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Considerations on the modelling and optimisation of resolution of ionisable compounds in extended pH-range columns

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Abstract

The problems associated to the modelling and optimisation of the chromatographic resolution of mixtures involving ionisable solutes at varying pH and acetonitrile content are discussed. Several retention models that separate the contributions of solute, column and stationary phase, were used. The retention was predicted with low errors in large pH domains (2–12), which was an essential requirement to face the optimisation of resolution. The selected mixture was particularly problematic under the viewpoint of resolution, owing to the excessively diverse acid–base behaviour of solutes. This variety led to sudden drops in retention at different pH for each solute, yielding numerous peak crossing, which made finding shared regions of high resolution especially difficult. Conventional resolution diagrams for these situations are scarcely informative, since both the overall and the worst elementary resolutions drop to zero if at least two compounds remain overlapped, even when all the others are baseline resolved. A new chromatographic objective function is proposed to address this drawback. This function, called "limiting peak count", is based on the limiting peak purity concept, and measures the success in the resolution focusing on the resolved solutes, in contrast to conventional resolution assessments that attend mainly to the least resolved solutes. Limiting peak count yields the same result as conventional assessments when full resolution is possible, but it is also able to discriminate the maximal resolving power in low-resolution situations. It offers a different perspective to that given by the complementary mobile phases approach, and the computation is far simpler.

Keywords: Retention modelling; Optimisation; Polarity measurement; Peak purity; Mobile-phase composition; pH

1. Introduction

Tuning of selectivity is a major topic in chromatography. Not surprisingly, numerous reports have been published on methodologies to optimise the separation of mixtures, mainly for non-ionisable compounds [1–3]. Generally, these approaches take advantage of the effects on retention and selectivity of changes in the amount of organic modifier in the mobile phase, since this continuous factor is the most convenient affecting any kind of solute. Although sequential trial-and-error methodologies are still frequent, other approaches based on a previous description of solute retention behaviour have proved to be advantageous in terms of comprehensive scanning of the system separation capabilities, possibility of reaching the true optimal conditions, and economy.

The simplest approach to predict the retention is an exponential decay relating the retention factor (k) to the volumetric fraction of organic modifier (φ):

$$\log k = c_0 + c_1\varphi \quad (1)$$

this model is, however, only valid for moderate solvent composition ranges. The inclusion of a quadratic term is usually necessary to achieve more accurate descriptions in wider solvent ranges or for particular modifiers, such as acetonitrile:

$$\log k = c_0 + c_1\varphi + c_2\varphi^2 \quad (2)$$

some other alternative models have been proposed. For instance, the following one describes linearly the retention of

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solutes as a function of parameters depending on the polarity of solute (p), mobile phase (P_m^N), and stationary phase ($(\log k)_0$ and P_s^N) [4,5]:

$$\log k = (\log k)_0 + p(P_m^N - P_s^N) \quad (3)$$

which can be rewritten as:

$$\log k = [(\log k)_0 - p\,P_s^N] + p\,P_m^N = q + p\,P_m^N \quad (4)$$

P_m^N is established as a function of mobile phase composition. When the organic modifier is acetonitrile, it is given by:

$$P_m^N = 1 - \frac{2.13\varphi}{1 + 1.42\varphi} \quad (5)$$

In previous work, Eq. (4) was found to yield better predictions of retention than Eq. (1), with an overall performance similar to that of the quadratic model (Eq. (2)) [5].

For ionisable compounds, since changes in solvent concentration affect the protonation constant and warp the retention surfaces, the interaction with pH demands more complex models. Also, any deviation of the target pH in the mobile phase will affect strongly the retention if mobile phase mispreparation concerns a pH region where the acid–base equilibrium is changing significantly. Therefore, mixtures containing ionisable compounds require careful buffering to guarantee reproducibility among injections.

Trying to minimise the impact of these problems, optimisation of the separation of such mixtures is often carried out at fixed pH. This makes the process similar to others applied in the separation of non-ionisable compounds. However, an optimisation developed at fixed pH will not take advantage of the benefits of this worthy experimental factor, and consequently, the chances to separate the mixture are reduced.

Several retention models depending on the organic modifier content and pH have been proposed for ionisable compounds [6–8]. The mechanistic models are mainly based on the combination of Eqs. (1) or (2) with an equation that considers the displacement of protonation equilibria with the modifier content. The polarity equation (Eq. (3)) has been also extended to ionisable solutes, as described below [9,10]. In these equations, the conditional character of the acid–base constants cannot be neglected, since the changes with the modifier content affect the observed pH.

On the other hand, it seems logical to think that the proper measurement of pH in aqueous–organic mixtures requires the use of reference buffers prepared in the same medium ($^{s}_{s}$pH, where the superscript indicates the solvent where the mobile phase pH was measured, and the subscript, the solvent where the reference buffers were prepared). Surprisingly, the most extended practice consists of measuring the pH in aqueous medium before the addition of the organic solvent, using reference buffers prepared in aqueous medium ($^{w}_{w}$pH). This means that the above-mentioned effects of the organic modifier on the pH are ignored. The strategy has, however, the obvious advantage of reducing the number of measurements, since the pH value will be the same for all mobile phases prepared with the same buffered solution, independently of the amount of organic modifier. Recently, aqueous buffers have demonstrated to be useful in the calibration step, when pH is measured in aqueous–organic solutions ($^{s}_{w}$pH) [11].

This work discusses the problems associated to the scanning of wide pH regions in the modelling and optimisation of the selectivity of mixtures involving ionisable solutes of widely different nature. The resolution capability of the chromatographic system is established by applying the concept of limiting peak purity. A new chromatographic objective function (COF), the limiting peak count, is proposed to tackle situations of low resolution where conventional measurements fail. This function is focused on the resolved compounds and not on the unresolved ones as conventional COFs do. It is able to discriminate the resolving power of the chromatographic conditions in the presence of coeluting compounds, where conventional COFs are dominated by the unresolved compounds.

2. Theory

2.1. Retention modelling

Ionisable compounds can be classified in three categories; acidic, basic and amphoteric:

$$A^- + H^+ \rightleftarrows AH \quad (6)$$

$$B + H^+ \rightleftarrows HB^+ \quad (7)$$

$$A^- + H^+ \rightleftarrows HA + H^+ \rightleftarrows H_2A^+ \quad (8)$$

For a solute with a unique acid–base equilibrium, the retention factor at a given pH can be described as a weighted mean of the retention factors of the two acid–base species (from now on, we will call k_A and k_{HA} to the retention factors of the basic and acidic species, respectively):

$$k = \frac{k_A + k_{HA} K h}{1 + K h} \quad (9)$$

where K is the protonation constant, and h, the concentration of hydrogen ion. By dividing all terms in Eq. (9) by k_{HA}, making $f_{A/HA} = k_A/k_{HA}$, and expressing the relationship in logarithmic form, the following results:

$$\log k = \log k_{HA} + \log\left(f_{A/HA} + \frac{K h}{1 + K h}(1 - f_{A/HA})\right) \quad (10)$$

If a linear relationship between $\log K$ and φ is assumed, the combination of Eqs. (4) and (10), yields:

$$\log k = q + p\,P_m^N + \log\left(f_{A/HA} + \frac{10^{\log K_0 + m\varphi} h}{1 + 10^{\log K_0 + m\varphi} h}(1 - f_{A/HA})\right) \quad (11)$$

K_0 being the protonation constant in water. Similarly, dividing by k_A and making $f_{HA/A} = k_{HA}/k_A$:

$$\log k = \log k_A + \log\left(1 + \frac{K h}{1 + K h}(f_{HA/A} - 1)\right) \quad (12)$$

$$\log k = q + p\, P_m^N + \log\left(1 + \frac{10^{\log K_0 + m\varphi} h}{1 + 10^{\log K_0 + m\varphi} h}(f_{HA/A} - 1)\right) \quad (13)$$

for an amphoteric solute, the retention factor is given by:

$$k = \frac{k_A + k_{HA}\beta_1 h + k_{H_2A}\beta_2 h^2}{1 + \beta_1 h + \beta_2 h^2} \quad (14)$$

where β_1 and β_2 are the cumulative protonation constants. By dividing all terms in Eq. (14) by k_{HA}, and expressing $\log k_{HA}$ as a function of the polarity parameters:

$$\log k = q + p\, P_m^N + \log\left(f_{A/HA} + \frac{10^{\log \beta_{1,0} + m_1\varphi} h(1 - f_{A/HA}) + 10^{\log \beta_{2,0} + m_2\varphi} h^2 (f_{H_2A/HA} - f_{A/HA})}{1 + 10^{\log \beta_{1,0} + m_1\varphi} h + 10^{\log \beta_{2,0} + m_2\varphi} h^2}\right) \quad (15)$$

$\beta_{1,0}$ and $\beta_{2,0}$ being the cumulative constants in water.

Since the intrinsic retentions of the acidic and basic species are different, a sudden change in solute retention happen at pH values close to the logarithm of the conditional acid–base constants. For this reason, the corresponding k versus pH curves are similar to those observed in titrations. For acidic solutes (Eq. (6)), a decreased retention is observed with pH, which is explained attending to the charge of both species in the acid–base pair. Acidic solutes are neutral at low pH and become negatively charged upon dissociation. The neutral species establishes hydrophobic interactions with the stationary phase, being retained according to its polarity. When the negative species dominates, the affinity towards the stationary phase is reduced, which makes the retention to decrease with pH. The retention of the ionic species is smaller than the neutral one, which allows simplifying Eq. (10), assuming that $f_{A/HA} \approx 0$:

$$\log k = \log k_{HA} + \log\left(\frac{K h}{1 + K h}\right) \quad (16)$$

therefore:

$$\log k = q + p_{HA}\, P_m^N + \log\left(\frac{10^{\log K_0 + m\varphi} h}{1 + 10^{\log K_0 + m\varphi} h}\right) \quad (17)$$

For basic compounds (Eq. (7)), the acidic species is positively charged and the basic species, neutral. The stronger interaction of the latter makes retention to increase with pH. Since k_{HA} is appreciably smaller than k_A, it can be assumed that $f_{HA/A} \approx 0$, giving rise to the following simplified equations:

$$\log k = \log k_A + \log\left(\frac{1}{1 + K h}\right) \quad (18)$$

$$\log k = q + p_A\, P_m^N + \log\left(\frac{1}{1 + 10^{\log K_0 + m\varphi} h}\right) \quad (19)$$

Amphoteric compounds exhibit a particular behaviour, showing small retention at low pH, where the cationic species dominates. At intermediate pH values, the neutral species becomes the most abundant, which increases the overall retention with pH, up to reach a maximum that coincides with the maximal abundance of this species. Beyond that point, the retention decreases again when the equilibrium is displaced towards the formation of the anionic species. Since k_{H_2A} and k_A are appreciably smaller than k_{HA}:

$$\log k = \log k_{HA} + \log\left(\frac{\beta_1 h}{1 + \beta_1 h + \beta_1 h^2}\right) \quad (20)$$

$$\log k = q + p_{HA}\, P_m^N + \log\left(\frac{10^{\log \beta_{1,0} + m_1\varphi} h}{1 + 10^{\log \beta_{1,0} + m_1\varphi} h + 10^{\log \beta_{2,0} + m_2\varphi} h^2}\right) \quad (21)$$

The coefficients of all these models can be obtained by non-linear regression.

