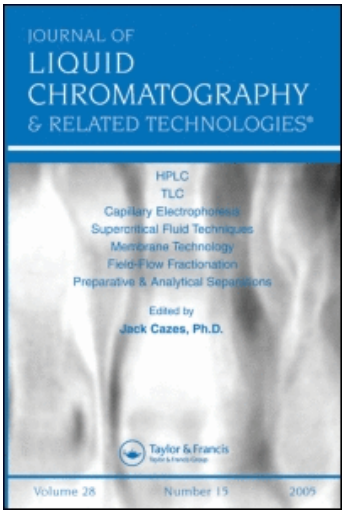


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# AUTOMATED PEAK RECOGNITION FROM PHOTODIODE ARRAY SPECTRA IN LIQUID CHROMATOGRAPHY

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ABSTRACT

The use of multivariate computation techniques for the treatment of data from multi-wavelength photo diode-array detectors is an important step in computer-guided optimization strategies in HPLC. The treatment should provide the user with data on the identity and retention times of the individual solutes. These data are essential for those optimization strategies that can be classified as "interpretive"-methods, since peak recognition of both pure peaks and overlapping peak systems is a prerequisite for these methods. The peak recognition is based on comparison of spectra using their correlation coefficient.

Three multivariate techniques used for deconvolution of overlapping peaks and determination of spectral information on the individual components are reviewed in this paper: Multi-component analysis, Target Factor Analysis and Iterative Target Transformation-Factor Analysis. The nature of the information and the quality of the results of the different multivariate techniques strongly influences the final result of the optimization procedure.

## INTRODUCTION

During the last years, different optimization procedures for reversed-phase high performance liquid chromatography (HPLC) have been developed (1,2). In general, systematic changes in selectivity have been explored by manipulating the mobile phase composition. This parameter has a large influence on retention and selectivity and is in general easy to vary. It is also possible to enlarge the dimensionality of the parameter space and to involve other factors such as the type of stationary phase, the temperature, the pH of the mobile phase, etc.

The definition of an optimization criterion is another difficult problem which has been addressed elsewhere (1). Here we will deal with the exact determination of the coordinates of the optimum in the vector space. An important point is the total amount of information already available on the sample. It is preferable to know the identity of the solutes present in the sample. Another important issue is the number of experiments one is willing to perform in order to locate the optimum.

The current optimization strategies can be divided into two classes. On the one hand we can distinguish the "non-interpretive"-methods. Different chromatograms of a sample are considered individually. The criterion-value is the only important value used to represent a chromatogram.

Rather than trying to predict the behaviour of the criterion over the parameter space, attention is focussed on its value in consecutive chromatograms. One typical method belonging to this group is the "brute force"-technique where a great number of predetermined grid points are measured and the one with the highest criterion value is selected. Another approach is the simplex-optimization strategy, which again operates without any model as far as the retention behaviour is considered and uses the results of earlier measured chromatograms to set the parameters for the next run.

Alternatively, one can make use of all the available knowledge on the chromatographic behaviour of the solutes, by applying so called "interpretive"- methods. The starting point is a model that can be understood and continuously updated. The retention behaviour of the solutes in the sample and their retention time is important rather than the criterion-value. On the basis of the expected retention behaviour, chromatograms can be calculated for different mobile phase conditions and the separation quality can be predicted. Again, two mainstreams can be considered: one can try to predict the "real" retention behaviour with sufficient accuracy from a quadratic model fitted to a sufficiently large number of experiments, or one can start from a few initial experiments and use a simple linear model which is updated after each new experiment. The position of the initially calculated optimum is then continuously adjusted on the basis of the new data.

An example of this iterative approach is given in figure 1 (3). The lower part of the figure represents the predicted retention behaviour from chromatograms 1 and 2 (see the chromatograms on the left and right of the figure). The upper part of the figure shows the quality of chromatograms calculated over the whole range of mobile phase compositions assuming linear retention behaviour (in this case the applied criterion is the resolution of the least separated peak pair, Rsmin). It should be clear that in order to calculate the criterion-value over the parameter space, the identity of the peaks in the two chromatograms must be matched. This means that the numbering of the peaks as given in chromatogram 1 should also be known for chromatogram 2, i.e. it is necessary that we are able to determine which peak in both chromatograms correspond to the same compound as indicated by the numbering in Fig.1.

