Journal of Chromatography A, 1218 (2011) 5829–5836

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

A complementary mobile phase approach based on the peak count concept oriented to the full resolution of complex mixtures

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ARTICLE INFO

Article history:
Received 20 April 2011
Received in revised form 13 June 2011
Accepted 22 June 2011
Available online 30 June 2011

Keywords:
Complex samples
Chromatographic optimisation
Complementary mobile phases
Peak purity
Peak count

ABSTRACT

Situations of minimal resolution are often found in liquid chromatography, when samples that contain a large number of compounds, or highly similar in terms of structure and/or polarity, are analysed. This makes full resolution with a single separation condition (e.g., mobile phase, gradient or column) unfeasible. In this work, the optimisation of the resolution of such samples in reversed-phase liquid chromatography is approached using two or more isocratic mobile phases with a complementary resolution behaviour (complementary mobile phases, CMPs). Each mobile phase is dedicated to the separation of a group of compounds. The CMPs are selected in such a way that, when the separation is considered globally, all the compounds in the sample are satisfactorily resolved. The search of optimal CMPs can be carried out through a comprehensive examination of the mobile phases in a selected domain. The computation time of this search has been reported to be substantially reduced by application of a genetic algorithm with local search (LOGA). A much simpler approach is here described, which is accessible to non-experts in programming, and offers solutions of the same quality as LOGA, with a similar computation time. The approach makes a sequential search of CMPs based on the peak count concept, which is the number of peaks exceeding a pre-established resolution threshold. The new approach is described using as test sample a mixture of 30 probe compounds, 23 of them with an ionisable character, and the pH and organic solvent contents as experimental factors.

1. Introduction

As the complexity of a sample increases, a single separation condition (e.g., isocratic mobile phase or gradient with one or several modifiers with one or several modifiers) becomes less suitable to provide an acceptable separation for all compounds in the sample. The challenge of resolving a complex mixture has led to the idea of using complementary situations, namely two (or more) different separation conditions to get full resolution. One of them allows the resolution of some compounds in the sample, while the other compounds are resolved using a second (or subsequent) separation condition(s) [1–9]. The optimisation is carried out in such a way that all compounds get satisfactorily resolved in one or another condition, which are devoted to resolve only some compounds, while the other compounds in the sample can remain overlapped. This strategy increases the separation space, being very useful to face situations of extremely low resolution.

Most published work on complementary situations deals with discrete experimental factors (i.e., column type or length, packing type or size, modifier nature, or HPLC mode), and frequently implies drastic changes in the separation system [1–4,7]. In this case, strictly, there is no optimisation but a selection of two rather different conditions. Situations of complementary resolution can be obtained in a simpler way, using the same column and specific sets of levels of experimental factors that can be varied continuously (e.g., organic modifier contents, ionic strength, or pH) [5,6,8]. This is attractive under both operative and economical points of view. In this case, a systematic selection of the experimental conditions is possible.

Complementary situations can be generated by running independent experiments under isocratic or gradient elution [5,6]. In this work, we will discuss the simplest case: isocratic separations, where the independent experiments imply mobile phases, which have been called “complementary mobile phases” (CMPs) [6].

In most cases, the direct search of optimal CMPs (i.e., set of mobile phases that maximises the separation of a given mixture), in a more or less random way (i.e., by trial and error assays), is too laborious and with few possibilities of success. This is especially true when the sample is complex. Computer assisted optimisation is the rational way to search systematically the best experimental conditions. In the literature, a huge effort has been made in the development of interpretive methodologies (i.e., based on models

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doi:10.1016/j.chroma.2011.06.087

built from experimental designs) to optimise single mobile phases and gradients for the analysis of samples of different complexity. The topic has been reviewed by several authors [10–14]. Some work has also been reported for the interpretive optimisation of CMPs [6,8], or complementary gradients [5].

We developed an approach to search CMPs based on the examination of the resolution of chromatograms simulated for a grid of hypothetical experimental conditions (e.g., mobile phases in a given range of acetonitrile contents, such as 20–60% in steps of 0.1%) [6]. The information (retention data and peak parameters) for simulating these chromatograms is provided by a few experimental runs in an experimental design. Performing the systematic examination of all possible combinations of complementary conditions can be a large effort. Thus, in a further work, we developed a hybrid genetic algorithm with local search (LOGA), which restricts the search space and expedites substantially the computation [8]. However, this approach cannot be implemented by non-experts in programming.

We describe here an alternative approach to search CMPs, which is far simpler and reduces the computing effort with respect to the approaches previously reported. The new approach is based on the "peak count" concept, which is the number of peaks exceeding a pre-established resolution threshold, as measurement of the success in the separation [15]. Therefore, the peak count is focused on the resolved compounds, in contrast to conventional resolution assessments that attend mainly to the separation of the least resolved compounds. The peak count resolution criterion yields the same results as conventional assessments when full resolution is possible, but it is also able to discriminate the maximal resolving power in low resolution situations.

The advantages and limitations of the new approach to search optimal CMPs are here compared with LOGA. As a test sample, a mixture of 30 probe compounds, 23 of them with an ionisable character (acidic, basic or amphoteric), was used. Two factors were considered: the pH of the mobile phase (covering a wide range, between 2 and 13), and the organic solvent contents (acetonitrile in the range 20–60% (v/v)). In previous work [15], we showed that this sample cannot be fully resolved using a single mobile phase.

