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Unique resolution of hidden minor peaks in multidetection chromatography by first-order differentiation and orthogonal projections

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Abstract

A new method for unique resolution of multiwavelength chromatograms with a minor analyte completely overlapped by a major one in time direction and with no selective wavelength regions for any of the analytes is derived in this paper. An orthogonal projection is first utilized to obtain the chromatographic profile of the minor analyte. Differentiation of the two-way data matrix in the time direction is subsequently performed to determine the retention time of the chromatographic peak maximum of the major analyte. Based upon these two pieces of information, two approaches for estimating spectra and chromatographic profiles of the pure analytes are developed. A direct approach, which assumes local symmetry in the proximity of the chromatographic peak maximum of the major analyte, and an optimization approach using the estimated peak maximum of the major analyte as objective function.

Keywords: Chromatography; Derivative chromatography; Minor peak detection; Multidetection; Peak detection method

Detection and resolution of a minor chromatographic peak completely hidden under a major one represent a task of major practical significance in analytical chemistry. One way to deal with the problem is to use spectroscopic detectors that are able to produce selective regions even for very similar compounds. Selective information can readily be detected by using latent-projective graphs/datascope [1] or eigenstructure tracking analysis [2] as implemented in the heuristic evolving latent projections (HELP) method. If both analytes possess selective spectral regions, the pooled elution profile for each analyte can be obtained by local principal component analysis at these wavelengths and the spectra of the pure analytes subsequently extracted by means of a least-squares procedure [1].

In the absence of spectral selectivity, only detection of the retention time intervals for elution of the minor and major analyte seems feasible with available approaches [1,3]. However, since selective chromatographic regions usually exist for the major analyte at the start and/or end of elution, the spectrum of the major analyte can be obtained by principal component analysis of these local regions. Making the assumption that the major analyte has at least one spectral maximum that is not coinciding with the maxima of the unknown spectrum of the minor analyte, the elution profile of the minor analyte can be obtained by using an approach developed by Milano et al. [4]. The first-order derivative of the absorbance

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with respect to wavelength, dA/dw, is by definition zero for an analyte at the wavelengths corresponding to local maxima in the absorbances for that analyte. Thus, the elution profile of the minor analyte can, at least in principle, be obtained in the time direction from the first-order derivative in the spectral direction at the local spectral maxima for the major analyte.

The approach developed by Milano et al. [4] has certain drawbacks. According to Fell et al. [5], the method suffers from lack of sensitivity, except for sharp peaks whose spectral maxima are close to the observation wavelengths. For this reason, we propose that the chromatographic profile of the minor analyte is estimated by use of orthogonal projections as developed by Lorber [6]. The contributions of the major analyte to the mixture spectra are removed by projecting on the spectrum of the major analyte. The relative chromatographic profile of the minor analyte is thus obtained. The procedure is based upon the observation that a chromatographic profile of a resolved analyte maps a straight line in multivariate wavelength space [1]. There is a one-to-one correspondence between the concentrations mapped by the elution profile and their positions on this straight line. A key observation used in the derivation of the chromatographic profile of the minor analyte is that any projection of the points on this straight line onto another straight line conserves the mutual relations between the concentrations.

Unfortunately, as the spectrum is unknown for the minor analyte, the chromatographic profile of the major analyte cannot be obtained neither by projections nor by the derivative procedure of Milano et al. [4]. In principle, the background correction procedure developed by Karstang and Kvalheim [7] could be used to locate the positions of the absorbance maxima of the minor analyte. The spectrum of an unknown analyte is modelled by using positivity constraints to refine the residual spectrum. Differentiation of the mixture spectra then provides the chromatographic profile of the major analyte at the absorption maxima for the minor analyte. However, this approach cannot be recommended for cases with similar spectra and with the concentration of the major analyte being more than an order of magnitude larger than that of the minor one.

It appears that only the spectrum of the major analyte and the chromatographic profile of the minor analyte can be obtained, and that we are facing a least-squares problem with no unique solution. There is, however, one characteristic of chromatographic elution that has so far been overlooked in the analysis, namely, that every eluting analyte provides a peak with one and only one maximum. With this information at hand, we shall proceed to find a unique solution to the problem.

The aim of the present work is thus to develop reliable methods especially addressing the resolution of a minor peak completely overlapped by a major one in chromatograms acquired on multidetector instruments. The outline of the work is as follows. First, necessary and sufficient assumptions for resolution in the case discussed above are given. Second, the absorbance matrix is differentiated in time and wavelength direction and some important properties of the derivative matrices are developed. Third, orthogonal projections are used to estimate the chromatographic profile of the minor analyte. Fourth, the derivative absorbance matrix obtained in the time direction is utilized to locate the maximum on the chromatographic profile of the major analyte. This information together with the characteristics of chromatographic elution, i.e., one maximum for each peak, is utilized to derive two approaches for estimating spectra and chromatographic profiles of the pure analytes. The performance of the developed methods is illustrated on simulated data.

THEORY

Assumptions

In the case where a minor analyte is completely overlapped by a major one in the chromatographic direction, and the spectra of the major and minor analyte overlap completely in the sense that no selective regions exist in the spectral direction, unique resolution can only be accomplished if certain assumptions are fulfilled.

First, the spectra must either be shifted or have slightly different shape. Furthermore, although the elution profile of the minor analyte is assumed completely overlapped by the major one, some selective chromatographic regions must exist in the beginning and/or end of elution for the major analyte from which the pure spectrum of the major analyte can be extracted. A final assumption is that the chromatographic peak maxima of the minor and major analyte do not coincide.

First-order two-way differentiation

Data from hyphenated chromatographic instruments are collected in time and wavelength direction. Let matrix A with N rows and M columns be the two-way data array of spectral intensities [absorbances in liquid chromatography

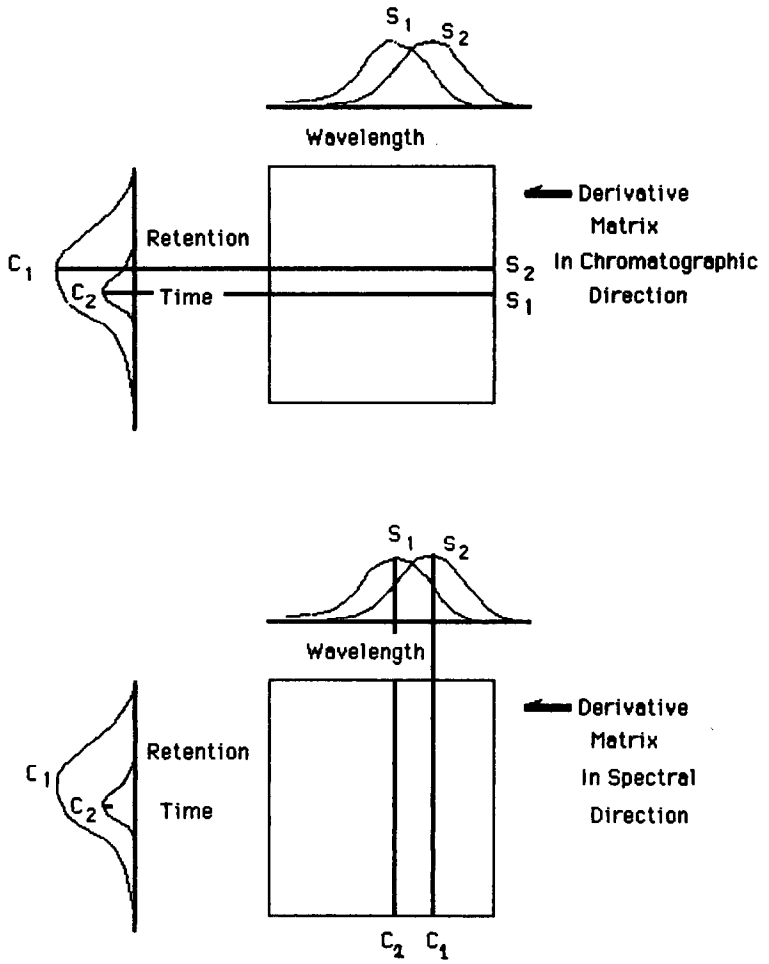


Fig. 1. The upper part of the illustration shows that the first-order derivative matrix in the time direction contains the pure spectra of the minor and major analyte at the retention times corresponding to peak maximum of the major and minor analyte, respectively. Similarly, the lower part illustrates that the first-order derivative matrix in the wavelength direction contains the pure chromatographic profiles of the minor and major analyte at the wavelengths corresponding to maximum absorbance of the major and minor analyte, respectively.

