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PEAK TRACKING AND SUBSEQUENT CHOICE OF OPTIMIZATION PARAMETERS FOR THE SEPARATION OF A MIXTURE OF LOCAL ANAESTHETICS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A peak tracking strategy based on multivariate analysis of multiwavelength spectral data was applied to four chromatograms of a mixture of eight local anaesthetics and extended to cope with coelution of components having identical spectral characteristics. The resulting characterization of the retention behaviour of the individual components was used to select a suitable parameter space for the subsequent improvement of the observed separation.

INTRODUCTION

During recent years a number of strategies for a systematic optimization of the mobile phase composition for high-performance liquid chromatographic (HPLC) separations has been developed[1,2]. Especially reversed-phase HPLC has been extensively studied, resulting in a number of so-called interpretive optimization methods, which base the prediction of the location of the optimum on a model of the retention behaviour of the individual components in a mixture[3,4]. By a suitable concentration of organic modifier, determined by means of a gradient scan, the retention range is fixed. By using isoeluotropic mixtures of different types of organic modifiers [methanol, acetonitrile or tetrahydrofuran (THF)] the specificity of the mobile phase is varied, hopefully resulting in the desired separation of all components. Depending on the number of modifiers in the mixture, a one- or two-parameter optimization is performed: with a mixture of two isoeluotropic solvents, the mixing ratio is the only parameter. When three solvents are involved two mixing ratios are varied and accordingly a two-parameter optimization is performed.

The above procedure is mainly restricted to mixtures containing uncharged solutes. When one is dealing with more complex samples, with both charged and uncharged species, different mobile phase additives, such as ion-pairing reagents, are required to ensure sufficient retention[5,6]. Consequently the number of parameters involved increases dramatically: apart from the type and concentration of organic modifier, the type and concentration of ion-pairing reagent and the pH will influence

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both the overall retention and the specificity of the separation. However, current strategies become extremely time consuming when a large number of parameters is involved. Furthermore, a large number of initial experiments will be required to gauge the influence of the parameters on the retention of the individual solutes in sufficient detail and to construct a response surface describing the quality of the separation for different values of the parameters. Consequently, the optimization is usually restricted to a smaller number of parameters, one of which is the ion-pair concentration[5]. This selection of parameters is done on the basis of experience.

Apart from practical considerations in the restriction of the parameter space with respect to the number of experiments and the limitations of the software, this restriction is often fundamentally correct, since large areas in the extended parameter space will never produce a suitable separation and will play no rôle of importance in the actual optimization. Recent studies by Low *et al.*[6,7] were directed at an efficient selection of parameters and determination of a suitable range of values based on a limited number of preliminary experiments. An important aspect is the characterization of the mixture: the type of components determine which parameters will influence the retention behaviour. For instance, when a (strong) acid is fully dissociated at low pH, inclusion of the pH as a parameter will be useless to influence the retention of this component and will only increase the number of experiments.

With this in mind, the following distinctions can be made between different types of solutes[6]: neutral solutes (N), not ionized within the specified pH range; strong acids and bases (SA/SB), fully ionized within the specified pH range; weak acids and bases (WA/WB) which change their ionic state in the specified pH range. Once all components in a mixture have been characterized according to this specification, a suitable selection of parameters for the optimization will be possible: the retention of neutral solutes is influenced solely by the type and concentration of the organic modifier(s). Weak acids and bases are sensitive to changes in the pH of the mobile phase. All charged solutes are influenced by the concentration of ion-pairing reagent. Acids are retained by a positively charged reagent such as quaternary ammonium salts, and bases by negatively charged ones, for instance sodium octanesulphonate. It is often possible to perform this characterization on the basis of a limited number of experiments, as will be demonstrated in the following discussion.

A separate problem, both in the interpretive optimization strategies and in the characterization of unknown mixtures, is peak recognition: in order to describe the influence of a change in the values of the parameters on the retention of the individual solutes, one must be able to identify the peaks between the chromatograms. Since one is not immediately interested in an identification of the solutes, but in which peaks in two chromatograms of the same mixture correspond to the same solute, this process is referred to as peak tracking. A number of methods are available to perform peak tracking based on relative areas[8,9] or wavelength ratios[10]. Introduction of linear photodiode array detection (LPDA) increased the potential of tracking considerably, since full spectra can now be compared[11].

Recently, we described a strategy for further automation of the tracking procedure based on a multivariate analysis of spectral data, collected by means of LPDA[12]. In order to compare the spectral characteristics of overlapping components in peak clusters, these characteristics must be extracted from a set of mixture spectra. In peak clusters with more than two more or less closely coeluting solutes, pure

component spectra are not observed directly and must be determined in some other way. By means of a multicomponent analysis or target factor analysis (TFA) deconvolution of peak groups is possible, but these techniques require library spectra[13]. Other techniques referred to as "self-modelling curve resolution", such as the iterative target transformation factor analysis (ITT-FA)[14], require no spectral information from other sources, but are more sensitive to the amount of chromatographic resolution[15]. We have demonstrated[12] for two examples of typical complexity that the combination of ITT-FA and TFA assisted by peak areas allowed unambiguous identification of nine and thirteen components, respectively. In both examples all solutes were uncharged.