This paper will concentrate on peak recognition techniques. We intentionally use the term "peak recognition" in stead of "peak identification" because, primarily, we are not so much interested

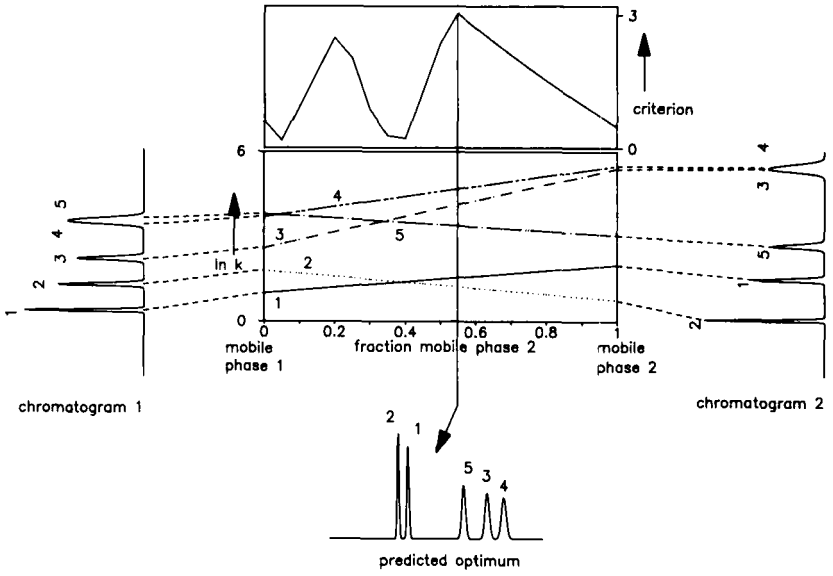


FIGURE 1 The first step in the iterative optimization procedure. Based on the (simulated) chromatograms A and B, capacity factors k are estimated for all components (1-5) in mixtures of mobile phases A and B. The location of the optimum is determined after evaluation of the predicted chromatograms on the basis of a criterion, in this case a minimum resolution observed in the predicted chromatograms.



in the identity of the solutes as in their retention behaviour. This process is also known as "peak-tracking".

## METHODS FOR PEAK RECOGNITION

When the composition of the sample is completely known, the retention behaviour of the individual solutes can be determined by separate injections, a rather time-consuming procedure. If we are faced with an unknown sample (not necessarely completely unknown) or, for time-saving reasons, want to inject the sample as it is, then it is necessary to use some form of peak tracking. Peak

tracking must be done by means of specific detection methods as illustrated in figure 2. The simplest form of peak recognition is applied in one-dimensional detection systems using a single wavelength or refractive index detection and considering peak area as being specific for the different solutes (fig. 2A). This method can be applied when changes in experimental conditions (mobile phase influences on absorption characteristics) do not influence the detection characteristics (peak area) too much. Apart from the fact that within one chromatogram several peaks can have almost the same area, and that the experimental conditions do have an influence on the spectral properties, peak overlap will cause severe problems. A possible solution can be found in using curve-fitting procedures with a predetermined peak model (4). The reliability of this method can be improved by using the "fuzzy-set"-theory (5).

It is possible to increase the specificity of the detection considerably by using more wavelengths and/or different detectors. The most extreme example is the use of a variable wavelength detector at a certain wavelength which is specific for one solute and does not detect the others. Such a detection is unambiguous. The use of two detectors at different wavelengths will increase the specificity considerably. The absorbance ratio over a peak consisting of a pure solute is a constant value independent of the concentration and only dependent on the absorptivities at the wavelengths of interest. The so-called ratio method (see fig. 2B) can be used as peak recognition technique because the value of the ratio is a unique quantity. However, this method also suffers from some serious drawbacks (6). For instance, it is difficult to estimate the ratio-value in the case of severe peak overlap.

