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SYSTEMATIC PROCEDURE FOR THE DETERMINATION OF THE NATURE OF THE SOLUTES PRIOR TO THE SELECTION OF THE MOBILE PHASE PARAMETERS FOR OPTIMIZATION OF REVERSED-PHASE ION-PAIR CHROMATOGRAPHIC SEPARATIONS

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SUMMARY

Separation selectivity of ionized solutes in reversed-phase ion-pair chromatography can be varied by manipulating a number of mobile phase variables. From a study of computer-simulated mixtures of differently charged solutes it became obvious that the selection of the parameter space for systematic solvent optimization is constrained principally by the nature of the charged species present in the mixture. For most sample mixtures there are preferred combinations of the mobile phase variables, leading to a significant reduction of the optimization search area. A systematic strategy is shown here for the determination of the charge type and the relative retention (hydrophobicity) of the components in samples for which this information is not known. The first part of the strategy identifies the weak acids and bases according to their retention behavior in two gradient separations at pH 2.5 and 7.5, respectively. The second part determines the presence of strong acids and bases by the same two gradients but "pulsed" with a negatively and a positively charged ion-pairing reagent, respectively. Solutes are classified according to their characteristic retention shifts using a sequential-elimination scheme. Solutes without retention shifts are classified as non-charged solutes.

INTRODUCTION

The use of computer-aided procedures for the optimization of separation selectivity in reversed-phase high-performance liquid chromatography (HPLC) has been extensively studied during the last few years[1–5]. The efforts of many research groups

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resulted in several commercial software packages[6–10]. However, the value and success of all these optimization strategies (including the trial-and-error aproaches) critically depends on the number and range of the mobile phase variables, which are selected to vary the retention and selectivity of the separation. The combination of these parameters and their limiting values defines the parameter space, in which the optimum separation conditions should be located. In all presently known HPLC optimization procedures[1–10], a preselected vector space is used. If the parameter ranges are too broad, many experiments may be required to find the optimum, while a too narrow parameter space often leads to a local (usually unsatisfactory) optimum.

The selection of an appropriate parameter space for sample mixtures containing non-charged solutes is relatively easy in reversed-phase HPLC, and involves almost exclusively the manipulation of either the type and/or the concentration of the organic modifier(s) in the mobile phase. The retention movement of the non-charged components is largely predictable with a decrease of solute retention when the organic modifier concentration is increased in the eluent. Simple isocratic or gradient scouting experiments can be used to determine the initial eluent compositions before starting the binary, ternary or quaternary solvent optimization procedure[6–12].

However, a wide range of typical samples such as ionic surfactants, drugs, reaction mixtures, environmental and biological samples often contain both non-charged and ionic or ionizable compounds. The separation of such sample mixtures usually needs the variation of a number of other mobile phase parameters (eluent pH, type and concentration of buffer, ionic strength, charge type, hydrophobicity and concentration of ion-pairing reagent). The increasing number (and/or range) of the mobile phase variables to be optimized necessitates the completion of many more chromatographic experiments and needs more complex instrumentation. Furthermore, most available optimization methods (except Simplex) permit the simultaneous optimization of only two or three parameters. Therefore, it is essential to reduce the parameter space as much as possible, by including (and varying) only those parameters which have a significant effect on the selectivity of the separation.

A number of recent publications have demonstrated the successful separation of sample mixtures containing solutes of different charge types using a mixture-design statistical approach along with predictive regression methods[13–15]. Generally, the three most important eluent parameters considered in these selectivity optimizations are the organic modifier, pairing ion concentrations and the eluent pH. The experimental designs described in refs. 13–15, depending on their philosophy, select different subspaces of the parameter space, as shown in Fig. 1 using a three-dimensional representation. However, the parameter space selected by these methods is correct for certain mixtures[16] and none of them is generally applicable.

Based on a study of the separations of many computer simulated sample mixtures of differently charged solutes it will be shown here that the optimization parameter space can be selected rationally if the nature (charge type and the relative hydrophobicity) of the sample components is known. A systematic and rapid procedure has been developed to obtain this information for solute mixtures, where it is not available *a priori*. The method is based on four specifically designed organic modifier gradients according to the unique retention shifts of charged solutes. The felicity of the scanning procedure is demonstrated by determining the solute types in complex synthetic solute mixtures.

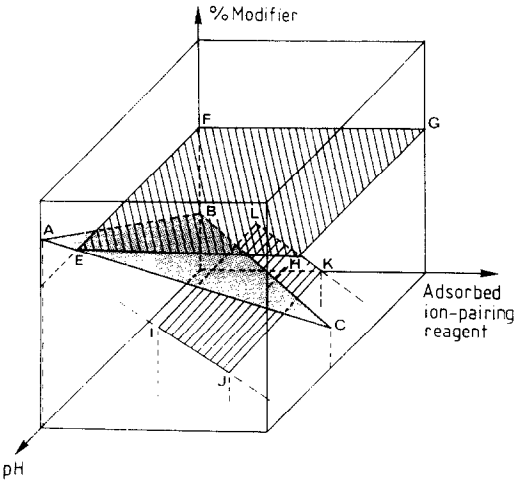

Fig. 1. Comparison of the parameter spaces selected for the optimization of reversed-phase ion-pair chromatographic separations according to the different mixture designs by (ABC) Goldberg *et al.*[13]; (ILKJ) Coenegracht *et al.*[14]; and (EFGH) Billiet *et al.*[15]. The three optimization parameters are eluent pH, organic modifier and ion-pairing reagent concentrations.

EXPERIMENTAL

Instrumental

Two HPLC systems were used in this work. The first consisted of two M6000A pumps, a M660 gradient controller, a M440 UV detector (all from Waters Chromatography Division, Milford, MA, U.S.A.), and a Rheodyne 7125 injector with a 20-μl loop (Rheodyne, Cotati, CA, U.S.A.). The second system was a HP 1090 liquid chromatograph with an autoinjector and a HP 1040A linear photodiode array detector. The latter was connected to a HP-85 desktop computer, equipped with a HP 7074A graphics plotter and a HP 9121 dual flexible disk drive (all from Hewlett-Packard, Waldbronn, F.R.G.).