The use of CMPs constitutes an alternative to apply when a single separation condition is demonstrated unable to get full resolution. The same information used to carry out the failed conventional optimisation, can be used in the search of optimal CMPs without additional experimental work. Often, two or three CMPs are enough to get a significant improvement in the resolution, regarding that found with a single mobile phase.

2. Theory

For the search of CMPs, a measurement that quantifies correctly the resolution level for each compound in the sample, for any combination of CMPs, is needed. An algorithm able to find in an efficient way the combination of mobile phases yielding the maximal resolution is also required. The first problem was solved satisfactorily in previous work, using the peak purity as resolution measurement [6,8,9]. However, the algorithm used to find the optimal CMPs was too complex. A simpler alternative with a similar performance should still be found to make the CMP strategy more accessible. We give below some information about the tools used in this work.

Before continuing, we would like to emphasize that the concept of complementary separation conditions can involve isocratic or gradient elution. It goes, therefore, beyond the elution mode. The chromatograms can be obtained using isocratic, gradient, or even mixtures of isocratic and gradient conditions. Furthermore, the conditions may involve different modifiers, different columns, separation modes or techniques. The only important requirement is having a set of chromatograms taken in different conditions, with differences in selectivity from which selecting the optimal complementary conditions. We will consider here the optimisation of isocratic mobile phases, involving two factors: acetonitrile content and pH.

2.1. Search of complementary mobile phases

The optimisation of CMPs can be considered as an intermediate level between a classical optimisation of the resolution of the whole mixture (where a single mobile phase is searched to resolve maximally all compounds), and the optimisation of individual compounds (the search of the best mobile phase for separating each compound from the others). The CMPs strategy will be closer to one or another, depending on the number of selected CMPs [13]. The CMPs strategy makes the resolution of more compounds possible, using fewer optimal mobile phases than the optimisation of individual compounds. It offers a reasonable compromise between the resolution capability of the system (which is not fully exploited, but up to a reasonable level), and the experimental effort (which in spite of not being as economical as in the case of using a single mobile phase, it can be still acceptable).

The search of optimal CMPs can be tackled with different algorithms. In any case, the calculation is obviously more complex with regard to the optimisation of a single mobile phase. The simplest way of implementing the search of optimal CMPs is through a systematic examination of all possible combinations of n (e.g., $n = 2$ or 3) mobile phases within a grid of hypothetical experimental conditions, to find out the combination yielding the maximal resolution [6]. This selection can be easily carried out by building, for each combination of mobile phases, a matrix with n resolution vectors (one vector by mobile phase) containing the elementary resolution of each compound in the analysed mixture. From the matrix, a new vector is built with the maximal resolution obtained for each compound (which may belong to any of the n mobile phases). The product of the elements in the combined vector will be the global resolution for that combination. The optimal combination (i.e., optimal CMPs) is that one yielding the highest global resolution.

For sufficiently simple problems, a comprehensive examination of all possible combinations of mobile phases that can be built within the grid of hypothetical experimental conditions is feasible. However, very often, the number of combinations is so large that the optimisation should be carried out by applying numerical procedures based on random searches, such as evolutionary algorithms [8]. In Section 4.2, we describe another perspective that allows a far simpler computation.

2.2. Peak purity and limiting peak purity

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

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

a'_s being the area under the peak overlapped by the chromatogram that would be obtained for the possible interferents, and a_s, the total area of the peak of interest. Peak purities are not accessible experimentally. They should be computed from simulations and require the prediction of the peak location and profile.

The most important parameter derived from the peak purity concept is the limiting elementary peak purity [13], which is the maximal peak purity that a compound can reach in the presence of the other compounds in the sample, in a certain experimental domain (e.g., range of pH values and levels of organic solvent contents in the mobile phase). The limiting elementary peak purity is

of high interest to determine the operative limits of the chromatographic system. When the peak purity for the optimal mobile phase composition coincides with the limiting purity, the separation system is exploited almost fully for that compound. The combination of the limiting purities for several compounds to obtain a global measurement (usually a product) indicates the maximal global resolution that can be achieved with the separation system.

2.3. Peak count

As commented, low (or extremely low) resolution is frequent with complex samples eluted with a single mobile phase: likely two or more compounds will show null or a rather small resolution. In these cases, the conventional global resolution measurements (as the resolution of the critical peak or peak pair, or the product of the elementary resolutions of all the compounds in the sample) are poorly informative, since they tend to be dominated by the low peak purities of the overlapped peaks [15]. A function oriented to the success, based on the compounds that have reached a sufficiently high resolution, is much more informative. Such is the case of the number of peaks that exceed an acceptable resolution threshold [15].

The threshold can be an absolute value (e.g., a minimal peak purity beyond which the peak is considered "well resolved"), or a relative value (the fraction or percentage of peak purity with regard to the limiting value for each compound). We have called peak count (PC) and limiting peak count (LPC) the number of peaks whose elementary peak purity exceeds the absolute or relative threshold, respectively. Both PC and LPC only consider the compounds that are resolved. They are not affected by the separation quality of the non-resolved compounds, in contrast to other conventional objective functions. In cases of low resolution and with complex samples, PC or LPC will likely not reach the number of total compounds in a sample, but they will be very useful to rank the separation performance of the different conditions in an optimisation domain.

When the limiting elementary peak purities for all compounds in a mixture is $p \approx 1$ (which means that all compounds can be fully resolved), PC = LPC. However, when some compounds cannot be resolved under any condition in the experimental domain, PC and LPC will differ, and LPC can be more informative about the exploitation level of the system separation capability.

