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Assessment of peak homogeneity in liquid chromatography using multivariate chemometric techniques

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ABSTRACT

The performance of three new chemometric techniques was evaluated systematically for assessment of peak homogeneity in liquid chromatography with photodiode-array detection. Multi-component analysis, window evolving factor analysis and the HELP method performed equally well in this study. Without making any assumption about peak shape or spectra, these methods were able to detect less than 1% of a spectrally similar impurity under a chromatographic peak, even if the chromatographic separation was low. The limitations of these techniques are also discussed.

INTRODUCTION

One of the most general and demanding problems in liquid chromatography (LC) is the assessment of peak homogeneity. After the synthesis of a drug, for instance, one has to ensure the absence of side products, isomers and degradation products. In practice, less than 1% of such impurities needs to be detected. As these impurities are often chemically related to the substance of interest, their chromatographic behaviour and spectra are often very similar. As a consequence, assurance of peak homogeneity is far from being trivial.

Single-wavelength UV detectors usually cannot solve such serious problems in LC, and a spectroscopic instrument is used instead. Most frequently, a photodiode-array detector (DAD) is coupled to the chromatographic system. Such a coupled instrument measures a complete UV spectrum at each chromatographic data point. Alternatively, one may say that many chromatograms are measured, each at a different wavelength. One common way of representation is a data table (matrix), where the rows correspond to the consecutively recorded spectra and the columns represent the chromatograms measured at different wavelengths. The amount of data obtained from a single experiment is very large and the problem is to extract the relevant information.

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Owing to its simplicity, the ratiogram method is probably the most popular technique for the assessment of peak homogeneity in LC–DAD [1,2]. This method works on the signals from two different wavelengths and can only be applied successfully if the spectra of all analytes are known in advance, which is generally not the case. Further, ratiograms are difficult to interpret. Visual inspection of spectra and chromatograms, spectral suppression using two discrete wavelengths and derivative chromatography have also been proposed for the assessment of peak homogeneity [3–6]. These methods have in common that they work on a very small part of the LC–DAD data only. As a consequence, they cannot solve the general problem, where a small amount of an unknown impurity with a similar spectrum has to be detected, even if chromatographic separation is low. Castledine *et al.* [7,8] have recently introduced very promising correlation-based algorithms. Using reference samples of known purity, they were able to detect as little as 1% of an impurity under a chromatographic peak, no matter how low the chromatographic separation was.

For cases where pure reference samples are not *a priori* available, multivariate methods have been developed that can be applied successfully for the assessment of peak homogeneity in LC–DAD.

THEORY

The main idea of all the three multivariate techniques compared in this study is to monitor the shape of the spectra as a function of analysis time. If the shape of the spectra remains constant within the noise, the chromatographic peak is considered to be homogeneous.

Multi-component analysis (MCA)

Multi-component analysis (MCA) is a least-squares deconvolution technique that has been known for many years [9,10]. Provided that the number of analytes and their spectra are known, MCA can be used to determine the concentration of the individual species from the spectrum of the mixture. Excoffier *et al.* [11] proposed MCA for rapid quantitative analysis in LC–DAD. They also introduced a modification of MCA for the assessment of peak homogeneity that works as follows.

First, an average spectrum is calculated for the peak of interest. Every measured spectrum is then compared with that reference spectrum using a root-mean-square (RMS) value:

$$\mathrm{RMS}_i = \sqrt{\frac{\sum_{j=1}^{n} (x_{ij} - c_i \bar{x}_j)^2}{n}} \qquad (1)$$

where RMS_i is the RMS value for spectrum i, x_{ij} is the signal for spectrum i and wavelength j, c_i is a scaling factor obtained by a least-squares fit ("reconstructed chromatogram"), $\bar{x}_j$ is the average signal for wavelength j and n is the number of wavelengths. This RMS_i is a measure of the difference between a single spectrum i and the average (reference) spectrum. For homogeneous peaks, where the consecutively recorded spectra change only within the noise, plotting RMS *vs.* analysis time (number of spectrum) will result in a more or less horizontal line. In the case of an impurity, however, such an RMS plot will indicate the magnitude and location of spectral differences. This procedure is equivalent to the results obtained with full spectral suppression of the average spectrum. To compare these RMS values more objectively with the noise of the system, an RMS threshold can be determined from the same experiment, using a region of the chromatogram where no substance elutes.