The computer simulation programs for building the library of synthetic solute mixtures were developed in PRO/BASIC on a Waters 840 data management system (Digital Equipment Corp., Maynard, MA, U.S.A.).

Two different reversed-phase columns were used. The first was a 200 × 4.6 mm I.D. column, slurry packed with 5-μm ODS-Hypersil (Shandon Southern Products, Runcorn, U.K.). The second was a commercial Nova-Pak C_{18} (3 μm, 150 × 4.6 mm I.D.) column, purchased from Waters. A flow-rate of 2 ml/min was used throughout this work. Column temperature was maintained at 35°C for the Hewlett-Packard system (Nova-Pak C_{18}), and at room temperature for the Waters system (ODS-Hypersil).

Chemicals

Methanol was purchased from Rathburn (Walkerburn, U.K.). Distilled, deionized water was prepared with a Milli-Q water purification system (Millipore, Molsheim, France). Sodium bromide, disodium hydrogenphosphate and citric acid (J. T. Baker, Deventer, The Netherlands); tetrabutylammonium bromide and anhydrous

sodium hexane- and octanesulfonate (Janssen Chimica, Beerse, Belgium); "Gold Label" quality triethylamine (TEA) and phosphoric acid (85%, w/w) (Merck, Darmstadt, F.R.G.) were used without further purification. The solutes were of the highest quality available. Individual sample solutions were prepared in methanol–water (50:50, v/v) and combined in appropriate proportions to form synthetic mixtures.

Mobile phases and gradient sequence

For the ODS-Hypersil column buffers were prepared from citric acid and disodium hydrogenphosphate, balanced with sodium bromide to maintain a constant 50 m*M* concentration of counterions in the mobile phase. The final eluents for this column also contained 10 m*M* triethylamine phosphate. For the Nova-Pak C_{18} column 15 m*M* triethylamine phosphate was used. Buffers of pH 2.5 and pH 7.5 were made by directly titrating the organic base with phosphoric acid (10%, w/w).

Above a methanol concentration of 20% (v/v) an appropriate correction was made to the apparent pH[17]. The buffer concentrations in the aqueous eluents and in the methanol rich eluents were identical. The solubility of the citrate–phosphate buffer (containing also sodium bromide) allowed a maximum of 70% (v/v) methanol concentration. A higher methanol concentration limit of 90% (v/v) could be used with the TEA–phosphate buffer. Mixing of different proportions of the aqueous and aqueous-methanolic solutions gave acceptable (±5%) errors in the expected pH values.

Solutions (0.5 *M*) of the ion-pairing reagents were prepared in methanol–water (50:50) for the "pulse" injection experiments. A volume of 20 μl of the selected reagent were injected 45 s prior to the injection of the sample mixture (the solvent gradient was always started at the injection of the sample). The gradient run consisted of four sequences: (i) a linear gradient from 0 to high methanol concentrations at a given pH (2.5 or 7.5) in 15 min, (ii) isocratic elution at high methanol concentration for 5 min, (iii) reverse linear gradient from high methanol to the aqueous buffer in 5 min, and (iv) reequilibration of the column with the aqueous buffer for 2 min. This procedure gave practically no "ghosting" effects or irreversible pairing ion adsorption.

COMPUTER SIMULATION OF SYNTHETIC MIXTURES

In order to develop a rational approach to the optimization of the separation of mixtures which contain differently charged solutes an extensive library of the possible separation problems has been built by computer simulation. The problems are represented by the retention *vs.* pH behavior of different solute types in each sample mixture. Retention profiles of acids and bases were obtained using the equations derived by Horváth *et al.*[18]. The following pK_a values were used: 3.5–5.0 for weak acids (WA) and bases (WB), <1.5 for strong acids (SA), and >9.0 for strong bases (SB). For hydrophilic compounds the capacity factors (k') of the ionized and non-ionized forms of the same solute were 0.3–0.5 and 4.3–4.8, respectively. For the hydrophobic ones the ranges 8.5–9.0 and 13.5–14.0 were used. The k' values of the hydrophilic and hydrophobic non-charged (N) solutes were assumed to be 0.5 and 13.5, respectively.

Mixtures containing 1:1, 1:2 and 2:2 permutations of WA, WB, SA and SB of

different hydrophobicity were formed to yield synthetic mixtures of increasing complexity. Non-charged compounds either hydrophilic, hydrophobic or both were then systematically included in each of these mixtures to increase the complexity of the sample. To account for very closely related compounds in the mixtures, each compound type was represented twice, with marginally (5–10%) different k' values. Therefore, the simplest mixture contained two components of a single compound type (*e.g.* hydrophilic strong acid), while the most complex mixture contained six different solutes types, a total of twelve components. Altogether 648 different types of mixtures were simulated and evaluated.

RESULTS AND DISCUSSION

Rational selection of the mobile phase optimization parameters

A study of the separation problems represented by a large number of computer-simulated synthetic mixtures of increasing complexity, revealed that the selection of the optimization parameter space in reversed-phase ion-pair HPLC can be rationalized, and it is constrained by the nature of the charged species in the sample mixture.

The primary variables for the optimization of ion-pair chromatographic separations considered in this study are the type and concentration of the organic modifier, the eluent pH and the charge type and concentration of the ion-pairing reagent. In this discussion we will show that the selection of these retention controlling parameters and their combinations depend, primarily, on the nature (charge-type and relative hydrophobicity) of the solutes in the sample mixture.