An obvious extension of this method is to use a multi-wavelength detector like the linear photo diode array detector (LPDA) (7). For fully separated peaks the characteristic spectra can be determined easily and compared with the spectra taken in other chromatograms (figure 2C). If the spectra of the solutes in the sample differ enough, a visual comparison can be sufficient to

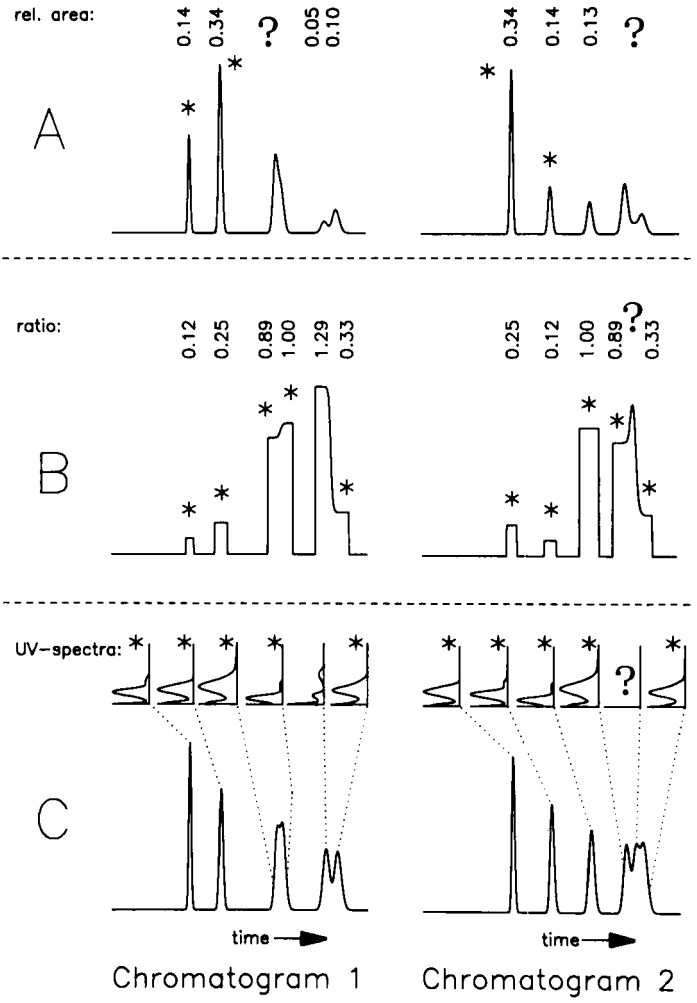


FIGURE 2 Three examples of peak-recognition by means of specific detection applied to two simulated chromatograms:
a) based on peak-area.
b) based on the ratio of the absorbances at 254 and 280 nm.
c) based on the spectra recorded during the elution.
Peaks marked with '*' are matched in both chromatograms. When the overlap is too severe to determine the characteristic value for a particular component, this is indicated by a question mark (?).

recognize the peaks. An objective method to compare spectra can be based on the correlation coefficient, which is equivalent with the sum of the squared differences between the normalised spectra. Normalisation is necessary to eliminate concentration effects.

Again, this technique runs into problems with severe peak overlap, since mixture spectra are generated during coelution of different solutes. Occasionally, spectra taken at the front of the peak and at the back can be used to represent "pure" spectra for the first eluting and last eluting solutes in the peak cluster, but only in the case of modest overlap. Solutes eluting somewhere in the middle of such an overlapping system can never generate "pure"-spectra unless the resolution from the neighbouring solutes is sufficiently large. Only for large differences in spectral characteristics visual interpretation may be performed as has been shown in the past (8). By plotting normalised spectra in overlay, taken at different places over the elution profile and watching characteristic changes in those spectra, full or partial recognition is sometimes possible.

Further expansion of the dimensionality of the data is possible when detection techniques such as fluorescence are used. Since the observed spectra are dependent on the excitation wavelength, there is an additional axis present (the others being time and absorption wavelength). Although the information content of these spectra is much higher than those of UV-VIS spectra, the main drawback is that only few solutes produce a fluorescence signal. Besides, to exploit the full power of fluorescence one has to use stop-flow techniques at present.

