the non-resolved peaks were normalised with respect to the threshold, so that the peaks with an elementary resolution slightly smaller than the threshold reached normalised values close to one.

Following the indicated criteria, we searched the experimental conditions that originated the highest resolution for groups of $n = 1$–4 peaks, among those that did not exceed the threshold $p = 0.85$. For this purpose, the elementary purities of these peaks were sorted for the whole domain, and the groups of n peaks that yielded the highest fPC values were selected. The f function was calculated as the geometric mean of the normalised purities with respect to the threshold of acceptable separation.

The latter approach was applied to the cases where 20, 21 or 22 peaks were resolved for a threshold of $p = 0.85$. The most interesting case corresponded to the resolution of 20 peaks (Table 3), which was fulfilled in around 80 experimental conditions within

Table 3
Selection of mobile phases with a peak count PC = 20 by optimising the separation of 1–4 "unresolved" compounds with $p < 0.85$.

Number of optimised unresolved compounds	Elementary peak purity[a]	Global resolution	Optimal mobile phase
One	$p(10) = 0.849$	fPC = 20.999	26.6% acetonitrile, pH 4.2
Two	$p(10) = 0.849$ $p(9) = 0.846$	fPC = 20.997	26.6% acetonitrile, pH 4.2
Three	$p(1) = 0.843$ $p(5) = 0.836$ $p(14) = 0.836$	fPC = 20.986	25.6% acetonitrile, pH 4.0
Four	$p(1) = 0.843$ $p(5) = 0.836$ $p(14) = 0.836$ $p(7) = 0.739$	fPC = 20.956	25.6% acetonitrile, pH 4.0

[a] The peak identities are given in parentheses (see Table 1).