Solutes can be classified according to their charge type within the 2.5–7.5 pH range. The constraint on the pH window is dictated by the chemistry of the currently available silica-based reversed-phase packing materials. In Fig. 2 the idealized reversed-phase retention behavior of different solute types is shown as a function of the eluent pH. Strong acids (SA) and bases (SB) are solutes which are fully ionized, whereas weak acids (WA) and bases (WB) are compounds which change their ionic state (and their retention) within this pH range. Compounds which are non-ionized within this pH gate are referred to as neutral (N) compounds. The terms "hydrophilic" and "hydrophobic" are relative terms referring to the order of elution of a solute in a given sample mixture. That is, the same compound can be classified as hydrophobic in one solute mixture, but as hydrophilic in another.

When examining a large number of simulated separation problems, we realized that a procedural strategy is needed to solve these problems rationally. First, by inspecting the problem, one must decide whether the retention gap between neighbouring solute peaks is to be decreased or increased. Second, one must select the optimization parameters which would affect the retention gap and provide the best overall selectivity (no more than three parameters are to be used at a time). Third, one must try to avoid very early and/or late elution of any of the components (all components are assumed to be of interest). Fourth, one must decide whether a reduced portion of the parameter space (which still contains the global optimum with respect to the selected mobile phase variables) could be used.

Several representative examples will be discussed below to demonstrate the advantages of this strategy, which selects the optimization parameter space according to the nature of the solutes in the sample mixture. The k' *vs.* pH plots are used to

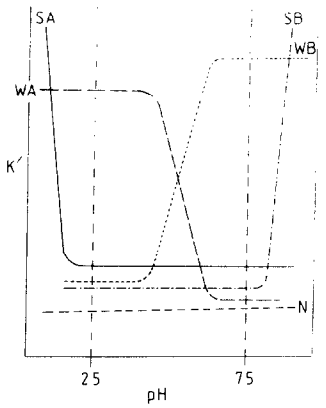

Fig. 2. Capacity factor (k') *vs.* eluent pH profiles of strong (SA) and weak (WA) acids, strong (SB) and weak (WB) bases and non-charged solutes (N) in an ideal reversed-phase chromatographic system.

illustrate the problem, and a three-dimensional representation of the selected combination of the optimization variables is used to visualize the resulting vector space.

The simple mixture shown in Fig. 3a consists of hydrophilic strong bases and hydrophobic neutrals. One of the important features of this sample is that there are no weak acids and bases present. Therefore, the eluent pH can be fixed at any practical value (*e.g.* low pH for basic compounds may give better peak symmetry). In order to close the retention gap between the early and late eluting solutes, a negatively charged ion-pairing reagent must be used to increase the retention of the lightly retained strong bases. (Alternatively, one could try another organic solvent, assuming that the eluent contains any at all, but experience shows that this option is more profitable with hydrophilic–hydrophobic non-charged solute combinations.) Once the bases have been moved away from the solvent front, the retention gap can be further decreased by increasing the concentration of the organic modifier. These considerations result in a simple line vector space (Fig. 3b). The search for an optimum composition can be simply performed by mixing the two low-pH eluents (lower and higher organic modifier concentrations, without and with an ion-pairing reagent, respectively) in different ratios.

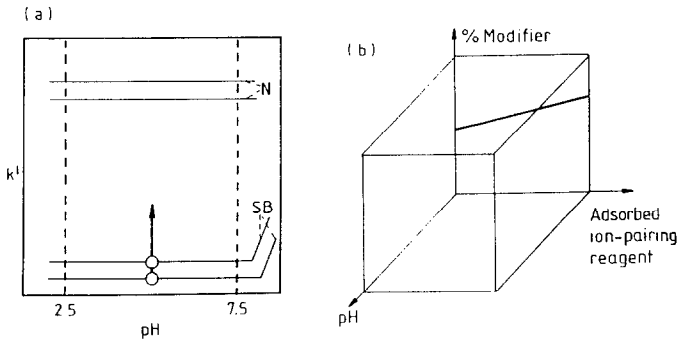

Fig. 3. Example 1. (a) k' *vs.* pH behavior of a simulated solute mixture, containing strong bases (SB) and non-charged (N) solutes; (b) the selected optimization parameter space (see text for discussion).

A more complex mixture is shown in Fig. 4a. This sample contains hydrophobic strong acids, hydrophilic weak acids and hydrophylic non-charged solutes. Again, the retention gap between the early and late eluting solutes should be closed. However, the organic modifier concentration cannot be increased, because this would shift the hydrophilic neutral solute to the solvent front. Therefore, the organic modifier concentration must be fixed at a level, which assures sufficient retention for the non-charged solutes. The retention gap can only be closed by decreasing the retention of the hydrophobic strong acid, with a similarly (negatively) charged ion-pairing reagent. Though in general the pH is varied to achieve separation of the weak acids, the high-pH region, where they are negatively charged, cannot be used in this case, because repulsion by the ion-pairing reagent will push these solutes to the solvent front. Again a fairly reduced optimization parameter space results (Fig. 4b).

It must be pointed out that none of the mixture designs shown in Fig. 1 is able to select these subspaces which, according to the reasoning given above, contain the global optimum.

Obviously, for most sample mixtures there are clear preferences as to which combinations of the mobile phase variables should be used, leading to a significant reduction of the optimization search area (see Figs. 3b and 4b). These preferences are directly related to the presence or absence of certain sample types, and can be described as rules. For example, the absence of weak acids and bases will always eliminate the need of pH variation. In this simplest form this rule reads: "if there is no WA and WB present then pH is fixed". A preliminary set of such rules has been derived in this study, as a part of a knowledge base of an expert system for ion-pair HPLC. Work is under way to develop a prototype expert system which can select the optimization parameter space by considering the solute types present.

