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GENERALIZED RANK ANNIHILATION METHOD

II. ANALYSIS OF BIMODAL CHROMATOGRAPHIC DATA

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SUMMARY

When confronted with the problem of overlapped peaks, the chromatographer's approach is to change the chromatographic process. However, this may entail considerable time in methods development, with no guarantee of achieving adequate resolution. The "generalized rank annihilation method" (GRAM) utilizes multivariate statistical methods to process a matrix of calibration data with a matrix containing the test data, and can be applied to the problem of completely overlapped peaks in chromatography.

Chromatographic analyses (both calibration and test) are carried out on either two (or more) columns of different stationary phases or on columns of similar stationary phase, but with different mobile phases. The results of each set of analyses are combined to create single, bilinear matrices (again, calibration and test). Rank annihilation is then performed on the combined matrices, and both the resolved elution profiles and spectra are generated, as well as quantitative information. The presence of components in the test mixture which are not in the calibration mixture does not interfere with the analysis for the desired analytes. Application of GRAM to bimodal liquid chromatography–ultraviolet detection data is demonstrated with simulated and real analyses of polynuclear aromatic hydrocarbons.

INTRODUCTION

The principal goal of chromatography is to resolve the components of a mixture so that they may be individually collected, detected, quantitated or otherwise characterized. When the chromatographic system under employ is incapable of adequately resolving the desired analytes, a suitable alternative must be sought. The chromatographer's traditional approach to inadequate resolution is to modify the chromatographic procedure. This is typically accomplished with a change in stationary or mobile phase. A recent extension of this approach is serial-column chromatography (often called multidimensional chromatography) in which heart-cuts are made, channeling a few selected components to a second column containing a different phase[1].

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True multidimensional techniques, which take advantage of chemometric methods, such as factor analysis, can largely eliminate the need for the additional methods development normally required by the above methods. In order to use factor analysis in single-column chromatography, the chromatographic detector must have multiple channels (*e.g.*, a mass spectrometer or a diode array UV detector). The advantages of chemometric methods of analysis derive from generating data in such a bilinear form, such as gas chromatography–mass spectrometry (*i.e.*, time *vs.* spectral channel).

When little is known about the sample, some form of curve resolution is probably the best multivariate approach[2–9]. However, most curve-resolution routines are limited in the number of components that can be reliably estimated from within a fused-peak set. Regression techniques, such as multiple linear regression and partial least squares[10], also have utility in chromatography and are preferred when all pure spectra are known and the sample has no more components than available pure standard spectra.

If a mixture of calibration standards is available for analysis by the same chromatographic procedures used for the test sample, the generalized rank annihilation method (GRAM)[11], based on rank annihilation factor analysis (RAFA)[12–14] can be used. The upper limit to the number of components which can be handled by current rank annihilation methods is as yet undefined, but is already comfortably beyond the number of components reasonably expected to occur in a single fused-peak —it has been shown that the majority of peaks in a (somewhat complex) chromatographic analysis are likely composed of multiple components[15], but at least 90% should contain fewer than 5 components.

Yet, both curve resolution and GRAM fail to resolve mixtures when component peaks co-elute exactly. A solution to this problem, called third-order chromatography (TOC), has been described[16]. TOC utilizes true multidimensional chromatography, with columns in parallel, to provide resolution of components never resolved in either of the individual chromatographic systems. But, again, as with curve resolution, the number of components which can be analyzed in a single fused-peak set is limited. We now wish to report that rank annihilation methods, when applied to parallel-column chromatographic methods, can solve the generalized overlap problem, for fused-peak sets of multiple components, even when some of the components are totally unresolved. The vehicle used to demonstrate this combination of GRAM and TOC is two-column liquid chromatography. The methods are applicable to other types of chromatography and more than two columns, with increasing effective resolution.

THEORY

Generalized rank annihilation

The method of RAFA has recently been improved, and the new method, called the generalized rank annihilation method (GRAM), provides several distinct advantages[11]: multiple overlapping analytes can be determined simultaneously and results include generation of both spectra and elution profiles, thus allowing quantitation. In addition, GRAM can be utilized in cases of unequal numbers of components in the calibration and test samples, *i.e.*, the test can contain fewer or more components

than the calibration. The description of generalized rank annihilation given here is necessarily brief; for additional details, see ref. 11. The following discussion will obey these conventions: lower case letters for scalars (*e.g.*, x_i); bold, lower case letters for vectors (*e.g.*, $\mathbf{x}_i$); bold, upper case letters for matrices (*e.g.*, **N**); and superscript T for transposed matrices or vectors.

GRAM requires that data be of a bilinear form: a bilinear "spectrum" of a single component can be expressed as the outer product of two vectors. Many analytical techniques which generate multiple channels of information in two domains are considered of bilinear form, such as, for example, the combination of gas chromatography (time domain) and mass spectroscopy (spectral domain). Such a bilinear spectrum, of a pure component, can be represented as a matrix ($\boldsymbol{\mu}_k$), of rank 1:

$$\boldsymbol{\mu}_k = \mathbf{x}_k \mathbf{y}_k^{\mathrm{T}} \tag{1}$$

For bilinear data of the class chromatography–spectroscopy, $\mathbf{x}_k = x_1, x_2, \cdots, x_m$ is a vector which corresponds to the normalized spectrum of the component, where x_i are the intensities of the individual spectral channels and m is the number of wavelengths; and $\mathbf{y}_k = y_1, y_2, \cdots, y_n$ is the normalized concentration profile (*i.e.*, chromatogram) of the component, where y_i are the intensities corresponding to each acquired scan and n is the number of these spectral scans in the chromatogram.