Window evolving factor analysis (WEFA)

Evolving factor analysis (EFA) is a general method for the analysis of multivariate data with an intrinsic order [12,13]. EFA is based on the concept of factor analysis and has been applied successfully in different fields of chemistry. In principle, EFA can be used to determine the number of substances in a mixture and their concentration profiles and spectra.

For the assessment of peak homogeneity in LC–DAD, Keller and co-workers [14–16] proposed a modified version of EFA, called window EFA (WEFA). This method works as follows. Starting with the first k spectra of the recorded data, one calculates the eigenvalues (EVs) of

these k spectra. These EVs represent systematic effects in the underlying data and their magnitude. In a next step, the EVs of spectra 2 to $k+1$ are determined. This procedure is then repeated until the whole data table has been analysed. The results are finally visualized by plotting these EVs *vs.* analysis time. To make such graphs easier to interpret, the points representing the largest (first) EV are connected with a line. In the same way, lines are also drawn to connect the other EVs. In a pure sample, where the spectrum of the analyte does not change across the chromatographic peak, there is only one source of systematic variation in the data. Consequently, a single peak in the EV *vs.* time plot will result. In the case of an impurity, however, one will observe a second peak in the WEFA plot. In analogy to MCA, a threshold value obtained from baseline spectra can also be set, providing a means to interpret WEFA graphs more objectively.

Heuristic evolving latent projections (HELP)

Related to WEFA is the concept of the heuristic evolving latent projections (HELP) method [17,18]. HELP first performs ordinary principal component analysis (PCA) on the whole data table. PCA is a standard method for analysis of multivariate data; its main purpose is data reduction by replacing the many measured variables (different wavelengths) by a small number of new, abstract variables, which are called principal components (PCs). These PCs are a linear combination of the original variables and still contain most of the information. HELP represents the spectra by their first two PCs. In such a PC plot, those parts of the chromatogram where only one analyte elutes appear as a more or less straight line, directed towards the origin of the coordinate system. Visual inspection of PC plots will therefore indicate regions where there is only one substance present.

The second part of HELP inspects these straight lines in more detail and ensures that there is only one compound present. To do this, the abstract spectra (loadings) from those parts of the chromatogram are calculated and inspected visually. The EVs are also determined and compared with those obtained from the baseline. An application of HELP for assessment of peak homogeneity in LC–DAD can be found in ref. 19.

In principle, neither WEFA nor HELP requires any special data pretreatment, and PCA can be performed directly on the raw data using the NIPALS algorithm [20,21]. However, to overcome instrumental or experimental difficulties, scan time correction and baseline correction may be needed, as discussed below.

It is the aim of this paper to compare the performances of these three fully multivariate methods for the assessment of peak homogeneity in LC–DAD.

EXPERIMENTAL SECTION

Apparatus

The liquid chromatograph was a Varian (Walnut Creek, CA, USA) LC Star system, consisting of a Model 9010 pump and a Rheodyne (Cotati, CA, USA) injection valve fitted with a 20-μl sample loop and connected to a Model 9100 autosampler. A 100 × 4.6 mm I.D. RP-18 column (Brownlee, 5 μm) (Applied Biosystems, San Jose, CA, USA) was used at ambient temperature. Spectra were collected on a Polychrom Model 9065 diode-array detector, covering the wavelength range 220–367 nm and scanning spectra at a frequency of 16 Hz.

Instrument control, data storage and analysis were performed on a Compaq (Houston, TX, USA) Deskpro 386/25m IBM-compatible personal computer with math coprocessor.