Of course, additional problems arise when two or more components have similar spectra. When these components are fully separated, additional information on peak areas can be used to track the peaks. However, if these components are present in the same cluster a correct analysis is no longer possible, as will be discussed in the following sections. More seriously, all methods indicated above assume that the spectral characteristics of the components do not change too much with varying experimental conditions. This is generally true for uncharged solutes, but is not necessarily the case for solutes with different states of dissociation in various mobile phases. However, peak tracking in chromatograms of mixtures containing ionized solutes is not *a priori* a waste of time, since some conclusions with respect to the uncharged or fully ionized solutes can always be drawn.

In order further to test the potential of the tracking procedure in other applications, this paper focusses on a mixture of eight local anaesthetics. With a minimum of knowledge regarding this mixture, *i.e.*, a reasonable retention in 50% methanol and the presence of basic solutes, we will try fully to characterize the mixture by means of peak tracking in order to select a suitable parameter space where improvement of the separation can be expected.

THEORETICAL

In order to describe some of the phenomena observed during the analysis of the mixture, a short description is provided here of the mathematical background. For further details the reader is referred to refs. 12–16.

Every mixture spectrum observed during the elution of a peak cluster can be described as a sum of the contributions of the individual pure component spectra, weighted by means of a concentration factor. When a component does not contribute to a spectrum, its concentration is equal to zero. When this decomposition is performed for every mixture spectrum of the cluster, the set of spectral data can be expressed by means of a matrix

$$\mathbf{D} = \mathbf{S}\,\mathbf{E}^{\mathrm{T}} \tag{1}$$

where the mixture spectra are the columns of the data matrix **D** (dimensions n_w wavelengths $\times$ n_s spectra). When n_c components are present, this decomposition results in a matrix **S** (dimensions $n_w \times n_c$) containing the pure component spectra and a matrix **E** (dimensions $n_s \times n_c$) with the contributions, *i.e.*, the elution profiles.

Without knowledge of the pure component spectra, an alternative decomposition is possible, based on a principal component analysis (PCA)[16]:

$$\mathbf{D} = \mathbf{R}\,\mathbf{V}^{\mathrm{T}} \tag{2}$$

The matrix **V** (dimensions $n_s \times n_s$) contains the eigenvectors of the covariance matrix of **D** and can be thought of as an abstract description of the elution profiles: every elution profile in the mixture can be described as a linear combination of the columns of **V**. The same holds for matrix **R** (dimensions $n_w \times n_s$) which can be used to reconstruct the pure component spectra. Since the columns of **V** and **R** are ordered according to the amount of variance they describe in the original data, only the first n_c columns are required to reconstruct the original data within the experimental error. The other columns describe the experimental noise. The reduced matrices, $\mathbf{R}'$ (dimensions $n_w \times n_c$) and $\mathbf{V}'$ (dimensions $n_s \times n_c$), can be transformed into their physical counterparts, **S** and **E**, by means of a suitable transformation, **T** (dimensions $n_c \times n_c$), resp. $\mathbf{T}^{-1}$:

$$\mathbf{D}' = \mathbf{R}'\,\mathbf{V}'^{\mathrm{T}} \tag{3a}$$

$$= \mathbf{R}'\,\mathbf{T}\,\mathbf{T}^{-1}\,\mathbf{V}'^{\mathrm{T}} \tag{3b}$$

$$= \mathbf{S}\,\mathbf{E}^{\mathrm{T}} \tag{3c}$$

Different techniques to determine **T** are available and described by the general term factor analysis. The target factor analysis (TFA) first determines the separate factors which are thought to contribute to the overall variance in the data by projecting an assumed factor, for instance a spectrum, on the relevant hyperspace, in this case $\mathbf{R}'$. When the target and projection coincide, the factor is accepted and the transformation required for the projection is used as an element of **T**. Once all factors (components) have been identified, the inverse transformation is calculated and the elution profiles are derived.

The ITT-FA[14] does not require pure component spectra or profiles to test as factors, but searches for solutions which conform to two boundary conditions, *i.e.*, non-negativity of the concentrations and unimodality of the derived profiles. Once all profiles have been determined, the inverse transformation is used to calculate the spectra.

One way to check whether the correct dimensionality, *i.e.*, the correct value of n_c, has been applied in the analysis, is to compare the reconstructed data matrix, $\mathbf{D}'$, with the original data matrix, **D**. The difference, expressed in the form of a sum of squares, was defined by Malinowski and Howery[16] as the extracted error, XE:

$$\mathrm{XE}^2 = \sum_{i=1}^{n_w} \sum_{j=1}^{n_s} (d_{ij} - d'_{ij})^2 / n_w n_s \tag{4}$$

When the analysis is performed with different values of n_c, that value is accepted as correct which yields the smallest extracted error.

EXPERIMENTAL

Mixture and chromatographic conditions

The composition of the mixture was selected on the basis of separation problems described in the literature[17,18]. The molecular formulas of the eight components are given in Fig. 1. Reference to the components is made by the numbers in this figure. The concentration of all components was 1 mg/ml, except for bupivacaine (0.5 mg/ml). An examination of the spectral similarities in a solvent of methanol–15 m*M* triethylamine (TEA) (50:50), pH 7 (Fig. 2), expressed by means of a correlation coefficient[19], indicated an extreme similarity between components 2 and 5 and between components 3, 4 and 7 (Table I). Comparison of the structures of the components (Fig. 2) endorses these conclusions, since the chromophoric groups (aromatic structures) are identical for the components specified.