There will likely be many conditions that will offer the same number of resolved peaks (i.e., PC and LPC values). In order to discriminate the performance of these experimental conditions, the addition of a fractional term (f) will indicate the global resolution of the set of peaks that exceed the threshold [15]. For example:

$$\mathrm{fLPC} = \mathrm{LPC} + f \quad (2)$$

In this work, we have used the product of elementary purities of the resolved peaks as fractional term added to the LPC values. We have called this function limiting peak count with fractional term (fLPC). The integer part indicates the number of compounds that exceed the threshold, and the fractional part qualifies the peak resolution. The elementary purities were normalised inside a range limited by the established threshold (which is the minimal value), and the limiting elementary peak purity (the maximal value), to make the fractional term values for different resolution thresholds comparable. The product of the normalised peak purities yields more uniform results, independently of the established threshold. Without normalisation, the fractional term would be biased towards small values for low thresholds.

Table 1
Probe compounds.

Code	Compound	Code	Compound
1[a]	Naphthoic acid	16[a]	*m*-Cresol
2[a]	2-Nitrobenzoic acid	17[c]	*N*-Ethylaniline
3[a]	3-Nitrobenzoic acid	18[c]	*N*,*N*-Dimethylbenzylamine
4[a]	4-Nitrobenzoic acid	19[c]	2,6-Dimethylaniline
5[a]	Benzoic acid	20[d]	Benzene
6[a]	Resorcinol	21[d]	Acetophenone
7[a]	Phenol	22[d]	Benzaldehyde
8[a]	2,4-Dichlorophenol	23[d]	Nitrobenzene
9[a]	2,4-Dinitrophenol	24[d]	Methylphenylether
10[a]	*β*-Naphthol	25[d]	Benzonitrile
11[a]	2-Nitrophenol	26[c]	2,4,6-Trimethylpyridine
12[a]	3,5-Dichlorophenol	27[c]	4-Chloroaniline
13[b]	3-Aminophenol	28[c]	Aniline
14[a]	3-Bromophenol	29[c]	*p*-Toluidine
15[a]	*p*-Chlorophenol	30[c]	Pyridine

[a] Acidic.
[b] Amphoteric.
[c] Basic.
[d] Neutral.

3. Experimental

The probe compounds were 15 acids, 8 bases, 6 neutral compounds and one amphoteric compound (Table 1). The experimental design consisted of 36 mobile phases: three levels of organic modifier contents (20%, 40% and 60% acetonitrile (v/v)) and eleven pH

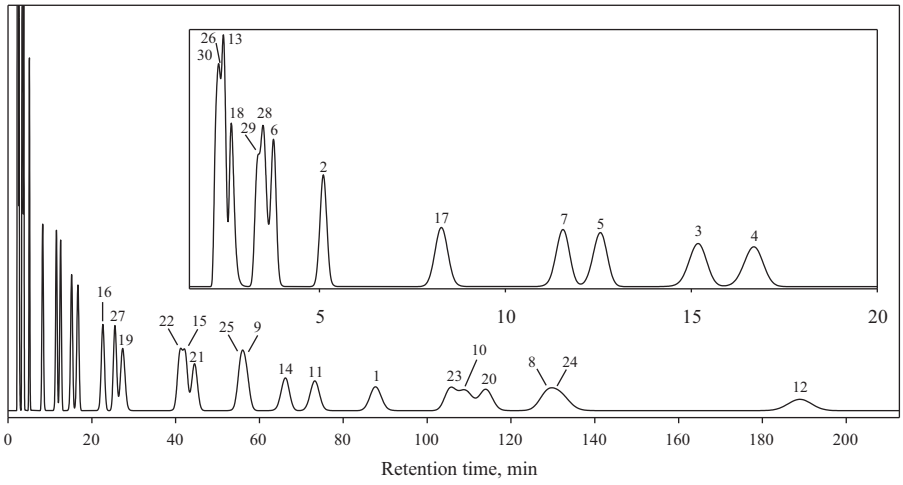

Fig. 1. Simulated chromatogram corresponding to the optimal single mobile phase. The composition of the mobile phase was: 21.4% acetonitrile/pH 3.4. Compound identities are given in Table 1.

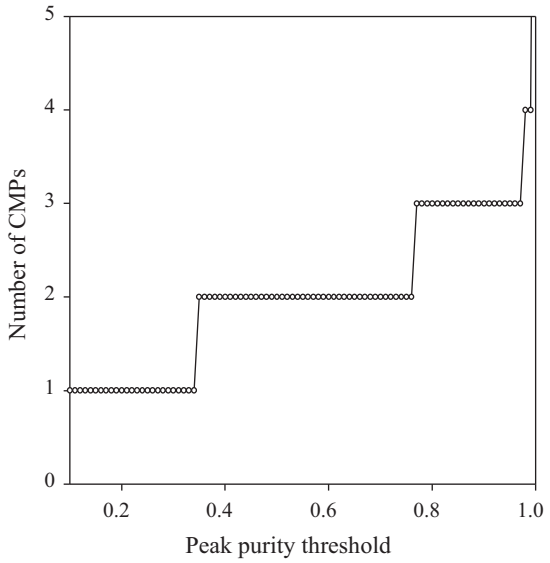

Fig. 2. Number of required CMPs according to the sequential search based on the fLPC function, against the resolution threshold, considering the full domain.

levels, covering the 2–13 range. This experimental design was carried out in a previous work to develop and test retention models for ionisable compounds [16]. Owing to its high complexity, this case of study was found particularly suitable to check the performance of the CMPs approaches.