For a mixture of p components, a matrix (**M**) of rank p can be expressed as a linear combination of p matrices $\boldsymbol{\mu}_k$:

$$\mathbf{M} = \sum_{k=1}^{p} \beta_k \boldsymbol{\mu}_k \tag{2}$$

where the β_k are the relative concentrations of the p components. Combining eqns. 1 and 2 yields

$$\mathbf{M} = \sum_{k=1}^{p} \beta_k \mathbf{x}_k \mathbf{y}_k^{\mathrm{T}} \tag{3}$$

which is the basic description, in vector form, of a bilinear data matrix. This can also be expressed in matrix form:

$$\mathbf{M} = \mathbf{X}\, \boldsymbol{\beta}\, \mathbf{Y}^{\mathrm{T}} \tag{4}$$

where the kth column of matrix **X** ($m \times p$) corresponds to the spectrum $\mathbf{x}_k$, the kth row of matrix $\mathbf{Y}^{\mathrm{T}}$ ($p \times n$) corresponds to the chromatogram $\mathbf{y}_k^{\mathrm{T}}$, and $\boldsymbol{\beta}$ is a diagonal matrix ($p \times p$) with $\beta_{kk} = \beta_k$ is the concentration of the kth component.

If **M** is defined as a test sample matrix, then define **N** as a calibration matrix:

$$\mathbf{N} = \mathbf{X}\, \boldsymbol{\xi}\, \mathbf{Y}^{\mathrm{T}} \tag{5}$$

where ξ is a diagonal matrix of concentration factors, similar to $\boldsymbol{\beta}$. As a simplification, assume that **X** and $\mathbf{Y}^{\mathrm{T}}$ are the same for **M** and **N**. These equations will still be valid even if the samples do not contain exactly the same mixture of components. If some components are not present in one of the samples, eqn. 4 (or 5) still models the

data so long as the corresponding diagonal elements of β (or ξ) are zero.

Thus, by rearranging and multiplying eqn. 4 by ξ and eqn. 5 by β,

$$\mathbf{X}\,\beta\,\xi = \mathbf{M}(\mathbf{Y}^{\mathrm{T}})^{+}\xi \tag{6}$$

$$\mathbf{X}\,\xi\,\beta = \mathbf{N}(\mathbf{Y}^{\mathrm{T}})^{+}\beta \tag{7}$$

where $(\mathbf{Y}^{\mathrm{T}})^{+}$ represents the pseudoinverse of the matrix $\mathbf{Y}^{\mathrm{T}}$. Thus,

$$\mathbf{M}(\mathbf{Y}^{\mathrm{T}})^{+}\xi = \mathbf{N}(\mathbf{Y}^{\mathrm{T}})^{+}\beta \tag{8}$$

Of the terms in eqn. 8, **M**, **N** and ξ are known, therefore, we must solve for $(\mathbf{Y}^{\mathrm{T}})^{+}$ and β. The solution to this matrix system can be expressed as an eigenvalue–eigenvector equation for which a set of four discrete cases has been defined[11].

Bimodal chromatography

When two components cannot be resolved with a particular chromatographic system, such as in liquid chromatography, it is usually possible to modify the resolution by either a change in mobile phase or a switch to a different stationary phase. However, if the components are totally unresolved —which the analyst may be unaware of— prescription of an alternate system by adjustment of selectivity parameters (*cf.* ref. 17) is not possible. Even if co-elution could be detected, solving this problem chromatographically would likely entail considerable effort in methods development, especially if the desired goal is total resolution of the components.

If the two components considered above actually comprise only a small part of an otherwise complex mixture, it may occur that, after finally achieving the desired separation, one of the components now overlaps a third component; in the worst case, the overlap would again be total. This latter situation is pictured in Fig. 1a and c. If the analyst was certain that the new separation produced some, if not complete, separation of the components, deconvolution could be attempted by a curve resolution technique (if there were not too many components present). GRAM can be used to accomplish the deconvolution, regardless of the degree of overlap.

As described above, GRAM compares the bilinear data generated from an analysis of a calibration sample and an analysis of a test sample. The procedure is not dependent on the structure of the data array so long as it is bilinear in form (*i.e.*, detector response is a function of both time and wavelength). If an attempt was made to apply GRAM to a calibration and sample having chromatographic profiles like those in Fig. 1a and b, only two components would be recognized: pure C as one component and the mixture of A and B as a second "component". Similarly, the samples having profiles like those in Fig. 1c and d would give equally misleading results. However, if the data arrays from both profiles were added, GRAM could predict correct results because there would then be sufficient information to identify the principal components, corresponding to the three chemical components in the data. The result of combining the data from two profiles would be a single chromatogram containing two peaks for each component —thus the name "bimodal chromatography"— and would appear as shown in Fig. 1e and f.