2.2. Resolution measurement

Peak purity can be defined as the area fraction of a peak which is not interfered by the chromatogram of the accompanying solutes. This assessment was selected to appraise the separation quality of a given peak in each predicted chromatogram. The optimisation process is based on the simulation of chromatograms within a predefined set of experimental conditions (pH and solvent content). Within each of these chromatograms, the global peak purity or global absence of interference, measured as:

$$P = \prod_{i=1}^{n} p_i = \prod_{i=1}^{n}\left(1 - \frac{o_i}{o_i'}\right) \quad (22)$$

is monitored to seek which of the scanned conditions yields the best global resolution. In the equation, p_i is the elementary peak purity of solute i, o'_i the total area under the peak of that solute, and o_i the area of that peak overlapped by a hypothetical chromatogram constituted by the remaining peaks, which are taken as interferents of the considered solute [12]. Peak profiles were simulated considering variations in efficiency and peak tailing with mobile phase composition, since changes in the ionic nature of solutes give rise to drastic alterations in their adsorption/desorption processes. Normalised areas were used throughout this work. Other details about the optimisation procedure are described elsewhere [12,13].

Several advantages are associated to the use of peak purity as resolution assessment: it measures the separation quality of each peak (and not each peak pair), its meaning is straightforward, it is quantitation-oriented, and finally,

it allows special optimisation strategies, including simultaneously more than two mobile phases, several kinds of eluents and/or columns, or even separation techniques (complementary optimisations) [12,14]. In this work, we exploit another feature: the possibility of anticipating the maximal resolution capability of the separation system, which allows the development of special resolution assessments for low-resolution situations, where conventional criteria fail.

3. Experimental

The mobile phases were prepared by mixing several aqueous buffer solutions with acetonitrile (HPLC grade, Merck, Darmstadt, Germany) up to get concentrations of 20, 40 and 60% (v/v) of the organic solvent. The buffer components were selected according to the desired pH value, from the following acid–base systems (reagents used to prepare the buffers are given between parenthesis): phosphoric (phosphoric acid, potassium dihydrogenphosphate, disodium hydrogenphosphate and sodium phosphate), citric (citric acid, potassium dihydrogencitrate, potassium sodium hydrogencitrate and sodium citrate), boric (boric acid and sodium borate), and butylammonium (butylamine and hydrochloric acid). The concentration of the buffer systems was in all cases 0.01 M (concentration after mixing the organic solvent).

The probe compounds (15 acidic and 8 basic) are detailed in Table 1. An amphiprotic compound (3-aminophenol) was also considered to study its particular retention behaviour. All reagents and probe compounds were at least reagent grade, obtained from Fluka (Buchs, Switzerland), Aldrich (Milwaukee, WI), Merck (Darmstadt, Germany), or Carlo Erba (Milan, Italy). Water was purified using the Milli-Q plus system from Millipore (Billerica, MA).

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 system (Isco Model 2350, Lincoln, NE), a 20 μl injection loop and an UV–vis detector (Shimadzu Model SPD-10Avp, Kyoto, Japan), set at 254 nm for the acids and bases, and 282 nm for the phenols, was used. Potassium bromide (0.01%) was chosen as hold-up time marker, being detected at 200 nm. 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 retention data were measured in a 15 cm × 4.6 mm i.d. column (15–20 μm, Polymer Labs. Model PLRP-S 100 Å). The potentiometric cell and the column were thermostated at 25 °C using water jackets. All measurements were triplicated and the mean values processed.

Table 1
Protonation constants in water and acetonitrile–water mixtures, using the $^{s}_{w}$pH scale

	Compound	Water			Acetonitrile–water		
		Literature[a]	Extrapolated[b]	Global[c]	20%[d]	40%[d]	60%[d]
1	2-Nitrobenzoic acid	2.19	2.26	2.21	2.93	3.59	4.26
2	3-Nitrobenzoic acid	3.47	3.34	3.35	3.91	4.38	5.00
3	4-Nitrobenzoic acid	3.43	3.20	3.21	3.79	4.29	4.92
4	Naphthoic acid	3.69	3.71	3.69	4.41	5.09	5.80
5	2,4-Dinitrophenol	4.10	3.65	3.66	4.04	4.36	4.79
6	Benzoic acid	4.19	4.22	4.11	4.73	5.28	5.77
7	2-Nitrophenol	7.23	6.64	6.77	7.37	7.91	8.74
8	2,4-Dichlorophenol	7.85	7.36	7.33	8.15	8.86	9.69
9	3,5-Dichlorophenol	8.18	8.13	7.98	8.68	9.34	9.83
10	3-Bromophenol	9.01	9.08	8.77	9.60	10.43	10.79
11	4-Chlorophenol	9.38	9.55	9.31	10.05	10.85	11.20
12	2-Naphthol	9.52	9.66	9.32	10.24	11.23	11.61
13	1,3-Dihydroxybenzene	9.81	10.17	9.77	10.48	11.11	11.26
14	3-Methylphenol	10.01	10.50	10.36	11.03	11.78	12.20
15	Phenol	10.09	10.24	10.01	10.77	11.61	11.98
16	2,6-Dimethylaniline	3.89	3.98	3.94	3.57	3.22	2.78
17	4-Chloroaniline	3.98	3.82	4.03	3.55	3.11	2.92
18	Aniline	4.60	4.75	4.80	4.35	3.97	3.56
19	4-Methylaniline	5.08	5.25	5.18	4.83	4.58	4.07
20	*N*-Ethylaniline	5.12	5.54	5.36	4.95	4.57	3.87
21	Pyridine	5.22	5.40	5.45	4.92	4.60	4.04
22	2,4,6-Trimethylpyridine	7.43	7.50	7.58	7.03	6.59	6.10
23	*N*,*N*-Dimethylbenzylamine	8.91	8.93	8.98	8.51	8.11	7.68

[a] From Refs. [16,18,19].
[b] Extrapolated from the values obtained in acetonitrile–water.
[c] Calculated with Eqs. (17) and (19).
[d] Calculated with Eq. (9).

4. Results and discussion

4.1. Measurement of pH and retention

The mobile-phase pH was determined by calibrating the electrode system with the usual aqueous reference buffers of potassium hydrogenphthalate (${}^{w}_{w}pH = 4.00$) and potassium dihydrogenphosphate/disodium hydrogenphosphate (${}^{w}_{w}pH = 7.02$). The pH was measured before and after mixing the aqueous buffer with the organic modifier, which gave ${}^{w}_{w}pH$ and ${}^{s}_{w}pH$values, respectively. Values of ${}^{s}_{s}pH$ were calculated from ${}^{s}_{w}pH$, instead of calibrating the system with buffers prepared in the same mobile phase. The reason is that both pH scales (${}^{s}_{s}pH$ and ${}^{s}_{w}pH$) differ in a δ term, which includes the primary medium effect for hydrogen ion, directly related to the Gibbs energy of transference of hydrogen ion from water to the acetonitrile–water mixture [15]. If the electrode system is designed in such a way that the difference between the liquid-junction potentials (E_j) of the two solvents (water and the acetonitrile–water mixture) is negligible, the δ term will only depend on the solvent composition, and one pH scale will be easily obtained from the other. For acetonitrile–water mixtures, ${}^{s}_{w}pH - {}^{s}_{s}pH = -0.03$, -0.14, and -0.46 for 20, 40 and 60% acetonitrile, respectively [15].

Several compounds showing acid–base properties were eluted in a polymeric column, according to an experimental design consisting of three acetonitrile concentration levels (20, 40 and 60%), each of them sampled at 10 pH values (separated about one pH unit). The studied pH ranges for the three scales were (acetonitrile percentage is given in parenthesis): ${}^{w}_{w}pH = 2.00–12.04$ (0%); ${}^{s}_{w}pH = 2.07–12.38$ (20%), 2.20–12.41 (40%) and 2.24–13.19 (60%); ${}^{s}_{s}pH = 2.10–12.41$ (20%), 2.34–12.84 (40%) and 2.70–13.65 (60%).

4.2. Modelling of retention

In previous work, the retention of non-ionisable solutes was successfully related to mobile phase (P^N_m) and solute (p) polarity parameters through Eqs. (3) and (4) [4,5]. These models are also able to describe variations in retention for ionisable solutes at fixed pH, when the organic modifier concentration is changed. We examine now the performance of global models considering simultaneously changes in solvent composition and pH. Extension of Eq. (4) to ionisable solutes leads to Eqs. (11), (13) and (15), and the respective simplified models (Eqs. (17), (19) and (21)).

This work was focused to the search of optimal experimental conditions (solvent composition and pH) in the chromatographic separation of ionisable compounds, trying to take advantage of the expanded pH range provided by polymeric columns. Such an optimisation requires the description of the retention behaviour with the highest possible accuracy level. The accomplishment of this aim implies enhancing the fitting behaviour, which means sacrificing some of the features of the polarity models (Eq. (3) and related). One of the features to be sacrificed is the transference of retention data between columns or solvents [5]. The exploitation of this capability implies setting unique column polarity parameters for all solutes (in the polymeric column used in this work they were estimated to be $P^N_s = -0.02$, and $(\log k)_0 = -1.22$ [16]). Forcing common contributions have the interesting advantage of leading to a greater level of generalisation of the retention behaviour. Also, the number of parameters in the models is decreased. However, this strategy diminishes the adaptability, which is detrimental for prediction purposes. The fittings are reasonably, but not maximally accurate.

Alternatively, Eqs. (11), (13) and (15) can be fitted solute-by-solute. This treatment yields individual estimations of the column polarity (gathered in the P^N_s and $(\log k)_0$ terms) not collecting exclusively column contributions, but also those coming from the solute nature. This feature is undesirable for system characterisation purposes, but makes equations more adaptable. Moreover, the solute-particularised models present the additional advantage of being better fitting-behaved, as will be further discussed.

In previous work [10], a model similar to Eq. (11), but using the column polarity parameters instead, was applied to the separation of a group of diuretics. The prediction errors were somewhat larger than those obtained with an equation derived from the combination of Eqs. (2) and (9). Eq. (11) had, however, the advantage of including a smaller number of parameters. This would allow, in theory, the use of simpler experimental designs, and consequently, requiring smaller experimental effort.