Measurement of pH was carried out with a potentiometer Crison (Model MicropH 2002, Barcelona, Spain) with a precision of ±0.002 pH units, using a Ross electrode (Orion Model 8102, a combination of a glass electrode and a reference electrode with 3.0 M KCl aqueous solution as salt bridge). A chromatograph equipped with a dual pump and a UV–visible detector was used. The flow-rate was 1 ml min^{-1} for the 40% and 60% acetonitrile mobile phases, and 3 ml min^{-1} for 20% acetonitrile. The separation was carried out with a 15 cm × 4.6 mm i.d. polymeric C18 column with 15–20 μm particle size from Polymer Labs (Model PLRP-S 100 Å). All measurements were performed at 25 °C.

The routines to compute the CPMs approaches were developed in MATLAB 2010b (The MathWorks Inc., Natick, MA, USA). Other details are given in Ref. [16].

4. Results and discussion

The first part of the study shown in this work was carried out considering only the resolution performance. The objective was obtaining the best separation inside a pre-fixed experimental domain. However, the analysis time has an unquestionable importance in the viability of an analytical chromatographic method. As other secondary factors, the analysis time can be considered in an optimisation process, in combination with the resolution in a multicriteria decision-making function. Another possibility is to make a pre-selection of mobile phases that will provide sufficiently short analysis times. This option is perhaps preferable to the inclusion in the fLPC function (Eq. (2)) of a term that restricts the analysis time, since this would make the interpretation of the results more difficult.

4.1. Resolution of the sample with a single mobile phase

As indicated, we had a detailed description of the retention behaviour for each compound in the studied sample. The retention models developed in a previous work attended to the chemical nature of each compound, and were checked to yield accurate

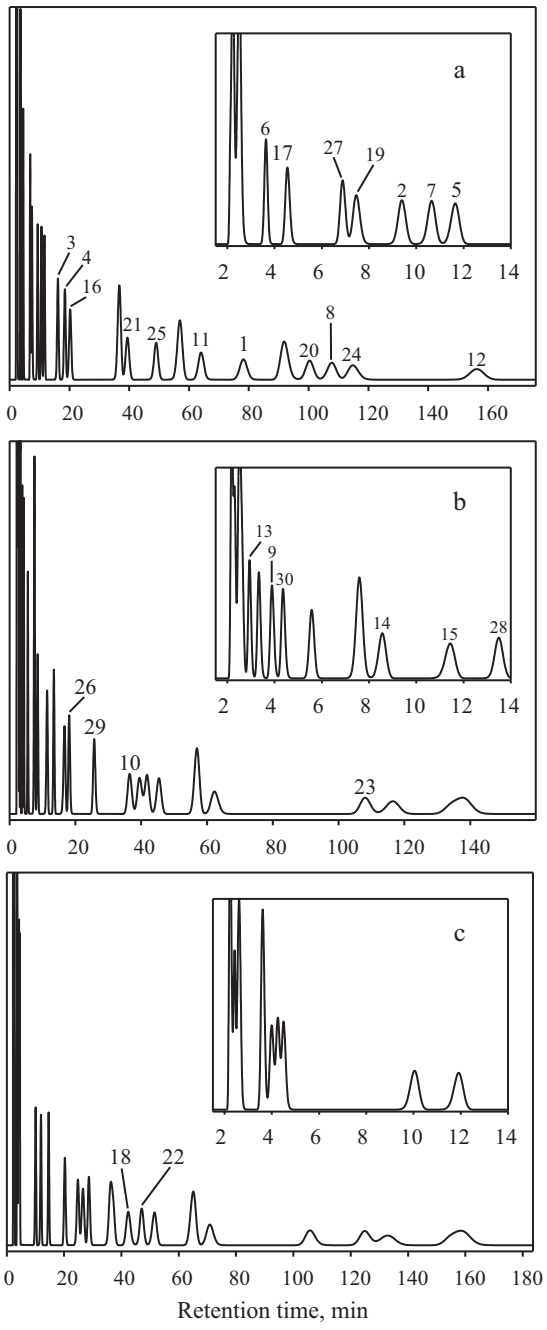

Fig. 3. Simulated chromatograms corresponding to the best combination of three CMPs for a 97% threshold, found in the sequential search inside the full domain. The composition of the CMPs was: (a) 22.6% acetonitrile/pH 2.5, (b) 21.2% acetonitrile/pH 10.7, and (c) 20% acetonitrile/pH 9.7. The compounds assigned to each CMP are indicated (see the identities in Table 1).

predictions of the retention behaviour for the probe compounds, satisfactory for optimisation purposes [16]. These models were used to predict the retention for each compound, scanning a domain constituted by a regular grid of 101 × 111 = 11,211 hypothetical conditions (solvent content × pH, with a grid step of 0.1 units for the pH and 0.2 units for the solvent content, expressed as v/v percentage).

The resolution contour maps drawn for the individual compounds, based on the simulation of chromatograms at varying pH and acetonitrile contents, revealed that the compounds did not share regions of common resolution [15]. The acidic compounds were more retained with an acidic mobile phase, while

Table 2
Required CMPs and number of compounds assigned to each CMP, according to the sequential method, considering the full experimental domain.