Reagents and samples

All solvents were of HPLC grade (Rathburn, Walkerburn, UK, and Merck, Darmstadt, Germany). Water was purified using a Milli-Q system (Millipore) and analytical-reagent grade diammonium hydrogenphosphate and phosphoric acid (Merck) were used to prepare a 0.01 *M* phosphate buffer of pH 2.5. The sample consisted of an acidic, pharmacologically active drug (1.0 μg on-column) and its spectrally similar isomer, used as available in the laboratory (Fig. 1).

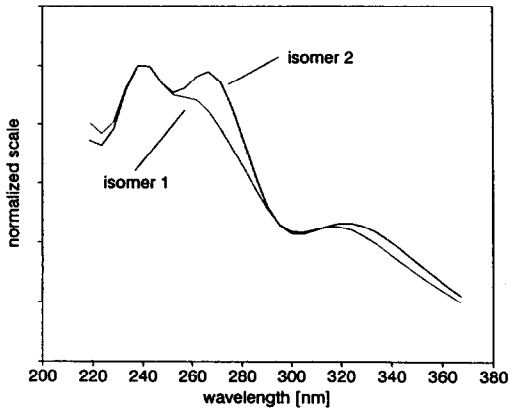

Fig. 1. Normalized spectra of the two isomers ($r = 0.980$).

Procedure

All analyses with the two isomers were carried out isocratically at a flow-rate of 2.0 ml min^{-1}. To obtain the different degrees of separation, the mobile phase consisted of 40–70% acetonitrile in 0.01 *M* phosphate buffer (pH 2.5). Chromatographic resolution between the two analytes could thereby be adjusted in the range of $R_s = 0.1$–1.0 (calculated on the basis of a concentration ratio of 100:1).

To separate the two isomers completely, a 250 × 4.6 mm I.D. ODS-Hypersil column (5 μm) (Bischoff, Leonberg, Germany) was used in combination with gradient elution [28–52% acetonitrile in 0.01 *M* phosphate buffer (pH 2.5) within 22 min at a flow-rate of 1.5 ml min^{-1}].

Data analysis

After collection, data were analysed with MCA using PolyView 2.0 software (Varian). A program written in-house in BASIC 7.0 (Microsoft, Redmond, WA, USA) was used for data analysis with WEFA and HELP. WEFA was performed on $k = 7$ consecutive spectra, which was found to be appropriate for most applications studied so far.

RESULTS AND DISCUSSION

Results obtained from a heterogeneous peak

To illustrate the three different techniques, the results obtained from a sample containing 2% of the impurity at $R_s = 0.6$ are given in Figs. 2 and 3. In the MCA error plot (Fig. 2b), one observes the highest RMS values around 2.5 min. At that point on the chromatogram, the measured spectra differ most from the average spectrum. In