If, in fact, GRAM was consistently successful in resolving those components which were overlapped, as shown in Fig. 1, then the generalized co-elution problem would be solved. However, when the same two components co-elute exactly in both

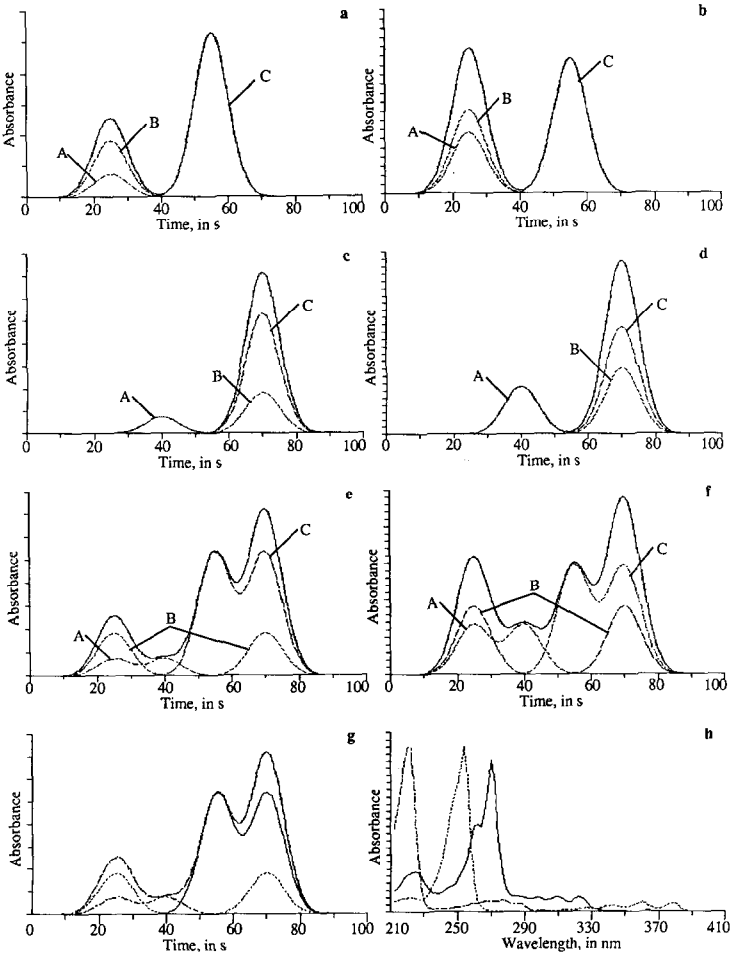

Fig. 1. Results of simulation T1A: a three-component mixture with two components overlapped (see Table I for details). (a) and (b) are from chromatographic column 1, (c) and (d) are from chromatographic column 2; (a) and (c) represent the test sample, while (b) and (d) represent the calibration sample; (e) and (f) are the corresponding bimodal chromatograms; (g) is the set of GRAM-resolved elution profiles of the test sample, and (h) the corresponding extracted spectra. The chromatograms are labeled for clarity: profile A = naphthalene, B = anthracene and C = chrysene. Upper chromatographic trace in each plot is the total wavelength chromatogram (TWC). Also, for the results in (g) and (h), ———— = naphthalene, - - - - = anthracene and —— = chrysene.

chromatographic sysems, the mixture will be recognized as a single component and deconvolution will be unsuccessful. Similarly, if two components have identical spectra, as certain isomers would, then the method will again fail to produce the correct results. Resolution of the underlying profiles in these two scenarios cannot be achieved without additional information. If the analyst is certain that there are only two components *and* the pure spectrum for each is accessible and different, then a regression technique such as partial least squares or principal components regression[10] will likely succeed.

This report describes the feasibility of applying GRAM to bimodal chromatographic data. Data are acquired from two different chromatographic systems simultaneously and combined to form one data matrix. A single calibration standard is prepared from a mixture of known analytes. The procedure then operates on both the calibration and sample data acquired using the same conditions, yielding the following results: elution profiles of all components, overlapped or not, are resolved; spectra for each resolved component are generated; and, quantitation of each component in the sample also present in the calibration is achieved.

EXPERIMENTAL

Equipment

The chromatographic hardware consisted of two Beckman (Berkeley, CA, U.S.A.) 114M pumps, a Beckman 340 μflow mixer, a Valco (Houston, TX, U.S.A.) 10-port valve used for solvent switching, another Valco 10-port, electrically-actuated injection valve fitted with two 10-μl injection loops, and two Hewlett-Packard (Palo Alto, CA, U.S.A.) 1040A diode array detectors. A single detector multiplexed between the two columns could also be used with some advantage. Program control was provided by a Beckman 421A LC controller, while data acquisition and storage were accomplished via Hewlett-Packard 85B computers and 9121 dual floppy disc drives.

Reagents

The mobile phase solvents, UV-grade acetonitrile and water, were obtained from Burdick & Jackson (Muskegon, MI, U.S.A.). Polynuclear aromatic hydrocarbon (PAH) standards were purchased from Chem Services (West Chester, PA, U.S.A.), and included: benz[*a*]anthracene (B*a*A), naphthalene (Naph), anthracene (Anth), chrysene (Chry), dibenz[*a,h*]anthracene (DBAn), benzo[*e*]pyrene (B*e*P), benzo[*b*]fluoranthene (B*b*Fl), benzo[*k*]fluoranthene (B*k*Fl), indeno[1,2,3-*c,d*]pyrene (InPy), and perylene (PER).