Threshold[a]	Number of CMPs	Number of compounds resolved by each CMP					
		CMP 1	CMP 2	CMP 3	CMP 4	CMP 5	CMPs 6 to 11
0.34	1	30					
0.50	2	29	1				
0.76	2	24	6				
0.80	3	23	6	1			
0.85	3	22	6	2			
0.90	3	20	9	1			
0.91	3	20	9	1			
0.92	3	19	10	1			
0.93	3	19	9	2			
0.94	3	19	9	2			
0.95	3	18	10	2			
0.96	3	18	10	2			
0.97	3	18	10	2			
0.98	4	17	10	2	1		
0.99	4	15	10	3	2		
1.00	11	9	7	3	3	2	1

[a] Fraction of peak purity with regard to the limiting value for each compound. For the full domain, the limiting peak purities of all compounds were $p = 1.00$.

those of basic character showed longer retention times with a basic mobile phase. Thus, in terms of separation space, an acidic mobile phase was predictable to be more appropriate to resolve the acidic compounds, while a basic mobile phase would separate the basic compounds. Therefore, both groups of compounds showed a complementary behaviour. The studied mixture also contained some neutral compounds, whose retention was not affected by the pH. As a consequence, multiple peak crossings with the ionisable solutes should happen at varying pH in the mobile phase. A complete separation of the mixture of these compounds using a single mobile phase was, thus, unfeasible (Fig. 1). The use of gradient elution would reduce the analysis times, but the compounds would remain unresolved if the scanned solvent and pH ranges are the same as those used to optimise the isocratic elution. This is an example among many others, of what may happen when complex samples are chromatographed. Another optimisation strategy was, therefore, needed to face the extremely low resolution.

4.2. Unsupervised sequential search of CMPs based on the peak count concept

The approach developed in this work to search CMPs needs the establishment of an arbitrary resolution threshold, which represents the resolution target to be reached for each compound (Section 2.3). The mobile phases that separate groups of compounds, with a resolution exceeding (or matching) the established threshold, are searched sequentially inside a selected experimental domain. Each mobile phase is focused to the resolution of the compounds not resolved by mobile phases previously selected. According to the resolution demands, the number of CMPs can be higher or smaller.

The first selected mobile phase will be that one that resolves the maximal number of compounds in the experimental domain. The process goes on by restricting the search to the compounds that were not resolved by the first CMP, to find a second CMP. This will be that one providing again the maximal number of resolved compounds among those unresolved by the first CMP. Note that some compounds resolved with the first CMP can also exceed the threshold with the second one (i.e., a compound already separated in a previously selected CMP can appear resolved in another, even with better resolution). The process is repeated with the remaining compounds to find a third CMP, and follows until all compounds have been resolved with additional CMPs. All compounds in the mixture will, eventually, be resolved above the pre-established threshold, when all CMPs are considered altogether. Subsequent CMPs will have associated a decreasing number of compounds, since the process leaves out those ones formerly selected. Therefore, there will be fewer unresolved compounds for the subsequent CMPs.

The proposed method is unsupervised, since it works without the need of any user input (i.e., decision along the process). Note that the number of CMPs needed to resolve the mixture is not established in advance, and can grow as the threshold value becomes more demanding. If the resolution demand is high, and some compounds require specific mobile phases to be resolved, the number of CMPs can become unpractical.

4.2.1. Search in the full domain

All compounds should reach its limiting elementary peak purity under a certain condition. Consequently, the approach will necessarily find a solution. However, the number of required CMPs is not directly controlled by the user, but depends on the threshold: the larger the threshold, the larger the number of CMPs. This limitation is easily overcome by performing a scan of thresholds: the best solution will be that one yielding the highest threshold with still a reasonable number of CMPs (e.g., 2 or 3).

Fig. 2 shows the results found in the scan of resolution thresholds for the mixture of 30 compounds, considering the full domain of experimental conditions in both factors (pH and organic modifier). In the figure, the number of CMPs required to separate the 30 compounds is plotted as a function of the resolution threshold. There is no practical interest in working with thresholds below certain values, but the examination of the whole variation range helps to understand the approach behaviour, and allows evaluating the complexity of the sample. It can be seen that thresholds of up to 0.34 for all compounds are fulfilled using a single mobile phase. With two CMPs, elementary resolutions of at least 76% of the separation capability of the system (i.e., a threshold of 0.76) can be reached. Considering the work and consumption of additional time and reagents for each additional CMP, it is advisable to evaluate if these conditions are good enough for the purpose of the analysis, since in practice, many compounds in the mixture will be resolved far above the minimal threshold.

As observed in Fig. 2, a good compromise between the separation quality and the experimental effort is achieved using three CMPs, for which at least 97% of the separation capability of the system is reached for all compounds. An additional mobile phase is required (a fourth CMP) to increase the resolution up to 99%, which is not practical. In order to fully exploit the separation performance of the chromatographic system (virtually up to 100%) 11 CMPs would be required, six of them devoted specifically to separate only one compound each, as can be seen in Table 2. In this table, the required number of CMPs and the number of resolved

Table 3
Elementary peak purities for the best combination of three CMPs found by the sequential method taking a 97% threshold, considering the full experimental domain.[a]