The mixture was eluted on a Novapak C_{18} column, particle size 5 μm, 7.5 cm × 3.9 mm I.D. (Millipore Waters, Milford, MA, U.S.A.) using a mobile phase of methanol–15 m*M* TEA (50:50). The flow-rate was set at 1 ml/min. The pH of the buffer was fixed at 3 and 7. At each pH value two experiments were performed, one without the ion-pairing reagent, the other with 5 m*M* sodium octanesulphonate added to the mobile phase. The four chromatograms resulting from these preliminary experiments are shown in Fig. 3; only the one at pH 7 with the ion-pairing reagent reveals eight significantly, but widely differently, resolved peaks.

Instrumentation

The chromatographic system consisted of an HP 1090 chromatograph, equipped with an HP 1040A linear photodiode array detector (Hewlett-Packard, Waldbronn, F.R.G.). The detector was connected to an HP-85 desktop computer, equipped with an HP-IB IEEE-488 interface and RS-232C serial interface. The data were temporarily stored on 5¼-in. flexible disks using an HP82910M disk-drive.

The data files collected by means of the detector were transferred to an Olivetti M24 personal computer (Olivetti, Ivrea, Italy) by means of serial interfaces on both

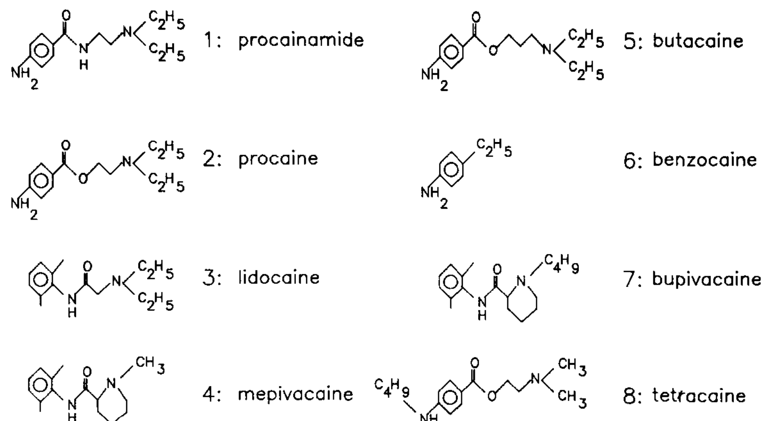

Fig. 1. The molecular structures of the eight local anaesthetics in the mixture.

Fig. 2. UV spectra between 210 and 400 nm of the eight local anaesthetics of Fig. 1.

computers. Further analysis of the data was performed on an IBM-PC (IBM, U.S.A.), equipped with two $5\frac{1}{4}$-in. disk-drives, an expansion unit with two 10-Mbyte hard disks, the Intel 8087 mathematical coprocessor and an HP-7470A graphics plotter with serial interface.

The spectra were recorded between 210 and 400 nm. The sampling frequency was set at 30 spectra per min.

Software

All software used was developed using the Turbo Pascal compiler version 4.0 (Borland International, CA, U.S.A.). The algorithm for the TFA was adapted from the description by Malinowski and Howery[16], the ITT-FA was adapted from the description by Vandeginste *et al.*[14]. Correlation coefficients were calculated according

TABLE I

THE SPECTRAL SIMILARITY OF THE EIGHT COMPONENT IN THE MIXTURE, EXPRESSED BY MEANS OF THE CORRELATION COEFFICIENTS

The spectra were recorded in methanol–15 m*M* triethylamine (50:50), pH 7. Two sets of components (2, 5 and 3, 4, 7) with a very high similarity are observed and indicated by underlining. The numbers refer to Fig. 1.

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1	1.000	0.93005	0.66438	0.66365	0.94787	0.97463	0.66316	0.64652
2		1.00000	0.51212	0.51187	0.99789	0.98027	0.51151	0.81042
3			1.00000	0.99998	0.52209	0.54496	0.99996	0.45219
4				1.00000	0.52171	0.54456	0.99999	0.45208
5					1.00000	0.99091	0.52142	0.77808
6						1.00000	0.54419	0.70918
7							1.00000	0.45159
8								1.00000

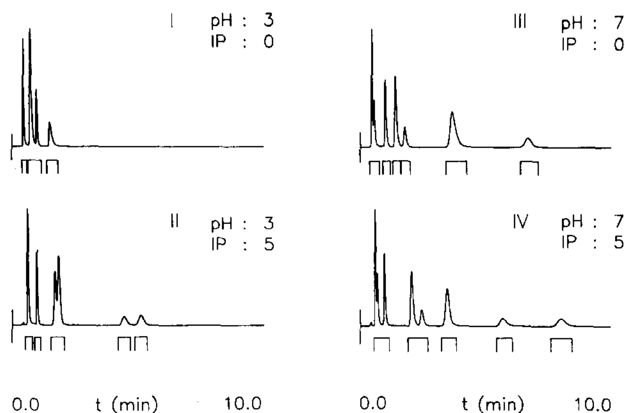

Fig. 3. The four preliminary chromatograms of the mixture at 210 nm, recorded in 50% methanol at pH 3 and 7, without and with 5.0 m*M* sodium octanesulphonate (IP). Further experimental details in the text. The clusters selected for further analysis according to the peak tracking procedure are indicated by brackets.

to Reid and Wong[19]. The eigenvectors and eigenvalues of the covariance matrix of the data matrix were determined by the HQRII algorithm[20].