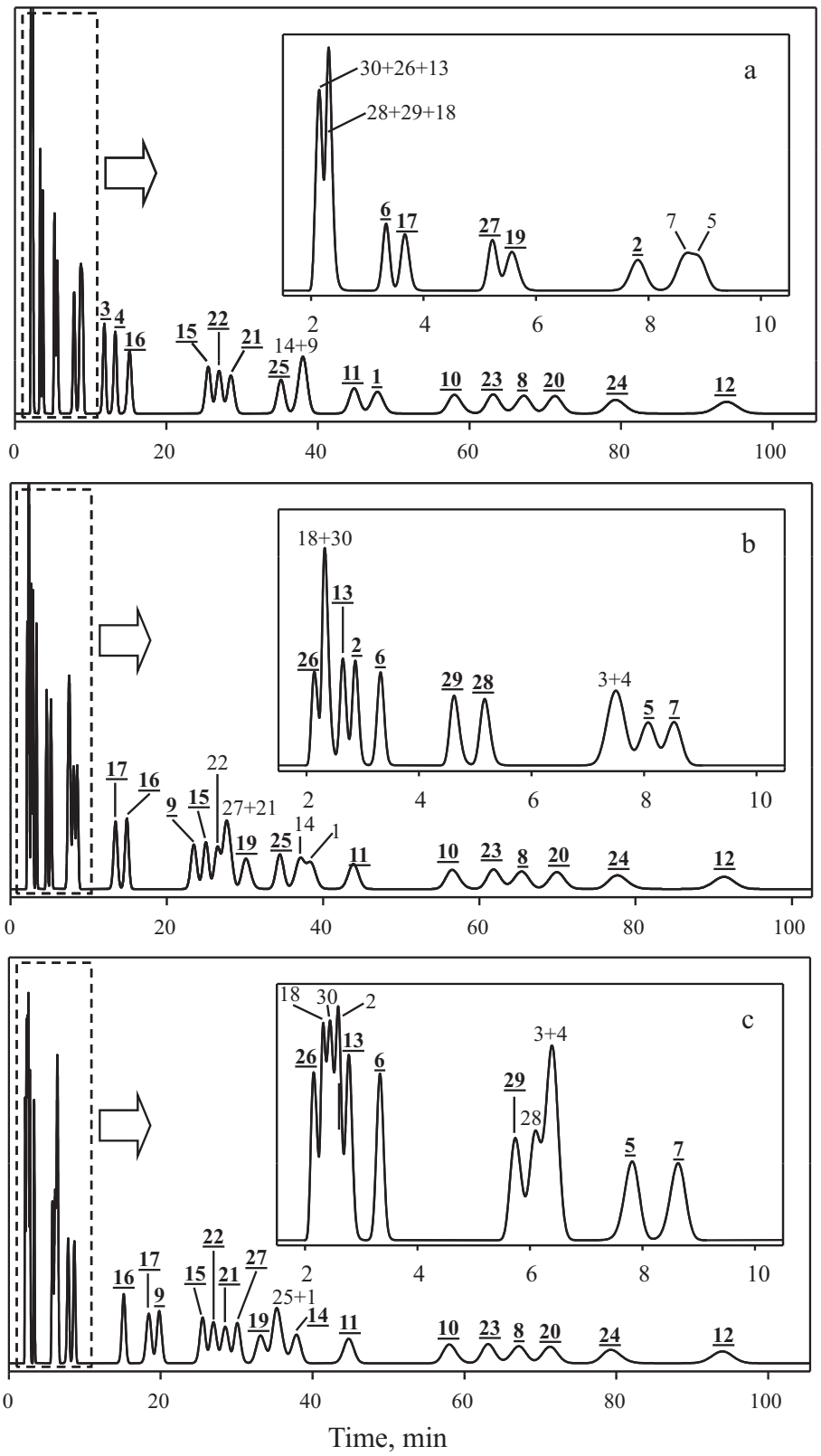

Fig. 7. Optimal chromatograms for a peak count (PC) of: (a) 20 (26.0% acetonitrile, pH 2.4), (b) 21 (26.2% acetonitrile, pH 4.0), and (c) 22 (26.0% acetonitrile, pH 4.2). The compounds for a threshold $p = 0.85$ are indicated in underlined bold characters.

the synthetic experimental design. As observed, three peaks that did not exceed the threshold yielded purities close to it, using a mobile phase of 25.6% acetonitrile and pH 4.0. In contrast, in the situations where 21 or 22 peaks exceeded the value $p = 0.85$, the peak purity for the best resolved peak under the threshold only reached $p = 0.794$ and 0.736, respectively. Fig. 8 depicts the chromatograms corresponding to the selected conditions indicated in Table 3, in which the peaks that have improved their separation according to this optimisation are indicated with arrows.

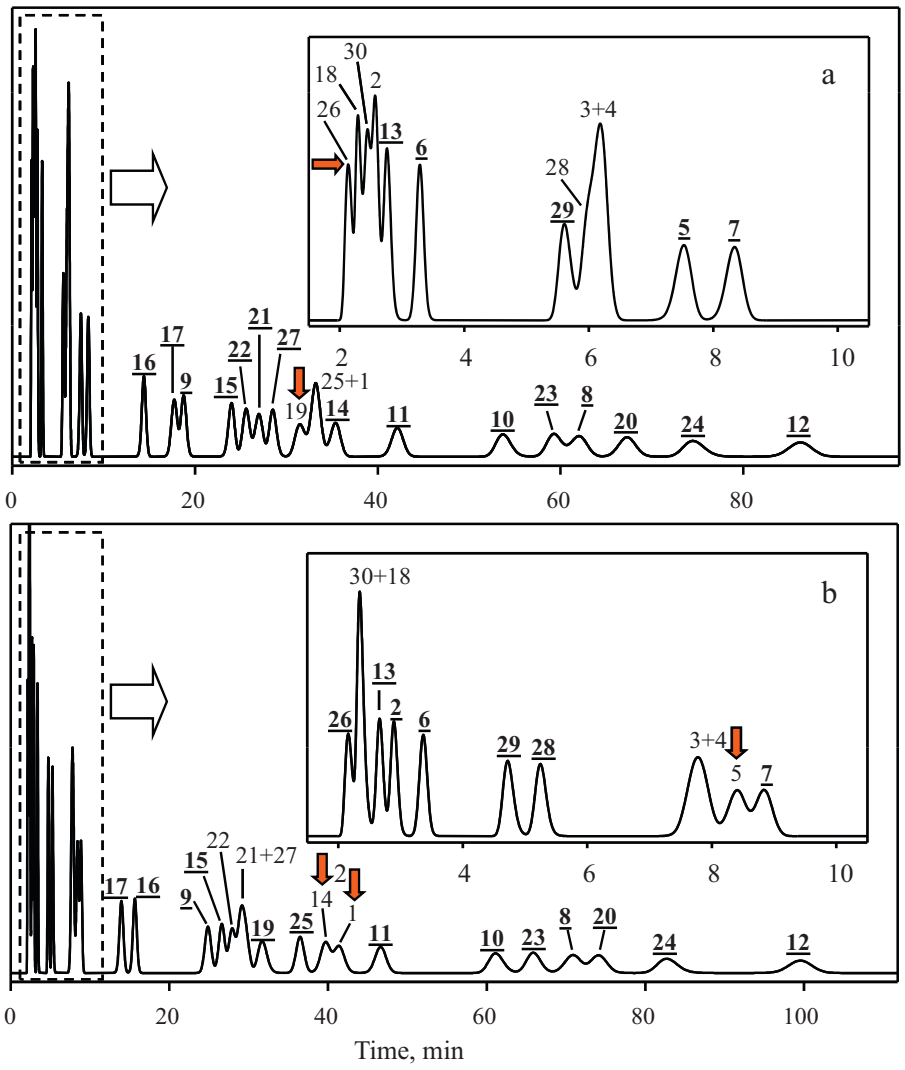

Fig. 8. Optimal chromatograms for a peak count of PC = 20, that resolve: (a) two (26.6% acetonitrile, pH 4.2) and (b) three (25.6% acetonitrile, pH 4.0) additional compounds with peak purities slightly below the threshold (see Table 3). The fractional peak count was fPC = 20.997 and 20.986, respectively. The compounds for a threshold $p = 0.85$ are indicated in underlined bold characters, and the peaks with purities below the threshold having the best scores are pointed out with arrows.