In the next section of the paper, we will limit ourselves to the use of the LPDA. Direct comparison of absorption spectra taken from well separated peaks in an automatic way is no real problem (9). Since one of the purposes of the optimization is the separation of coeluting components, sets of mixture-spectra will be generated. Multivariate statistical techniques are needed to tackle this problem. In the remainder of this paper we discuss three possible methods with emphasis on their potential for the

chromatographic optimization procedures. Further emphasis will be given to the necessary performance conditions of the different mathematical techniques. The principles of the methods can be found in the literature (10,11,12,13).

## PEAK RECOGNITION USING MULTIVARIATE STATISTICS

### Treatment of each Mixture Spectrum Individually

This is the simplest approach because the data are considered in one direction only, i.e. the wavelength domain. First, a mixture spectrum observed at a particular point in time is subjected to a multi-component analysis using a set of reference spectra. The reference spectra are summed after multiplication with a weighting factor chosen such that the resulting sum-spectrum matches the observed mixture spectrum as closely as possible. The weighting factor can be seen as the contribution of a particular solute to the total spectrum. This contribution can be zero which means that the corresponding solute is absent. In order to find reliable concentrations, a first prerequisite is to have the spectra of all solutes in the peak profile available as reference spectra. If this is not the case, the contribution of the missing solutes will be attributed to an apparent contribution of the other known solutes.

The next step is an evalution of the derived contributions with repect to time. Since an elution-profile can be described as an ordered sequence of concentration in a time frame, a chronological ordering of subsequent contributions will result in an elution profile of a given component. Again, if all contributions of a component are equal to zero, this component is simply not present in the peak group.

This approach is very elegant and simple, provided the qualitative composition of the sample is completely known and

adequate spectra are available for the solutes in the profile. This stringent condition also indicates the weakness of the technique (14). As mentioned before, a missing component in the reference set will influence the result related to all other components. Second, if several compounds have almost the same spectral characteristics, the result will be uncertain because it is not clear which solutes are responsible for a given contribution. Even more serious in the practice of liquid chromatography is the fact that small changes in the spectrum caused by the use of a different mobile phase than the one used to record the reference spectrum, have a dramatic influence on the results of the multi-component analysis. A small shift in absorption maximum of the spectrum tends to be described by an incorrect contribution of a non-present compound. From a few cases studied by us, we conclude that the applicability of multi-component analysis is so limited that it is of very little practical value.



## A Successive Evaluation of Separate Reference Spectra

By a simultaneous evaluation of all spectra observed over an elution profile, considerably more conclusions can be drawn using less apriori information. The collective data are subjected to a principal component analysis (PCA) (15) which indicates how many solutes are needed to describe the variation in the mixture spectra taken over the peak profile within the experimental error. In addition, this analysis produces a general spectral description of these compounds and their elution profiles. Although these descriptions do not resemble true spectra, it is still true that every mixture spectrum can be reconstructed by a linear combination of the elements of this abstract model.

The same applies to a reference spectrum: the corresponding solute is present in the mixture, if and only if, the spectrum fits the model given by the PCA. This method is called "target

testing", the reference spectrum fulfilling the role of the target.

Target testing enables us to determine in which peak group a given compound can be found, independently of the other compounds in the sample or in the peak group. The method is less sensitive to small changes in spectral characteristics (due to changes in mobile phase properties) than multi-component analysis. In addition, it suffers less from strong spectral similarity. Indeed, spectral similarity may cause us to find more than one possible peak group for a given compound but the number of possible locations is strongly reduced.

However, contrary to multi-component analysis which does not need any resolution, the overall result of target testing is dependent on the chromatographic resolution although this can be as small as 0.1 (14).



It should be emphasized that target testing only reveals the presence of a particular solute in a peak cluster, but not its elution profile. Indeed, elutions profiles can only be derived if all solutes in a cluster have been identified. It would thus appear that target testing is subject to the same limitation as multi-component analysis, i.e. spectra must be available for all solutes. However, because the demands are less strict, target testing will be more generally applicable : When a solute is completely separated in one chromatogram, then the spectrum extracted from this pure peak can be used for target testing in another chromatogram, where the solute coelutes with others. In this way target testing provides a complete solution to the recognition problem when all solutes in the sample are either spectrally known beforehand or elute as pure peaks in at least one of the available chromatograms. In this case a library of spectra can be composed during the analysis and used to generate target spectra for all peak clusters with unsatisfactory separation. This results in the selection of the correct compounds to derive the individual elution profiles.