Procedures

Two 5-μm C_8, 150 × 4.6 mm columns (Brownlee, Santa Clara, CA, U.S.A.) were used in the liquid chromatographic (LC) analyses. The chromatographic analyses followed a basic procedural outline. An events table was first created in the 421A controller, including mobile phase flow-rate, time of injection, and time for initiating data acquisition. The mobile phases were pumped through the columns independently, and sample injections were made into both of two parallel columns simultaneously by using the 10-port injection valve. Calibration and test samples were analyzed in exactly the same manner, preferably on the same day, to minimize error in retention time reproducibility.

The diode array detectors were operated in "periodic spectra" mode, in which full spectral scans from 210–400 nm, with a bandwidth of 2 nm, were acquired at a rate of *ca.* 1 scan/s. Data acquisition was initiated upon a command from the 421A controller and was terminated at the time entered for stop-time in the HP 85B system.

Computation

Raw data, stored on disc, were translated to ASCII format, then transferred to a Micro VAX II system (Digital Equipment Corporation, Marlboro, MA, U.S.A.). The data processing routines were implemented on the Micro VAX station, and included routines for generating simulated chromatograms; for creating simulations of bimodal chromatograms; a principal components regression routine which allows verification of underlying chromatographic profiles based on a knowledge of the input components; a program to adjust retention times of the calibration and test samples[18]; and GRAM[11].

DISCUSSION

Simulations

To demonstrate the feasibility of implementing GRAM for parallel-column

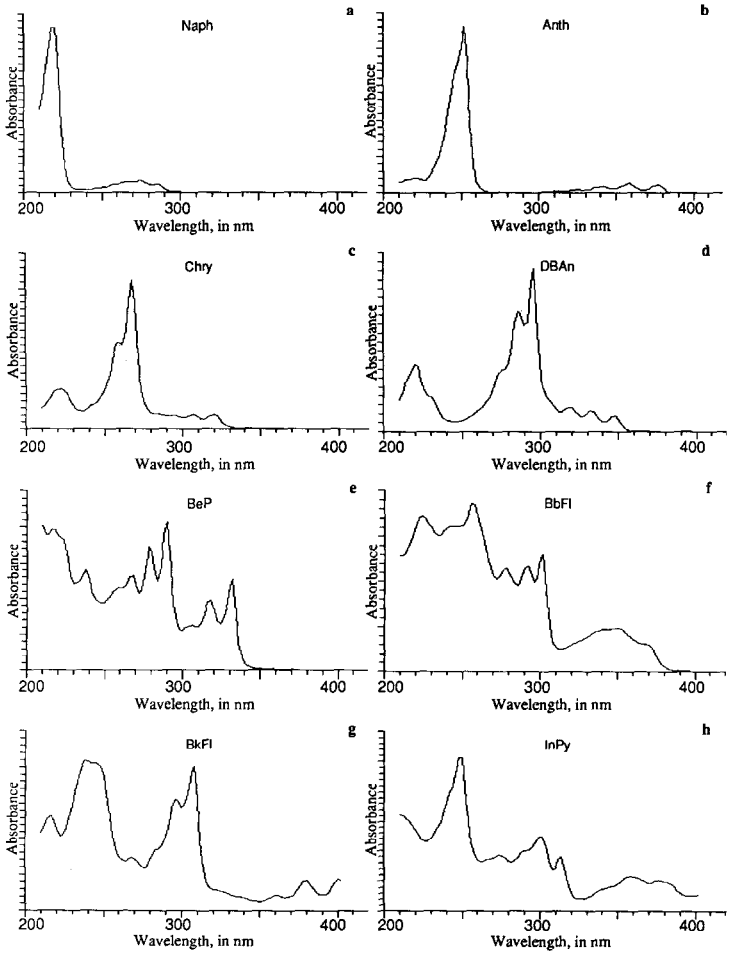

Fig. 2. Pure spectra of PAH standards used in the GRAM studies.

LC–UV data, simulations of chromatograms were generated which were designed to include several possible scenarios of varying complexity in overlap. The chromatograms consisted of Gaussian concentration profiles, which were created from authentic spectra stored in the data system. Fig. 2 shows the pure spectra used in the simulations. Sample systems consisting of three and four components were assembled, although GRAM has been successfully used for systems containing many more components[19].

In the simple three-component case, the worst-case co-elution problem can be described as follows: two components elute at exactly the same retention time on the first column, and one of those components co-elutes with a third component when the sample is analyzed on a second column (see Fig. 1). For four components, overlaps can occur in three basic ways: two components may be overlapped on one column, and two other components on a second; two pairs of components overlap on the first column, and a pair from each of these might overlap on the second; or three components overlap on the first column, while one of these and a fourth component overlap on a second column. Tables I and II summarize the simulations created for this study. Note that other, more complex, situations of multiple overlaps can occur (*e.g.*, four components totally overlapped on the first column and two on the second), but these cannot be solved completely, as discussed earlier, because some combination of two or more components are never resolved from each other on any of the columns.

In real LC, one would expect that components would elute with substantially different retention times when columns with two different stationary phases are used. To minimize computation time and facilitate visual interpretation of the data, the simulations were made so that retentions for the two columns would be of similar capacity factors (k') where $k' = (V_R - V_0)/V_0$ (V_R is the analyte retention volume and V_0 is the column void volume).