4.5. Comparison of the fractional peak count with the Berridge, and Duarte and Duarte functions

At this point, it is interesting to compare the new approach with the functions proposed by Berridge (Eq. (1)), and Duarte and Duarte (Eq. (2)), which are based on the counting of apparent peaks. The Berridge function allows the qualification of chromatograms, and the optimisation of the experimental conditions by means of a simplex algorithm, without knowing the identity of the individual peaks, neither the number of compounds associated to each peak. In order to discriminate among the performance of chromatograms that resolve the same number of peaks, it uses a function that measures the resolution among adjacent observed peaks (R_S). Since this is a non-normalised criterion, its maximal value is limited to $R_S = 2$ for each peak pair, so that those pairs exceeding this resolution do not contribute excessively to the COF. Berridge also provided the possibility of increasing the weight of the number of observed peaks, with an arbitrary exponent, so that the number of peaks could be made the most important requirement at the expense of the resolution among adjacent peaks. It should be kept in mind that the sum of the R_S values for the observed peak pairs can exceed their number. Also, the measurement of the peak width needed to evaluate the R_S function is difficult if the peaks are partially overlapped. This problem was solved by Duarte and Duarte [8], who proposed a COF that included a normalised resolution criterion (the valley-to-peak ratio) to measure the resolution (Eq. (2)).

An important difference between the peak count based on the peak purity, and the Berridge function (or that of Duarte and Duarte), is that the number of peaks in the latter functions (Eqs. (1) and (2), respectively) is referred to those detected, which can have one or more compounds associated. These strategies are especially advisable to qualify chromatograms used in the authentication of unknown samples, for which standards are not available. In our approach, the identity of each peak is known, and therefore, the peak count corresponds to visible peaks of individual compounds. To make this possible, a criterion that associates a resolution measurement to each peak (as the peak purity) is needed. Note that the computation of peak purities requires previous modelling of the chromatographic behaviour using standards, which is anyway necessary if the optimised chromatogram will be used with quantitative purposes.

Finally, since the peak purity is a normalised measurement, the value of the global resolution function (the *f* function) that is added to the peak count (Eq. (8)) is always less than one. Therefore, the combined fPC function does not need the inclusion of weights and

always informs in a clear way about the number of peaks that have exceeded the threshold (i.e. the resolved peaks), and the best resolution associated to these peaks (or, alternatively, to those below the threshold). This is not possible with Eqs. (1) and (2).

5. Conclusions

The peak purity, as any resolution criterion that qualifies individually the peaks in a chromatogram, is more easily interpretable than the criteria that measure the resolution of peak pairs. Also, it provides exhaustive information about the resolution expectancies: it is possible to orient the search to find what one wishes among a large number of situations (i.e. how to separate an individual compound, a group of selected compounds, or the whole mixture). With the criteria associated to peak pairs, this is not feasible, except if individual measurements are assigned artificially to each peak.

On the other hand, the COFs used routinely in the optimisation of the chromatographic conditions (e.g. the worst resolution value and the product of the resolutions for all peaks) attend to the failure in the separation. Therefore, they are only useful in situations of satisfactory resolution, which hardly happens with complex samples. When the peaks of one or more compounds cannot be resolved under any condition, and no alternative exists for improving the separation by means of a change in the nature of the column or the modifier, it can be still of interest to get the maximal possible resolution. In this case, the optimisation should be carried out using a COF that looks for the situations offering the maximal number of resolved peaks, that is, a COF that rewards the success in the separation. This is the case of the peak count, which is intuitively applied when a chromatogram is examined.

The peak count based function (fPC) we have developed yields the same result as conventional assessments when full resolution is possible, but it is also able to discriminate among chromatograms in low resolution situations. In fact, the conventional optimisation based on the product of peak purities can be considered as a particular case of the fractional peak count strategy, when the f term in Eq. (8) is focused to the well resolved peaks and the threshold is set to $p = 0$.

In principle, COFs as those proposed by Berridge, and Duarte and Duarte, can be used to carry out an interpretive optimisation, by adding the chromatograms of the individual compounds in each condition in an experimental design, and qualifying each chromatogram by means of the respective multicriteria resolution function. However, the peaks should be detected by monitoring the derivatives of the artificial signals. As commented, the observed peaks would not necessarily correspond to individual compounds.

Finally, it has to be mentioned that the Berridge function has been criticised, since the poorly resolved peaks have a minor influence on the function value and the quality of a chromatogram is determined by the well resolved peaks [3]. The fPC function developed in this work is more flexible to be adapted to the chromatographer aims and/or the particularities of the analysed mixture, and can attend to the peaks that do not exceed the established resolution threshold. Also, the scanning of thresholds (see Fig. 6) reveals the capability of the chromatographic system, and allows the analyst to choose a compromise solution.

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