However, the prerequisite to have ultimately a pure spectrum for each solute can be relaxed further by using the iterative target transformation factor analysis.

Treatment of Peak Clusters whithout external Spectral Information

The major problem to tackle is the situation where a peak cluster contains solutes not completely separated in any chromatogram and no further spectral data with regard to these compounds is available. The method is similar to the preceeding one. We first derive the number of solutes in a peak cluster from a principal component analysis. However, rather than testing for a particular spectrum (that we do not have) we test for an elution profile, that we can reasonably estimate by imposing suitable boundary conditions, e.g. non-negativity of the elution profiles and absorptivities. Among these techniques are the self-modeling curve resolution, iterative target transformation and "evolving factor analysis". The important differences in these methods are the applied constraints and the way spectra and/or elution profiles fulfilling the constraints have been chosen. For instance, the iterative target transformation starts with a pulse-like elution target, subsequent targets are adjusted for negative concentration and secondary maxima until the result approaches a true elution profile (fig. 3). This continues until there is no marked improvement of the profile. This process is repeated for all components in the cluster.

These methods will be especially of use when, during a chromatographic optimization, a certain compound coelutes in different peak clusters in all available chromatograms. In this case, no pure spectrum can be collected, hence neither multi-component analysis nor target testing (for spectra) can be performed. Iterative target transformation (for elution profiles) is then the method of choice because peak clusters containing more than three solutes can be tackled, which is not the case with the

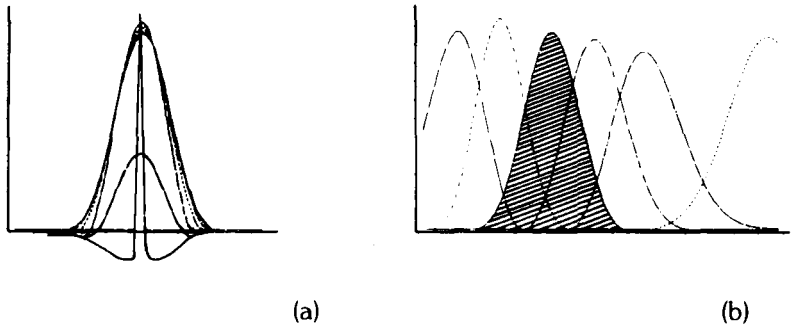


FIGURE 3 An illustration of the iterative target transformation factor analysis:
a) Starting with a pulse-like target, subsequent targets are adjusted for negative concentrations until the result approaches a true elution profile.
b) The result of the analysis for the third component in a chromatogram of a mixture of six polycyclic aromatic hydrocarbons.



self-modeling curve resolution technique. The most important limitation of this method is the rather extensive resolution required to obtain reliable elution profiles and consequently, the real retention behaviour of the individual compounds. Admittedly, at low resolution (< 0.4) the deviation from the true elution profiles does not seem too severe and the accuracy of the calculated retention time seems to be adequate. However, a second effect is of importance: as the calculated spectra are derived from calculated retention profiles, errors in the elution profiles propagate into the spectra. Since the spectra are to be used in the peak recognition procedure, high demands are placed on their accuracy. This is especially true when spectrally similar components are well separated from each other but coelute with other compounds in all chromatograms. In order to match the peaks unambiguously we need reliable spectra. It is for this reason that iterative target testing requires a minimum resolution of at least 0.4.

A Practical Approach to Peak Recognition

If we try to compare the different multivariate techniques for their advantages and disadvantages, they appear to be complementary. The result of a multi-component analysis is independent of the chromatographic resolution but the reference spectra must be very accurate, hence for all solutes we need a spectrum recorded in the eluting solvent. The target factor analysis needs some resolution (0.1) and good quality (but not necessarily perfect) spectra. The iterative target transformation does not need any spectral pre-knowledge but requires more resolution (>0.4) than target transformation. Figure 4 summarises the statements in the text. From the top down in the figure, the demands with respect to the required information on the pure spectra decrease. In contrast, the demands on the resolution within the peak cluster increase from top to bottom. A fully automated system for solute recognition will use a combination of the different techniques guided by rules to conclude which technique should be applied in a particular case. Such a rule-based selection path can be a first step in an expert-system.