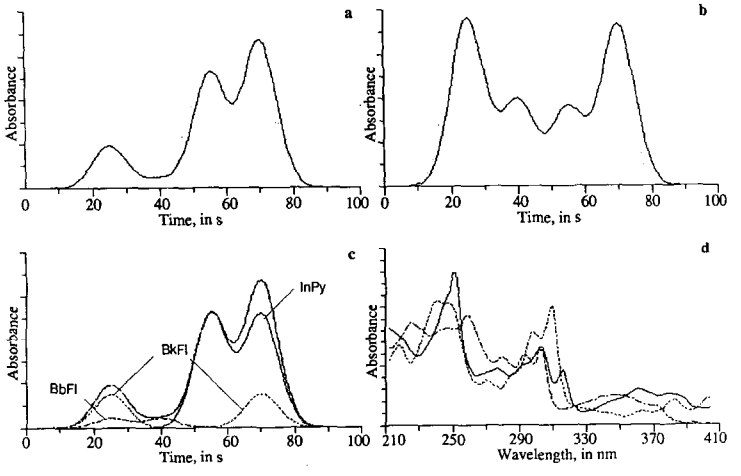

Fig. 3. Results of simulation T1ANL: a three-component mixture with two components overlapped, with noise and wide concentration range (see Table II for details): (a) and (b) bimodal TWC of sample and calibration, respectively; (c) GRAM-resolved elution profiles; (d) extracted spectra: ——— = B*b*Fl, - - - - = B*k*Fl, —— = InPy.

Variations can be envisioned in retention patterns for each of the cases outlined above, in which overlaps also occur in the bimodal representation, *i.e.*, peaks from the first column have exactly the same retention as other peaks from the second column. Although these are admittedly worst-case scenarios and unlikely to occur under normal chromatographic conditions, they serve to demonstrate the power of the GRAM technique in handling complex situations as well as to help identify the point at which the technique begins to fail. The various combinations used in the simulations are shown in Tables I and II.

Lack of reproducibility of retention times can pose problems for rank annihilation, because the technique relies on matching elution profiles of the (predicted) components as well as their spectra. A solution to this problem has been proposed[18], and this technique was used to correct retention times in the real analyses before the data matrices from the two columns were added.

Resolution *per se* is not a factor in trying to understand the limitations of GRAM with bimodal LC–UV data. In fact, if there is *any* significant chromatographic resolution, then deconvolution can be achieved by applying GRAM to data from a single column[19]. Evaluation of other constraining parameters should include spectral similarity, relative concentration and noise.

Evaluation of concentration effects

Simulation samples consisted of components present in various relative amounts. The concentration factors had little or no effect on the accuracy of the results, including cases in which the components were in a ratio of 1:4:16 (see simulations T1AL and T1ALN in Tables I and II). Even when noise of 1%, relative to an average peak height (and Gaussian distributed), was added, the results were accurate (see Fig. 3): the correlation coefficient computed between each estimated spectrum and its known pure spectrum was in every case greater than 0.9999, and the error in estimating the concentration of the test components was never greater than 1%.

Evaluation of noise effects

A number of the simulations were run with no noise added and the predictions which were produced by GRAM were accurate in every case. For example, the bimodal profiles for the sample in simulation T1A (Fig. 1e) were compared by GRAM to the corresponding calibration sample (Fig. 1f). The predicted profiles for the sample, shown in Fig. 1g, are essentially perfect.

Various amounts of noise were added to other simulations, the greatest being 4% (see Table II). Only at this high level of noise, rather unrealistic for usual chromatographic conditions, were the spectral correlations lower than 0.999. Nevertheless, the basic spectral structures predicted in this case differed from the true spectra only because of the unusual amount of added noise. The remaining simulations, in which noise was added, yielded accurate spectral predictions when computed with GRAM (see Table II). The concentrations, when compared to the input concentrations, differed from the true value by amounts varying from 0.33 to 0.88%.

Evaluation of spectral similarity and resolution

To evaluate the effects that similar spectra might have on the results predicted

TABLE I

DESCRIPTION OF SIMULATIONS AND PREDICTED RESULTS FOR CHROMATOGRAPHIC PROFILES OF DISSIMILAR SPECTRA

Simulation	*No. of components*	*No. of overlaps*	*Noise (%)*	*Retention times (s)*								*Average correlation*	*Avg. error (%) concentration*
				Column 1 components				*Column 2 components*					
				Naph	*Anth*	*Chry*	*DBAn*	*Naph*	*Anth*	*Chry*	*DBAn*		
Concentration ratios (Naph:Anth:Chry:DBAn): calibration 1:1.1:1.2:1.3; sample 2:1:3:2													
F1A	4	2	–	20	20	35	50	30	45	60	60	1.0000	0.00
F1B	4	2	–	20	20	35	50	30	50	60	60	1.0000	0.00
F1C	4	2	–	20	20	35	50	35	50	60	60	***	***
F1CR	4	2*	–	20	20	35	50	34.5	50	60	60	1.0000	0.00
F1CJ**	4	2	–	20	20	35	50	35	50	60	60	1.0000	0.00
F2A	4	2 pair	–	20	20	35	35	30	45	45	55	1.0000	0.00
F2B	4	2 pair	–	20	20	35	35	35	45	45	55	1.0000	0.00
F2C	4	2 pair	–	20	20	35	35	20	35	35	50	***	***
F3A	4	3	–	40	40	50	40	30	45	45	55	1.0000	0.00
Concentration ratios (Naph:Anth:Chry): calibration 0.9:1:1.1; sample 1:2:4													
T1A	3	2	–	25	25	55		40	70	70		1.0000	0.00
T1B	3	2	–	25	25	55		40	55	55		1.0000	0.00
Concentration ratios (Naph:Anth:Chry): calibration 0.9:1:1.1; sample 1:4:16													
T1AL	3	2	–	25	25	25		40	70	70		1.0000	0.00

* Some inter-column resolution ($R_s \approx 0.025$).