In conclusion, the knowledge of the nature (not the exact identity) of the components in the mixture is decisive in the rational selection of the optimization parameter space. In some cases, this information is known *a priori*, but in most cases (reaction mixtures, new products, mixtures of metabolites) one might have only limit-

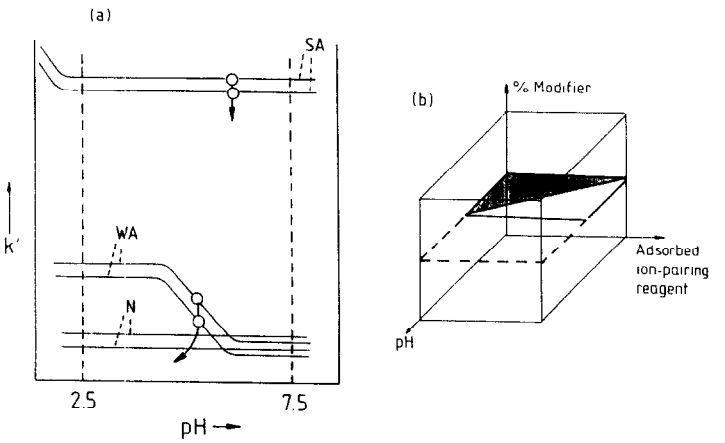

Fig. 4. Example 2. (a) k' *vs.* pH behavior of a simulated solute mixture, containing weak (WA) and strong (SA) acids and non-charged (N) solutes; (b) the suggested optimization parameter space (see text for discussion).

ed or no information about the solute mixture. Therefore, an efficient and easy to use method is needed to obtain rapid information about the nature of the components in the sample.

Determination of the nature of the components

In order to aid the rational selection of the optimization parameters in reversed-phase HPLC, we developed a systematic and rapid scouting procedure to determine the nature (hydrophobic or hydrophilic, non-charged or charged, weak or strong acid or base) (though not the exact identity) of the solutes in the mixture. The strategy is based on the unique retention shifts of the differently charged solutes (see Fig. 5), which occur when a positively or a negatively charged ion-pairing reagent is added to the eluent at a given pH. The retention behaviors of the different solute types in pH 2.5 and pH 7.5 eluents are shown on the two middle bars in Fig. 5. The bars on the left and the right sides show the retention shifts of the same solutes when negatively and positively charged ion-pairing reagents are used at pH 2.5 and pH 7.5, respectively. For example, the retention of a weak acid will be lower at high pH where it is ionized, but it will increase if a positively charged ion-pairing reagent is added to this high-pH eluent. Obviously, if retention data of a given solute are subsequently measured in all the four eluents, its charge type can be determined by matching the retention shifts with one of the patterns.

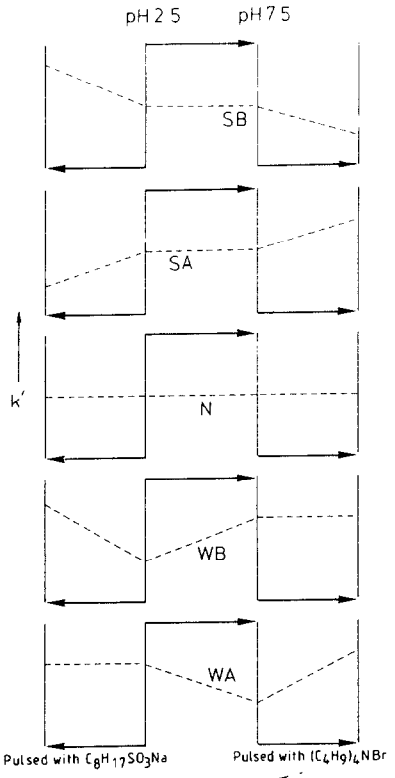

Fig. 5. Idealized retention shift patterns of different solute types, in the solute-type determination strategy proposed here.

The retention data can be collected either in isocratic or gradient mode, with respect to the organic modifier. The advantage of the gradient mode is that the column can be reequilibrated rapidly and *a priori* knowledge of the sample mixture is not required. Thus, four separate 0–90% organic modifier (methanol) gradient runs are used. Two gradients have their pH fixed at 2.5 and 7.5, respectively. They are expected to show retention shifts of the chromatographic peaks only if weak acids and/or bases are present. The two other gradients which involve a "pulse" injection (a technique described by Berry and Shansky[19] with a negatively and a positively charged ion-pairing reagent, respectively, will show retention shifts when strong acids and/or bases are present. The retention of the non-charged solutes is unaffected in all the four gradients.

The solutes are classified sequentially by an eliminative algorithm shown by its flowchart in Fig. 6. The strategy can be divided into two main parts: (i) differentiation between strong and weak (acid/base) solutes; (ii) differentiation between acids and bases and finding the non-charged solutes by simply eliminating the other possible solute types.

The fourth gradient may seem somewhat superfluous, since all solute types are already assigned. However, at low pH both SB and WB are positively charged, their retention increases with a negatively charged pairing ion, which prevents the unambiguous discrimination between these solute types. Furthermore, in complex mixtures containing very hydrophilic ionic components (eluting close to the solvent front) the repulsion effect of the "pulsed" pairing ion cannot be observed. In such cases the "pulse" with an oppositely charged pairing ion can produce positive retention shifts. The fourth gradient seems to eliminate this problem and also enhance the chances of discriminating all solutes from the non-charged ones.

In order to realize the benefits of this procedure, the majority of the solutes (more exactly their shifts) must be recognized in the sequential chromatographic runs. One can inject standards (if available) separately for peak identification, but this can be time and solvent consuming. Although this method was used in this study to validate the scanning strategy, it should be considered as a last resort, especially for the gradient method.

Peak tracking procedures based on the solute UV spectra can only be used when the spectra do not change with the eluent composition. An extensive use of mathematical techniques allowed for the ready identification of the components in a mixture of local anaesthetics, when an "isocratic" version of the scanning procedure was used[20].

However, the UV spectra of weak acids and bases can change significantly with the eluent pH. Therefore gradients at pH 2.5 and 7.5 can give different chromatograms at a constant detection wavelength. An example is shown in Fig. 7 for a mixture of a weak base (N-methylaniline) and a weak acid (phthalic acid). Both the magnitude of the UV signal and the UV spectra change dramatically with the ionization of the solutes. Therefore, a simple comparison of retention times and peak areas (and/or spectra) will not reveal peak identity in the two chromatograms.