Code[b]	CMP 1 22.6% acetonitrile pH 2.5	CMP 2 21.2% acetonitrile pH 10.7	CMP 3 20% acetonitrile pH 9.7
1	**1.000**	0.579	0.771
2	**1.000**	0.126	0.310
3	**1.000**	0.333	0.435
4	**0.996**	0.082	0.542
5	**0.997**	0.350	0.399
6	**1.000**	0.552	0.153
7	**0.997**	0.235	1.000
8	**0.977**	1.000	1.000
9	0.130	**0.998**	0.777
10	0.064	**0.981**	1.000
11	**1.000**	0.998	0.877
12	**1.000**	0.233	0.963
13	0.000	**0.992**	0.168
14	0.130	**0.998**	0.617
15	0.177	**1.000**	0.616
16	**0.996**	0.996	0.968
17	**1.000**	0.602	0.589
18	0.119	0.876	**0.998**
19	**0.980**	0.998	0.997
20	**0.988**	0.980	0.929
21	**0.984**	0.993	0.997
22	0.188	0.888	**0.996**
23	0.067	**0.980**	0.930
24	**0.985**	0.602	0.589
25	**1.000**	0.216	0.392
26	0.077	**0.996**	1.000
27	**0.980**	0.215	0.389
28	0.229	**1.000**	1.000
29	0.000	**1.000**	0.995
30	0.494	**0.998**	0.883
P_{CMP}[c]	0.886	0.943	0.994
P[c]	0.831		

[a] The elementary peak purities for the compounds assigned to each CMP are marked in bold.
[b] Compound identities are given in Table 1.
[c] The partial purities for each CMP (P_{CMP}) were obtained as the product of the elementary peak purities for the compounds assigned to that CMP, and the global purity (P) as the product of the best elementary purities for the 30 compounds.

compounds are indicated, as a function of the threshold value with regard to the limiting purity of each compound. Thus, for instance, all compounds will be resolved with four CMPs for a threshold of 99% (the number of compounds resolved by each CMP is 15, 10, 3 and 2). For thresholds between 80% and 97%, three CMPs are required. As commented, a good compromise can be a threshold of resolution of 97%, for which the separation capability of the system is almost completely exploited using three CMPs. The number of compounds resolved by each CMP was 18, 10 and 2. The reduction in the number of CMPs down to two leads to an unacceptable resolution, since the threshold needs to be set only at 76% of the limiting elementary peak purities.

A peak purity threshold of $p = 0.8$ can be enough for certain purposes (e.g., screening), since it will show clear valleys between peaks. However, the acceptance of such a threshold depends on the analyst demands or experience, and the type of problem. If the selected threshold is not considered finally appropriate, an enhancement will be achieved by introducing an additional CMP. The chromatographer should decide whether this change is practical or not.

The elementary peak purities for the optimal combination of three CMPs with a threshold of 97% are given in Table 3. The mobile phase compositions (one CMP was acidic and two basic), and the partial and global resolutions are indicated, as well as the compounds assigned to each CMP marked in bold (note that the elementary peak purities for the selected compounds are well above $p = 0.97$). Fig. 3 depicts the corresponding chromatograms. It should be observed that for the second and subsequent CMPs, although a CMP is assigned by the algorithm to a certain group of compounds, this does not mean that other compounds are necessarily overlapped.

4.2.2. Search in a restricted analysis time domain

A drawback of the search of CMPs described above is that it can lead to mobile phases yielding an excessively long analysis time, when this is not considered along the optimisation. The concentration of acetonitrile in the selected CMPs for the test sample was low. This increased the retention, obviously enhancing the separations, but with an adverse effect on the analysis time.

Next, we decided to restrict the analysis time to a maximal value of 45 min. As observed in the contour map in Fig. 4, this time restriction implied that the organic solvent fraction should be above ~32%, while the full pH domain continued being available to carry out the optimisation. Therefore, the factor that affected the analysis time was the organic solvent contents.

We carried out the sequential search of CMPs in the new restricted domain. This means that the possibilities of finding

Table 4
Required CMPs and number of compounds assigned to each CMP, according to the sequential method considering the restricted domain (analysis times below 45 min).[a]

Threshold[b]	Number of CMPs	Number of compounds resolved by each CMP						Unresolved[c]
		CMP 1	CMP 2	CMP 3	CMP 4	CMP 5	CMP > 6	
0.21	1	30						–
0.22	2	28	2					–
0.75	3	22	4	2				2
0.76	4	22	4	1	1			2
0.77	4	22	4	1	1			2
0.90	5	18	4	4	1	1		2
0.91	6	18	4	3	1	1	1	2
0.92	6	17	5	3	1	1	1	2
0.93	5	16	5	3	2	1		3
0.94	5	16	5	2	2	2		3
0.95	5	14	5	4	2	2		3
0.96	5	14	5	4	2	2		3
0.97	5	14	5	4	2	2		3
0.98	8	13	4	3	2	2	1	3
0.99	7	11	4	2	2	1	1	8
1.00	9	7	3	2	2	1	1	11

[a] The limiting elementary peak purities were calculated within the restricted domain.
[b] Fraction of peak purity with regard to the limiting value for each compound.
[c] Compounds not reaching the threshold under any condition.