Curve fitting, performed to estimate resolutions and concentrations, was performed on the basis of bigaussian profiles and a non-linear procedure described by Bevington[21]. This procedure was preferred to a direct calculation of the peak width and areas based on the calculated profiles, because of the limited sampling frequency over the chromatographic peaks, and the inherent uncertainty in the derived results.

RESULTS AND DISCUSSION

The following discussion is split into two parts. The first section will concentrate on the peak tracking in the four chromatograms of Fig. 3, referred to as I–IV. Consequently, we will assume that no further prior knowledge of the mixture is available apart from the assumptions mentioned in the Introduction. In the second part, the results of the peak tracking will be used to select the appropriate parameters to improve the separation.

Peak tracking

The results of the peak tracking strategy are summarized in Fig. 4. The strategy consists of a number of steps, performed sequentially and aimed at a combination of the results derived for the individual chromatograms. The first step is the selection of clusters on the basis of an absorbance threshold. The clusters selected are indicated in Fig. 3. The next step consists of a separate analysis of all peak clusters by means of a PCA, followed by the ITT-FA. The results of the ITT-FA, estimates of the individual elution profiles and pure component spectra, are corrected for negative concentrations and absorbances and used to calculate a reconstructed data matrix, $\mathbf{D}'$ (eqn. 3c). The difference between the reconstructed data matrix and the original one is a measure of the success of the analysis. By repeating the ITT-FA a number of times with different dimensionalities, *i.e.*, assuming a different number of components, and selecting the

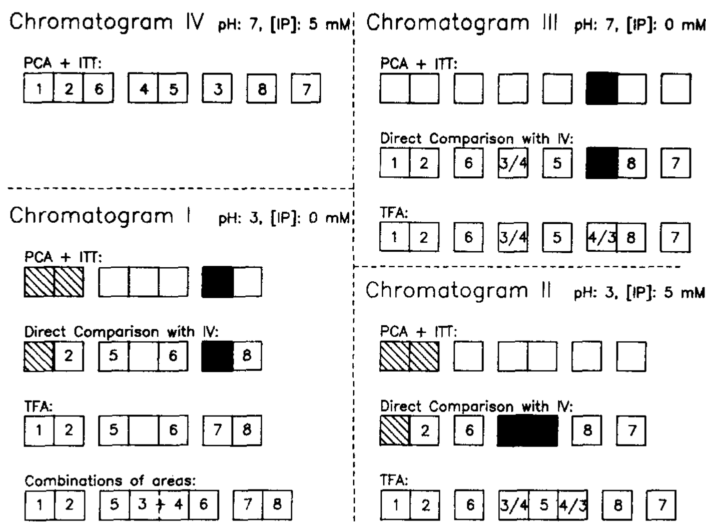

Fig. 4. A schematic representation of the results of the peak tracking procedure. Components in clusters are categorized with respect to the quality of the corresponding spectral and quantitative data as reliable (□), tentative (▧) or unreliable (■). The component numbers refer to the components in Fig. 1. Chromatograms are indicated by Roman numbers corresponding to Fig. 3. Chromatogram IV is the reference to which the other three are correlated as discussed in the text.

best result indicated by the extracted error (eqn. 4), the number of components in the cluster best described by unimodal profiles and physically realistic spectra is selected. On the basis of a 1% concentration threshold, possible impurities, described by an additional unimodal profile in negligible quantity, are rejected. The conclusions of this step, the number of components, N_C, in each cluster, are summarized in the third column of Table II.

The first observation is that more components are found in chromatograms III and IV than in chromatograms I and II, *e.g.*, eight and seven respectively. However, all concentrations exceeded the threshold value of 1%, so that none can be considered as an impurity. There are two possible explanations to account for this observation: two components are eluted at exactly the same time, *i.e.*, the observed mixture spectra all contain the same ratio of pure component spectra and consequently one of the degrees of variation is removed from the observed data. Only one profile and one corresponding mixture spectrum is formed which will not be found in any of the other chromatograms (if this coelution occurs in every chromatogram the problem passes undetected since the analysis will simply be performed with one component less then those thought to be present in the mixture). However, the chances of a coelution as extreme as this (resolution below approximately 0.01) are remote and will probably only be observed early in the chromatogram close to the dead-volume.

A second explanation, which will occur more frequently, is the coelution of two components with an high spectral similarity ($p > 0.999$). Even when two separate peak maxima may be observed, the variation in the spectral data will still be reduced, and the number of components derived for the cluster will be one less than the true number of components. Two examples will be discussed later.

TABLE II

THE CONCLUSIONS OF THE FIRST AND SECOND STEPS OF THE PEAK TRACKING PROCEDURE

For every cluster in each chromatogram (identification according to Fig. 3), the number of relevant components, N_C, as determined after PCA, ITT-FA and evaluation of concentrations is listed. In addition the minimum observed extracted error, XE_{min}, is displayed. The final column presents the number of reliable or tentative spectra in every cluster, N_S, based on an evaluation of resolution and spectral similarity (*cf.*, Fig. 4).