4.3. Calculation of solute polarities and protonation constants

For the current study, several probe compounds showing a wide acidity behaviour (Table 1) were selected: 15 acidic (with $\log K_0$ in the range 2.2–10.1), 8 basic ($\log K_0 = 3.9-8.9$), and an amphiprotic compound (3-aminophenol, with $\log K_{0,1} = 9.9$ and $\log K_{0,2} = 4.3$). Estimations of the retention factors for the acidic (k_{HA}) and basic (k_A) species of the probe compounds, at three concentration levels of organic modifier (20, 40 and 60% acetonitrile), are given in Table 2. The retention factors of the neutral and ionic species were calculated by fitting all available retention factors in mobile phases containing the same amount of organic modifier, using Eq. (9) for the acidic and basic compounds, and Eq. (14) for the amphiprotic one. For the latter compound, the retention factors of the three acid–base species (not included in the table) were extremely low: $k_{H_2A} = 0.26$, 0.11 and -0.00, $k_{HA} = 1.07$, 0.47 and 0.24, $k_A = 0.06$, 0.09 and -0.02, at 20, 40 and 60% acetonitrile, respectively.

The difference in retention between the neutral and ionic species for some acidic compounds was in some cases rather large, especially at 20% acetonitrile (for instance, compare in Table 2, k_{HA} and k_A for naphthoic acid, 2,4-dichlorophenol, 2-naphthol and *N*-ethylaniline). For this reason, at this acetonitrile concentration, the flow-rate was

Table 2
Retention factors for the acidic and basic species at several acetonitrile concentrations[a]

Compound	k_{HA}			k_A		
	20%	40%	60%	20%	40%	60%
2-Nitrobenzoic acid	7.12	1.21	0.43	0.24	0.13	0
3-Nitrobenzoic acid	11.2	1.50	0.51	0.42	0.16	0.01
4-Nitrobenzoic acid	13.3	1.61	0.53	0.46	0.17	0.01
Naphthoic acid	65.0	4.26	1.14	0.34	0.16	0.01
2,4-Dinitrophenol	44.6	4.57	1.24	1.35	0.29	0.06
Benzoic acid	7.20	1.20	0.51	0.21	0.13	0
2-Nitrophenol	50.1	6.50	1.97	1.16	0.28	0.05
2,4-Dichlorophenol	87.3	6.66	1.67	2.24	0.54	0.01
3,5-Dichlorophenol	131	8.35	1.94	2.60	0.45	0.02
3-Bromophenol	43.2	4.51	1.29	0.98	0.21	0.03
4-Chlorophenol	26.8	3.32	1.02	0.79	0.17	0.02
2-Naphthol	73.6	5.91	1.55	3.37	0.10	0.02
1,3-Dihydroxybenzene	1.18	0.47	0.22	0.03	0.07	0
3-Methylphenol	13.4	2.34	0.82	0.05	0.01	0
Phenol	6.08	1.52	0.62	0.20	0.08	0
2,6-Dimethylaniline	0.91	0.21	0.08	38.4	6.05	2.01
4-Chloroaniline	0.45	0.18	0.46	35.3	5.01	1.65
Aniline	0.31	0.17	0.11	7.11	2.01	0.95
4-Methylaniline	0.40	0.23	0.11	14.9	2.99	1.21
N-Ethylaniline	1.72	0.30	0.06	87.7	12.0	3.32
Pyridine	0.19	0.15	0.10	1.51	0.65	0.45
2,4,6-Trimethylpyridine	0.31	0.18	0.13	10.3	1.74	0.82
N,*N*-Dimethylbenzylamine	0.51	0.27	0.12	16.2	3.87	1.52

[a] Acetonitrile content in volumetric fraction percentage.

increased to $3\,ml\,min^{-1}$, avoiding thus excessive retention for the most hydrophobic solutes. The retention factors for most ionic species were below $k = 0.5$, being as reduced as 0.01 for some compounds at 60% acetonitrile.

Solute polarity values for the acidic (p_{HA}) and basic (p_A) species are given in Table 3. These values were estimated according to two approaches. The first approach consisted of fitting the retention factors of the acidic (k_{HA}) and basic

Table 3
Polarity parameters for the acidic and basic species

Compound	p_{HA}		p_A	
	Eq. (4)	Eq. (17)	Eq. (4)	Eq. (19)
2-Nitrobenzoic acid	3.58	3.55	2.03	–
3-Nitrobenzoic acid	4.03	4.04	2.52	–
4-Nitrobenzoic acid	4.25	4.25	2.58	–
Naphthoic acid	5.52	5.54	2.16	–
Benzoic acid	3.56	3.58	1.92	–
1,3-Dihydroxybenzene	1.95	1.96	0.45	–
2,4-Dichlorophenol	5.22	5.24	3.25	–
2,4-Dinitrophenol	4.62	4.63	3.23	–
2-Naphthol	5.11	5.12	–	–
2-Nitrophenol	4.13	4.02	3.07	–
3,5-Dichlorophenol	5.59	5.61	3.81	–
3-Bromophenol	4.57	4.58	3.03	–
4-Chlorophenol	4.21	4.23	3.38	–
3-Methylphenol	3.52	3.35	2.98	–
Phenol	2.80	2.81	2.57	–
2,6-Dimethylaniline	2.96	–	3.73	3.75
4-Chloroaniline	0.09	–	3.93	3.95
Aniline	1.22	–	2.52	2.53
4-Methylaniline	1.38	–	3.22	3.23
N-Ethylaniline	3.67	–	4.05	4.06
Pyridine	0.64	–	1.58	1.59
2,4,6-Trimethylpyridine	1.04	–	3.48	3.48
N,*N*-Dimethylbenzylamine	1.48	–	2.90	3.64

(k_A) species to Eq. (4), at the three solvent modifier levels (Table 2). In the second one, the retention factors, obtained at all mobile phase compositions and pH values, were simultaneously fitted for each acidic and basic compounds to Eqs. (17) and (19), respectively. It should be noted that *p*-values obtained with the global equations (Eqs. (17) and (19)) correspond to the retained neutral species of the acid–base pair. Thus, for instance, Eq. (17) does not allow the calculation of *p*-values for the anionic species of the acidic compounds, since its contribution to retention is negligible. Similarly, Eq. (19) cannot provide *p*-values for the cationic species of the basic compounds. Taking into account this limitation, it can be checked that both approaches give rise to virtually identical *p*-values (Table 3). The range of *p*-values for the retained species is 1.96–5.54 for the acidic compounds, and 1.59–4.06 for the basic ones.

It should be mentioned that *p* can be considered as a conditional parameter that depends on pH. However, the calculation of *p* is highly inaccurate when it is obtained at pH values where the unretained species dominates, using retention data measured in mobile phases containing different amounts of organic modifier. Finally, it should be reminded that the *p*-values listed in Table 3 were not calculated considering unique column polarity parameters. They, consequently, gather partially the contribution of the column.

Solute protonation constants for the assayed acetonitrile concentration levels can be obtained by fitting the retention data to Eq. (9) in mobile phases with the same modifier content at several pH values. The values listed in Table 1 were calculated using the ${}^{s}_{w}$pH scale. Protonation constants increase and decrease with the modifier concentration for acidic and basic compounds, respectively. Linear relationships of log *K* with the modifier content have been reported in the 20–80% acetonitrile concentration range [17]. Similar – although poorer – relationships have been obtained with P^N_m (mobile phase polarity) [16]. Based on this observation, we calculated the protonation constants in water by linear extrapolation with φ. Eqs. (11), (13), (17) and (19) contain a term showing this linear relationship. Therefore, these equations allowed another estimation of the protonation constants in water (K_0), which is also given in Table 1. As observed, the water-extrapolated log *K* values and those obtained from the global fittings (Eqs. (17) and (19)) agree satisfactorily with the literature values. This similarity is even closer when the latter values are compared with those obtained through the global fitting. This observation, together with the similarity of *p*-values obtained from the partial and global fittings, demonstrate the coherence of the proposed models, and constitute an indication of the reliability of both the results and the models.

4.4. Experimental designs and quality of predictions

Table 4 shows the errors in the prediction of retention obtained with Eqs. (11), (13) and (15), and Table 5, the

Table 4
Prediction errors for different retention models and experimental designs, using Eqs. (11) (acidic compounds), (13) (basic) and (15) (amphiprotic)

Compound	Experimental design														
	30			4 × 3			10 + 2			6 + 6			6 + 4 + 2		
	RE	RE_{max}	R_{adj}	RE	RE_{max}	R_{adj}	RE	RE_{max}	R_{adj}	RE	RE_{max}	R_{adj}	RE	RE_{max}	R_{adj}
2-Nitrobenzoic acid	0.97	3.1	0.9975	2.4	29	0.9426	1.0	3.3	0.9971	1.4	17	0.9814	1.4	17	0.9823
3-Nitrobenzoic acid	0.64	2.4	0.9993	1.12	9.0	0.9940	0.73	2.8	0.9988	0.66	2.8	0.9988	0.74	2.9	0.9987
4-Nitrobenzoic acid	0.54	2.2	0.9994	1.7	17	0.9820	0.65	2.9	0.9989	0.59	2.9	0.9999	0.64	3.0	0.9989
Naphthoic acid	0.54	3.8	0.9990	0.80	10	0.9946	0.68	3.8	0.9986	0.62	6.5	0.9980	0.49	6.5	0.9984
2,4-Dinitrophenol	0.54	3.3	0.9992	0.97	11	0.9931	0.61	3.3	0.9990	0.54	5.6	0.9986	0.57	5.6	0.9986
Benzoic acid	0.92	2.9	0.9988	0.93	6.1	0.9982	1.2	4.7	0.9975	1.0	4.6	0.9975	0.89	4.4	0.9986
2-Nitrophenol	1.5	16	0.9924	4.4	37	0.9293	1.6	16	0.9922	2.2	21	0.9835	2.6	20	0.9840
2,4-Dichlorophenol	0.90	4.0	0.9992	0.90	3.4	0.9991	1.2	4.0	0.9988	1.1	4.5	0.9988	0.87	5.1	0.9990
3,5-Dichlorophenol	0.45	1.6	0.9998	0.60	3.1	0.9996	0.72	2.5	0.9995	0.71	2.2	0.9996	0.47	2.0	0.9998
3-Bromophenol	0.59	2.8	0.9997	0.72	3.0	0.9996	0.87	2.8	0.9994	0.91	3.1	0.9994	0.62	3.1	0.9997
4-Chlorophenol	0.75	3.5	0.9996	3.9	66	0.9271	1.06	3.4	0.9992	1.03	4.6	0.9991	0.79	4.7	0.9994
2-Naphthol	1.1	5.2	0.9987	3.5	55	0.9477	1.5	5.2	0.9982	1.6	12	0.9969	1.2	12	0.9975
1,3-Dihydroxybenzene	1.4	4.7	0.9981	3.2	33	0.9719	4.8	27	0.9650	1.7	8.0	0.9965	1.6	7.0	0.9972
3-Methylphenol	0.90	6.5	0.9992	1.8	31	0.9876	1.1	6.4	0.9989	1.01	7.0	0.9989	0.83	7.1	0.9991
Phenol	0.72	5.3	0.9994	2.4	42	0.9700	0.91	5.3	0.9991	0.81	7.1	0.9990	0.78	7.1	0.9990
2,6-Dimethylaniline	0.85	5.1	0.9993	0.91	5.8	0.9991	1.0	5.0	0.9990	1.1	6.0	0.9987	0.89	6.0	0.9989
4-Chloroaniline	0.70	3.0	0.9997	0.97	5.0	0.9992	1.0	3.0	0.9992	0.99	3.6	0.9992	0.69	3.6	0.9996
Aniline	0.84	1.8	0.9996	1.1	7.7	0.9985	1.1	4.0	0.9989	1.0	3.8	0.9991	0.81	2.0	0.9996
4-Methylaniline	0.90	1.4	0.9996	0.99	2.5	0.9994	1.3	6.1	0.9984	1.1	2.9	0.9990	0.85	2.8	0.9995
N-Ethylaniline	0.77	3.4	0.9994	0.77	6.7	0.9989	0.85	3.4	0.9993	0.87	7.1	0.9989	0.79	7.1	0.9989
Pyridine	2.3	4.1	0.9958	2.6	11	0.9929	3.1	13	0.9864	2.7	13	0.9893	2.5	9.6	0.9938
2,4,6-Trimethylpyridine	1.2	2.4	0.9989	1.8	9.6	0.9961	2.7	14	0.9893	1.6	5.8	0.9963	1.3	2.4	0.9988
N,*N*-Dimethylbenzylamine	0.74	2.1	0.9994	0.77	3.0	0.9993	1.1	4.9	0.9979	0.93	4.9	0.9984	1.6	8.1	0.9957
3-Aminophenol	2.2	6.3	0.9939	4.8	48	0.911	–	–	–	2.8	9.4	0.9884	2.6	10	0.9902