On the other hand, one must realize that not all solutes have to be identified in order to reduce the range and/or number of the mobile phase optimization variables (*e.g.* the presence of only one hydrophilic non-charged solute may be enough to limit the organic modifier concentration of the mobile phase). The nature of the first and

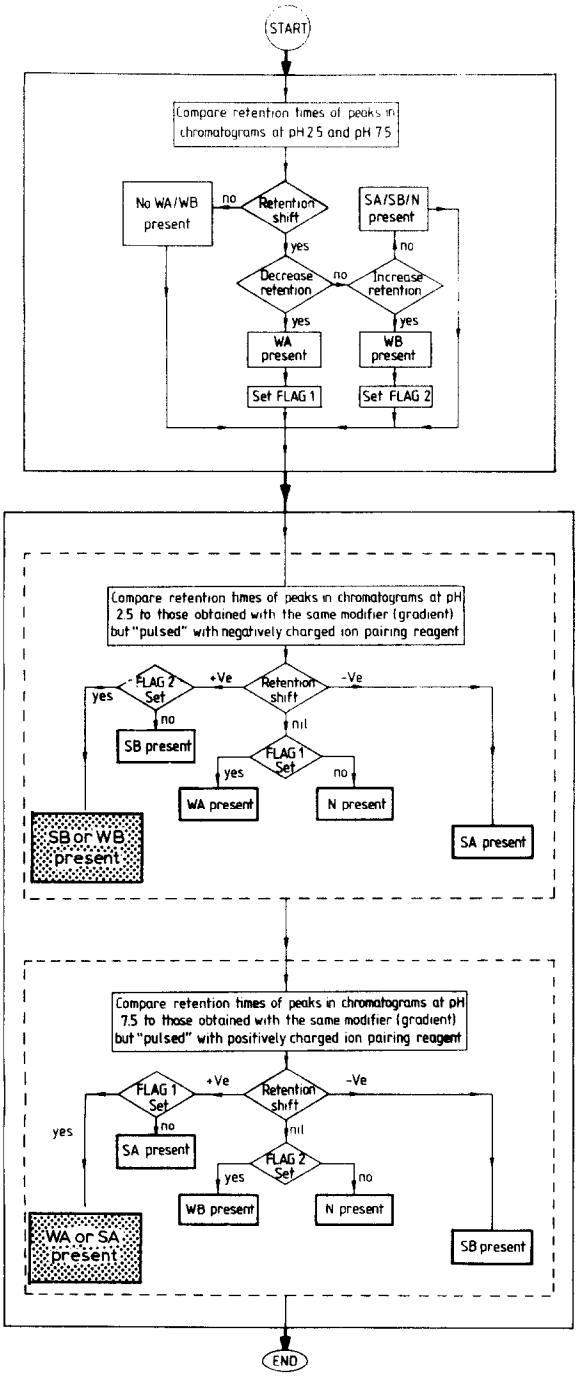

Fig. 6. Flow chart of the solute-type determination strategy.

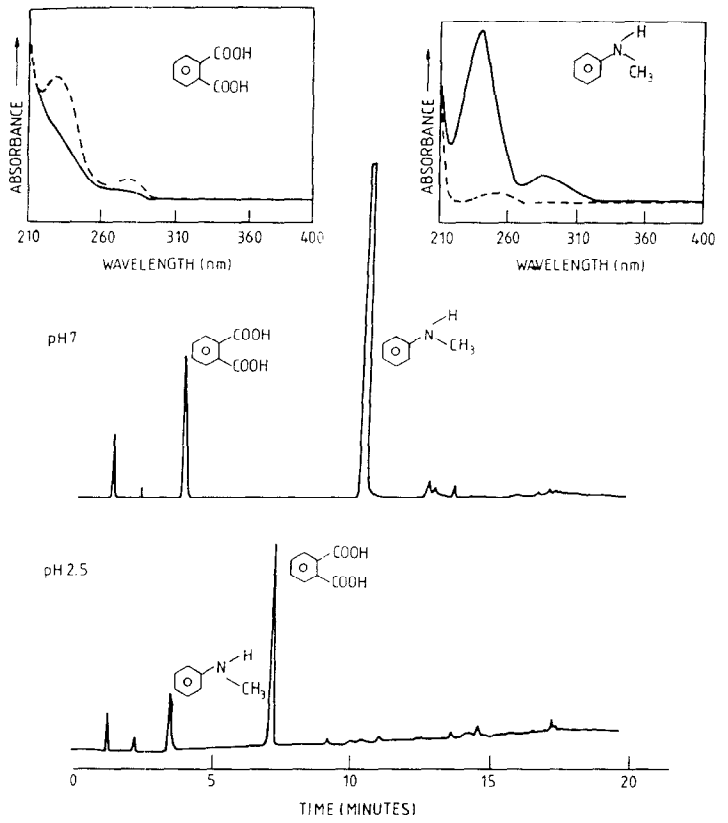

Fig. 7. Examples of changes of UV absorbance signals and spectra (insets obtained by a diode-array detector) of a weak acid (phthalic acid) and a weak base (N-methylaniline) with the eluent pH (solid lines, pH 7.0; broken lines, pH 2.5). Chromatograms were measured at 254 nm wavelength, using the triethylamine–phosphate buffer (15 m*M*) with a Nova-Pak C_{18} column.

last eluting peaks (at pH 2.5 and 7.5) is very important for the selection of the initial mobile phase conditions.

In the case of mixtures containing one or two solute types, the retention shifts can be easily recognized and solute-type classification is relatively simple[21]. More complex sample mixtures require a retention shift-based successive elimination type computer program (currently under development[22]).

Experimental requirements for the solute-type determination

A number of experimental requirements must be fulfilled before the proposed strategy can be used to classify the different solute types in an unknown mixture using the procedure outlined in Fig. 6: (a) the reversed-phase column must behave "ideally" towards the different classes of compounds in all chromatographic runs; (b) the retention of the charged solutes must be sufficiently altered by ionic repulsion and attraction when the ion-pairing reagent is added to ("pulsed" into) the eluent, throughout the whole of the chromatographic run; (c) the organic modifier concentration in the gradient scan must be sufficiently high so that very hydrophobic solutes can also be eluted, and the pH (2.5 or 7.5) during the modifier gradient must be stable.