with diode array detection (LC–DAD)]. Retention time is defining the rows and wavelength the columns in the matrix. Neglecting the error term, the matrix **A** for a mixture of two analytes can be decomposed as a sum of two bilinear matrices, one for each analyte

$$\mathbf{A} = \mathbf{c}_1\mathbf{s}_1^{\mathrm{T}} + \mathbf{c}_2\mathbf{s}_2^{\mathrm{T}} \quad (1)$$

In Eqn. 1, $\{\mathbf{c}_i;\ i = 1, 2\}$ and $\{\mathbf{s}_i^{\mathrm{T}},\ i = 1, 2\}$ are the (presently unknown) chromatographic and spectral profiles, respectively, of the two analytes. Transposition T is used to symbolize a row vector as opposed to a column vector.

Assuming both w and t to be continuous, the element $a(t, w)$ of the data matrix **A** at time t and wavelength w can be written as

$$a(t, w) = c_1(t)s_1(w) + c_2(t)s_2(w) \quad (2)$$

Thus, every element in **A** can be described by the functions $\{c_i(t);\ i = 1, 2\}$ and $\{s_i(w);\ i = 1, 2\}$. Equation 2 describes the bilinearity of the matrix **A**. Furthermore, Eqn. 2 shows that the absorbance matrix can be differentiated in either time or wavelength direction:

$$\mathrm{d}a(t, w)/\mathrm{d}t = s_1(w)\ \mathrm{d}c_1(t)/\mathrm{d}t + s_2(w)\ \mathrm{d}c_2(t)/\mathrm{d}t \quad (3a)$$

$$\mathrm{d}a(t, w)/\mathrm{d}w = c_1(t)\ \mathrm{d}s_1(w)/\mathrm{d}w + c_2(t)\ \mathrm{d}s_2(w)/\mathrm{d}w \quad (3b)$$

The derivatives may be expressed in vectorial form:

$$\mathrm{d}\mathbf{a}_i^{\mathrm{T}}(t)/\mathrm{d}t = \mathbf{s}_1^{\mathrm{T}}\ \mathrm{d}c_1(t)/\mathrm{d}t + \mathbf{s}_2^{\mathrm{T}}\ \mathrm{d}c_2(t)/\mathrm{d}t \quad (i = 1, 2, \ldots, N-1) \quad (4a)$$

$$\mathrm{d}\mathbf{a}_j(w)\mathrm{d}w = \mathbf{c}_1\mathrm{d}s_1(w)/\mathrm{d}w + \mathbf{c}_2\mathrm{d}s_2(w)/\mathrm{d}w \quad (j = 1, 2, \ldots, M-1) \quad (4b)$$

Here $\{\mathrm{d}\mathbf{a}_i^{\mathrm{t}})/\mathrm{d}t;\ i = 1, 2, \ldots, N-1\}$ are the row vectors in the derivative matrix $\mathrm{d}\mathbf{A}/\mathrm{d}t$ obtained by differentiation in chromatographic (time) direction, while $\{\mathrm{d}\mathbf{a}_j(w)/\mathrm{d}w;\ j = 1, 2, \ldots, M-1\}$ represents the column vectors for the derivative matrix $\mathrm{d}\mathbf{A}/\mathrm{d}w$ obtained by differentiation in the spectral (wavelength) direction.

The derivative function has a very attractive feature: it becomes zero when the original function has a maximum. This fact means that the local chemical rank (number of analytes contributing in a specific wavelength or retention time region) in the two derivative matrices drops from two to one at the maxima. If the sampling rate in chromatographic direction and the digital spectral resolution is high it should be possible to locate the maxima directly by evaluating the local rank of the two derivative matrix by means of eigenstructure tracking analysis (ETA) [2]. By ETA, the evolving number of analytes in time and wavelength directions is revealed by moving a window of size $2 \times M$ and $N \times 2$, respectively, through the derivative matrices obtained by Eqns. 4a and 4b, starting from the first wavelength and retention time. The chromatographic profiles $\{\mathbf{c}_i;\ i = 1, 2\}$ and spectra $\{\mathbf{s}_i^{\mathrm{T}};\ i = 1, 2\}$ of two overlapping analytes might thus be found directly from the two derivative matrices by locating the maxima on the chromatographic and spectral profiles of the analyte. This is illustrated in Fig. 1.

As discussed in the introduction, the pure spectrum $\mathbf{s}_1$ of the major analyte can easily be extracted by principal component analysis of the selective chromatographic regions for the major analyte [1]. Thus, Eqn. 4b offers a possibility for directly determining the chromatographic profile of the minor analyte since, at the spectral maxima of the major analyte, Eqn. 4b shows that the derivative in the spectral direction is proportional to the chromatogram of the minor analyte, i.e., $\mathrm{d}\mathbf{a}_j(w)/\mathrm{d}w = \mathbf{c}_2\mathrm{d}s_2(w)/\mathrm{d}w$. However, we shall develop another approach for this task in the next section.

Chromatographic profile of the minor analyte

The chromatographic profile of the minor analyte can be estimated by utilizing the orthogonal projection technique introduced by Lorber [6]. Neglecting the error term, the mixture spectrum $\mathbf{a}_i^{\mathrm{T}}$ at the ith retention time in the matrix **A** can be expressed by

$$\mathbf{a}_i^{\mathrm{T}} = c_{i1}\mathbf{s}_1^{\mathrm{T}} + c_{i2}\mathbf{s}_2^{\mathrm{T}}\ (i = 1, 2, \ldots, N) \quad (5)$$

The coefficients $\{c_{ij};\ i = 1, 2, \ldots, N;\ j = 1, 2\}$ are the (presently unknown) values of the chromatographic profiles of the two analytes. As shown in Ref. 1, the pure spectrum of the major analyte $\mathbf{s}_1^{\mathrm{T}}$ can be obtained by means of the HELP method.