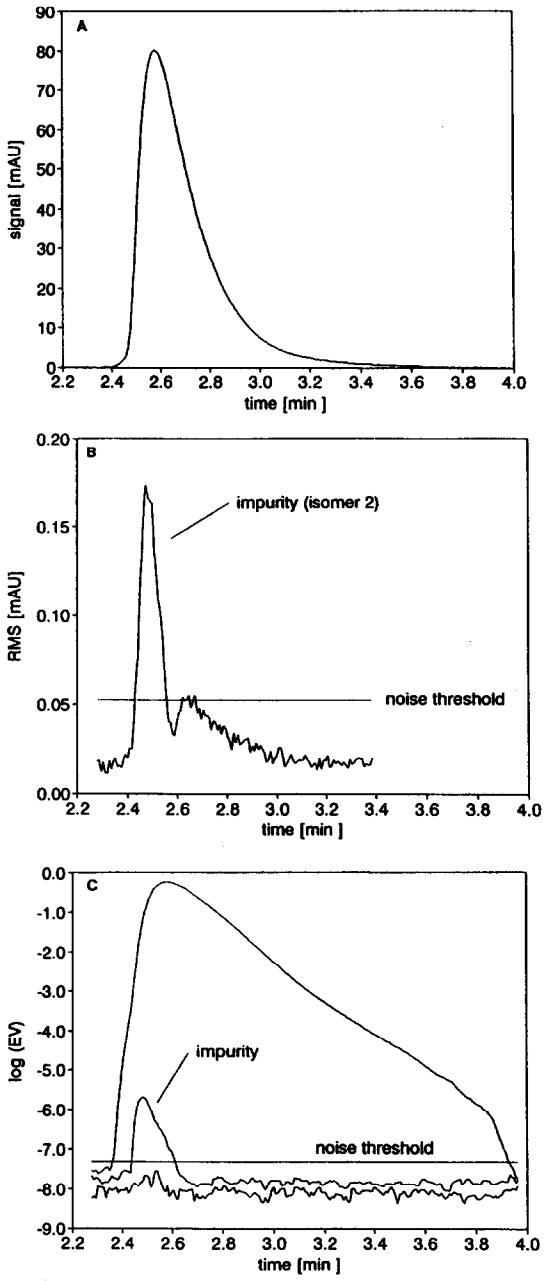

Fig. 2. Results obtained from a sample containing 2% of impurity at $R_s = 0.6$. (A) Chromatogram at 239 nm; (B) RMS plot of MCA; (C) WEFA plot.

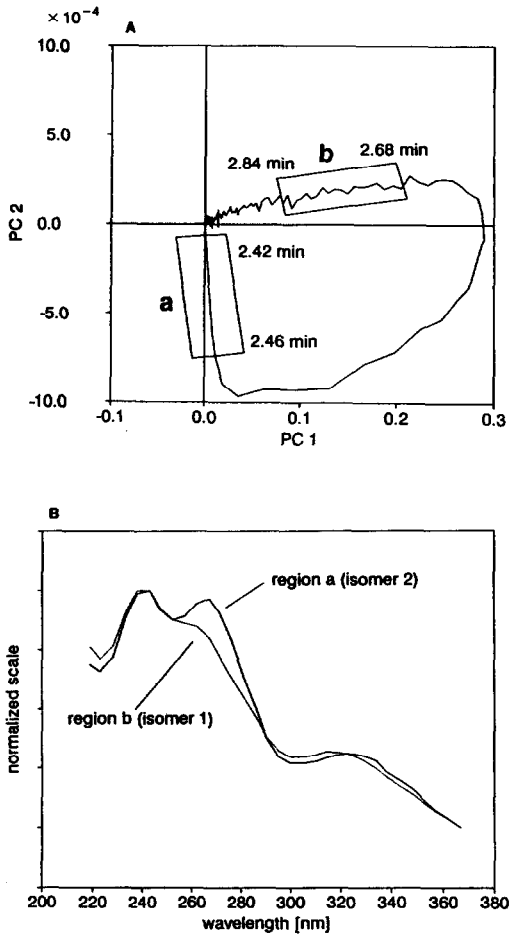

Fig. 3. HELP plots obtained from a sample containing 2% of impurity at $R_s = 0.6$. (A) Each point representing a spectrum is characterized by its scores on the first two PCs (PC_1, PC_2). Those parts of the chromatogram where only one analyte elutes appear as more or less straight lines directed towards the origin of the coordinate system (a, b). Note that PC_2 is scale expanded. (B) The loadings obtained on PC_1 from these two regions a and b correspond to the spectra of the two analytes.

fact, these high RMS values exceed the threshold obtained from noise and therefore indicate a significant deviation from the average spectrum. In this case, the impurity has a retention time of about 2.5 min and is situated on the upslope of the chromatographic peak (Fig. 2a). The spectra measured before and after compare with the average spectrum within the noise, which is represented by RMS values well below the threshold line.

To summarize the results of WEFA, the logarithms of the EVs are plotted *vs.* analysis time (Fig. 2c). Up to about 2.35 min no substance elutes from the column. The first (largest) EV, EV_1, represents noise; its level is about $\log(EV_1) = -7.5$. Later in the chromatogram, one observes two peaks that are caused by the two underlying substances. Owing to the tailing of the chromatographic peak and the logarithmic representation of the results, EV_1 seems to be high after 3.0 min; it falls to the noise level only at 4.0 min. The second peak in the WEFA plot appears to be relatively small. Still, a comparison of the maximum of the second EV [$\log(EV_2) = -5.7$] with the noise threshold [$\log(EV_2) = -7.3$] shows that this EV_2 is more than an order of magnitude larger than the noise.