Chromatogram	*Cluster*	N_C	XE_{min}	N_S
I	1	2	1.41	2
	2	3	1.64	3
	3	2	0.12	1
	Total	7		6
II	1	2	0.24	2
	2	1	0.05	1
	3	2	3.73	2
	4	1	0.02	1
	5	1	0.01	1
	Total	7		7
III	1	2	0.54	2
	2	1	0.02	1
	3	1	0.02	1
	4	1	0.02	1
	5	2	0.02	1
	Total	8		7
IV	1	3	1.05	3
	2	2	0.01	2
	3	1	0.01	1
	4	1	0.01	1
	5	1	0.01	1
	Total	8		8

In order to perform the actual peak tracking by a comparison of pure component spectra, the next step consists of a judgement of the reliability of the spectra resulting from the ITT-FA. Due to the fact that errors are introduced in the reconstruction of the profiles when the resolution becomes too low (below approximately $R = 0.4$ for components present in equal concentration, *i.e.*, equal UV activity), not all calculated spectra are sufficiently free from errors to be used directly. Since the extent of the observed distortion also depends on the spectral similarity of the components and on their concentrations, a further analysis of these quantities is required to select the appropriate spectra. Such an analysis has been presented previously using two-component clusters[15] where the observed resolution, the observed spectral similarity and the apparent concentrations were used to judge the reliability of the derived spectra. The expected similarity between the derived spectra and the true spectra is estimated and expressed by means of a correlation coefficient. However, the demand

that this quality index exceeds for instance 0.995 does not guarantee that the calculated concentrations are accurate. Consequently, three types of components are distinguished after the ITT-FA: reliable components, accurately described with respect to both the spectral characteristics and concentration; tentative components, of which the spectra are sufficiently accurate, but the derived concentration is suspect; and unreliable components, which are present in too low a concentration or coelute too closely with other components to rely on the corresponding calculated spectra. Unreliable spectra can be determined only in a second step involving the TFA where the demands on the resolution for a successful analysis are greatly reduced (the amount of information provided during the analysis is increased since spectra derived from the other chromatograms are used as targets). Tentative components are accepted as such but are candidates for further improvement by means of TFA.

The results of the classification are indicated in Fig. 4 by means of open (reliable), shaded (tentative) or dark (unreliable) blocks. The resolution of the first cluster in chromatograms I and II is fairly low (estimated 0.36) but the spectra are tentatively accepted because of the high spectral similarity of the components, and hence the reduced influence of the error introduced by the distortions in the calculated profiles. The resolutions, in the third cluster of chromatogram I and in the fifth cluster of chromatogram III are definitely too low to rely on the spectrum of the fronting component due to the low relative concentration in comparison with the tailing component.

The resolution in chromatogram IV is sufficient to derive reliable spectra of all components, and thus enables us to decide that we are indeed dealing with an eight-component mixture (Fig. 4). Indeed, a comparison of the spectra, as illustrated in Table I and Fig. 2, gives an indication of the spectral complexity of the mixture. Since three components do have extremely similar spectra, the reduced dimensionality of chromatograms I and II is probably caused by coelution of two of these components in one cluster.

Since chromatogram IV produces the largest number of components and the largest number of reliable spectra (Table II), the resulting library of eight spectra is used as a basis for a direct comparison of pure component spectra and in the TFA performed on unresolved clusters in the other chromatograms. The components can therefore be numbered as indicated in Fig. 4. Although we proceed on the assumption that we are dealing with an unknown mixture, for the convenience of the reader and to facilitate the discussion we use the same numbering as in Fig. 1.

The comparison between the chromatograms III and IV is straightforward. A direct comparison of spectra and concentrations identifies all components, except 3 and 4. Due to the fact that both components have equal spectral characteristics and similar concentrations, an unambiguous identification is not possible. Component 7, though also spectrally identical to 3 and 4, is identified since its concentration is different. Consequently, cluster 3 of chromatogram III contains either component 3 or 4. Since the other is not detected by a direct comparison of spectra, it must be situated in cluster 5 (dark block). This is verified by means of the TFA. A definite choice of the location of components 3 and 4 is discussed in the next section.

The comparison between chromatograms I and IV is more difficult, because of the lower resolution in chromatogram I. A direct comparison of spectra and concentrations tracks the components 2, 5, 6 and 8. Although there is a high

correlation between component 1 and the first component in the first cluster of chromatogram I, no positive identification is possible because the concentrations are too dissimilar. The same is true for components 3, 4 and 7 that correlate highly with the central solute in the second cluster of chromatogram I. The decision on the location of component 1 can be made on the basis of its spectral uniqueness (Table I), although the procedure implies a verification by means of TFA. In the case of components 3, 4 and 7 there is the additional problem of extreme spectral similarity that makes it impossible to determine which of the three components matches. This spectral similarity also complicates the application of TFA: when virtually identical spectra are used as targets they cannot be distinguished. To put it differently: components with identical spectra will not produce meaningful elution profiles. In technical terms, such targets produce closely similar transformations of the columns of $\mathbf{R}'$ (eqn. 3b). Consequently, in the inverse transformation resulting after a selection of all matching components, the determinant of $\mathbf{T}$ is very small (an ill conditioned matrix), resulting in large errors in the calculated profiles.