With this information at hand, the chromatographic profile of the minor analyte can be obtained by using the projection technique introduced by Lorber [6]. An orthogonal projection matrix is constructed by using the pure spectrum $\mathbf{s}_1^T$ of the major analyte:

$$\mathbf{P}_1 = \mathbf{I} - \mathbf{s}_1\mathbf{s}_1^T/(\mathbf{s}_1^T\mathbf{s}_1) \quad (6)$$

In Eqn. 6, $\mathbf{I}$ is the identity matrix with dimension $M \times M$. The orthogonal projection matrix $\mathbf{P}_1$ can be used to obtain a new vector which is orthogonal to $\mathbf{s}_1$ and proportional to the chromatographic profile of the second (minor) analyte. Application of the projection matrix $\mathbf{P}_1$ (Eqn. 6) on the mixture spectrum at retention time i (Eqn. 5) gives

$$\begin{aligned}\mathbf{a}_i^T\mathbf{P}_1 &= (c_{i1}\mathbf{s}_1^T + c_{i2}\mathbf{s}_2^T)[\mathbf{I} - \mathbf{s}_1\mathbf{s}_1^T/(\mathbf{s}_1^T\mathbf{s}_1)] \\ &= c_{i2}[\mathbf{s}_2^T - (\mathbf{s}_2^T\mathbf{s}_1)/(\mathbf{s}_1^T\mathbf{s}_1)\mathbf{s}_1^T] \\ &= c_{i2}(\mathbf{s}_2^T - \beta\mathbf{s}_1^T) \quad (7)\end{aligned}$$

The scalar β in Eqn. 7 is defined as the ratio $(\mathbf{s}_2^T\mathbf{s}_1)/(\mathbf{s}_1^T\mathbf{s}_1)$. Equation 7 shows that the contribution of the major analyte to the mixture spectrum at retention time i is deleted by the projection. The procedure can be repeated for all the mixture spectra $\{\mathbf{a}_i^T;\ i = 1, 2, \ldots, N\}$. In this way, we obtain a chromatographic profile which is proportional to the profile of the minor analyte. The profile proportional to $\mathbf{c}_2$ is obtained simply by calculating the norm of the row vectors $\{\mathbf{a}_i^T\mathbf{P}_1;\ i = 1, 2, \ldots, N\}$ obtained by Eqn. 7 and rearranging:

$$c_{i2} = \|\mathbf{a}_i^T\mathbf{P}_1\| / \|(\mathbf{s}_2 - \beta\mathbf{s}_1)\| \quad (i = 1, 2, \ldots, N) \quad (8)$$

Defining $d_i = \|\mathbf{a}_i^T\mathbf{P}_1\|$ and $\mu = \|(\mathbf{s}_2 - \beta\mathbf{s}_1)\|$ and rewriting Eqn. 8 in vectorial form, we obtain

$$\mathbf{c}_2 = \mu^{-1}\mathbf{d} \quad (9)$$

Here $\mathbf{d} = (d_1, d_2, \ldots, d_N)^T$ and $\mathbf{c}_2 = (c_{12}, c_{22}, \ldots, c_{N2})^T$.

Retention time for peak maximum of the major analyte

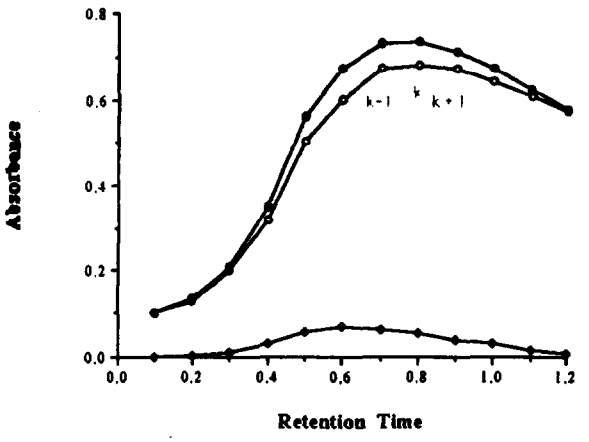
With the spectrum of the major analyte and the chromatographic profile of the minor analyte at hand, Eqn. 4a shows that if the chromatographic peak maximum of the major analyte can be located, the spectrum of the minor analyte can be derived. The chromatographic profile of the major analyte can subsequently be estimated by simple least squares (Eqn. 12 below).

The estimation of the chromatographic peak maximum of the major analyte can be done without making any further assumptions than those discussed at the beginning of the Theory section. The positivity constraint on the pure spectra of the two analytes shows that the sign of the elements of the vectors in the derivative matrix in chromatographic direction is completely determined by the sign of $dc_1(t)/dt$ and $dc_2(t)/dt$. From the relative chromatographic profile for the minor analyte, obtained by orthogonal projections, one knows already the sign of $dc_2(t)/dt$ and thus the second term in Eqn. 4a for every retention time $(1, 2, \ldots, N-1)$. Furthermore, in case of a minor chromatographic peak completely overlapped by a major one, there are only two possible overlapping modes: either the chromatographic peak maximum of the minor analyte appears before the chromatographic peak maximum of the major analyte, or the other way round. For both possibilities, the retention time corresponding to the chromatographic peak maximum for the major analyte can be determined. We shall examine in detail the case where the maximum of the minor peak appears before the maximum of the major peak.

In the proximity of the chromatographic peak maximum of the major analyte, the contribution of the minor analyte to Eqn. 4a is negative in the case where the maximum of the minor peak appears before the maximum of the major one. This is obvious since the concentration of the minor analyte is decreasing in this region. After the retention time corresponding to peak maximum of the major analyte, the first-order derivative mixture spectrum $d\mathbf{a}_i^T(t)/dt$ becomes negative at every wavelength, since after peak maximum the concentration of the major analyte is decreasing and thus its contributions to the derivative mixture spectrum becomes negative at all wavelengths. Before peak maximum, however, the contribution from the first term in Eqn. 4a, i.e.,

$s_1^T dc_1(t)/dt$, is positive at all wavelengths. Accordingly, before peak maximum of the major analyte, the derivative mixture spectrum will be positive in some part since the first term represents the major analyte. Thus, the retention time at which the derivative mixture spectrum obtained in chromatographic direction changes from positive or partially positive to negative, represents the chromatographic peak maximum of the major analyte. The situation is illustrated in Fig. 2. This piece of information is crucial for successful resolution in case of complete chromatographic overlap and no spectral selectivity.

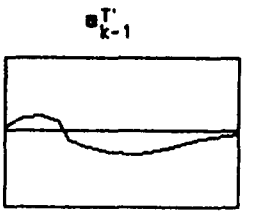
With the retention time of chromatographic peak maximum for the major analyte at hand the present problem can be solved. Both approaches



$$a'_{k-1} = d\,a^T_{k-1}(t)/dt = s_1^T\, dc_1(t)/dt + s_2^T\, d(t)/dt$$

$$= s_1^T \frac{c_{1,k} - c_{1,k-1}}{t_k - t_{k-1}} + s_2^T \frac{c_{2,k} - c_{2,k-1}}{t_k - t_{k-1}}$$

positive negative



$$a'_k = d\,a^T_k(t)/dt = s_1^T\, dc_1(t)/dt + s_2^T\, d(t)/dt$$

$$= s_1^T \frac{c_{1,k+1} - c_{1,k}}{t_{k+1} - t_k} + s_2^T \frac{c_{2,k+1} - c_{2,k}}{t_{k+1} - t_k}$$

negative negative

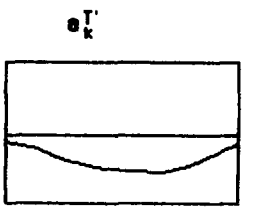


Fig. 2. Upper part shows parts of the resolved elution profile for a minor analyte, a major overlapping analyte and the observed elution profile of both analytes (before resolution). The retention time k corresponds to the chromatoraphic peak maximum of the major analyte. The two nearest neighbouring retention times are denoted $k-1$ and $k+1$. The derivatives obtained in the time direction (using Eqn. 4a) for the retention time intervals $[k-1, k]$ and $[k, k+1]$ are shown together with a display illustrating the change in the derivatives from partial positive to negative at all wavelengths after passing the retention time of peak maximum for the major analyte.

developed below for estimating the chromatographic and spectral profiles of the pure analytes are based on this information. If the chromatographic sampling rate is not extremely high, interpolation techniques [8] may be used to accurately determine the peak maximum of the major analyte. Interpolation is performed upon the original two-way data matrix, i.e., before differentiation. A 5-point Lagrange interpolation was used in our examples to insert some values around the peak maximum of the major analyte estimated from the two-way derivative matrix.

Resolution by estimating the spectrum of minor analyte

Knowing the retention time for the peak maximum of the major analyte, and the chromatographic profile of the minor, differentiation of the chromatographic profile of the minor analyte and insertion into Eqn. 4a, should provide an estimate of the spectrum of the minor analyte at the retention time of peak maximum for the major analyte. A more reliable estimate of the spectrum of the minor analyte is obtained by using symmetry constraints on the chromatographic peak of the major analyte.