The requirements in point c can be fulfilled by the judicious selection of the buffer system (see Experimental for details). Points a and b are discussed below.

(a) Realization of "ideal" retention behaviour of charged solutes on reversed phase columns. The success of our strategy critically depends on whether the different solute classes follow the idealized retention behavior shown in Fig. 2. To ascertain this, the retention data of weak/stong acids/bases and non-charged solutes were measured as a function of the eluent pH (2.5–7.0) using isocratic (12.5% methanol) solvents buffered with citric acid and disodium hydrogenphosphate on the ODS-Hypersil column (see Fig. 8a).

The capacity factors of amphetamine and norephedrine (both strong bases with pK_a values above 9) gave the largest deviation from the expected retention profile, showing a minimum at around pH 3.3 rather than constant retention over the entire pH range. Increased retention in the high-pH region is usually attributed to an ion-exchange interaction of the positively charged amines with the dissociating silanol groups[23–25]. The less pronounced increase of the retention of SBs at the lower pH region (pH < 3.3) is more likely due to the citrate ions, which may act as ion-pairing reagent with respect to the protonated base molecules. This reasoning is further supported by the decreasing retention of the negatively charged *p*-toluene sulfonic acid in the same region, presumably caused by ionic repulsion between citrate and SA ions. The retention profiles of N,N-dimethylaniline (WB) and 3,4-dihydroxyacetic acid (WA) were as expected.

The inclusion of organic amines (such as diethyl- or triethylamine) in the buffer system was successfully used to suppress the anomalous behaviour of basic solutes caused by the silanol groups on the surface of the octadecylsilica stationary phases[23–25]. The mobile phase concentrations of these additives appear to vary in a range of 5 to 25 m*M*, the upper limit being dependent on the peak asymmetry[13,14]. In our

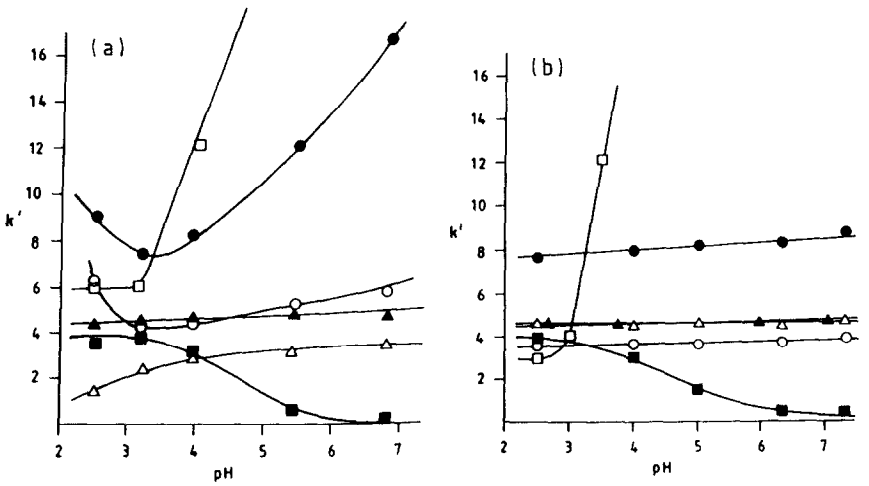

Fig. 8. Retention behavior of differently charged solutes on ODS-Hypersil column, using 12.5% (v/v) methanol in 50 m*M* citrate–phosphate buffers (a) without the addition of triethylamine, and (b) with the addition of 20 m*M* triethylamine phosphate. Solutes: □ = N,N-dimethylaniline (WB); ● = amphetamine (SB); ▲ nitropropane (N); ○ = norephedrine (SB); △ = *p*-toluenesulfonic acid (SA); ■ = 3,4-dihydroxyphenylacetic acid (WA).

case, 25 m*M* triethylamine phosphate completely eliminated the adverse silanol effects. However, it was also found to act as a positively charged ion-pairing reagent, having a serious impact on the second part of our strategy. It considerably toned down the expected retention shifts of charged solutes, when additional positively or negatively charged ion-pairing reagents were "pulsed" in the organic modifier gradients. Therefore, a lower (20 m*M*) triethylamine concentration was used finally, to realize the "ideal" retention behavior of the charged solutes, as shown in Fig. 8b. This eluent system, however, occasionally caused band broadening and/or peak splitting in the methanol gradients at pH 2.5, and allowed for the use of "pulsed" injections only at pH 7.0.

A more simple buffer system, prepared from 15 m*M* triethylamine and phosphoric acid was sufficient to normalize the retention behavior of the charged solutes on the other reversed-phase column (Nova-Pak C_{18}). With this organic buffer alone, higher final methanol concentration (90%), v/v) could be achieved in the gradient runs. However, due to the higher organic modifier concentration reduced ionic interactions were observed between the charged solutes and the ion-pairing reagents in the later part of the gradient. This additional problem will be discussed in section b below.

The procedure followed with these two columns can easily be generalized to evaluate whether other columns behave "ideally" in the selected buffer system (and allow for the use of our strategy). The retention of slightly and strongly retained strong acids and bases (see Fig. 8) must be determined at three different pH values (2.5, 5.0, 7.5), which could give immediate information on the behavior of the column. It is also advisable to include several non-charged solutes in the set, since their retention shifts can indicate inaccuracies of eluent preparation.