The results obtained with HELP appear to be different, because there is no time axis (Fig. 3a). The individual spectra (points in time) are represented on the first two PCs instead. From 2.42 to 2.46 min only one isomer elutes (region a). As the spectral shape does not change in that part of the chromatogram, points representing the consecutively recorded spectra fall on a line that is directed towards the origin of the coordinate system. Thus, visual inspection of such a scores plot permits parts of the chromatogram to be selected where only one compound elutes. A second pure compound region can be found, *e.g.*, from 2.68 to 2.84 min (region b). Comparison of regions a and b might lead to the wrong conclusion that noise is larger in b. This is not the case, however, and can be explained with the expanded scale on PC_2. In the second part of HELP, the EVs of these pure compound regions are determined and compared with those from noise. Table I shows that in the two pure compound regions EV_1 is much larger than the noise. Hence the spectra change systematically. As EV_2 and EV_3 of the two regions a and b are comparable to the EVs of noise, only one substance is present in each of the two regions. In a last step, the abstract spectra (loadings) obtained from the two regions are plotted and visually compared with each other. Fig. 3b demonstrates that the loadings obtained from the two pure compound regions are different. Consequently, there are two analytes present in the sample.

TABLE I

EIGENVALUES FROM NOISE AND PURE COMPOUND REGIONS

Region	Time range (min)	EV_1	EV_2	EV_3
Baseline (noise)	2.25–2.30	$2.9 \cdot 10^{-8}$	$2.1 \cdot 10^{-8}$	$5.8 \cdot 10^{-9}$
Region a	2.42–2.46	$2.8 \cdot 10^{-4}$	$1.5 \cdot 10^{-8}$	$1.0 \cdot 10^{-8}$
Region b	2.68–2.84	$4.0 \cdot 10^{-1}$	$3.5 \cdot 10^{-8}$	$2.2 \cdot 10^{-8}$

Also, the loadings compare closely with the spectra obtained from pure samples (Fig. 1).

The main purpose of this study was to compare the performances of new multivariate techniques for the assessment of peak homogeneity in LC–DAD. Therefore, both the chromatographic separations between the analytes and the relative concentrations were changed systematically. As the third variable that is highly relevant for the performance of all methods, *i.e.*, the spectral similarity between the analytes, cannot easily be changed, it was held constant. The signal-to-noise ratio for the isocratic runs was found to be 4000.

Results obtained from a homogeneous peak

To demonstrate the results obtained from a homogeneous peak, an illustrative example is given below. For a homogeneous peak, the shape of the spectra varies only within the noise. Consequently, the average spectrum in MCA matches the spectra that were measured across the chromatographic peak. In such a case, the RMS values represent noise and remain below the threshold, as shown in Fig. 4b. Analogously, in WEFA there is only one peak; EV_2 represents noise and does not exceed the threshold either (Fig. 4c). As there is only one systematic effect present, PC_1 of the HELP method explains virtually all variations in the data (Fig. 5). PC_2 is much smaller and represents only noise. In this case, the line indicating a pure compound region is identical with PC_1, while random noise causes the points representing spectra to be located to a small degree on either side of PC_1. As the peak is homogeneous, a second straight line cannot be found and there is no need to determine the loadings. As completely pure reference material was not available and as a complete separation of the two isomers studied here could not be obtained with isocratic elution, a gradient system was used to generate a homogeneous peak. Although, owing to gradient elution, the noise level was higher in the latter example, Figs. 4 and 5 clearly illustrate the results obtained from a homogeneous peak.