The nearest neighbour (times $k-1$ and $k+1$ in Fig. 2) at each side of the retention time k corresponding to chromatographic peak maximum for the major analyte is of prime importance in the further development. If the chromatographic peak of the major analyte is symmetric in the proximity of maximum, the spectrum of the minor analyte can be obtained directly from the derivative matrix. Symmetry of the major peak just around maximum means that the derivatives in time direction calculated for the retention time intervals $[k-1,\ k]$ and $[k,\ k+1]$ obey the following relation:

$$(c_{k,1}-c_{k-1,1})/(t_k-t_{k-1}) = -(c_{k+1,1}-c_{k,1})/(t_{k+1}-t_k) \qquad (10)$$

Thus, the spectrum $\mathbf{s}_2$ of the minor analyte can be obtained by inserting Eqn. 10 in Eqn. 4a for $[k-1,\ k]$ and $[k,\ k+1]$, combining and rearranging:

$$\mathbf{s}_2^T = \left[\mathbf{da}_{k-1}^{\mathrm{T}}(t)/\mathrm{d}t + \mathbf{da}_k^{\mathrm{T}}(t)/\mathrm{d}t\right] / \left[(c_{k,2}-c_{k-1,2})/(t_k-t_{k-1}) + (c_{k+1,2}-c_{k,2})/(t_{k+1}-t_k)\right] \qquad (11)$$

Note that the derivation of Eqn. 11 does not require symmetry of the chromatographic peak of the major analyte in the whole elution region. It is sufficient that local symmetry exists for the major peak just around maximum. For a non-symmetric chromatographic peak, the interpolation technique may be helpful to approximate symmetry just around the peak maximum for the major analyte.

With both $\mathbf{s}_2^{\mathrm{T}}$ and $\mathbf{s}_1^{\mathrm{T}}$ available, the chromatographic profiles for the two analytes can be estimated by a least-squares procedure,

$$\mathbf{C} = \mathbf{AS}(\mathbf{S}^{\mathrm{T}}\mathbf{S})^{-1} \qquad (12)$$

In Eqn. 12, the matrices $\mathbf{C}$ (dimension $N \times 2$) and $\mathbf{S}$ (dimension $M \times 2$) contain the chromatographic profiles $\{\mathbf{c}_1,\mathbf{c}_2\}$ and spectral profiles $\{\mathbf{s}_1,\mathbf{s}_2\}$, respectively. Note that Eqn. 12 provides a second estimate of the chromatographic profile of the minor analyte which can be compared with the estimate obtained by orthogonal projections (Eqns. 7–9).

If the scalar denominator in Eqn. 11 is very small, the estimate of $\mathbf{s}_2$ will inevitably be contaminated by noise. In this case, the target transformation technique developed by Malinowski and Howery [9] can be used to reduce random noise. If the concentration of the minor analyte is not too small, iterative target transformation factor analysis as developed by Vandeginste et al. [10] may be used to refine the chromatographic profiles of the analytes. In this case, least-squares can be used to recalculate the spectrum of the minor analyte, i.e., by using Eqn. 17 below, and thus provide a check on the consistency of the results.

Resolution by estimating the chromatographic profile of the major analyte

An alternative road to resolution is to estimate the chromatographic profile of the major analyte.

This can be carried out by optimization using the already estimated chromatographic peak maximum of the major analyte as objective function.

Every column of the original data matrix is a linear combination of the chromatographic profiles of the two pure analytes. The major analyte

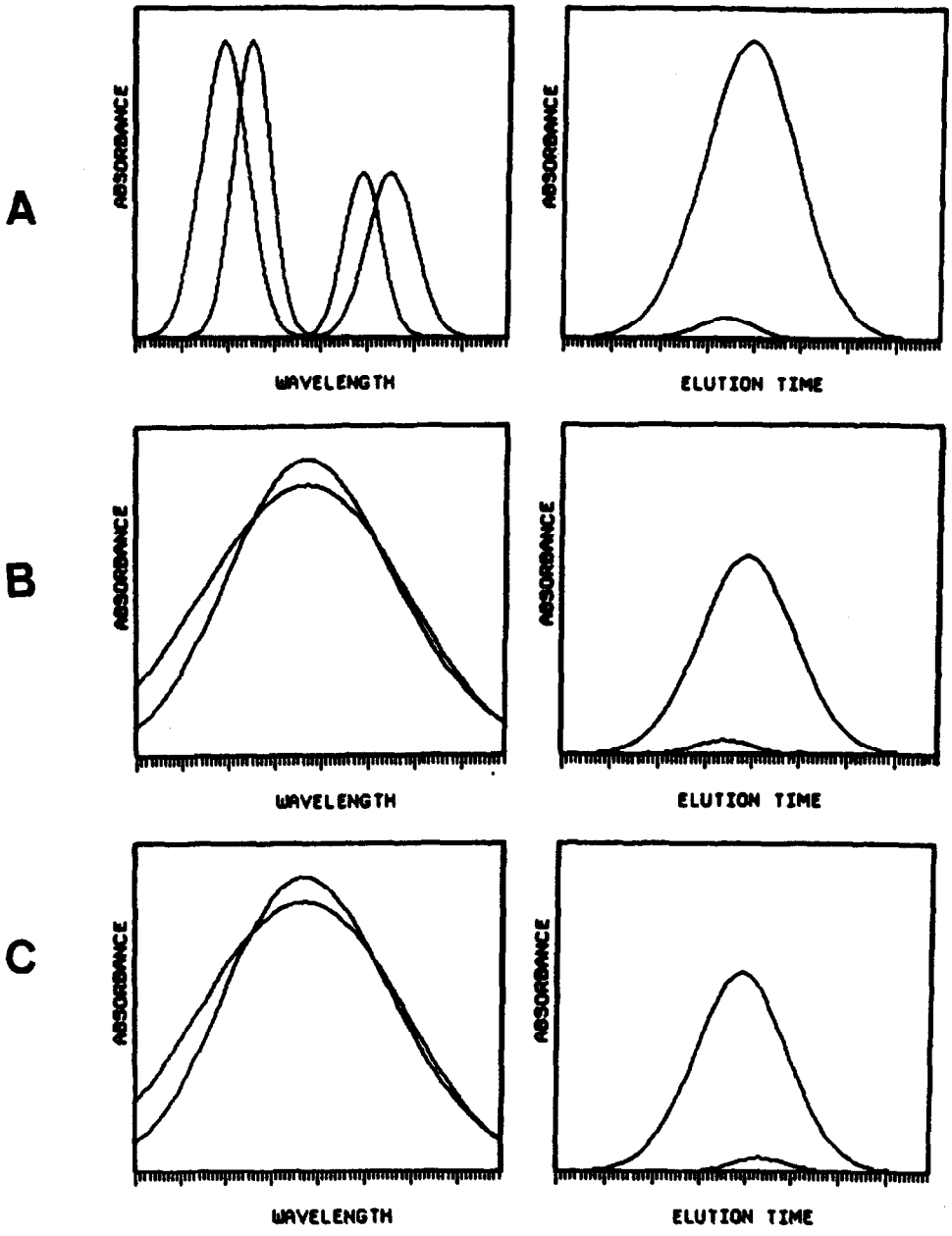


Fig. 3. Three simulated mixtures of two analytes acquired on a chromatograph with multiwavelength detector. In all cases the minor analyte is completely overlapped in the time direction. Case A differs from cases B and C by the presence of selective wavelengths regions. The mixtures B and C are composed of the same analytes in the same relative concentrations, but with a shift towards later elution times for mixture C compared to B.

at wavelength j is thus the difference between the chromatographic profile of the mixture and the chromatographic profile of the minor analyte:

$$s_{1j}\mathbf{c}_1 = \mathbf{a}_j - b\mathbf{d} = s_{1j}\mathbf{c}_1 + s_{2j}\mathbf{c}_2 - b\mu\mathbf{c}_2 \quad (j = 1, 2, \ldots, M) \tag{13}$$

Equation 13 holds if and only if b is chosen as s_{2j}/μ. The relative chromatographic profile $\mathbf{d}$ of the minor analyte is already estimated by orthogonal projections (Eqns. 7–9) so that by Eqn. 13 the resolution problem is changed into a one-dimensional optimization search problem: find the coefficient b that provides the chromatographic profile of the major analyte. The already estimated retention time for chromatographic peak maximum of the major analyte is used as objective function. The multiplication of $\mathbf{c}_1$ with the absorbance s_{1j} of the major analyte at wavelength j (see Eqn. 13) does not change the position of the peak maximum. Thus, the performance of the different choices of b can be assessed with respect to the deviation they produce from the already established peak maximum of the major analyte.