** Matrices adjoined rather than added.

*** Erroneous results: only three components found.

TABLE II

DESCRIPTION OF SIMULATIONS AND PREDICTED RESULTS FOR CHROMATOGRAPHIC PROFILES OF SIMILAR SPECTRA

Simulation	*No. of components*	*No. of overlaps*	*Noise (%)**	*Retention times (s)*								*Average correlation*	*Avg. error (%) concentration*
				Column 1 components				*Column 2 components*					
				BeP	*BbFl*	*BkFl*	*InPy*	*BeP*	*BbFl*	*BkFl*	*InPy*		
Concentration ratios (BeP:BbFl:BkFl:InPy): calibration 1:1.1:1.2:1.3; sample 2:1:3:2													
F1AN	4	2	1	20	20	35	50	30	45	60	60	0.9997	0.50
F1A4N	4	2	4	20	20	35	50	30	45	60	60	0.9972	4.30
F1BN	4	2	1	20	20	35	50	30	50	60	60	0.9998	0.50
F2AN	4	2 pair	1	20	20	35	35	30	45	45	55	0.9999	0.58
F2BN	4	2 pair	1	20	20	35	35	35	45	45	55	0.9999	0.88
F3AN	4	3	1	40	40	50	40	30	45	45	55	0.9999	0.50
Concentration ratios (BbFl:BkFl:InPy): calibration 0.9:1:1.1; sample 1:2:4													
T1AN	3	2	1		25	25	55		40	70	70	1.0000	0.50
T1BN	3	2	1		25	25	55		40	55	55	1.0000	0.33
Concentration ratios (BbFl:BkFl:InPy): calibration 0.9:1:1.1; sample 1:4:16													
T1ANL	3	2	1		25	25	55		40	70	70	0.9999	0.40

* Noise relative to signal, based on a peak of arbitrary intensity = 1, and Gaussian distributed.

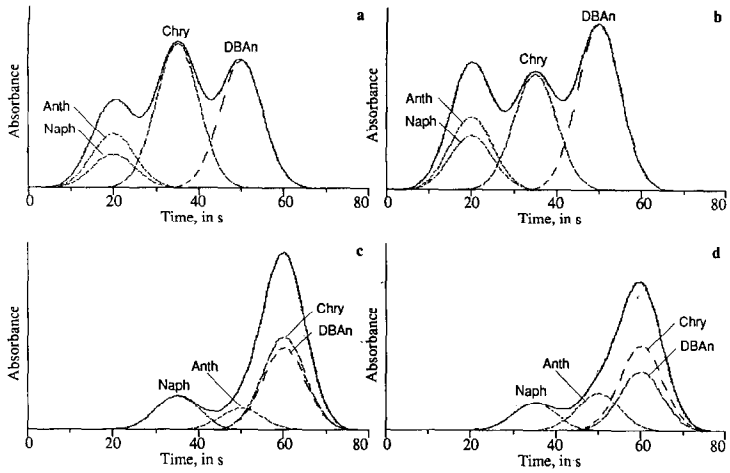

Fig. 4. Chromatographic profiles used in simulations in F1C series (Fig. 5): (a) and (c) two simultaneous analyses of test sample; (b) and (d) two simultaneous analyses of calibration sample. Components are shown; upper trace in each case is the TWC.

by GRAM, simulations were made with spectra that were both dissimilar and similar. Similarity was measured by computing the correlation coefficient between various spectra in our laboratory's spectral file. The most and least similar spectra were then chosen: dissimilar spectra chosen had inter-spectral correlations which varied from 0.15 to 0.47, while similar spectra were in the range 0.78 to 0.95. In addition, cases were examined in which various numbers of overlaps, between components within a column and between components on the different hypothetical columns, were prepared.

For the simulations F1A, F1B, and F1C, the same degree of overlap is present within each chromatographic process (see Fig. 4), but the amount of confounding between the two processes due to retention time overlap is varied. The correct predictions were made by GRAM for the first two cases, but simulation F1C failed. This occurred because the number of overlaps between columns (in addition to those within a given column) became excessive; as a result, GRAM predicted only three components (see Fig. 5a), whose spectra were actually linear combinations of the true spectra (Fig. 5b). Similarly, the additional between-column confounding present in simulation F2C also proved unsolvable, while the predictions for the related simulations F2A and F2B were accurately computed by GRAM (see Table I).

If the inter-column overlap could be avoided, then GRAM should be able to make the correct predictions. The inter-column overlap of one pair of components was reduced by only a small amount (equivalent to a resolution of *ca.* 0.025), and the system regained its ability to predict spectra and concentrations correctly, as shown in Fig. 5c and 5d.