Performance of the different methods

To quantify the performance of the methods to be evaluated, we chose the detection limit of an impurity as the criterion. For MCA the criterion selected to detect the impurity was an RMS value exceeding the threshold, which was calculated as three times the noise. Analogously, the threshold value for WEFA was set to the mean EV_1 plus three standard deviations obtained from the noise; an EV_2 exceeding that threshold was considered to indicate the impurity. For the HELP method more subjective criteria were used. Two (more or less) straight lines were selected, and the loadings obtained from these regions were compared visually. Different loadings indicated an impurity.

The limits of detection of one isomer in the other are summarized in Fig. 6. Above and to the right of the line the impurity was detected. One observes for instance, that the impurity could be detected at the 0.5% level for R_s values of at least 0.3. Lower relative concentrations were not detected. Similarly, if the chromatographic separation drops below 0.3, the impurity could only be detected at a higher relative concentration. Interestingly, all three methods performed equally well in detecting one isomer under the other. One should also note that none of the tested methods makes any assumption about peak shape, chromatographic separation or similarity of the spectra. Further, the pure compound spectra need not be known in advance. It should be noted, however, that the

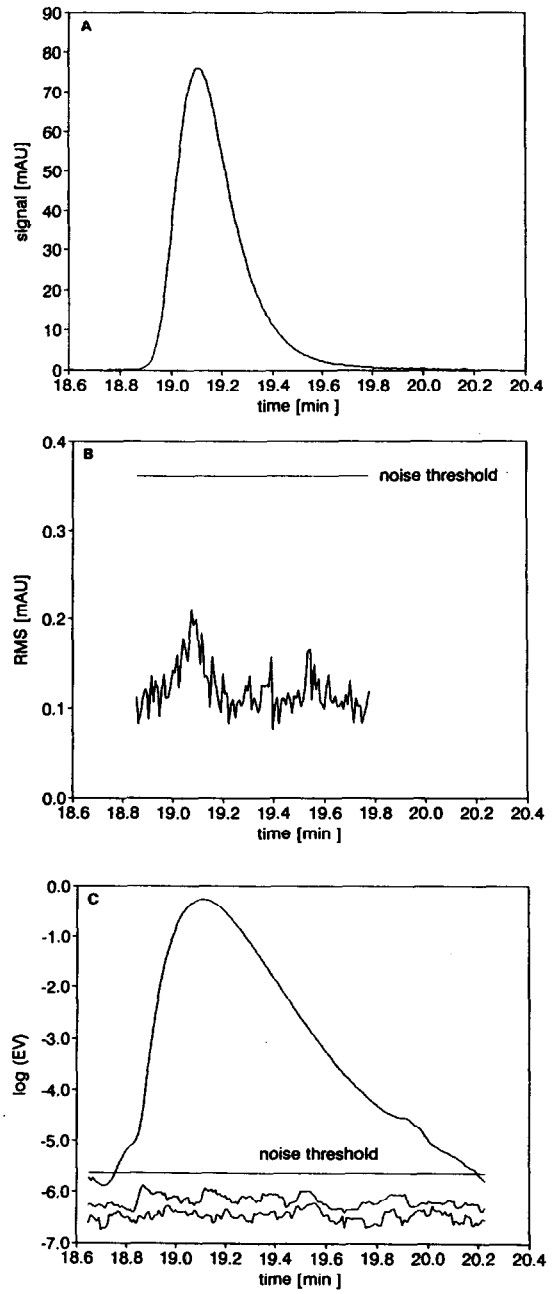

Fig. 4. Results obtained from a homogeneous peak. (A) Chromatogram at 239 nm; (B) RMS plot of MCA; (C) WEFA plot.

degree of spectral similarity will strongly affect the results obtained with the different methods. Although the influence of the similarity of the spectra cannot be quantified at present, one may