(b) Ionic attraction and repulsion of charged solutes by "pulsed" injection of the ion-pairing reagent. The basis of the "pulsed" injection method is to load a concentrated "slug" of ion-pairing reagent on the top of the reversed-phase column before the sample is introduced (and the organic modifier gradient is started)[19]. The ion-pairing reagent adsorbs on the hydrophobic surface of the packing material, and alters the retention of the charged solutes through ionic interaction. Bartha and co-workers[26–27] have demonstrated previously that the adsorption of the ion-pairing reagent decreases substantially with the increase of the organic modifier concentration of the mobile phase. Therefore, the ionic attraction/repulsion effect of the adsorbed pairing ion drops off significantly in the later part of the gradient, where it is increasingly removed from the column by methanol rich eluent. This phenomenon is clearly demonstrated by the retention data shown in Table I. For example, a more retained solute (which elutes also at higher methanol concentrations) such as N-ethylnaphthylamine shows marginal retention shift in the gradient at pH 2.5 when "pulsed" with sodium hexanesulfonate. The retention shift was considerably larger, when a more hydrophobic, more strongly adsorbed ion-pairing reagent, sodium octylsulfonate was used (see Table I). The retention of naphthalenesulfonic acid (SA) was also decidedly more affected in this latter case.

The results in Table I also indicate that even more hydrophobic pairing ions might be needed to effect significant retention movements for very highly retained ionic solutes. Work is in progress to explore this possibility[22]. Higher injection volumes and/or more concentrated solutions of the reagents have been tried with limited

TABLE I

TYPICAL RETENTION MOVEMENTS OF DIFFERENTLY CHARGED SOLUTES IN GRADIENT RUNS "PULSED" WITH DIFFERENT ION-PAIRING REAGENTS

Mobile phases buffered with triethylamine phosphate. Maximum methanol concentration during the gradient is 90% (v/v).

Compound	*Solute classification*	*Modifier gradient at pH 2.5*			*Modifier gradient at pH 7.5*	
		Non-pulsed	*Pulsed*		*Non-pulsed*	*Pulsed*
			$C_6H_{13}SO_3Na$	$C_8H_{17}SO_3Na$		$(C_4H_9)_4NBr$
Arginine phenylthiohydantoin	SB	7.25	8.38	10.85	7.54	6.34
N-Methylaniline	WB	3.38	6.64	9.55	10.26	10.33
N-Ethyl-naphthylamine	SB	9.40	9.57	11.67	10.59	9.68
Naphthaleneacetic acid	WA	12.78	12.73	12.72	9.56	10.55
1-Naphthalenesulfonic acid	SA	8.15	7.41	6.02	8.10	9.89
Ethyl iodide	N	12.34	12.41	12.40	12.34	12.35

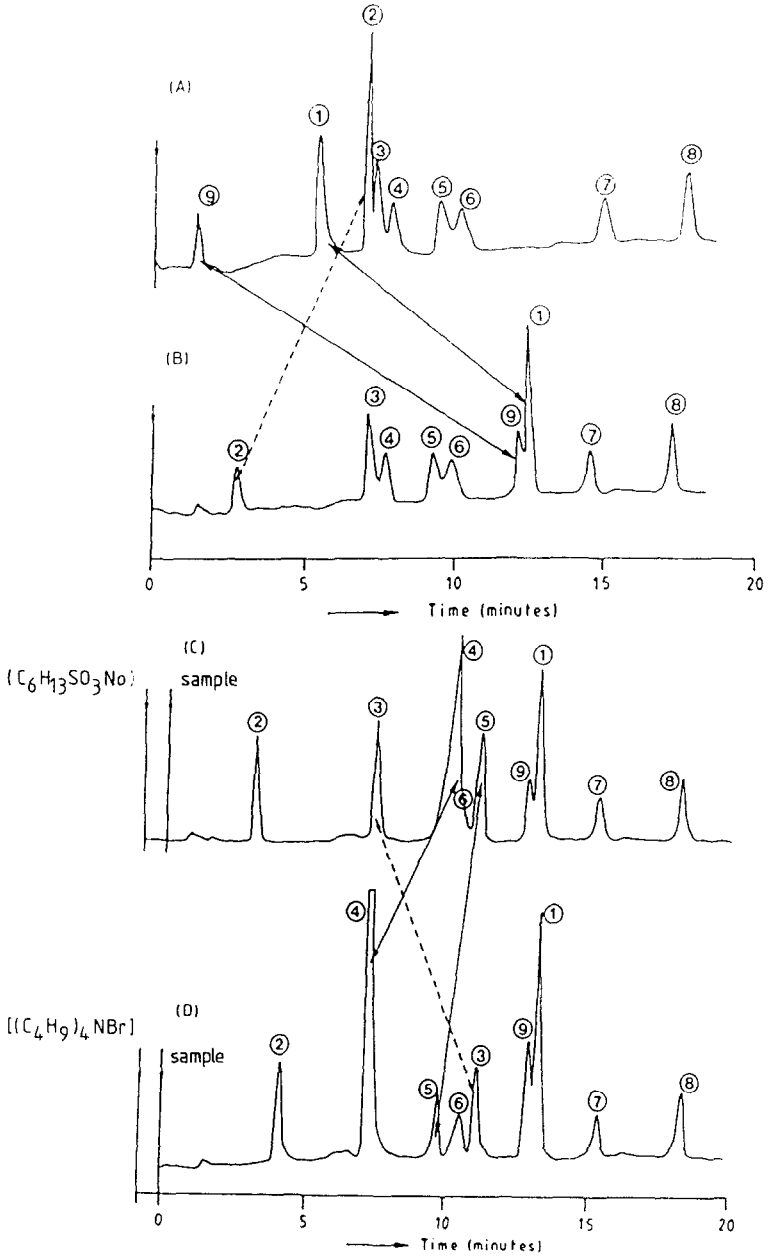

Fig. 9. Application of the solute-type determination strategy to an "unknown" mixture using the 50 m*M* citrate–phosphate (containing 20 m*M* triethylamine) buffer eluents on the ODS-Hypersil column. Chromatograms were obtained with 0–70% (v/v) methanol gradients at (A) pH 2.5; (B) pH 7.0; (C) pH 7.0 and "pulsed" with sodium hexylsulfonate; (D) pH 7.0 and "pulsed" with tetrabutylammonium bromide. Solutes: 1 = N-methylaniline (WB); 2 = 3,4 dihydroxyphenylacetic acid (WA); 3 = *p*-toluenesulfonic acid (SA); 4 = norephedrine (SB); 5 = amphetamine (SB); 6 = methyl iodide (N); 7 = ethyl iodide (N); 8 = propyl iodide (N); 9 = an impurity from N-methylaniline, which appears to be a WB.

success. Injection volumes larger than 20 μl were found to disturb the retention of the early eluting solutes (*e.g.* adrenaline, $k' < 1.5$), because of the disturbance effect caused by the solvent (methanol–water, 50:50) of the pairing ion slug. Limited solubility and long column equilibration times prevented the use of ion-pairing reagents in concentrations higher than 0.5 *M*.