Initial attempts to analyze real experimental data with GRAM were largely unsuccessful. The confounding of information between columns when the data matrices were added proved to be limiting. This problem of confounding was eliminated when data sets were adjoined rather than added. For example, the simulation which earlier failed, F1C, was modified by recombining the data sets from the two

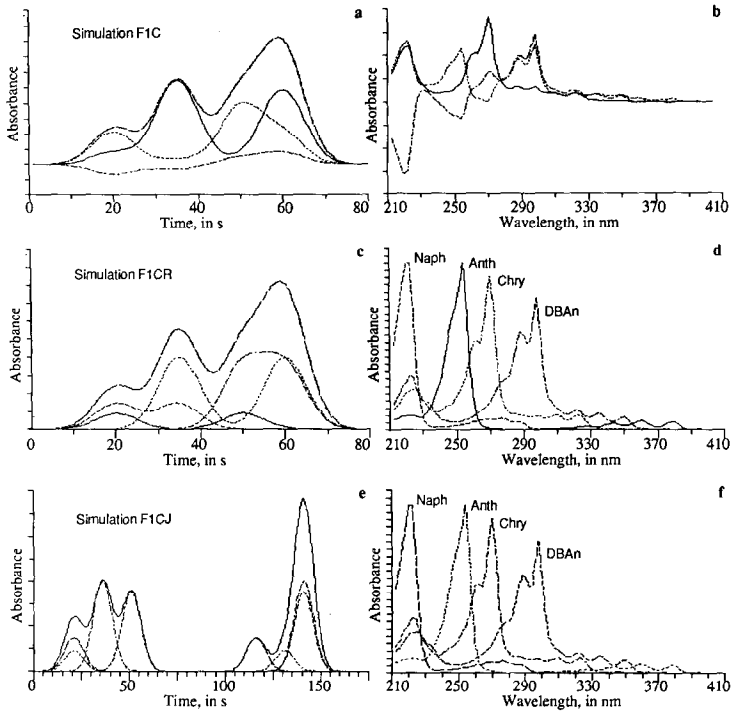

Fig. 5. Results of related simulations F1C, F1CR and F1CJ: all use a four-component mixture with two components overlapped. F1C also has two overlaps between columns; in F1CR, one overlap between columns has R_s = 0.025; F1CJ has matrices adjoined rather than added. (a) GRAM-resolved elution profiles of test sample in F1C and (b) extracted spectra. The spectra generated are actually linear combinations of the pure spectra. (c) GRAM-resolved elution profiles of test sample in F1CR and (d) corresponding extracted spectra. (e) GRAM-resolved elution profiles of test sample of F1CJ and (f) corresponding extracted spectra.

chromatographic processes: the data sets were adjoined rasther than added, yielding new matrices. The change was dramatic: results computed by GRAM for the adjoined matrices resulted in accurate predictions for both the spectra and concentrations (see Fig. 5e and f).

Experimental results

Two samples, containing different amounts of several PAHs, were analyzed by the bimodal chromatographic approach, using GRAM for the deconvolution. Simultaneous analyses of each sample were made on two parallel columns, with water–acetonitrile (5:95) used in the first column and water–acetonitrile (24:76)in the second. The total wavelength chromatograms for these samples are shown in Fig. 6a–d. After determining the appropriate adjustment to retention times, the two analyses of the calibration sample were joined into a single matrix, and the test sample analyses were joined into another (Fig. 6e). Analysis was made by GRAM (see Table III), and the resolved profiles for the test sample are shown in Fig. 6f. In the first portion of the chromatogram, corresponding to the analysis on the first column, the peaks for B*b*Fl

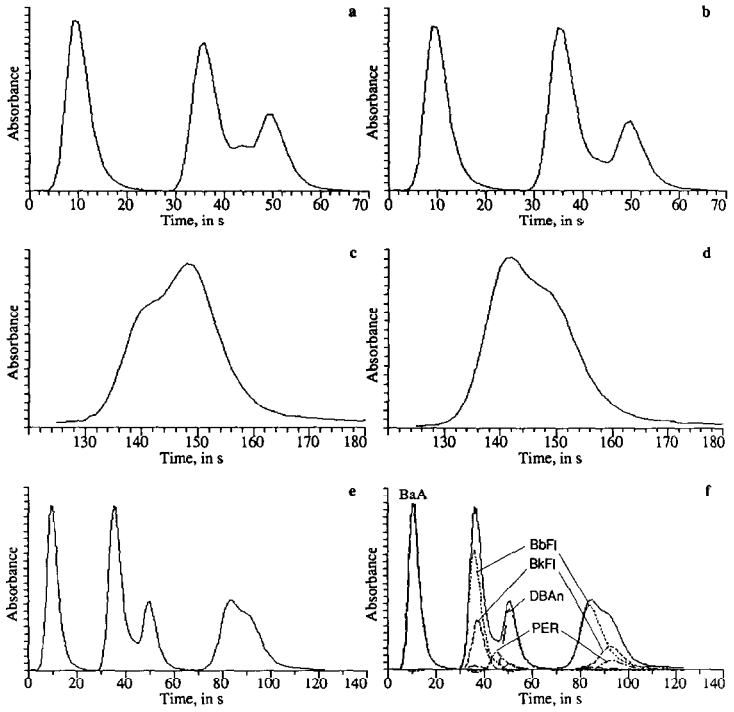

Fig. 6. Results of LC–diode array-UV analysis of PAH mixture (four components with two components overlapped): (a) and (b) analyses with first chromatographic system: mobile phase water–acetonitrile (5:95); (c) and (d) analyses with second chromatographic system: mobile phase water–acetonitrile (24:76). (a) and (c) are analyses of the calibration sample, while (b) and (d) are the analyses of the unknown sample. (e) is the total wavelength bimodal chromatogram of the adjoined matrices from (b) and (d). (f) shows the resolved elution profiles of the adjoined data from the unknown sample.