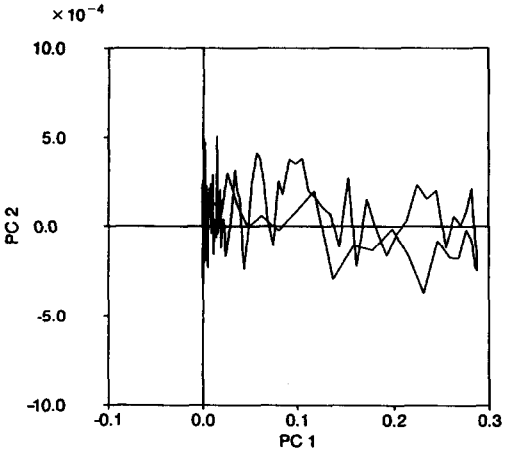

Fig. 5. HELP scores plot obtained from a homogeneous peak. Each point representing a spectrum is characterized by its scores on the first two PCs (PC_1, PC_2). Note that PC_2 is scale expanded.

state that the methods tend to perform better the more dissimilar are the spectra, whereas the performance must be expected to decrease for more similar spectra. Still, we consider the example given here as being representative for problems observed in our laboratory. Work is in progress to understand better the effect of this parameter.

As the problem dealt with in this study is the assessment of peak homogeneity, results were shown for both a homogeneous and a heteroge-

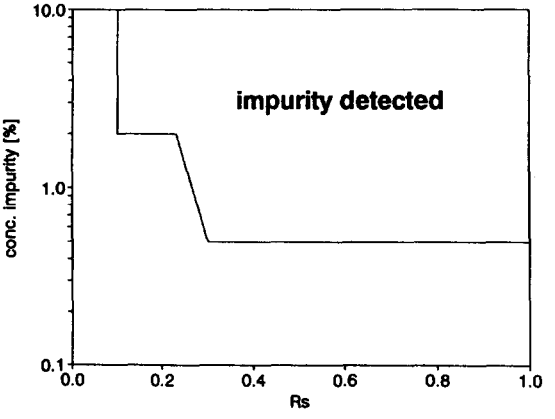

Fig. 6. Limits of detection of isomer 2 in isomer 1, obtained with all three methods and given as a function of relative concentration and chromatographic separation. Above and to the right of the line an impurity was detected with MCA, WEFA and HELP.

neous peak. The latter instance is the simplest and most representative example for a non-homogeneous peak. Very similar results are obtained for situations where more than two substances co-elute. It is important to remember, however, that detection of one or more impurities demands a (minimum) difference in both time and spectra.

Possible limitations

Generally, the smaller the chromatographic separation, the lower the relative concentration of the impurity, the more similar the spectra and the higher the noise level, the more difficult the problem is. Consequently, all methods fail to detect the very lowest concentration at $R_s = 0.1$.

On the other hand, instrumental and experimental difficulties must also be taken into account. MCA, WEFA and HELP all rely on linear mathematical techniques, *i.e.*, Beer's law has to be valid. Non-linearities may be due to different phenomena, such as a non-linear calibration graph, a non-zero or sloping baseline, polychromatic radiation, the DAD scan rate and noise [22].

In practice, one therefore has to be certain to work in the linear range of the calibration graph, which depends on both the instrument and the absorbance value. Non-zero or sloping baselines will seriously affect the results; adequate baseline correction before applying linear mathematical methods is therefore very important. As reported by Dose and Guiochon [23], the polychromatic radiation measured at each diode can also cause non-linearities, because Beer's law is valid for monochromatic radiation only. For cases where a spectrum changes considerably within the optical bandwidth of the diode-array detector, non-linearities can also be introduced. The time required to measure a spectrum by DAD can also lead to non-idealities, as discussed elsewhere [24]. For the system used in this study, scan time correction was performed automatically in the detector using the method reported in ref. 24. For some instruments, the noise level is a function of the signal, which is known as heteroscedasticity. This phenomenon is less obvious but can lead to serious problems in practice. A detailed discussion can be found in ref. 15. Additionally, one should also be aware of possible pH effects and solvent effects, which could change the spectrum of a pure analyte during the chromatographic run.

For the present study, which was carried out under isocratic conditions and at relatively low concentrations of the analytes (signals not exceeding 100 milliabsorbance), the above limitations appeared not to be relevant for the instrument used. The only requirement was adequate baseline correction, implemented in the following way. First, two average spectra were calculated from the spectra measured before and after the chromatographic peak. The baseline was then estimated by linear interpolation between these two average spectra and subtracted from the raw data. After such a baseline correction there was no sign of any experimental or instrumental limitation of the methods discussed in this paper.