Table 5
Prediction errors for different retention models and experimental designs, using Eqs. (17) (acidic compounds), (19) (basic) and (21) (amphiprotic)

Compound	Experimental design														
	30			4 × 3			12 + 2			6 + 6			6 + 4 + 2		
	RE	RE_{max}	R_{adj}	RE	RE_{max}	R_{adj}	RE	RE_{max}	R_{adj}	RE	RE_{max}	R_{adj}	RE	RE_{max}	R_{adj}
2-Nitrobenzoic acid	1.7	6.9	0.9907	3.7	42	0.8764	2.1	6.9	0.9882	2.3	23	0.9636	2.3	23	0.9640
3-Nitrobenzoic acid	1.4	6.4	0.9955	1.9	11	0.9908	1.6	6.4	0.9948	1.5	6.4	0.9951	1.6	6.4	0.9950
4-Nitrobenzoic acid	1.3	5.9	0.9960	1.9	10	0.9902	1.5	5.9	0.9950	1.4	5.9	0.9955	1.4	5.9	0.9955
Naphthoic acid	0.64	3.8	0.9990	0.87	8.3	0.9962	0.79	3.7	0.9985	0.77	6.5	0.9981	0.63	6.5	0.9985
2,4-Dinitrophenol	1.2	4.6	0.9970	1.3	6.4	0.9958	1.4	4.6	0.9966	1.2	4.9	0.9965	1.3	4.9	0.9965
Benzoic acid	1.4	6.1	0.9972	1.5	6.1	0.9970	1.6	6.1	0.9962	1.6	6.1	0.9960	1.4	6.1	0.9971
2-Nitrophenol	1.61	16	0.9924	4.5	37	0.9329	1.8	16	0.9922	2.4	22	0.9835	2.7	21	0.9830
2,4-Dichlorophenol	1.05	3.9	0.9990	1.1	3.8	0.9989	1.3	3.9	0.9986	1.3	4.5	0.9986	1.0	5.1	0.9988
3,5-Dichlorophenol	0.62	2.1	0.9997	0.75	3.1	0.9995	1.1	3.6	0.9992	0.86	2.3	0.9995	0.63	2.4	0.9997
3-Bromophenol	0.71	2.8	0.9996	0.83	2.9	0.9995	1.1	2.8	0.9992	1.2	7.0	0.9988	0.90	6.8	0.9991
4-Chlorophenol	0.86	3.6	0.9995	2.4	28	0.9828	1.2	3.5	0.9991	1.1	6.1	0.9989	0.90	6.1	0.9992
2-Naphthol	1.1	6.2	0.9985	2.0	21	0.9902	1.5	6.2	0.9981	1.6	13	0.9969	1.2	12	0.9975
1,3-Dihydroxybenzene	1.5	5.7	0.9980	2.9	37	0.9751	5.3	28	0.9596	1.8	7.8	0.9966	1.6	6.8	0.9972
3-Methylphenol	0.92	6.4	0.9992	1.0	5.6	0.9990	1.1	6.4	0.9990	1.0	6.9	0.9990	0.85	7.1	0.9992
Phenol	0.79	5.7	0.9993	1.3	15	0.9948	0.89	5.7	0.9992	0.88	7.3	0.9989	0.84	7.3	0.9990
2,6-Dimethylaniline	0.89	5.1	0.9992	1.1	6.0	0.9984	1.1	5.1	0.9990	1.2	6.1	0.9987	0.98	6.2	0.9990
4-Chloroaniline	0.73	3.0	0.9997	1.0	6.6	0.9990	1.0	3.0	0.9992	1.0	3.6	0.9992	0.72	3.6	0.9997
Aniline	1.1	4.1	0.9991	1.6	15	0.9953	1.4	4.4	0.9984	1.3	4.6	0.9986	1.1	4.5	0.9991
4-Methylaniline	1.0	3.1	0.9994	1.2	5.2	0.9990	1.5	6.6	0.9981	1.4	6.1	0.9984	1.0	3.3	0.9993
N-Ethylaniline	0.81	3.9	0.9993	0.82	7.0	0.9988	0.94	3.9	0.9992	0.91	7.4	0.9988	0.82	7.4	0.9989
Pyridine	3.4	13	0.9872	3.6	13	0.9847	4.2	16	0.9775	3.8	15	0.9806	3.5	13	0.9859
2,4,6-Trimethylpyridine	1.6	3.6	0.9982	2.6	19	0.98990	3.2	14	0.9886	2.0	6.1	0.9957	1.6	3.6	0.9982
N,*N*-Dimethylbenzylamine	1.1	3.2	0.9988	1.1	3.2	0.9987	1.3	4.7	0.9980	1.3	5.0	0.9978	1.9	8.3	0.9951
3-Aminophenol	3.8	19	0.9780	5.6	44	0.911	5.0	22	0.9596	4.0	21	0.9705	4.2	21	0.9722

errors with Eqs. (17), (19) and (21), always using the $^{s}_{w}$pH scale. The models were fitted with all available experimental data (10 pH levels, each one sampled at three acetonitrile concentrations), but also with simpler experimental designs. Errors in the prediction of retention factors were quantified as the mean (Eq. (23)) and maximal (Eq. (24)) relative deviations:

$$\mathrm{RE} = \frac{\sum |\hat{k}_i - k_i|}{n\, k_{i,\max}} \tag{23}$$

$$\mathrm{RE}_{\max} = \frac{|\hat{k}_i - k_i|_{\max}}{k_{i,\max}} \tag{24}$$

where $\hat{k}_i$ and are the predicted and experimental retention factors, and $k_{i,\max}$ the maximal experimental value for each solute. These error definitions were selected because the conventional RE is too design-dependent (i.e. given two compounds with protonation curves exhibiting similar scattering around the fitted equation, that one having more experimental points in the pH region where the unretained species dominates will yield oversized RE values). Since the magnitude of the error in Eqs. (23) and (24) is referred to the retained species, the proposed definitions will not be affected by this problem, avoiding thus the influence of the experimental design, and shape of the drop in the k versus pH curve, in the interpretation of the results.

Also, in order to avoid biased interpretations when models including a different number of parameters (i.e. the full and simplified models) are compared, errors are also given in Table 4 as adjusted correlation coefficients:

$$R_{\mathrm{adj}} = \sqrt{1 - \frac{(1 - R^2)(n-1)}{n-m}} \tag{25}$$

where R^2 is the conventional squared correlation coefficient, n the number of experiments, and m the number of model parameters.

As commented above, Eqs. (17), (19) and (21) were obtained from Eqs. (11), (13) and (15), respectively, by neglecting the contribution of the ionic species to retention. Since the number of parameters is smaller in the former equations, the treatment is simpler, giving rise nevertheless to satisfactory predictions. With regard to other reported equations used to describe the retention as a function of pH and mobile phase composition [8], Eqs. (11), (13), (17) and (19) include a significantly smaller number of parameters.

The drastic reduction in the number of parameters in the retention models suggests that minimal experimental designs can be appropriate. However, it must be considered that the design should provide adequate information to infer properly the retention behaviour for each solute of concern. Note that our purpose was the separation of a mixture. Thus, a common design should be applied, providing information of quality for all solutes simultaneously. As a consequence, minimal designs including five or six experiments, although apparently adequate considering the number of model

parameters, will not be sufficient if the purpose is developing an optimisation. Only when the involved solutes share similar protonation constants, minimal designs will be valid to infer properly the retention behaviour of each solute. Otherwise, designs apparently adequate attending to the quality of the fitting in the training set will lead to incorrect predictions for external experiments. To sum up, the inclusion of points sampling for each compound the transition between acidic and basic species at varying pH is mandatory. This implies strong limitations in terms of feasibility of minimising the experimental work, when the involved compounds exhibit extremely variable acid–base behaviour and a wide pH domain is covered, as is our case.

Tables 4 and 5 list the prediction errors for some of the simplified designs assayed in this work. The three columns labelled "30" correspond to the fitting of the whole set of available data (30 experiments), whereas the other columns show the results obtained with some simplified 12 point designs, selected according to geometrical and chemical considerations. Designs 4×3 and $6 + 4 + 2$ include points at the three assayed levels of organic modifier, whereas designs $10 + 2$ and $6 + 6$ only comprise experiments at the two extreme levels of organic modifier. The ${}^{w}_{w}$pH values were approximately 2, 5, 8 and 12 for four pH levels and 2, 4, 6, 8, 10 and 12 for six pH levels. According to the fitting quality estimators, the most satisfactory design was $10 + 2$, which with regard to the others, has the feature of sampling more comprehensively the pH at the lowest organic modifier level (i.e. that level giving rise to the highest retention), apparently sampling poorly the remaining factor space. In spite of this, this design was sufficient to establish properly the influence of the modifier on retention for the acidic and basic compounds. However, the model fitted for the amphiprotic 3-aminophenol using the $10 + 2$ design lead to poor results in the prediction of external data, since the double acid–base equilibrium required sampling more comprehensively the upper level, in order to establish properly the influence of the organic modifier.