Application of the solute-type determination procedure

The practical application of the solute-type determination strategy for two synthetic solute mixtures is illustrated in Figs. 9 and 10. Peaks which have moved during the scans were identified by the injection of standards in this validation of our procedure. The characteristic shifts of some solute types are indicated by arrows.

Results obtained on the ODS-Hypersil column with the citrate–phosphate buffer and using an earlier scheme of the solute-type determination strategy are shown in Fig. 9A–D. Chromatograms A and B were run at pH 2.5 and 7.0, respectively, without pairing ions. When these two chromatograms are compared, the increased retention of peaks 1 and 9 can be observed with the eluent pH, indicating that they must be weak bases. The decreased retention of component 2 reveals the presence of a weak

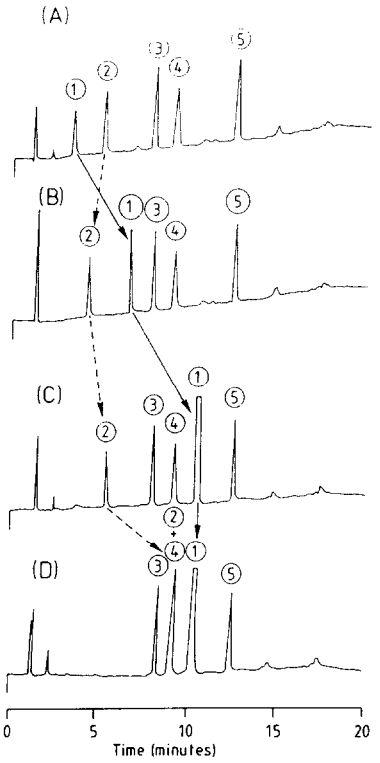

Fig. 10. Application of the solute-type determination strategy to a simple mixture using the triethylamine–phosphate buffered mobile phase on a Nova-Pak C_{18} column. The chromatograms were obtained with 0–90% (v/v) methanol gradients at (A) pH 2.5; (B) pH 2.5 and "pulsed" with sodium hexylsulfonate; (C) pH 7.5; (D) pH 7.5 and pulsed with tetrabutylammonium bromide. Solutes: 1 = N-methylaniline (WB); 2 = *p*-toluenesulfonic acid (SA); 3 = phenol (N); 4 = methyl iodide (N); 5 = ethyl iodide (N).

acid in this mixture. The remaining solutes do not change their retention with the pH and can be either strong acids/bases or non-charged solutes. A "pulsed" injection of a sodium hexanesulfonate in the gradient run at pH 7.0 (see chromatogram C) produced a positive retention shift of peaks 4 and 5 (compared to pH 7.0 without pulse) indicating that these components are strong bases. Similarly, a "pulsed" injection of tetrabutylammonium bromide at pH 7.0 (see chromatogram D) gave a pronounced positive shift for peak 3, indicating that this component is a strong acid. Therefore, out of nine components, two WBs (1, 9) and SBs (4, 5), one WA (2) and SA (3), and three Ns (6, 7, 8) are in the mixture. The most hydrophobic compound is peak 8 (N), and the least retained solute is either peak 2 (WA) or 9 (WB), depending on the final pH of the eluent.

Fig. 10A–D show the application of the solute-type determination strategy for a simple mixture, as given by the flowchart of Fig. 6. Triethylamine–phosphate buffer was used with the Nova-Pak C_{18} column. The highest methanol concentration at the end of the gradient is 90% (v/v). The buffer–methanol gradient was pulsed with sodium hexylsulfonate at pH 2.5 (Fig. 10B) and with tetrabutylammonium bromide at pH 7.5 (Fig. 10D). A notable feature of this example is that peak 1 (N-methylaniline) followed exactly the retention movement pattern of a WB, as outlined in Fig. 5. It is also noted that the confirmation of peak 2 as a SA is not conclusive until the completion of the fourth gradient, where a large retention increase occurs. Nevertheless, the consistent trend of the first three chromatograms indicated that peak 2 was likely an SA. The remaining solutes (3, 4, 5) in the mixture are non-charged (N) compounds.

Other applications of this solute-type determination strategy along with the extensive discussion of the problems of peak tracking, optimization parameter selection and subsequent mobile phase optimization can be found in refs. 20 and 21.

CONCLUSIONS

From the study of simulated mixtures of differently charged compounds we found that the nature (*i.e.* charge-type and relative hydrophobicity) of the components (not their exact identity) is important to decide what combination of eluent pH, organic modifier and pairing-ion concentration is to be selected for systematic optimization. Adapting the design of the eluent composition to the nature of the sample mixture often leads to a significant reduction of the optimization search area.

A systematic strategy, along with a sequentially eliminative algorithm was suggested and experimentally realized to determine the nature of the components in mixtures where this information in unavailable. A novel combination of aqueous buffer-methanol gradients at two different pH (2.5 and 7.5) values with the "pulsed" injection of ion-pairing reagents was used in this method. Since the classification of solute types (strong/weak acid/base, non-charged) is based on their "ideal" retention behavior in the reversed-phase chromatographic system, certain experimental requirements must be fulfilled for the successful application of this strategy. Both the reversed-phase column and the buffer system must be selected carefully, as shown for two commercial C_{18} colums of the same generic type.

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