In order to avoid unfortunate choices of the wavelength j and thus b, the marker-object projection technique [11] is used to define a new chromatographic profile representing all the mixtures. The marker-object projection technique as used here additionally reduces the influence from random noise. The estimated spectrum of the major analyte is used as a "marker object" and the chromatographic profile at each retention time is projected on this spectrum:

$$\mathbf{a}_i^{\mathrm{T}}\mathbf{s}_1 = (c_{i1}\mathbf{s}_1^{\mathrm{T}} + c_{i2}\mathbf{s}_2^{\mathrm{T}})\mathbf{s}_1 = c_{i1}\mathbf{s}_1^{\mathrm{T}}\mathbf{s}_1 + c_{i2}\mathbf{s}_2^{\mathrm{T}}\mathbf{s}_1 = r_1c_{i1} + r_2c_{i2} \quad (i = 1, 2, \ldots, N) \tag{14}$$

The scalars r_1 and r_2 are defined as the projections of the pure spectra $\mathbf{s}_1$ and $\mathbf{s}_2$ on the "marker"-spectrum $\mathbf{s}_1$, i.e., $\{r_i = \mathbf{s}_i^{\mathrm{T}}\mathbf{s}_1;\ i = 1,2\}$. The projection defined by Eqn. 14 is performed for all the mixture spectra $\{\mathbf{a}_i^{\mathrm{T}};\ i = 1, 2, \ldots, N\}$. In this way, a chromatographic profile is obtained that is a linear combination of the chromatographic profiles of the two analytes, but where the part of the minor analyte's spectrum orthogonal to the spectrum of the major analyte is removed. Collecting the projections in a vector $\mathbf{m}$ gives

$$\mathbf{m} = r_1\mathbf{c}_1 + r_2\mathbf{c}_2 \tag{15}$$

and

$$\mathbf{m} - b\mathbf{d} = r_1\mathbf{c}_1 + r_2\mathbf{c}_2 - b\mu\mathbf{c}_2 \tag{16}$$

Optimization with the chromatographic peak maximum of the major analyte as objective function, provides the chromatographic profile of the major analyte for $b = r_2/\mu$. The converged value of the parameter b using Eqn. 16 for the optimization differs from the value obtained by use of Eqn. 13 due to the use of the marker projection to reduce the contribution of the minor analyte. With estimates of $\mathbf{c}_1$ and $\mathbf{c}_2$ available, the pure spectra for the two analytes can subsequently be obtained by a least-squares calculation

$$\mathbf{S}^{\mathrm{T}} = (\mathbf{C}^{\mathrm{T}}\mathbf{C})^{-1}\mathbf{C}^{\mathrm{T}}\mathbf{A} \tag{17}$$

The main advantage of this approach compared to the previous one is that the estimates do not suffer from contamination of random noise. Thus, refinement by use of the target transformation technique is no longer necessary. If slightly different estimates of the chromatographic profile of the major analyte provide the maximum for the major analyte at the same retention time, the average profile is taken as the final result. Therefore interpolation [8] is useful also in this method to narrow the region around the peak maximum of the major analyte.

EXPERIMENTAL

The method has been implemented in VAX FORTRAN as a part of the heuristic evolving latent projections (HELP) program [1]. The implementation is running on a VAXstation 2000. Three different simulated two-way mixture data with the minor peak completely overlapped by the major chromatographic peak were created, denoted by the letters a–c in Fig. 3. The concentration of minor analyte is in all cases 7% of the

major one. The data were generated with random noise of standard deviation of 0.0001 which is comparable to that commonly observed in real LC–DAD data.

RESULTS AND DISCUSSION

Figure 3 shows three simulated mixtures of two analytes. Figure 3, right side, shows that all systems contain a minor analyte that is completely overlapped by a major one in the time direction. Systems B and C are mixtures of the same analytes as observed from the spectra (Fig. 3, left side) in the same proportions (Fig. 3, right side). The chromatographic conditions are different, however, as the minor analyte is shifted towards later retention times for system C compared to B. Actually, the two systems are similar around the retention time corresponding to peak maximum of the major analyte and thus we shall only resolve system B. First, however, we resolve system A.

Resolution of systems with selective spectral regions

There is a major difference between system A and systems B and C. Thus, Fig. 3 (left side) suggests that mixture A has selective spectral regions for both analytes, while the spectra of the two analytes in systems B and C overlap completely. Although this observation can only be done after the system has been resolved, selectivity in either the spectral or chromatographic domain can easily be revealed by means of procedures developed in the heuristic evolving latent projections (HELP) method [1]. Figure 4 shows the latent-projective graphs mapping the evolution in chromatographic direction (left side) and spectral direction (right side) on the two major principal analytes for mixture A in Fig. 3. The evolution implied by the latent-projective graph in chromatographic direction suggests the presence of a minor chromatographic peak being completely overlapped by a major one. This follows from the fact that there is only one straight-line segment in the latent-projective graph (Fig.

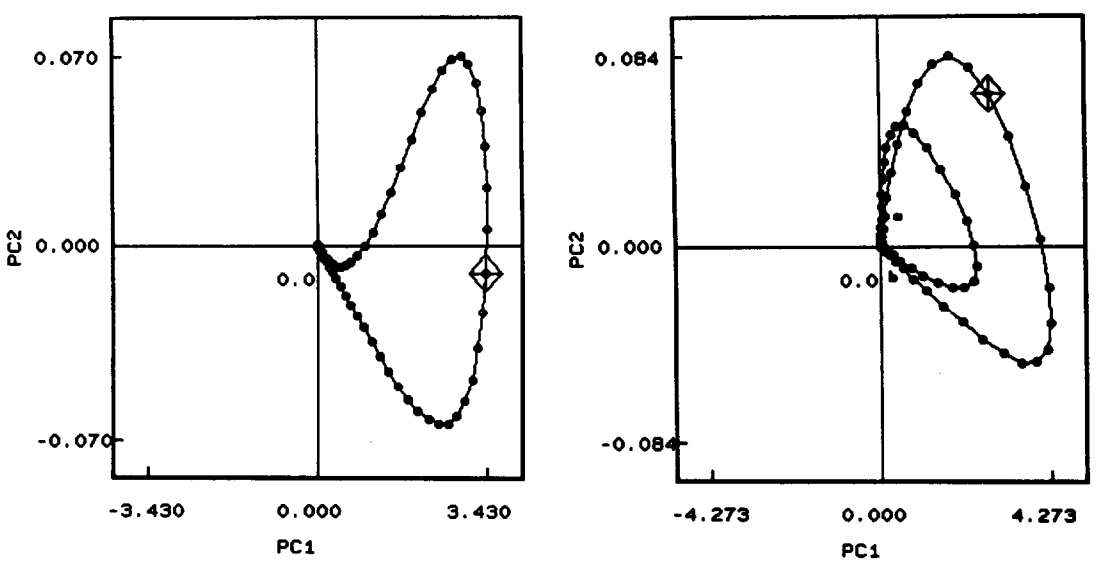


Fig. 4. Latent-projective graphs in time (left side) and wavelength (right side) domain for mixture A. The graphs are obtained by principal components analysis of the two-way data. In the time domain (left side) the chromatographic elution is described. Each point corresponds to the spectrum at a particular retention time. The continuous character of the graph reflects the continuous nature of the chromatographic profile. The first and last spectra projects in origin indicating the start and end of elution. In the spectral domain (right side), each point describes the chromatographic profile at a particular wavelength. Straight-line segments pointing towards origin are indicative of selective regions.