Designs with less than 12 experiments were found risky for scanning the full domain of the polymeric column (some solutes were poorly described). Simplifications of this design sampling less comprehensively the lowest pH level led to predictions good for some solutes, but deficient for others. The other two possible $10 + 2$ designs built by sampling the 10 pH levels at 40 or 60% acetonitrile (results not shown) did not perform as well as the outlined design. In addition, pH increments of ca. one unit were found necessary to predict appropriately external data owing to the variability of the protonation constants. Finally, 12-points designs developed in such a way that the experiments in the training set are allocated within the experimental domain as distant each other as possible, yielded also poorer results.

It should be considered that the studied mixture is a very particular case, where $\log K$ values (see Table 1) are spread along the entire pH domain, which obliges to sample comprehensively pH values in the range 2–13. Such a situation is not so common in practice. Chromatographers often dispose literature values of $\log K$ that help to bracket the meaningful pH domain ($\log K$ values in an aqueous–organic solvent can be estimated from $\log K = \log K_0 + m\varphi$, adopting $m = 0.03$ for acidic and $m = -0.02$ for basic solutes, if φ is given as volume percentage). The pH domain to be scanned can be set extending ca. one pH unit the maximal and minimal $\log K$ values obtained considering the minimal and maximal concentration of organic solvent in the experimental design.

When the pH is measured in the mobile phase after mixing the aqueous buffer with acetonitrile, independently on the way the pH-meter was calibrated (with aqueous buffers, ${}^{s}_{w}$pH scale, or with buffers prepared in the same medium as the mobile phase, ${}^{s}_{s}$pH scale), good fittings were obtained for all compounds. With the common procedure of measuring the pH in the aqueous buffer solution, before adding the organic solvent to the mobile phase (${}^{w}_{w}$pH), the errors were somewhat larger, although not substantially (Table 6). Fig. 1 plots the predicted retention factors versus the experimental ones for the acidic and basic compounds and the whole set of experimental mobile phases. The results were considered satisfactory enough to be able to face the optimisation of selectivity with guarantee.

4.5. Predictions forcing common polarity parameters for all solutes

As commented, Eqs. (11), (13) and (15) can be also expressed in terms of column polarity parameters (i.e. $(\log k)_0$ and P^N_s), instead of q. In practice, both approaches lead to predictions of similar quality, but the use of column polarity terms presents some fitting problems owing to collinearity between parameters, which slows down the convergence and

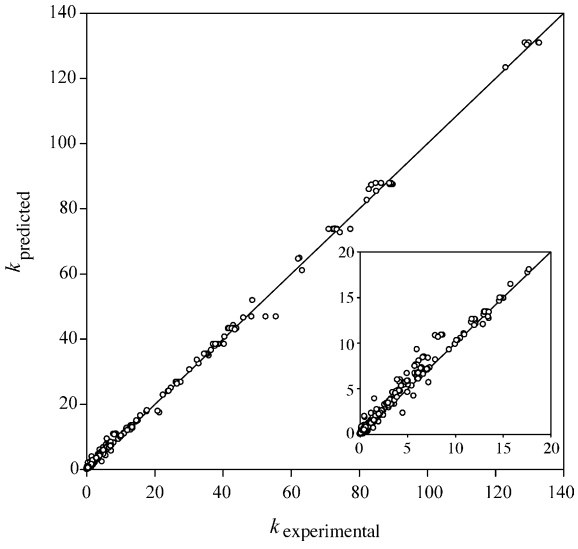

Fig. 1. Prediction of retention according to Eqs. (11) and (13) for the 23 acidic and basic compounds. A $10 + 2$ experimental design was used for the training set, but predictions were carried out for the 30 available mobile phases ($n = 690$, $r = 0.9992$, $F = 426000$).

Table 6
Prediction errors for Eqs. (11) and (15) and the three pH scales

Compound	pH scale								
	${}^{s}_{s}$pH			${}^{s}_{w}$pH			${}^{w}_{w}$pH		
	RE	RE_{max}	R_{adj}	RE	RE_{max}	R_{adj}	RE	RE_{max}	R_{adj}
2-Nitrobenzoic acid	1.0	3.1	0.9975	0.97	3.1	0.9975	0.94	2.9	0.9977
3-Nitrobenzoic acid	0.66	2.4	0.9992	0.64	2.4	0.9993	0.72	2.6	0.9990
4-Nitrobenzoic acid	0.56	2.2	0.9994	0.54	2.2	0.9994	0.63	2.4	0.9992
Naphthoic acid	0.55	3.8	0.9990	0.54	3.8	0.9990	0.64	4.3	0.9986
2,4-Dinitrophenol	0.55	3.3	0.9992	0.54	3.3	0.9992	0.59	3.8	0.9989
Benzoic acid	0.92	2.9	0.9988	0.92	2.9	0.9988	1.0	3.8	0.9985
2-Nitrophenol	1.5	16	0.9924	1.5	16	0.9924	1.6	16	0.9924
2,4-Dichlorophenol	0.90	4.0	0.9992	0.90	4.0	0.9992	1.1	3.9	0.9990
3,5-Dichlorophenol	0.45	1.6	0.9998	0.45	1.6	0.9998	0.76	4.3	0.9995
3-Bromophenol	0.59	2.8	0.9997	0.59	2.8	0.9997	0.74	3.8	0.9995
4-Chlorophenol	0.75	3.5	0.9996	0.75	3.5	0.9996	0.71	3.7	0.9996
2-Naphthol	1.1	5.2	0.9987	1.1	5.2	0.9987	1.2	6.1	0.9985
1,3-Dihydroxybenzene	1.4	4.6	0.9982	1.4	4.8	0.9981	1.8	9.1	0.9963
3-Methylphenol	0.90	6.5	0.9992	0.90	6.5	0.9992	1.1	7.0	0.9989
Phenol	0.70	5.3	0.9994	0.72	5.3	0.9994	1.0	6.3	0.9988
2,6-Dimethylaniline	0.83	5.1	0.9993	0.85	5.1	0.9993	0.77	4.8	0.9994
4-Chloroaniline	0.68	3.0	0.9997	0.69	3.0	0.9997	0.72	3.2	0.9997
Aniline	0.83	1.7	0.9996	0.84	1.8	0.9996	0.81	2.5	0.9996
4-Methylaniline	0.86	1.4	0.9996	0.90	1.4	0.9996	0.92	1.9	0.9995
N-Ethylaniline	0.75	3.4	0.9994	0.77	3.4	0.9994	0.69	3.3	0.9995
Pyridine	2.2	4.2	0.9961	2.3	4.1	0.9958	2.2	4.2	0.9961
2,4,6-Trimethylpyridine	1.2	2.4	0.9989	1.2	2.4	0.9989	1.2	2.4	0.9989
N,*N*-Dimethylbenzylamine	0.70	2.1	0.9995	0.74	2.1	0.9994	0.92	3.7	0.9990
3-Aminophenol	2.2	6.3	0.9938	2.2	6.3	0.9939	2.5	8.9	0.9903

introduces instabilities. As a consequence, after convergence, the parameters may present larger uncertainties, although the quality of predictions is equally excellent.

A plot of q versus p obtained from Eqs. (11) (retained acidic species) and (13) (retained basic species) is shown in Fig. 2 (3-aminophenol was not included since it was the only available amphiprotic solute). Attending to the acid–base nature of the compounds, two distinct trends, converging at low p-values, are evidenced. Also, within the acidic category, at least two parallel trends can be detected, corresponding to carboxylic acids and most phenolic compounds (1,3-dihydroxybenzene and 2-nitrophenol deviate from this behaviour). These results indicate that a rigorous treatment using equations including a unique column parameter set – $(\log k)_0$ and P_s^N – will require a previous classification of the compounds according to their acidity.

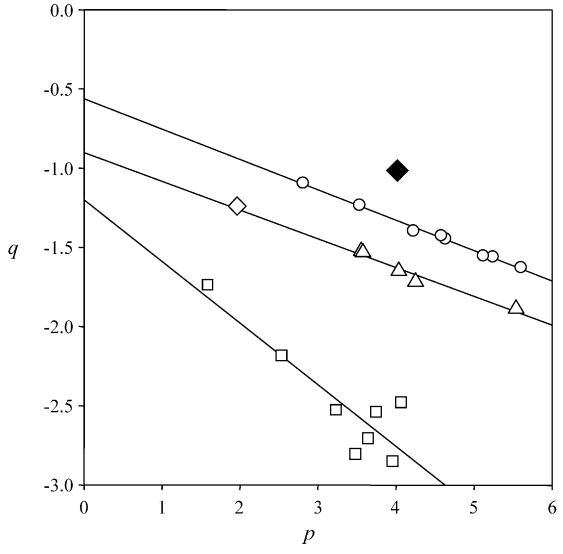

Fig. 2. Correlation of q vs. p, obtained from Eqs. (11) and (13), for several basic compounds (□), carboxylic acids (△), phenols (◯), 1,3-dihydroxybenzene (◇) and 2-nitrophenol (◆).

Based on these observations, an iterative regression with the aim of fitting common $(\log k)_0$ and P_s^N values for the compounds of a given category was applied. The procedure consists of two steps that were alternated. In the first one, individual regressions of Eq. (11) for acidic (or Eq. (13) for basic) solutes were fitted. The second step consisted of fitting q versus p values obtained in the previous step, this time including simultaneously all acidic (or basic) solutes. The purpose was to determine the column parameters for that kind of solute. As a result, new individual q-values were obtained as $q = (\log k)_0 - p\ P_s^N$. The first step was then repeated using the p-values from the individual regressions and the new calculated q-values as regression seeds, iterating this process up to convergence. This procedure is an extension of the approach previously developed for non-ionisable compounds [5].

As expected, once got convergence, predictions of retention factors were found somewhat poorer than those

obtained with the individual models for each solute, with typical $R_{adj} = 0.995$. According to these results, we decided to use exclusively individual fittings for next studies, since our objective was finding optimal separation conditions, and this requires the highest possible accuracy level in predictions.

4.6. Examination of the system resolving capability

We considered next a hypothetical mixture including the 23 acidic and basic compounds, for which the transition in retention happens at different pH values for each compound. The probe mixture was selected to get a representative set in terms of variability in acid–base properties. As a consequence, achievement of optimal separation conditions was particularly difficult. In this case, trial-and-error approaches were completely inadequate, and even interpretive approaches presented serious limitations.

The pH is indeed a worthy factor in the government of selectivity for weakly acidic or basic solutes, but if the involved mixture is too complex, the purity surfaces become considerably unrugged, which makes the practical application of this factor rather problematic. For all these reasons, the use of pH requires highly accurate predictions of retention, and the pH should be often corrected in the translation of optimal predicted conditions to the chromatograph. Another problem intrinsically related to the pH is that different solutes demand different pH regions for resolution, which makes finding shared regions of high resolution increasingly difficult with the number of components in the mixture.