Hence, a library is constructed containing only the spectral characteristics of components 1 and 3, with the latter being representative of solutes 4 and 7 as well. This library is extended with component 2 (already identified but required for the determination of the correct inverse transformation) and used in the TFA performed in cluster 1. Component 1 is now identified unambiguously and is situated in this cluster. The same procedure is followed for cluster 2, after extension of the library with components 5 and 6, and on cluster 3 after extension of the library with the spectrum of component 8. In both cases an high correlation between the spectrum of component 3 and the projections on the spectral description of the clusters (matrix $\mathbf{R}'$) is observed, indicating that all three components, 3, 4 and 7, are located in clusters 2 and 3. The concentration of the peak in cluster 3 corresponds with the concentration of component 7, which leaves components 3 and 4 to be situated in cluster 2. When the concentrations of 3 and 4 are summed, the result equals the concentration of the combined profile derived by means of both the ITT-FA and TFA. Since the two components are poorly resolved, the combined profile can be described by a unimodal profile, as illustrated in Fig. 5, and both the profile and the derived spectra correspond

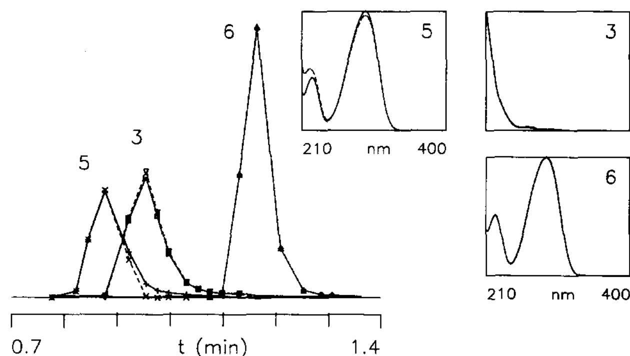

Fig. 5. The results of the ITT-FA performed on cluster 2 of chromatogram I (Fig. 3), indicated by the dashed line, as compared to the results of the TFA (solid line) performed with the pure component spectra of components 3, 5 and 6. The left side of the figure displays the elution profiles, the right side shows the spectra derived by means of ITT-FA (dashed line) compared with the true pure component spectra (solid line).

with the ones derived by means of the TFA. The minor distortion in the spectrum of component 5 derived by means of the ITT-FA is caused by the incorrect reconstruction of the tail of the profile and is not sufficient to hinder a correct identification. In this case the result of the ITT-FA together with a combination of areas can be applied to track all components in the second cluster. The exact identification of components 3 and 4 is not relevant since they have approximately the same retention time.

Similar problems are encountered with the comparison of the spectral data of chromatograms II and IV. The tracking of components 2, 6, 7 and 8 is unambiguous. Component 1 is identified in the same way as above using a library consisting of spectra from components 1, 2 (already identified), 3 and 5. We are left with components 3–5 which must all reside in the third cluster of chromatogram II. It might be surprising that component 5 is not found in this cluster from ITT-FA, despite its reasonable resolution from the fronting component (estimated resolution 0.86). An explanation is found after cluster 3 has been analysed by means of a TFA using the spectra of components 3 and 5, illustrated in Fig. 6. The TFA correctly reconstructs a combined profile of components 3 and 4, containing two distinctive maxima. It should be remembered, however, that ITT-FA assumes an unimodal profile, and thus results in the incorrect profile indicated by the dashed line coinciding with the first peak maximum of the bimodal profile. Through the inverse transformation, this error is introduced in the spectrum derived from the second component, which thus deviates strongly from the spectrum of component 5 (see Fig. 6, dashed *versus* solid line in the spectrum of solute 5). Consequently this distorted spectrum is not observed in the other chromatograms and should not be included in libraries used for peak tracking. By contrast, the results of the TFA can be used to estimate the concentration of the individual profiles, for instance by means of a perpendicular division of the profile, yielding the identification displayed in Fig. 4, with again the exact location of components 3 and 4 uncertain.

The detection of the occurrence of a bimodal profile must be based on one of the following points: when it is observed that one or more spectrally identical solutes are missing in a chromatogram, clusters producing spectra similar to the spectra of these components are suspect and must be checked by means of TFA. If the resulting profiles

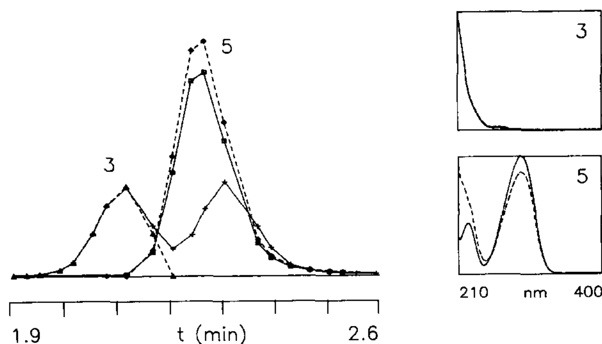

Fig. 6. The results of the ITT-FA performed on cluster 3 of chromatogram II (Fig. 3), indicated by the dashed line, as compared to the results of the TFA (solid line) performed with the pure component spectra of components 3 and 5. Other details as in Fig. 5.

are identical to the ones derived by means of ITT-FA, a direct match or a combination of areas will produce one or more possible solutions. However, when the TFA results in bimodal profiles, other spectra reconstructed previously by ITT-FA for this cluster will become unreliable and must be removed from libraries derived for the chromatogram in question. The tracking of the corresponding solutes can be performed only by means of TFA. It is for this reason that the third cluster of chromatogram II is darkened after the direct comparison of the spectra. Usually, the occurrence of bimodal profiles can also be detected by an increase in the minimum extracted error (Table II) since the removal of part of a profile can be compensated for only partly by other components in the cluster.