and B*k*Fl are almost completely overlapped ($R_s \approx 0.09$), while in the last portion, corresponding to analysis on the second column, B*k*Fl overlaps with PER ($R_s \approx 0.003$). Although this degree of overlap could not be adequately resolved by multivariate curve-resolution techniques on a single-column data matrix, and in fact might not be noticed by the chromatographer without prior information, it was possible to

TABLE III

RESULTS OF ANALYSIS OF POLYNUCLEAR AROMATIC HYDROCARBONS BY LC–DIODE ARRAY-UV AND GRAM

Compound	*Concentration in calibration (ng/μl)*	*Concentration in sample (ng/μl)*	*Concentration predicted for sample (ng/μl)*	*Correlation of predicted spectrum*
B*b*Fl	6.00	10.00	8.57	0.9991
B*k*Fl	4.12	3.09	2.99	0.9982
PER	5.40	3.24	3.02	0.9957

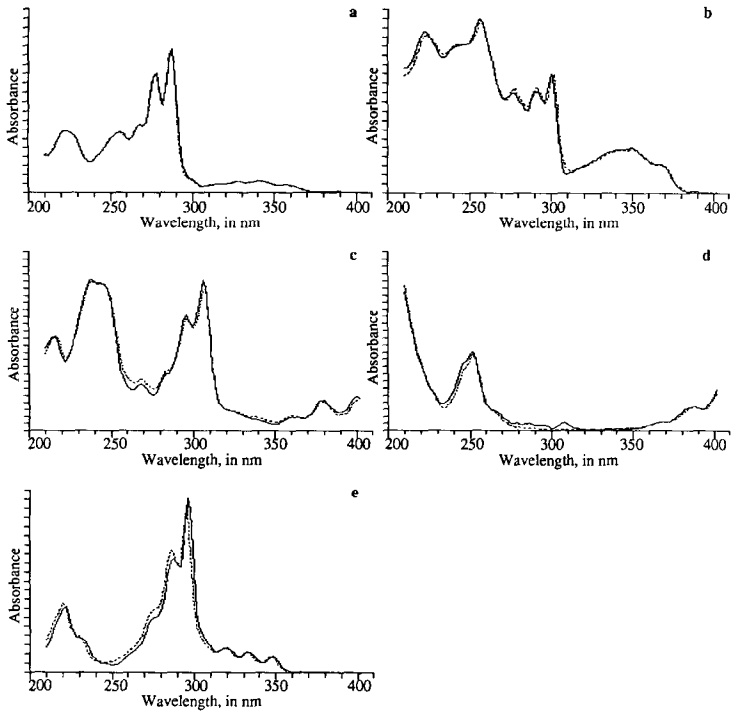

Fig. 7. Reconstructed spectra from analysis of PAH mixtures (see Fig. 6). (a) B*a*A, used as a reference standard, (b) B*b*Fl, (c) B*k*Fl, (d) PER and (e) DBAn. ——, predicted spectra; -----, spectra from pure standards.

perform the deconvolution with bimodal chromatography and GRAM analysis. The spectra which were extracted by the procedure are shown in Fig. 7, overlayed with the corresponding pure spectrum. The calculated correlations between the extracted and pure spectra (see Table III) are very reasonable considering the minimal resolution between components. This accuracy in extracting profiles and spectra is particularly striking for B*k*Fl which was not resolved on either column.

The overlapping components in this sample could have been partially resolved, on the selected column, by using a mobile phase composition midway between those chosen for the analysis. Complete resolution, however, would be unlikely without changing columns. The distinct advantage which bimodal chromatography–GRAM offers in such a situation is that a only single analysis of both a calibration and a test sample is required. Rather than devote valuable resources to seek an optimal *chromatographic* resolution of the desired analytes, GRAM provides a *mathematical* resolution of chromatographically unresolved components, and with minimal effort. Although the data resulting from analysis on one column may, by chance, be adequate to the needs of the analyst, there is no guarantee that this will always occur. By choosing a system of appropriately different chromatographic parameters for the bimodal analysis (such as very different mobile phases) such that resolution of the desired analytes is sure to be *different,* even if not complete, GRAM can then be called upon to supply the necessary resolution, without concern for the degree of separation.

CONCLUSIONS

GRAM has proven to be a powerful means of solving the problem of unresolved components in chromatography. When coupled with chromatographic analysis employing columns in parallel, GRAM can accurately predict spectra and concentrations of components that are totally overlapped. A bimodal chromatogram, a result of third-order chromatography, has a much lower probability of having both peaks for one component completely overlap with the two peaks of a second component. The approach has greater intrinsic informing power than single-column chromatography. However, due to the extreme cases of overlap investigated in the foregoing simulations, bimodal chromatography can become visually crowded, with two peaks per component, and some failures can be expected. By adjoining the matrices from each chromatographic analysis instead of simply adding them, this confounding problem can be eliminated, at the expense of increased computation time. The simulations described in this paper represent a minimum performance level for GRAM when applied to third-order chromatography. These preliminary experiments indicate that the adjoined-matrix approach will be favored in actual practice. Additional studies on applying bimodal chromatography and GRAM to real environmental samples are already underway and will be reported in a future communication.

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