The concept of peak purity gives rise to interesting tools to prospect the separation difficulties of any mixture. One of these tools are individual diagrams of purity for each solute (i.e. in the case of concern, purity surfaces as a function of organic modifier concentration and pH), whose maximal value is the so-called "limiting purity" of the considered solute. Therefore, the limiting purity is the highest purity value that a given solute can reach when the other accompanying solutes are considered interferents. When a mobile phase leads to a purity value equal to the limiting one, the capability of the chromatographic system to resolve that particular solute will have been fully exhausted (i.e. no further improvement will be obtained at any other experimental condition). On the contrary, if the limiting value is small, no mobile phase will allow the separation of that solute. Thus, elementary and limiting purities constitute worthy measurements for establishing the maximal separation capability of a chromatographic system.

Fig. 3 shows contour maps for each of the 23 examined compounds (15 acidic and 8 basic), where the shadowed regions depict mobile phases exploiting 90% or more of the resolving capability of the chromatographic system for isolating the considered compound. Even if a solute could not be fully resolved under any condition, the limiting plot will denote the best possible separation conditions for that chromatographic system. In this example, all limiting purities reach $p_L = 1.000$, which seems very promising. The chosen threshold percentage of elementary peak purity was therefore 0.9 for all solutes (i.e. 90% of the peak area is free of interference for each of them).

One of the most evident conclusions to be extracted from Fig. 3 is that, in spite of the high maximal elementary values, the resolution requirements for different solutes are incompatible. For instance, for the most acidic solutes (2-, 3- and 4-nitrobenzoic, naphthoic and benzoic acids), the capability of the chromatographic system can be only adequately exploited at very low pH. Meanwhile, the regions of good resolution for the most basic compounds (*N*,*N*-dimethylbenzylamine and 2,4,6-trimethylpyridine) are located mainly at pH > 7. These different requirements are translated in the practical impossibility of finding a single eluent able to separate mixtures containing extreme solutes in terms of acid–base behaviour: a hypothetical mixture including the 23 ionisable compounds cannot be resolved with a unique mobile phase, at least with the studied column and scanned experimental conditions. The plots also have the intrinsic ability of revealing the most problematic solutes, which in this case are the mentioned five acidic solutes, especially 3- and 4-nitrobenzoic acids.

4.7. A chromatographic objective function oriented to low-resolution situations

Unfortunately, in situations involving incompatible solutes, conventional resolution diagrams are scarcely informative, since both overall and worst elementary resolution will drop to zero if at least one compound is overlapped, even when all the others were baseline resolved. These diagrams will not rank adequately the true separation power of the different chromatographic conditions. However, common sense tells us that even conditions not being able to resolve all compounds can show evident differences in terms of resolving power. These situations can be still worthy for the chromatographer. Think, for instance, in a chromatogram where all compounds except two are baseline resolved. Conventional resolution criteria in such a case are blind, and just qualify the system as completely useless, independently of the number of resolved compounds. Hence, the development of a specific resolution assessment for low-resolution situations is of interest.

We propose the use of alternative plots, where the count of "well resolved" solutes as COF is used instead of the global purity or resolution value. A solute will be considered here as "well resolved" when the elementary purity is close enough to the limiting one, so that the system capability for resolving that compound has been almost fully exploited. These diagrams are drawn by counting how many solutes in a given mixture exceed a particular threshold of purity, established individually for each solute. The result of this procedure extended to the whole factor domain can be represented as a contour map. The threshold of "proper resolution"

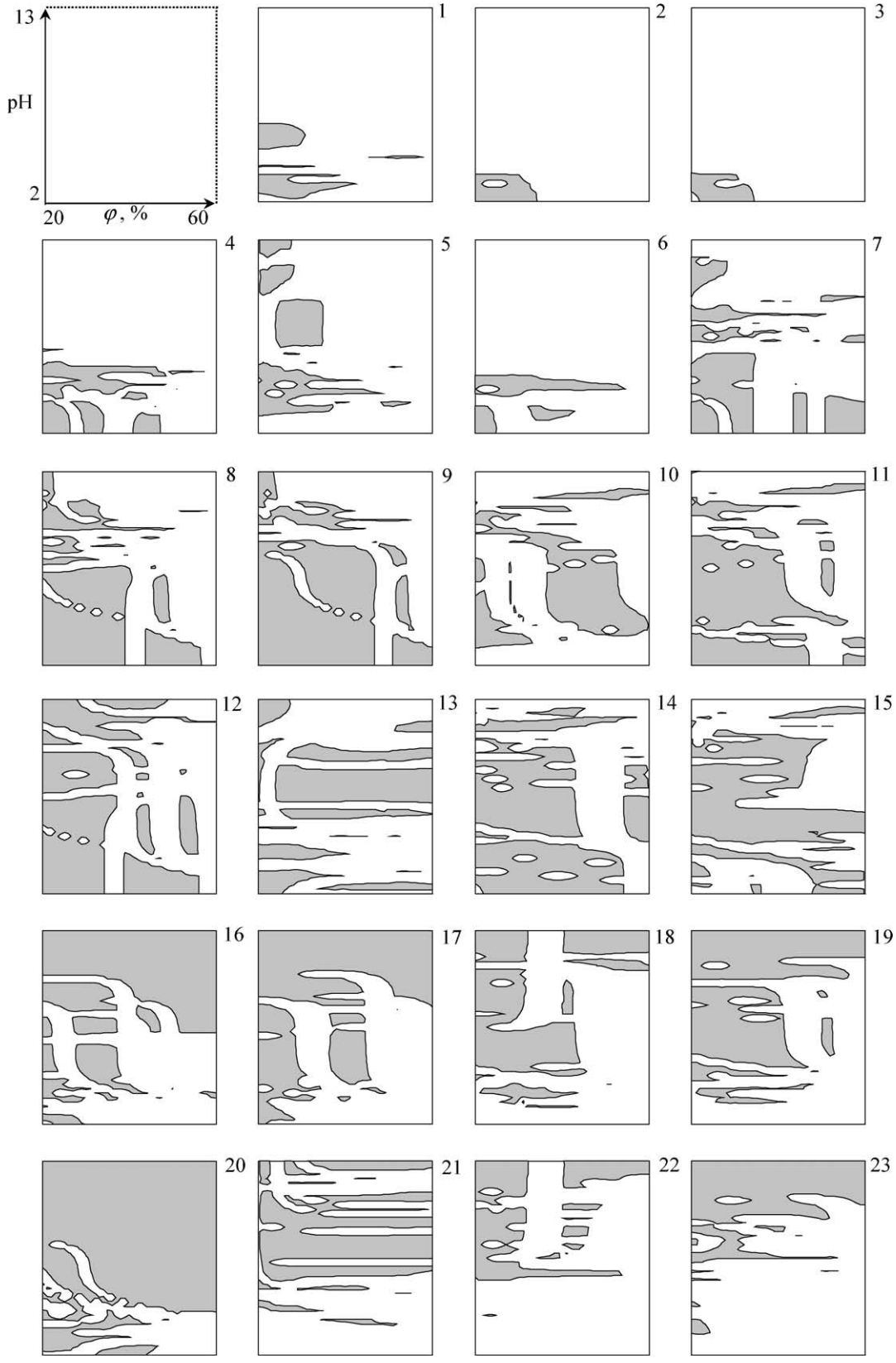

Fig. 3. Contour maps of elementary peak purities, considering a mixture involving the 23 probe compounds (15 acidic and 8 basic). The shadowed regions for each solute bracket the acetonitrile–water mixtures yielding 90% or more of the respective limiting resolution value. The numbers indicate the identity of compounds given in Table 1.

for each compound can be established by attending either to a predefined absolute value of purity (e.g. 0.9), or to a fraction of its maximal elementary purity, which was the adopted approach. The interest of this diagram is to find out the regions that allow the separation of a maximal number of solutes, in the presence of incompatible solutes.

Note that the "limiting purity-assisted peak count" criterion (which will be abbreviated from this point on as "limiting peak count") is focused on the successfully resolved compounds, in contrast to conventional resolution diagrams, that attend only to the unresolved or least resolved solutes (the resolved solutes do not affect these measurements). Consequently, classical assessments are largely influenced by the least resolved solutes (the worse the separation, the larger their influence), whereas in limiting peak count the influence is the opposite (the worse the resolution, the smaller their influence).

Fig. 4 shows the limiting peak count diagram for the mixture of 23 ionisable compounds, which was drawn by checking, for a given compound and mobile phase, if its purity exceeded a threshold that was arbitrarily set as 90% of the maximal purity. In this case, the compound was considered as "well resolved" and counted. In the diagram, a certain number of neighbouring phases with the same limiting peak count appear. Within the established regions, secondary criteria can be applied, such as the absolute values of purity and/or the analysis time. The latter was the selected secondary criterion in this work.

As mentioned, in our example, the limiting values for all solutes were the highest possible ($p = 1.000$), therefore, the drawn function gave straightforwardly the number of resolved peaks, with less than 10% of interference. The limiting peak count map indicates that only 18 out of the 23 solutes will be resolved within this system. In contrast, the classical assessments only indicated that the mixture of 23 compounds cannot be resolved under any condition.

Two significant regions can be observed in Fig. 4 (one at low pH and the other, a smaller one, at basic pH), where 18 solutes can be satisfactorily resolved. All mobile phases within the former region resolve the same 17 compounds (14 acidic, and the 3 basic *N*-ethylaniline, 2,6-dimethylaniline and 4-chloroaniline). The identity of the 18th resolved compound depended on the selected mobile phase: 1,3-dihydroxybenzene (compound 13) and *N*,*N*-dimethylbenzylamine (compound 23). Fig. 5a and b show chromatograms within the low pH region (20.0% acetonitrile at pH 3.0, and 21.5% acetonitrile at pH 3.5, respectively),

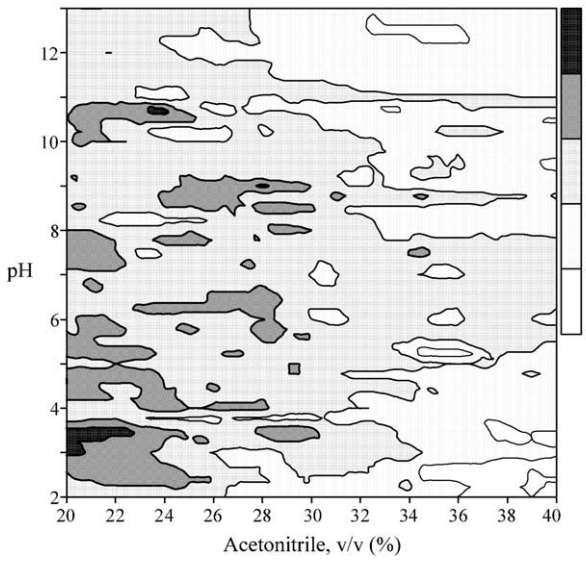

Fig. 4. Contour map of limiting peak count for the mixture of 23 probe compounds. The intensity of the shadowed regions is proportional to the number of resolved compounds. The best resolving mobile phases separate 18 compounds.