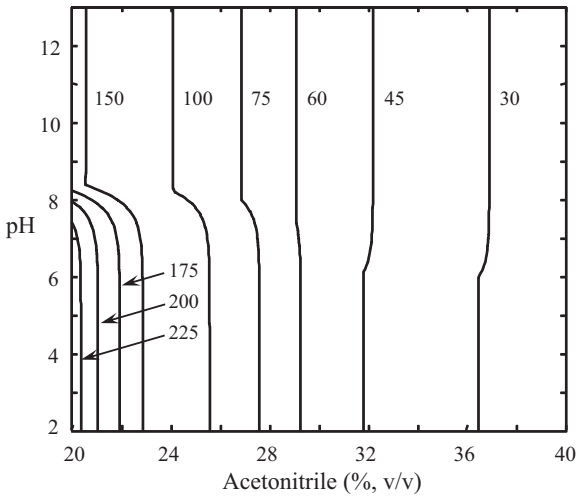

Fig. 4. Contour map depicting the analysis time (min) for the mixture of 30 compounds.

separation conditions were decreased (substantially for some compounds). Thus, the pair acetophenone–benzaldehyde showed a maximal resolution slightly below 0.7, and in a lesser extent, the possibilities of resolution for the amphoteric compound (3-aminophenol) were decreased.

Table 4 shows the results obtained from the scanning of resolution thresholds in the restricted domain, where the maximal thresholds for each number of CMPs are included, together with other representative cases. The number of compounds that did not reach the threshold is also indicated. The optimal combination of three CMPs with a threshold of 75% was (% acetonitrile/pH) 32.0/3.2, 36.2/6.8, and 31.8/3.9. The partial resolutions were $P_{CMP} = 0.125$, 0.469 and 0.923, respectively, and the global resolution $P = 0.054$. The number of resolved compounds was 22, 4 and 2, respectively, and two compounds remained unresolved. Even with five CMPs the resolution remained low. If maximal resolution was demanded, fixing the threshold at 1.0, only 19 from the 30 compounds could reach the threshold using 9 CMPs. Note that the peak capacity in the restricted domain was reduced from 70 to 48.

4.3. Systematic search of optimal CMPs and genetic algorithm with local optimisation (LOGA)

The CMPs search described in Section 4.2 is a sequential approach that attends to the maximal number of resolved compounds, where the CMPs are established independently, one by one. In previous work, we developed a systematic approach to find the optimal combination of CMPs. This approach carries out a comprehensive inspection of the global resolution of all possible combinations of mobile phases in a selected domain, in order to find the best [6]. There are significant differences between both approaches. The sequential search is based on the measurement of elementary peak purities. In contrast, the systematic search is governed by the global resolution of the whole mixture, measured as the product of elementary peak purities. Also, whereas the chromatographer does not have a direct control of the number of CMPs in the sequential search, the establishment of the number of mobile phases before starting the process is needed in the systematic search.

The optimisation strategy through a comprehensive search assures that the best combination will be found, but it involves a massive computation volume that grows exponentially until becoming impractical when the number of analysed compounds and/or mobile phases is large (it would take days to years of computation). The number of possible combinations of mobile phases

Table 5
Number of combinations to be examined in the systematic search of optimal CMPs within the full domain, and computation times.[a]

Number of CMPs	Combinations	Time
2	5.37×10^8	14.6 days
3	3.43×10^{13}	2.56×10^3 years
4	4.80×10^{16}	3.58×10^6 years
5	7.71×10^8	5.75×10^8 years
6	2.99×10^{20}	2.23×10^{10} years

[a] The computation times are extrapolations from reduced assays in a personal computer equipped with an Intel CoreDuo 2.53 GHz and 4 GB RAM.

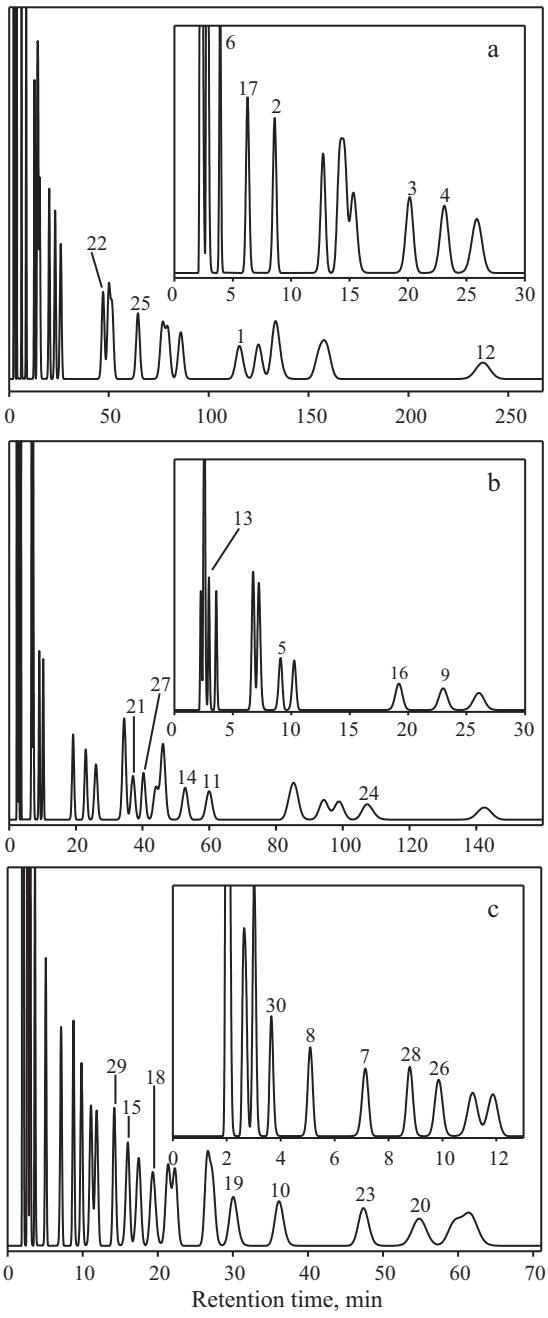

Fig. 5. Simulated chromatograms corresponding to the best combination of three CMPs, found by LOGA. The composition of the CMPs was: (a) 20.0% acetonitrile/pH 2.9, (b) 23.2% acetonitrile/pH 4.3, and (c) 28.8% acetonitrile/pH 9.8. The compounds assigned to each CMP are indicated (see the identities in Table 1).

to examine, and the estimated computation time for sets of two to six CMPs in the analysis of the mixture of 30 compounds, considering a space with 11,211 mobile phases (the same studied for the sequential method in the full domain) are given in Table 5.