4, left side) mapping the chromatographic profile of the major analyte at the beginning and end of elution. The straight line segment represents the chromatographic retention time regions where the major analyte elutes alone. This follows from the fact that only the concentration changes for a single eluting analyte, while the spectrum remains the same. Thus, the chromatographic profile of a pure analyte maps a line in multivariate space. The straight-line segment in Fig. 4 (left side) is a

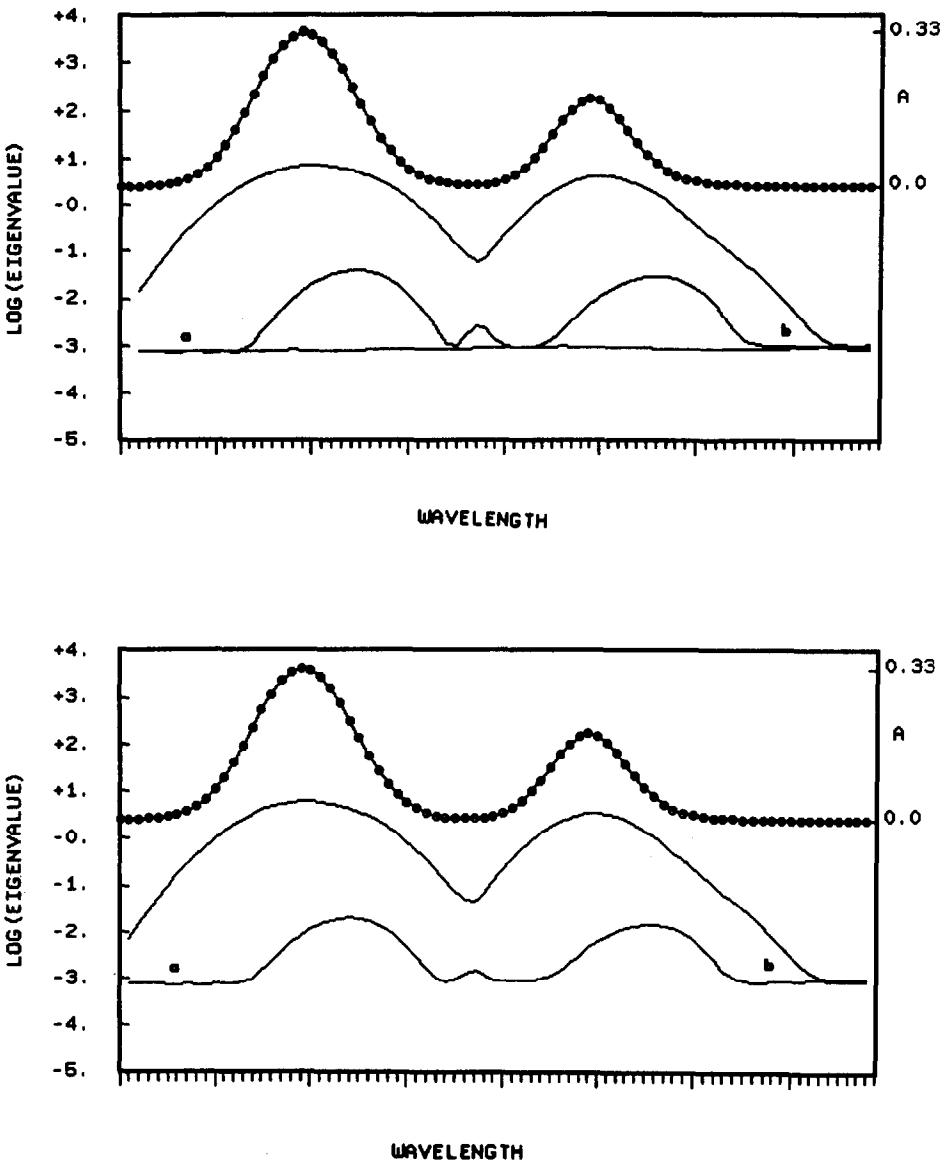


Fig. 5. Eigenstructure tracking analysis in wavelength direction for mixture A. Lower and upper part display the evolving eigenvalues when moving a window containing 2 and 3 neighbouring wavelengths, respectively, one wavelength at-the-time from the first until the last wavelength. The upper part (window size 3) reveals that system A is a two-analyte mixture with selective spectral regions a and b for the two analytes. Note that the third evolving eigenvalues (upper part) implies the noise level in the data.

projection of this line. The loop maps the chromatographic region where the two analytes coelute.

Although the analysis has revealed selective chromatographic regions for the major analyte, the system cannot be resolved using the procedure developed in HELP [1,2]. This is due to the lack of any zero-concentration region [12] for the major analyte. However, the latent-projective graph mapping the evolution in the spectral direction (right side), reveals straight-line segments, denoted (a) and (b) in plot, pointing towards origin. As shown previously [1], this pattern is indicative of selective wavelength regions. The results from ETA [2] where an evolving window is moved in either time or wavelength direction, support the same conclusion (Fig. 5). The figure shows the evolving eigenvalues in wavelength direction with window sizes of 2 (lower) and 3 (upper). This dynamic (and two-way) procedure is an extension of a window approach introduced by Keller and Massart [3]. The selective regions (a and b, Fig. 5) for the two eluting analytes are easily identified in the plots. Extracting the chromatographic profiles by principal component analysis on the two selective regions, provides the result shown in Fig. 6 for the two analytes, which should be compared with system A in Fig. 3. This result shows that if one can find selective information in spectral direction for both analytes, resolution becomes almost trivial.

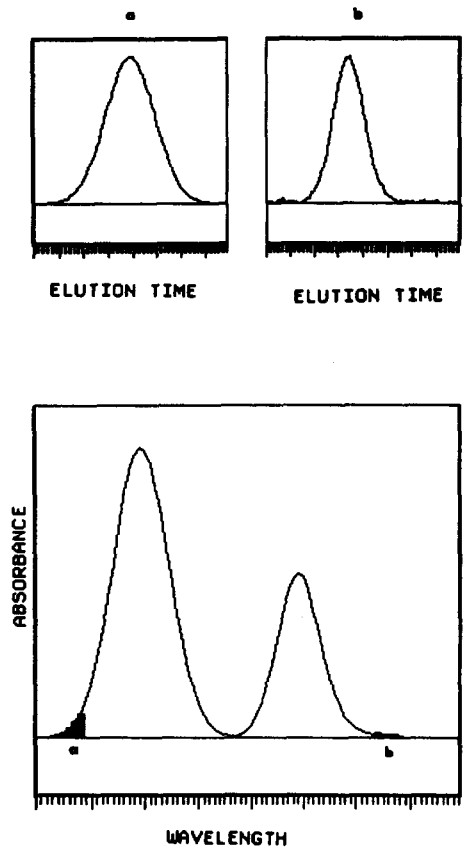


Fig. 6. The resolved elution profiles of the two analytes (upper part) estimated by principal component analysis of the selective spectral regions a and b (lower part).