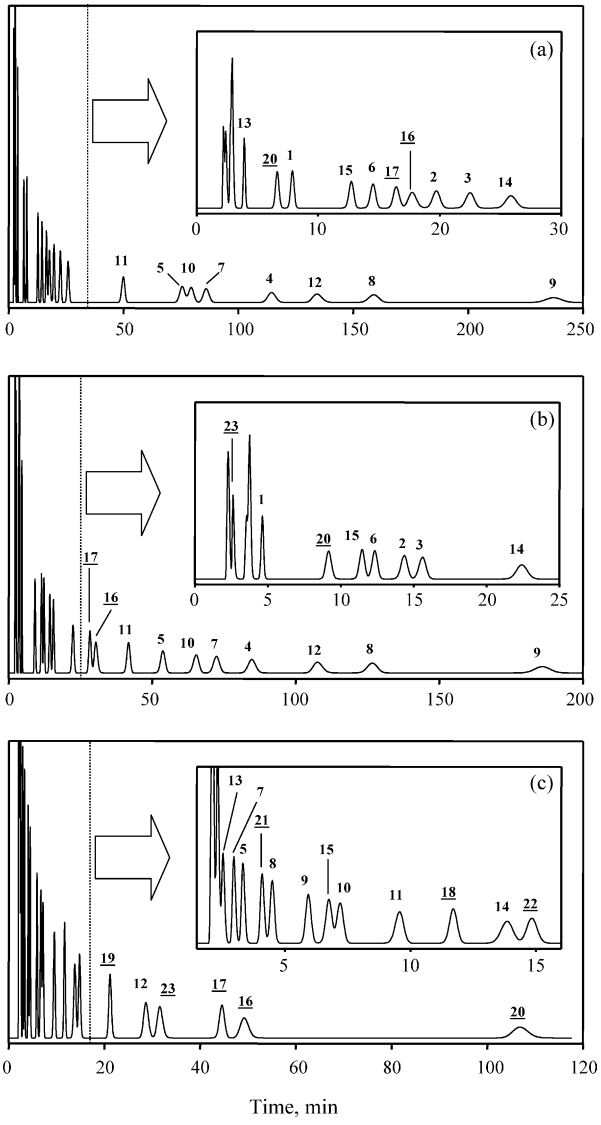

Fig. 5. Chromatograms simulated at a flow-rate of 1 ml min^{-1} for a mixture of the 23 acidic and basic compounds. In each case, 18 compounds were resolved according to the limiting peak count criterion: (a) 20.0% acetonitrile/pH 3.0; (b) 21.5% acetonitrile/pH 3.5; and (c) 23.5% acetonitrile/pH 10.75. Only the resolved compounds are labelled (see Table 1 for peak identities). Underlined numbers correspond to the basic compounds.

illustrating the change of identity of the 18th resolved compound.

On the other hand, in the basic region, 10 of the 15 acidic compounds (the 9 weakest and 2,4-dichlorophenol), plus the 8 basic, are resolved. Fig. 5c shows a chromatogram for 23.5% acetonitrile at pH 10.75, corresponding to this situation. As observed, the analysis times at 1 ml min^{-1} are too large. All these results indicate that, although the system is able to isolate each compound from all the others (see also Fig. 3), this requires particular conditions. Finding a shared condition for all compounds is largely unpractical.

4.8. Improvement of resolution considering more than one mobile phase

Situations like this (low resolution and/or long analysis time) can be tackled by searching two or more complementary chromatographic mobile phases (CMPs). This strategy selects two or more eluents (or, in general, chromatographic conditions), in such a way that each of them is devoted to get optimal separation of a given subset of compounds. The selection of CMPs is done in such a way that, when the results of all conditions are considered globally, all compounds should be maximally resolved, tending to the corresponding limiting purities. Details on the calculation of CMPs are given elsewhere [12]. Often, two CMPs are sufficient to get large improvements in resolution, depending on the variations of selectivity that is possible to generate within the chromatographic system.

In this example, the existence of two acid–base categories mutually exclusive in nature seems to lead us to conclude that the CMP approach is the ideal solution to this sort of problem. We found, however, that two CMPs were insufficient to reach good resolution, owing to the excessive incompatibilities amidst compounds, and a third CMP was required. The three optimal CMPs (see Fig. 6) were 33.0% acetonitrile at pH 3.0, 39.0% acetonitrile at pH 5.0 and 49.0% acetonitrile at pH 13.0. Note that the third CMP (Fig. 6c) is devoted to resolve basic compounds. The other two CMPs (Fig. 6a and b) resolve all the acidic compounds the best.

The incompatible behaviour of the compounds in the studied mixture can be further evidenced by examining the separation of simpler mixtures. Fig. 7 shows a double contour map overlaying the conventional overall purity and the maximal analysis time, in the case of separating mixtures including either the acidic (Fig. 7a) or the basic (Fig. 7b) compounds. The maximal limiting peak count isoline is also overlaid for the former compounds, as a shadowed region. In this case, the only possible region of acceptable resolution was located at pH ca. 3.5, which implies, however, unpractical analysis times. In contrast, basic compounds can be baseline resolved within 25 min at pH 6.75 (Figs. 7b and 8c). In addition, for basic compounds, the resolution surface is rather robust in the optimal conditions. Note also that above pH 10, the contour lines in Fig. 7b are remarkably parallel to the pH axis. This is a consequence of the log K values, that for the considered

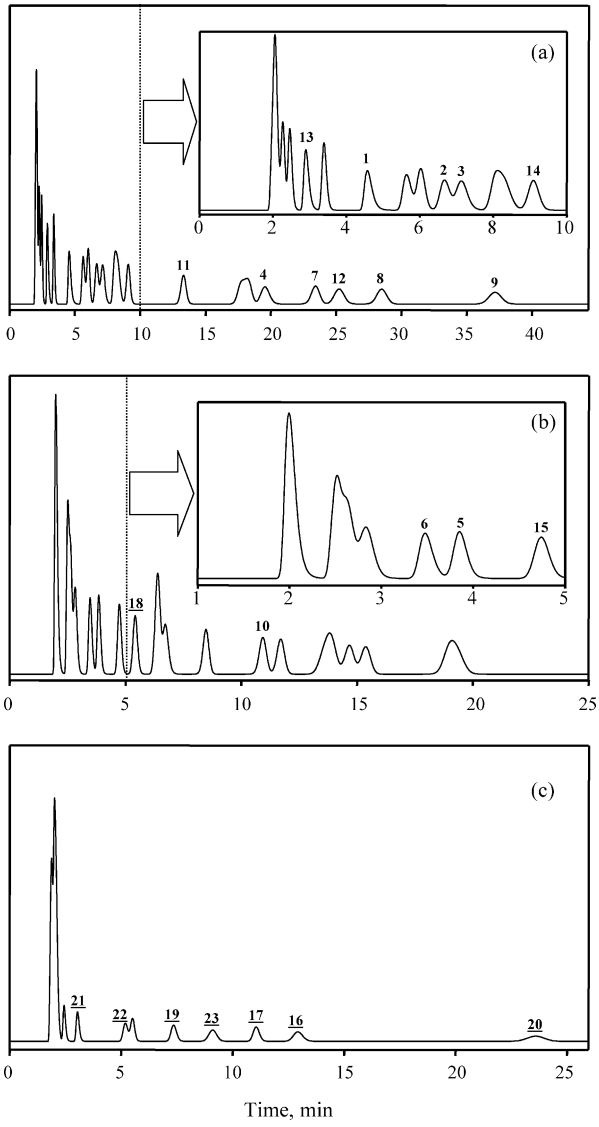

Fig. 6. Optimal chromatograms obtained according to the complementary mobile phase approach for a mixture of the 23 acidic and basic compounds: (a) 33.0% acetonitrile/pH 3.0; (b) 39.0% acetonitrile/pH 5.0; and (c) 49.0% acetonitrile/pH 13.0. See Fig. 5 for other details.

basic compounds were below ca. 8.5. At pH > 9.5, the peaks are unaffected by pH, so the resolution remains unchanged, which means that an experimental design focused on these compounds should only sample the pH region between 2 and 9.5. The lower pH region is, nevertheless, of low interest for resolution. To sum up, acidic compounds cannot be baseline resolved with a single mobile phase under any isocratic condition, whereas basic compounds present no problem.

The limiting peak count criterion offers a different picture of the separation of acidic compounds (Fig. 7a), pointing out a wide region where only 3- and 4-nitrobenzoic acids remain overlapped. Therefore, although conventional resolution assessments indicate that a mobile phase containing 33.5% acetonitrile at pH 4.25 is useless, indeed, 13 of the

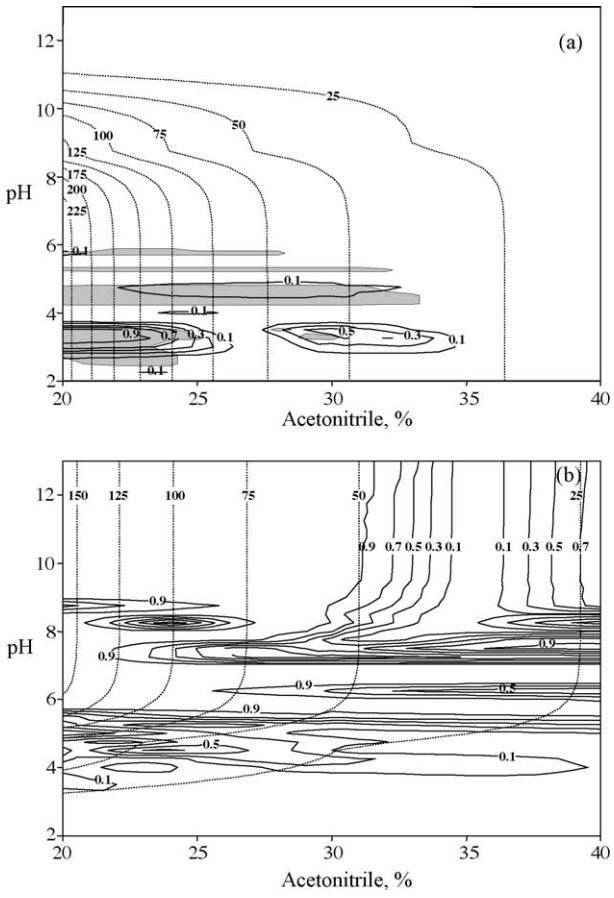

Fig. 7. Contour maps of overall peak purity (solid lines) and maximal analysis time in min (dotted lines) for a mixture involving: (a) the 15 acidic and (b) 8 basic compounds. Regions where 13 or more compounds are resolved at 90% free of interference are shadowed in (a).