Treatment of Separate Peak Clusters

The approach is illustrated in figure 5. The first step is the determination of the total number of solutes present in the selected peak cluster. The results of the PCA combined with a statistical technique like cross validation (16) can be used to serve this purpose: the absorbances are recalculated from the abstract model with a steadily increasing number of components until the calculated values approach the observed ones with a certain accuracy (the experimental error). The results of the iterative target transformation can be used in the same way answering the question: how many realistic elution profiles can be calculated from the original data set. After having performed this

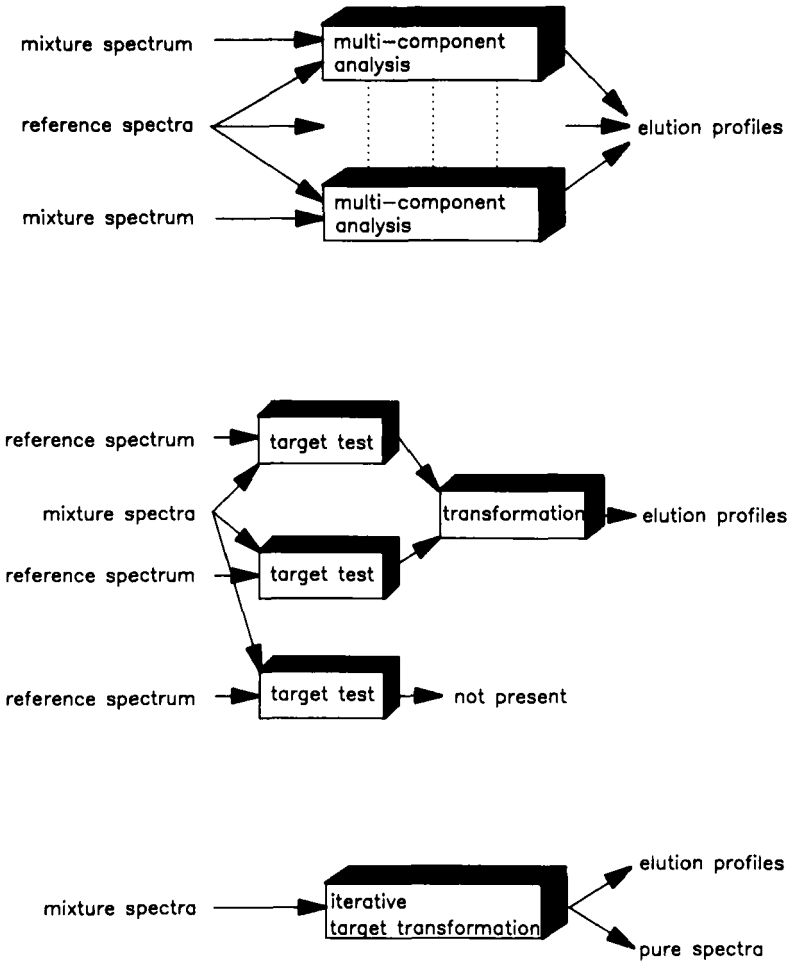


FIGURE 4 An overview of three methods which can be applied for deconvolution of overlapping peak profiles using multiwavelength detection.