Resolution of systems without selective spectral regions

We shall now turn our attention to the much more challenging resolution problem presented by the mixtures B and C (Fig. 3). Only the results for system B will be provided in detail since system C differs from system B only in a shift towards later elution times for the minor analyte. Figure 7 shows the two latent-projective graphs for system B. The latent-projective graph showing the evolution in chromatographic direction (Fig. 7, left side) suggests a similar chromatographic situation as for system A, namely a minor peak completely overlapped by a major one, but selective chromatographic regions for the major analyte at start and end of elution. The latent-projective graph mapping the spectral domain (Fig. 7, right side), however, reveals that the prospect for successful resolution and quantitation is worse for system B than for system A since no selective regions exist in the spectral domain. Apparantly, there are two straight-line segments in the graph, but, unfortunately, they do not point to the origin of the coordinate system. As shown in previous work [1], this picture means that the spectra of the analytes overlap at all wavelengths.

Figure 8 shows the results of component analysis upon the selective chromatographic regions for the major analyte at start of elution as indicated by the latent-projective graph of the chromatographic domain (Fig. 7, left side). The loadings on the first principal component represent the pure spectrum of the major analyte, while the

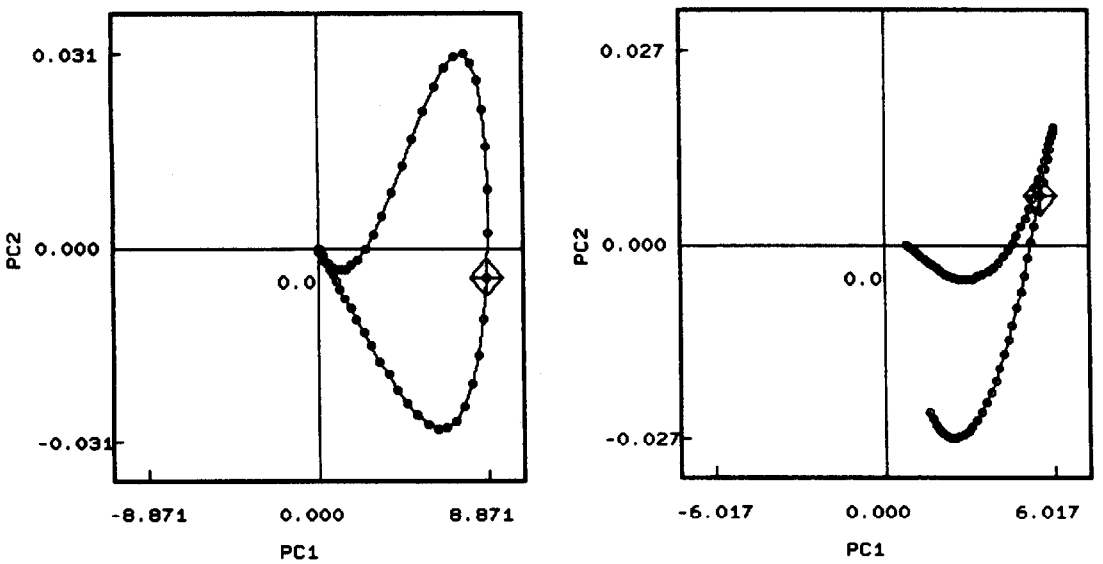


Fig. 7. Latent-projective graphs in time (left side) and wavelength (right side) domain for mixture B.

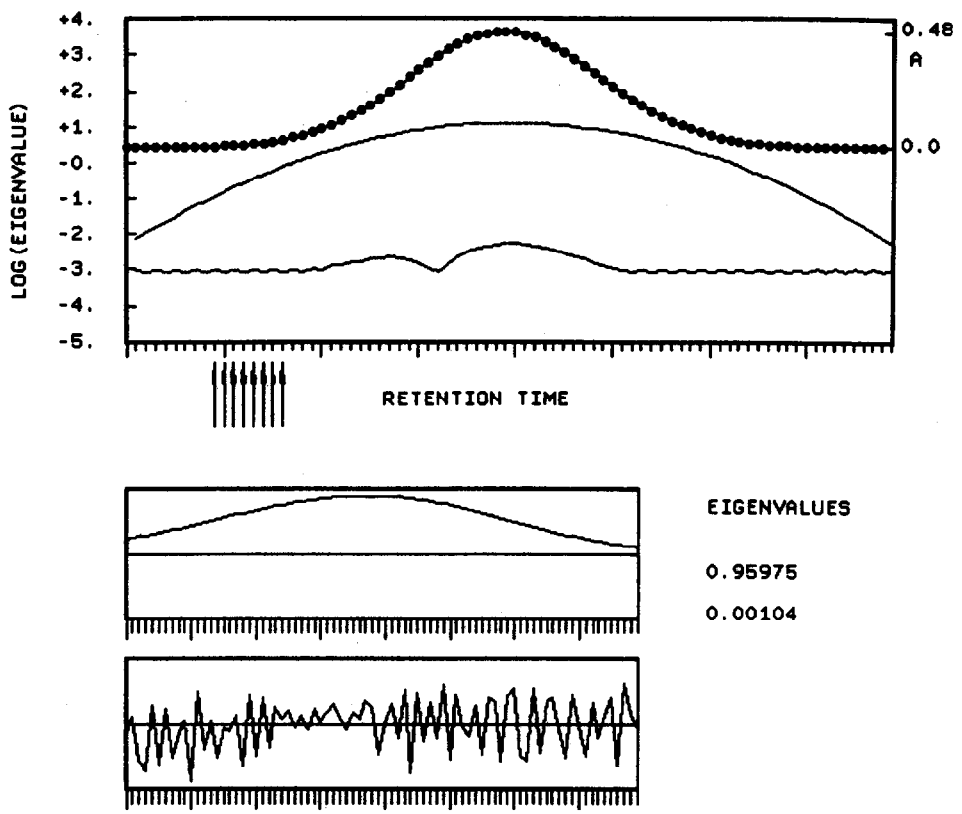


Fig. 8. Eigenstructure tracking analysis (upper part) of system B with window size 2 in the time direction. Local principal component analysis of the retention time interval marked with arrows (selective chromatographic regions for the major analyte) provide the loading patterns and the eigenvalues on the two first principal components shown in the lower part.

loadings on the second one displays a typical noise pattern. Principal component analysis upon the region at the end of elution showed the same picture: a single eluting analyte. As the spectra showed the same absorbances at all wavelengths, they definitely represent the same analyte.

In order to locate the maximum on the chromatographic profile of the major analyte, the original data matrix is differentiated in the time direction. Figure 9 shows the first-order derivated spectra at the two crucial retention times, i .e., times 39 and 40, for the investigated system. These derivated spectra imply that the chromatographic peak maximum of the major analyte is around retention time 40 (see also Fig. 2). The validity of this statement can readily be assessed by visual inspection of the derivated spectra, which reveal that up to retention time 39 the spectra differentiated in the time detection are all positive or partially positive and that after time 40 the spectra in the derivative matrix are negative at all wavelengths. With this information, the chromatographic profiles and spectra of

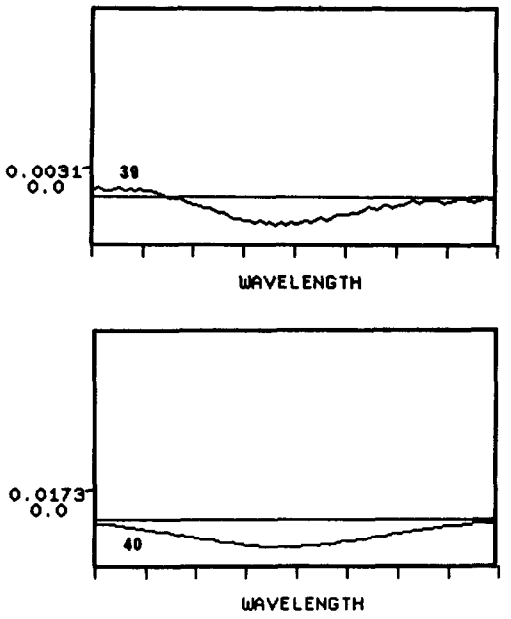


Fig. 9. The derivated spectra at retention times just before (time 39) and after (time 40) peak maximum of the major analyte obtained by differentiation in the time direction for mixture B.