After the above procedure, the chromatograms I and IV have been identified as far as the retention times of the components are concerned. In chromatograms II and III, the exact location of the components 3 and 4 remain interchangeable. If one or both of these components are available, a separate injection will solve the dilemma, as has been done to produce Fig. 7. If not, then consecutive experiments must be based on both alternatives. However, often the identity wil remain uncertain, even when the separation is complete.

Parameter selection

Now that the components have been localized in the four chromatograms, their response to the elution conditions can be analysed. An idealized representation of the observed behaviour is given in Fig. 8. The behaviour of the solutes in Fig. 8 is not measured as such but serves to help the reader better understand the following discussion.

Components 1 and 2 both display very low retention at both low and high pH and only display a (small) increase in retention with the addition of a negatively charged ion-pairing reagent. Apparently they are positively charged solutes, protonated over the full pH range examined with a weak dependence on the ion-pair concentration. Consequently, we are dealing with components which behave like strong bases in the parameter space examined.

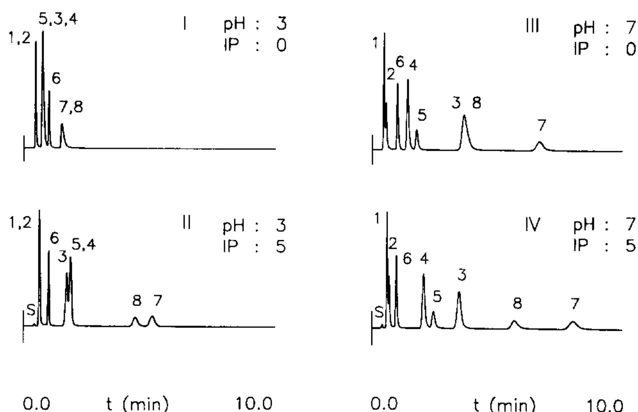

Fig. 7. The results of the peak tracking procedure. The component numbers refer to Fig. 1; the chromatograms are identical to those in Fig. 3. S = Solvent.

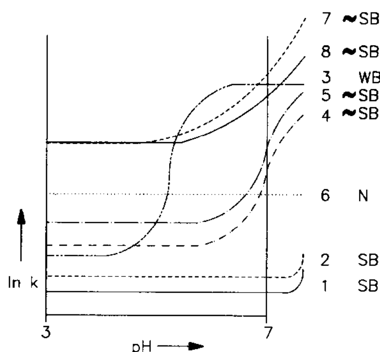

Fig. 8. The characterization of the components in the mixture. On the basis of the retention behaviours at low and high pH, taking the response of the ion-pair reagent concentration into account, the components are identified as behaving like strong bases (SBs), moderately strong bases (~SBs), weak bases (WBs) or neutrals (Ns).

Components 4, 5, 7 and 8 are substantially retained when the pH is increased from 3 to 7, hence the degree of protonation is changed. Since the components react to the addition of an ion-pairing reagent at both low and high pH, they are still charged at high pH. Because of this behaviour, the solutes are categorized as moderately strong bases.

The retention of component 6 is not influenced by either a change in pH or the addition of an ion-pairing reagent and consequently it is considered to be neutral.

The retention of component 3 increases at low pH with the addition of a negatively charged ion-pairing reagent, thus it is protonated at this pH. However, at high pH the (much larger) retention is no longer influenced by the addition of ion-pairing reagent and the component behaves like a neutral solute. Apparently we are dealing with a component with a retention behaviour like a weak base, which is protonated only at low pH. Here we have an additional indication of the identity of components 3 and 4 in chromatogram III, as compared to chromatogram IV. Due to an increase in retention with increasing pH, the components are identified as bases, but a protonated base will not suffer a decrease in retention with the addition of a negatively charged ion-pairing reagent. Consequently the elution order of the components does not change in chromatograms III and IV. This information is essential when interpretive optimization techniques are applied.

When we compare these conclusions with the molecular structures in Fig. 1, a few observations regarding the retention behaviour are immediately confirmed. Component 6, benzocaine, clearly contains no strong basic groups and can be considered as neutral. The tertiary amino groups in the other components are responsible for their basic behaviour, while the long hydrophobic aliphatic chains in the components 7, bupivacaine, and 8, tetracaine, cause the long retention times of these compounds. The reason why component 3 appears less basic than components 4 and 7 is the fact that the piperidine group is a stronger base than a tertiary amine group with alkyl chains[22,23].

One of the requirements of the applied peak tracking procedure is the invariability of the pure component spectra under varying experimental conditions.

Although this cannot always be expected for charged solutes, in this case the requirement is fulfilled since a direct comparison of the pure component spectra of well resolved components produces correlation coefficients ranging from 0.9996 to 1.0000. Apparently, despite the change in protonation (different behaviours at pH 3 and pH 7), the spectra of the components are fairly constant, probably because the chromophoric groups are not affected by the protonation of the basic tertiary amino groups.

Although most components are reasonably separated in chromatogram IV, the retention of components 1 and 2 is still limited and it is desirable that their separation is improved. An inspection of the four chromatograms recorded so far indicates that the changes of finding a better separation within the parameter space examined (pH from 3 to 7 and ion-pairing reagent concentration from 0 to 5 m*M*) are minimal, since the critical pair is coeluted in every chromatogram. However, from the characterization of the components we can also draw the following conclusions regarding the retention behaviour of the components outside the above parameter range: due to the limitations of the stationary phase, a further increase in pH will not be possible, and an increase in the retention of components 1 and 2 can be obtained only by an increase in the ion-pairing reagent concentration. However, this will result in a strong increase in the retention of components 8 and 7, leading to impractically long chromatograms. In order to decrease the retention of these latter components one could increase the methanol content, but this would result in a decrease in the retention of component 6, which would then be coeluted with either component 1 or 2. Therefore the best chromatogram is observed at pH 7 and an ion-pairing reagent concentration of 5 m*M* and further changes in the pH, methanol content or the ion-pairing reagent concentration are not expected to improve the overall separation or quality of the chromatogram. However, there is a possibility that a change in the nature of the organic modifier will provide additional specificity.