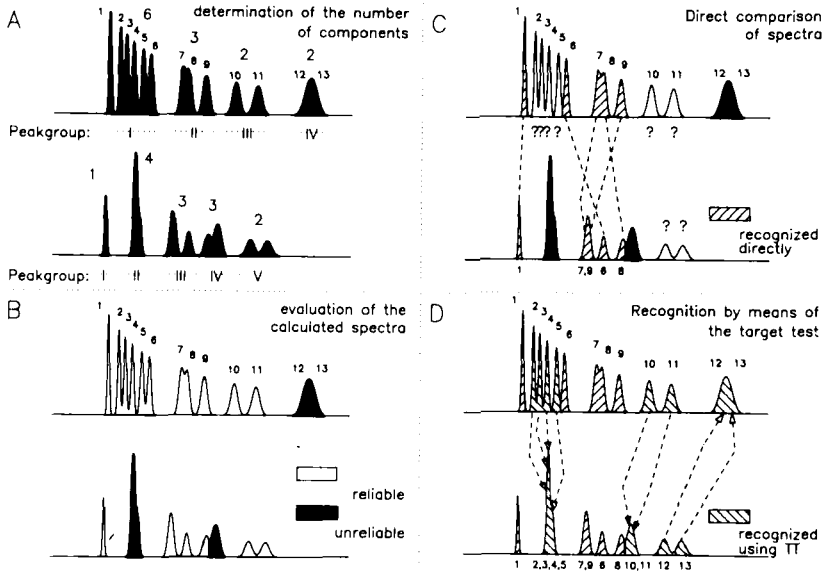


FIGURE 5 The four steps in the peak-recognition procedure illustrated with chromatograms (constructed from the retention times) of a sample containing 13 components: 1) acetanilide, 2) methylparaben, 3) benzaldehyde, 4) acetophenon, 5) cinnamylalcohol, 6) nitrobenzene, 7) methylbenzoate, 8) anisole, 9) diethylphtalate, 10) methylsalicylate, 11) ethyl-benzoate, 12) nitro-naphtalene, 13) benzophenon. The analysis was performed on two chromatograms resulting from elution on a NOVA-PAK (TM) C18 column (particles 5 µm, length 15 cm, I.D. 8 mm) coupled with a HP1040A photo diode array detector. Mobile phases: 60% methanol/ 40% water (upper chromatogram) and 40% tetrahydrofuran/ 60% water (lower chromatogram).

A. Iterative target transformation yields the number of solutes in each cluster and an estimate of their spectra

B. With reference to Fig. 6, the spectra are classified as reliable or unreliable.

C. Comparison of reliable spectra allows positive matching of five solutes in the bottom chromatogram.

D. Target testing of the remaining reliable spectra yields a complete match of all 13 solutes.

The peaks in the second chromatogram which have been matched with a peak in the upper chromatogram are marked by the corresponding number. Further explanation is given in the text.

first step, estimates of elution profiles and "pure" spectra become available, how imprecise these first estimates may be.

On the basis of the total observed concentration (total UV-VIS-activity per solute) a further division in main compounds and minor impurities, which are not taken into account for the further optimization, can be performed. Figure 5A shows two chromatograms from the same sample taken under different chromatographic conditions, analysed with PCA resulting in 13 compounds in either chromatogram. The solutes have been numbered in the top chromatogram and the question now is to locate these solutes in the bottom chromatogram.



## How to Judge the Results of the Iterative Target Transformation?

Although elution profiles and spectra have now been calculated for all solutes present in the chromatogram, part of the data may be unreliable due to errors originating from insufficient chromatographic resolution. The judgement on quality is more complicated than just a simple decision on the basis of a limiting value for the observed resolution (which does not necessarily correspond to the true resolution). The result of the transformation is dependent on a number of factors such as the similarity between the spectra, the concentration ratio, the total number of solutes in the profile and the experimental noise level (17). The more the spectra of the overlapping solutes differ, the stronger the propagated error of incorrect mixing up will influence the quality of each calculated spectrum. What we need is an estimate of the reliability of the spectrum derived from iterative target testing. From simulations of systems of two overlapping solutes, such an estimate is presented in Fig. 6. In this figure, R* respresents the observed resolution between the two components and $r^*_{1,2}$ the correlation coefficient between their two observed spectra. The curves now describe a prediction for the correlation coefficient $r_{1,1}$ of one observed spectrum with the true