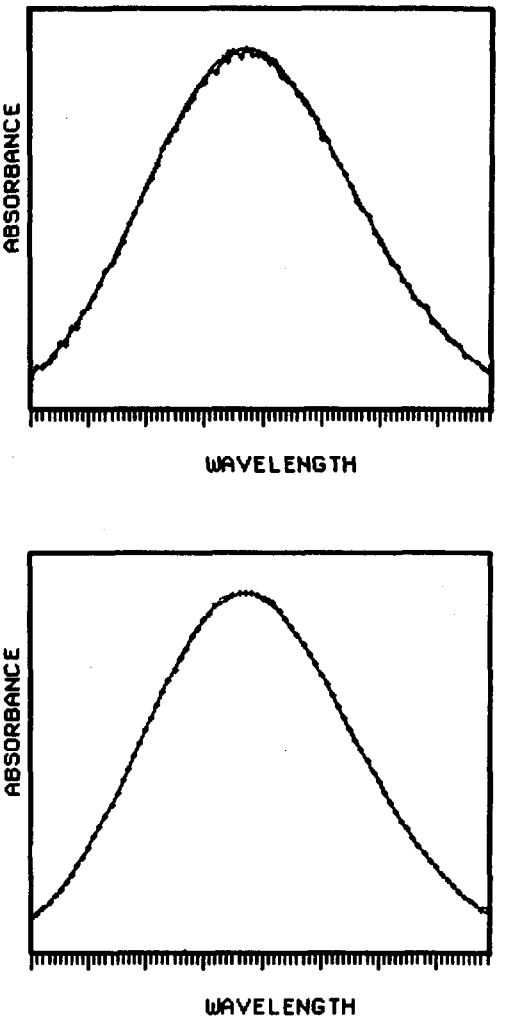


Fig. 10. Estimated spectrum of the minor analyte in mixture B. Upper part shows the first estimate, while the lower part shows the refined spectrum obtained by target transformation.

the analytes can be obtained by either of the two approaches derived in the theoretical section. The results obtained from the direct approach and the optimization approach, respectively, are shown in Fig. 10 and Fig. 11. In the direct approach, the pure spectrum of the minor analyte is first estimated. Figure 10, upper part, shows the first estimate of the minor analyte. The lower part shows the spectrum after refinement by using the target transformation technique. The agreement between the real one and calculated one is excellent.

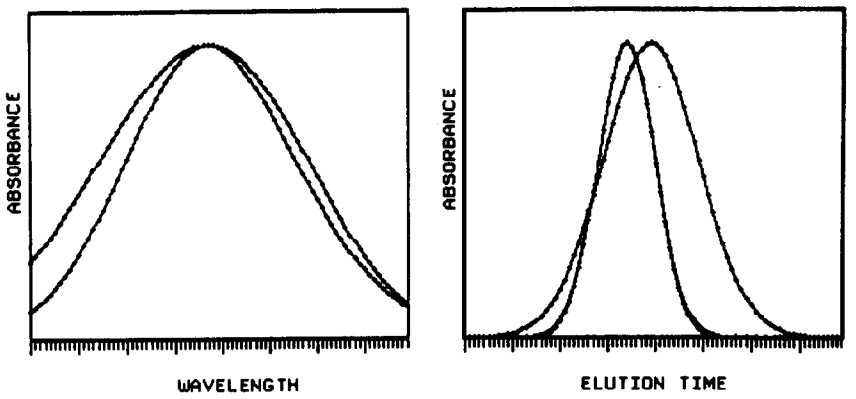


Fig. 11. The spectra (left side) and chromatographic profiles (right side) of the resolved analytes in mixture B using optimization with the retention time of peak maximum for the major analyte as objective function.

Figure 11 shows the estimates of both chromatographic profiles and spectra of the two analytes as calculated by the optimization approach described in the theoretical section. The chromatographic profile of the minor analyte was first estimated by orthogonal projections, then the chromatographic profile of the major one was obtained by optimization using the information about the retention time for peak maximum of the major analyte. The pure spectra of the analytes were obtained by the least-squares procedure defined by Eqn. 17. Interpolation around the retention time of peak maximum of the major analyte was used as described in the theoretical section.

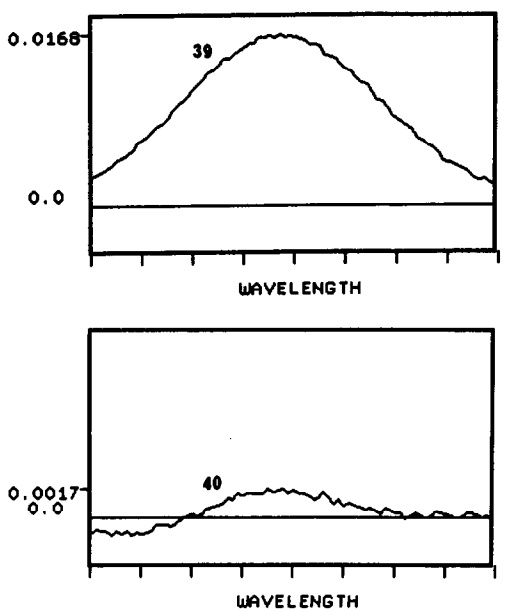


Fig. 12. The derivated spectra at retention times 39 and 40 obtained by differentiation in the time direction for mixture C.

Figure 12 shows the first-order derivative spectra of system C at retention times 39 and 40. The difference observed between Fig. 12 obtained for system C and Fig. 9 obtained for system B is due to the fact that the peak maximum of the minor analyte for system C appears *after* the peak maximum for the major one. Thus, for system C the first-order derivative spectrum in time direction changes from positive at all wavelengths up to time 39 to partial positive after this time, since in the neighbourhood of peak maximum of the major analyte the chromatographic profile of the minor analyte is always increasing. It should be pointed out that by doing the differentiation in time direction backwards, i.e., starting from the last retention time, the reversed order of peak maximum in C can be handled in complete analogy with system B.

Conclusions

In this work, we have shown that chromatography with multiwavelength detection may enable resolution and quantitation of a minor analyte even for the case where the overlap is complete

in both time and wavelength direction. By use of heuristic evolving latent projections [1] the diagnosis of such a case is readily made from visual inspection of the latent-projective graphs in the chromatographic and spectral domain. By utilizing the chromatographic selective regions, the spectrum of the major analyte can then be extracted by principal component analysis of the selective regions. With this information at hand, the problem is changed from one of resolving a "black" multicomponent system into one of resolving a "grey" system [13].

The key to successful resolution of the studied cases is the combination of projections and first-order differentiation. Thus, the present work represents a development and extension of the pioneering work of Milano et al. [4] and Fell et al. [5] on the use of derivatives in wavelength and time direction for resolution enhancement of seriously overlapping spectral and chromatographic profiles.

As the evaluation of the methods derived in this work is performed upon simulated systems, one may ask how the performance will be influenced by real noise. The answer to this is that correlated noise, represented by drifting baselines and systematic spectral background can be removed by analyzing the zero-component regions, i.e., the regions before elution starts and after elution is finished as shown in previous work [14]. This should leave us with random noise which seems to pose no major problem for the derived methods. However, the limit of resolution in the presence of real noise should be established for this case of systems and, indeed, suitable data are now generated in other laboratories [15].

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