The use of isoeluotropic mobile phases, changing the specificity of the mobile phase, but keeping the overall retention constant, has been described extensively[1,2]. When we determine the isoeluotropic mobile phase composition of 50% methanol by means of transfer rules, 38% acetonitrile or 32% THF is advised[24]. However, the retention times observed in chromatograms recorded with these compositions were too short, and corrections were required. The desired retention range was reached at 30% acetonitrile and 18% THF (Fig. 9). A number of changes in specificity are clearly observed. Especially the neutral component 6 shows a considerable change in retention behaviour, eluting in the proximity of component 4 in the chromatogram recorded in acetonitrile and coeluting with component 3 in the chromatogram recorded in THF. Although the retention of the charged solutes is influenced less dramatically by the change of organic modifier, some changes of the elution order are observed, for instance components 7 and 8 change their order in the chromatogram recorded in THF. In addition, the retention of components 1 and 2 is influenced such that the hoped for increase in resolution is obtained. Additional experiments showed that a simple increase in the water content of the mobile phase did not achieve a similar result but actually decreased the resolution of components 1 and 2. Unfortunately the overall separation in the chromatogram recorded in THF is insufficient, due to the coelution of components 3 and 6, but in the chromatogram in 30% acetonitrile sufficient separation of all components is observed. If desired a further optimization

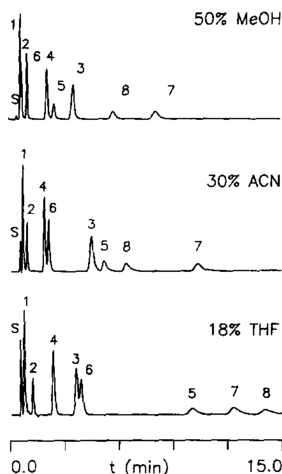

Fig. 9. Three chromatograms of the mixture recorded at 210 nm in isoeluotropic mobile phase compositions consisting of 50% methanol (MeOH), 30% acetonitrile (ACN) and 18% THF respectively. The aqueous phase contained 15 mM TEA and 5 mM octane sulphonate, pH 7. The numbers refer to Fig. 1. S = Solvent.

can be performed according to one of the established methods, for instance the iterative regression design[3].

An acceptable separation was reached on the basis of a limited number of experiments: four preliminary chromatograms, two chromatograms to determine isoeluotropic compositions and the two actual isoeluotropic binary compositions.

Table III summarizes the retention times of the solutes in the chromatograms of Fig. 9. The exact chromatographic conditions are given in the caption.

TABLE III

RETENTION TIMES OF THE EIGHT LOCAL ANAESTHETICS (SEE FIG. 1 FOR THE IDENTIFICATION) IN THE CHROMATOGRAMS OF FIG. 9 UNDER THE CHROMATOGRAPHIC CONDITIONS GIVEN IN THE CAPTION

Elution order	*Acetonitrile*		*18% THF*	
	Solute no.	t_R *(min)*	*Solute no.*	t_R *(min)*
1	1	0.63	1	0.70
2	2	0.88	2	1.18
3	4	1.84	4	2.33
4	6	2.09	3	3.63
5	3	4.52	6	3.90
6	5	5.23	5	10.20
7	8	6.48	7	12.60
8	7	10.60	8	14.30

CONCLUSIONS

Peak tracking is not only required in the final stage of interpretive optimization strategies, but can also play a useful role in preliminary scouting procedures, required for an intelligent selection of the optimization parameters. Based on the change in retention, detected by means of the peak tracking procedure in a limited number of chromatograms, the components can be categorized. When the characteristics of the components in the sample examined are known, the response to a change in one of the parameters can be predicted, and the desirability of this change will play a rôle in the decision to include the corresponding parameter in the actual optimization. In this way irrelevant parameters are removed from consideration, reducing the number of experiments required to find an acceptable separation.

Peak tracking procedures which can be applied to chromatograms of unknown mixtures are limited in the sense that the spectral characteristics of the components must not be influenced too much by the experimental circumstances. However, if this requirement is fulfilled the application of advanced detection techniques such as LPDA, in combination with extensive mathematical treatment, will be able to solve most problems related to peak tracking, even in the case of severe peak overlap in one or more chromatograms.

If coelution of components with identical spectra is observed, additional analysis of this cluster by means of TFA is required, since bimodal profiles cannot be derived by means of the ITT-FA and will introduce errors in the spectra derived for other components present in these clusters. Furthermore, a better estimate of the related areas will be possible using the TFA. When coelution of components with identical spectra results in unimodal profiles a systematic combination of areas will be required to affirm the exact location.

By a rigorous analysis of the retention behaviour of the components in a mixture of local anaesthetics, useless experiments involving pH or ion-pairing reagents can be avoided, and an acceptable separation was derived by a variation of the selectivity of the mobile phase.

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