A solution to the problem of the excessive computation time for the comprehensive search is the use of genetic algorithms (GAs), which reduce the number of combinations to be examined. GAs, however, can present convergence problems, or fail in the search of the global optimum. To overcome this limitation, we developed a modified GA with internal local search (LOGA) [8], which yields excellent results and reduces the computation time to the order of a few seconds (in cases that required hundreds or thousands of millions of years with the comprehensive examination). The compositions of the optimal CMPs found by LOGA considering the full domain were (% acetonitrile/pH): 20.0/2.9, 23.2/4.3, and 28.8/9.8, with partial resolutions P_{CMP} = 0.952, 0.958 and 0.974, respectively (Fig. 5). The global resolution was P = 0.889. The number of compounds resolved by each CMP was 9, 9 and 12, respectively. These figures should be compared with those obtained by the sequential search, which found a different combination of optimal CMPs (% acetonitrile/pH): 22.6/2.5, 21.2/10.7, and 20.0/9.7 with P_{CMP} = 0.886, 0.943 and 0.994, respectively, and a global resolution of P = 0.831. The global resolution for the sequential search was somewhat smaller. However, it can be observed that this approach was able to find two CMPs that resolved most compounds in the sample (18 + 8 = 26), whereas the solution found by LOGA only could resolve a maximal number of 21 compounds with two CMPs. With a single mobile phase, only 17 compounds could be resolved (Fig. 1).

5. Conclusions

One of the problems to be solved in the development of a liquid chromatographic method is the search of the optimal conditions in cases of minimal resolution. A possible solution is to increase the selectivity by implementing complementary experimental conditions. The easiest way to afford this is the analysis of the sample using two or more mobile phases (CMPs), each of them focused to resolve only some compounds in a mixture.

We have introduced three approaches to obtain optimal CMPs:

(i) the comprehensive examination of all possible combinations of mobile phases (or compounds),
(ii) the systematic search of optimal CMPs assisted by a genetic algorithm with local search (LOGA), and
(iii) the sequential search, based on the peak count concept.

In the two former approaches, the number of CMPs should be fixed a priori. In the latter approach, a resolution threshold is fixed and the CMPs needed to reach it are found. The sequential approach presents the advantage of a short computation time and simplicity: it only requires the examination of the data matrix and the scanning of thresholds. The approach based on LOGA also requires a short computation time, but the algorithm is considerably more complex and cannot be easily implemented [8].

We have demonstrated to which extent the sequential approach is viable to find suitable CMPs. It should be noted, however, that the CMPs found by this approach will probably not coincide, neither the number, nor the distribution of compounds resolved by the CMPs, with the result found by LOGA (or the comprehensive search approach). Therefore, the solution with the sequential approach is not necessarily the optimal one. Other combinations with the same number of CMPs may exist showing better global resolution. Meanwhile, in the search based on LOGA, the elementary resolution of each compound is not considered, but the global one. It is then possible that the elementary resolution for some compounds be below that obtained with the combination chosen by the sequential approach.

The global peak purities can be directly compared in both cases. In contrast, the partial peak purities for each CMP cannot be compared, since the number of resolved compounds is different. This number is more uniform with LOGA than with the sequential search, due to the nature of the latter approach, which tends to concentrate more compounds in the first CMPs.

Due to its simplicity and the structure of the algorithm, the sequential method is more "governable" via threshold tuning. Also, the selection of one or more compounds to be included in the first CMP is feasible, either based on their analytical interest (in our example, all phenols could be forced to be resolved with the same CMP), or on the separation complexity (as was the case of 3-aminophenol, acetophenone and benzaldehyde).

The CMPs approach requires two or more runs (in one or more chromatographs) by sample. Once a conventional optimisation has been checked to fail, the same experimental information (i.e., retention and peak shape data) can be used to compute in a few minutes the complementary conditions. The chromatographer can appraise if the solution found is practical enough, against the extra work of performing a fully new optimisation. Sometimes, this could be preferable to develop a full modelling and optimisation for another system (column, modifier(s), pH, buffer nature, etc.), which incidentally may fail.

The practical difficulty in a CMP approach can arise when the required number of mobile phases needed to exploit the whole resolution capability of the system is too high. In that case, some separation potential can be sacrificed, finding a reasonably number of CMPs (two or three), and reducing the resolution expectations. Also, the analysis time can be expedited optimising complementary gradients, which will be the subject of future work.

Acknowledgements

This work was supported by Projects CTQ2007-61828/BQU (Ministerio de Educación y Ciencia of Spain), and CTQ2010-16010/BQU (Ministerio de Ciencia e Innovación of Spain), and FEDER funds. A. Ortin is grateful to Polymer Char for the time allowance to develop this work.

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