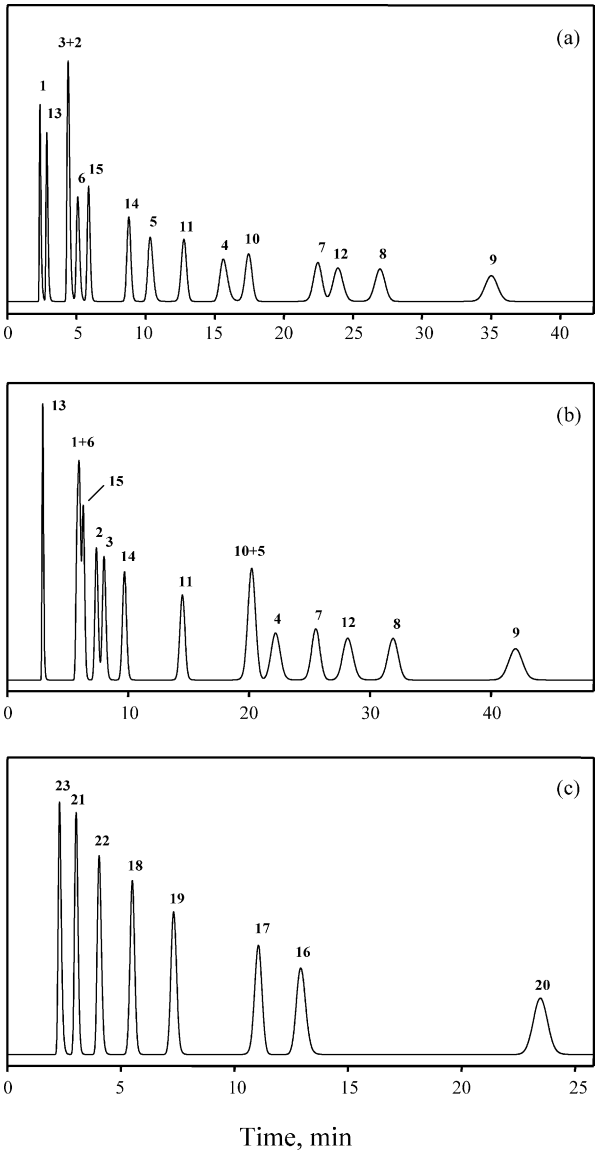

Fig. 8. Optimal chromatograms for: (a) a mixture involving the 15 acidic compounds (33.5% acetonitrile/pH 4.25); (b) 3- and 4-nitrobenzoic acids (compounds 2 and 3) interfered by the other 13 acidic compounds (32.0% acetonitrile/pH 2.0); and (c) the 8 basic compounds (40.0% acetonitrile/pH 6.75). The limiting peak count and minimal analysis time criteria were used in the optimisation. See Fig. 5 for other details.

15 acidic compounds can be well resolved (Fig. 8a): only the above mentioned solutes (numbered as 2 and 3) remain overlapped. Such a result demonstrates again the incapability of conventional assessments for situations where a few compounds are always overlapped.

These results suggest the convenience of checking whether a secondary phase could be successful in resolving the two problematic acidic solutes in the presence of the other 13. We found that a limiting peak count diagram similar to that shown in Fig. 4, but considering only 3- and 4-nitrobenzoic acids, showed a narrow plateau where both peaks are well resolved, whose faster analysis time is found at 32.0% acetonitrile and pH 2.0 (Fig. 8b).

As mentioned above, the CMP study concluded that the demands for resolution for acidic and basic compounds are incompatible: the resolution conditions for each solute category produce peak merging in the other (Fig. 6). This incompatibility suggests the need for resolving separately each category, in mixtures including the 23 compounds. In this study, the limiting peak count criterion was again selected, owing to the expected low resolution. It should be reminded that this problem is particularly difficult in terms of cluttering and peak crossing, which make the examination of the actual resolving power of the tested mobile phases hard.

Fig. 9a and b show the chromatograms in the optimal conditions recommended by the limiting peak count criterion, when the separation of the acidic compounds is optimised in the presence of the basic ones. The first chromatogram shows the optimal separation for the 15 acidic compounds, whereas the second one reduces the analysis times at expenses of decreasing the number of resolved compounds to 14. Fig. 9c is the optimal chromatogram for resolving the 8 basic compounds in the presence of the acidic ones. For this particular problem, thus, the CMP approach presents

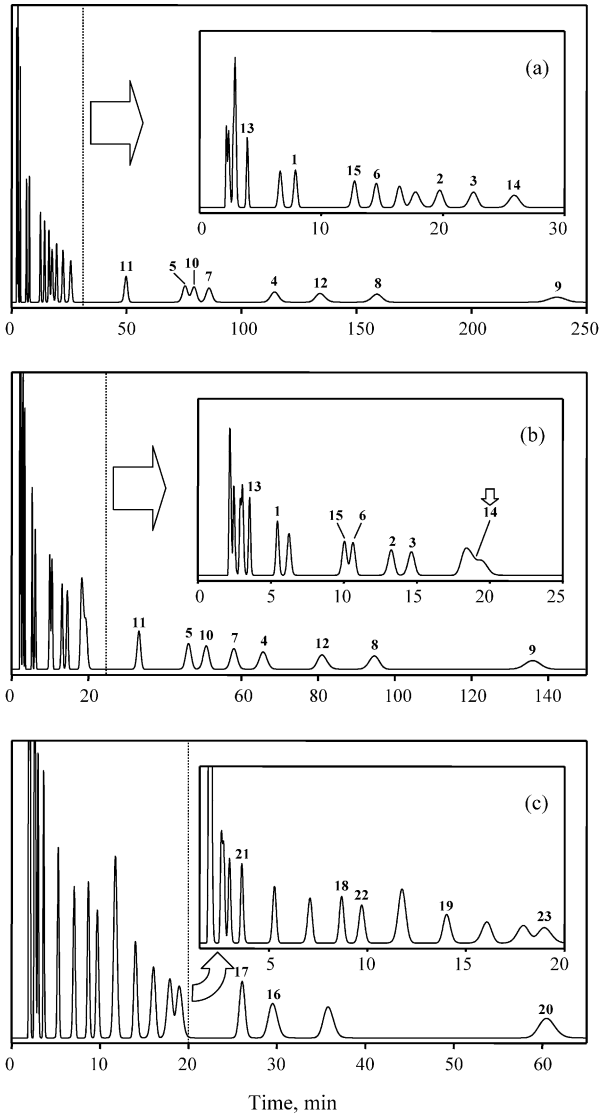

Fig. 9. Chromatograms for a mixture of the 23 acidic and basic compounds. Mobile phase compositions: (a) 20.2% acetonitrile/pH 3.0; (b) 23.5% acetonitrile/pH 3.25; and (c) 28.9% acetonitrile/pH 9.7. The optimisation was performed focused on the acidic compounds (a and b) and basic compounds (c). The unresolved acidic compound (overlapped with 4-chloroaniline and 2,6-dimethylaniline) is marked with an arrow in (b). See Fig. 5 for other details.

the advantage of yielding more practical analysis times (Fig. 6).

5. Conclusions

The benefits of pH should not be ignored when the separation of mixtures containing ionisable compounds is optimised. In spite of the difficulties associated to the modelling of retention in wide pH regions (i.e. $2 < pH < 13$), and the sudden drops of retention close to the logarithm of protonation constants in diverse pH regions, retention factors can be predicted with low errors. The mobile phase pH should be preferably measured after mixing the aqueous buffer and organic modifier (${}^{s}_{w}pH$), instead of measuring only the pH of the aqueous buffer (${}^{w}_{w}pH$). Because of the simplicity and higher accuracy of the derived predictions of retention, calibration with common aqueous buffers is recommended.

For acidic compounds, adequate retention (and hence resolution) requires acidic pH. This is incompatible with the requirements for basic compounds, for which neutral or basic pH is needed. The best separation conditions for most mixtures containing acidic compounds or relatively weak basic compounds can be achieved with conventional columns, working in a reduced pH range of 3–7. However, highly acidic compounds and most basic compounds require the extended pH ranges provided by special columns, which nowadays are being increasingly marketed.

The main problem associated to the use of pH in these separations is the difficulty in finding a common experimental design able to satisfy the requirements of different solutes, for achieving good predictions. The design should concentrate most of the effort in sampling the logarithmic variation of retention with pH, which requires measurements at pH intervals of ca. one unit. Designs covering narrow pH ranges can, however, be appropriate for simple mixtures. A 2×3 (modifier concentration $\times$ pH) design was, thus, found suitable in a reduced 0.5–1.0 pH range for resolving a complex mixture of ionisable compounds [20]. However, such a narrow design takes scarce benefit of the power of pH, even more when nowadays columns able to operate along 10 pH units are readily available. A selection of a pH range of only 0.5–1.0 units means that some compounds will remain protonated/unprotonated (i.e. they will be insensitive to pH changes), whereas only some of the others will experience the strong effects in eluent strength intrinsic to this factor. In addition, most of the separating power of the column will remain unexplored. However, the examination of the full pH domain presents some new challenging inconveniences: more complex retention models are needed, and the number of degrees of freedom in the experimental designs should be increased considerably, so that each solute is well described.

A resolution measurement, the “limiting purity-assisted peak count” or just “limiting peak count”, is here proposed to compensate the insufficiency of conventional assessments of being oriented to qualify positively only those situations where all compounds in the analyzed mixture are satisfactorily resolved. When at least one peak pair is overlapped, the conventional assessments neglect the acceptable resolution of the remaining compounds. In other words, when full resolution cannot be obtained under any chromatographic condition, differences in resolving power remain hidden. It can be said that these assessments are “negatively oriented”. The orientation of limiting peak count is the opposite: it is “positively oriented”. It accounts what works in the considered chromatogram, and not what fails (i.e. it weights positively the level of success in the resolution of peaks in the chromatogram). Therefore, it allows the exploitation of the

capability of the system in difficult situations. Note that when full resolution is possible, limiting peak count addresses the chromatographer to the same mobile phases as conventional assessments.

The limiting peak count concept is supported by the measurement of peak purity and complements the vision offered by limiting purities. The latter led to the development of the concept of complementary separations [14], and to the establishment of the maximal possible purity offered by the system, which is also of interest to set the maximal number of nodes in a gradient optimisation [21], as well as the maximal useful number of CMPs [14]. Finally, there are many situations where full resolution is needed for only some compounds, although the interference of the remaining should be taken into account. For these cases, both the peak purity and limiting peak count are useful as objective functions.

This work is based on isocratic elution, and consequently, long analysis times are obtained at elution conditions where the neutral species dominate. Analysis time can be shortened by applying a pH gradient that will sweep off the column the retained species [22,23]. The models and optimisation strategy described in this work can be easily extended to pH gradients.

Acknowledgements

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