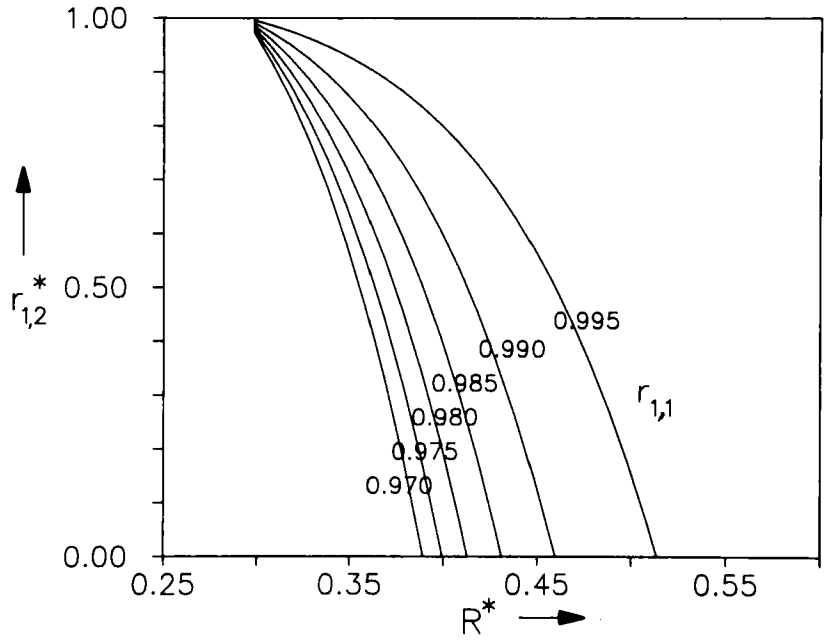


FIGURE 6 Judging the reliability of spectra derived by iterative target transformation of two overlapping solute peaks. R* and $r^*_{12}$ are the resolution and the correlation coefficient observed in the iterative target transformation; $r_{11}$ is the correlation coefficient between an observed spectrum and the true spectrum of that solute and, hence, provides an estimate of this reliability. This plot was derived using the results of the ITT-FA of a large number of simulated elution profiles constructed using the real UV-spectra of components 1, 3, 4, 8, 9, 10, 12 and 13 given in figure 5.

spectrum of the solute. Obviously, the closer $r_{1,1}$ approaches 1, the more reliable the observed spectrum will be. In a practical situation, the value $r_{1,1}$ can be compared with a user defined value in order to decide whether the calculated spectrum can be used for peak recognition. Since Figure 6 is derived from simulations for two compounds of equal concentration, one should use it as a first indication only.

Figure 5B shows the results for the two chromatograms of fig.5A that were found to contain 13 solutes. In the top chromatogram reliable elution profiles and hence spectra ($r_{i,i}$ >0.9) could be derived for solutes 1 through 11, but not for the final two. In the bottom chromatogram seven reliable spectra could be assigned, the other six remaining uncertain.

## Combination of Data from Different Chromatograms

To perform the peak recognition in practice, we start with calculated spectra, but observed peak areas can give additional information. First of all, the eleven reliable spectra from chromatogram A are compared with the seven from chromatogram B. A number of solutes, in this case five, can be matched directly as is indicated in fig. 5C. For the remaining spectra (2 at the top and 6 at the bottom) this is not possible. However we still have six reliable spectra in the top chromatogram and two more in the bottom chromatogram available. These reliable spectra can be used in a target test on the peak cluster with insufficient resolution for the iterative approach (fig. 5D). It is then found that solutes 2 to 5 coelute in peak group II in the bottom chromatogram, whereas the uncertain solutes at the end of the top chromatogram can be matched with the corresponding ones in the bottom chromatogram. In this case a complete match of all solutes has been realized. Their retention times can be used for further optimization (cf. fig.1).

In the case that not all peaks could be identified, a limited number of possible solutions can usually be generated, which can be used as alternatives in the next steps of the optimization procedure. The quality of the unreliable spectra can also be improved during the course of the optimization procedure, when hopefully better resolution will be obtained. The results can also be used to design a specific experiment to gain the necessary spectral information. The largest problem will remain to be the

occurence of severely overlapping peaks of the same subset of solutes in all chromatograms (in varying configurations), in which case accurate spectra cannot be obtained. It is then doubtful, however, whether optimization within the chosen boundaries will lead to a satisfactory chromatogram.

## CONCLUSIONS

Multiwavelength detection and application of advanced data treatment provide powerful tools for automated peak recognition in optimization procedures for liquid chromatography, even for completely unknown samples. For more complex mixtures, a combination of different techniques may be applied to solve the problem. The limits of the different techniques should be clearly outlined in order to make a rational selection. In the near future, the scope of the multivariate techniques in the case of reversed phase chromatography will be extended, first to clusters of more solutes at varying concentration and thereafter to other forms of chromatography such as ion pairing, were the influence of experimental conditions on the spectra will be even larger.



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