Comparison of the different methods

Although the three methods performed equally well, one should also note their merits and limitations. MCA has the advantage of being less complex and therefore easier to understand than the other techniques. In principle, MCA is an automated method. Still, one should bear in mind that a good peak detection algorithm is required, because inclusion of too many baseline spectra would reduce the quality of the average (reference) spectrum and thereby reduce the performance of the method.

WEFA and HELP both rely on the concept of PCA and are therefore similar. Among the three techniques WEFA can be automated most easily because no user interaction is required. A commercial example of this is Beckman's System Gold, which corresponds closely to WEFA and which can be used for the automated assessment of peak homogeneity in real time. As the selection of straight lines in the HELP method can be a difficult task in some instances, much experience and user interaction are required for this method. On the other hand, HELP is at the same time the most flexible and powerful method, because pure compound spectra can easily be found.

CONCLUSIONS

MCA, WEFA and HELP can be applied successfully for the assessment of peak homogeneity in LC–DAD. Without making any assumption about the chromatograms or spectra, less than 1% of a spectrally similar impurity could be detected under a chromatographic peak. As could be expected, for very small amounts of the impurity and very low R_s values the methods fail. Although the tested methods worked well, one should also be aware of possible instrumental and experimental difficulties.

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REFERENCES

1 A.C.J.H. Drouen, H.A.H. Billiet and L. de Galan, *Anal. Chem.*, 56 (1984) 971.
2 F.V. Warren, Jr., B.A. Bidlingmeyer and M.F. Delaney, *Anal. Chem.*, 59 (1987) 1897.
3 G.T. Carter, R.E. Schiesswohl, H. Burke and R. Yang, *J. Pharm. Sci.*, 71 (1982) 317.
4 A.F. Fell, H.P. Scott, R. Gill and A.C. Moffat, *J. Chromatogr.*, 282 (1983) 123.
5 B.J. Clark, A.F. Fell, H.P. Scott and D. Westerlund, *J. Chromatogr.*, 286 (1984) 261.
6 T. Alfredson and T. Sheehan, *J. Chromatogr. Sci.*, 24 (1986) 473.
7 J.B. Castledine, A.F. Fell, R. Modin and B. Sellberg, *Anal. Proc.*, 29 (1992) 100.
8 J.B. Castledine, A.F. Fell, R. Modin and B. Sellberg, *J. Chromatogr.*, 592 (1992) 27.
9 R.P. Bauman, *Appl. Spectrosc.*, 13 (1959) 156.
10 M.J. Milano, S. Lam, M. Savonis, D.B. Pautler, J.W. Pav and E. Grushka, *J. Chromatogr.*, 149 (1978) 599.
11 J.L. Excoffier, M. Joseph, J.J. Robinson and T.L. Sheehan, *J. Chromatogr.*, 631 (1993) 15.
12 M. Maeder, *Anal. Chem.*, 59 (1987) 527.
13 H.R. Keller and D.L. Massart, *Chemometr. Intell. Lab. Syst.*, 12 (1992) 209.
14 H.R. Keller and D.L. Massart, *Anal. Chim. Acta*, 246 (1991) 379.
15 H.R. Keller, D.L. Massart, Y.Z. Liang and O.M. Kvalheim, *Anal. Chim. Acta*, 263 (1992) 29.
16 H.R. Keller, D.L. Massart, J.O. de Beer, *Anal. Chem.*, 65 (1993) 471.
17 O.M. Kvalheim and Y.Z. Liang, *Anal. Chem.*, 64 (1992) 936.
18 Y.Z. Liang, O.M. Kvalheim, H.R. Keller, D.L. Massart, P. Kiechle and F. Erni, *Anal. Chem.*, 64 (1992) 946.
19 H.R. Keller, D.L. Massart, Y.Z. Liang and O.M. Kvalheim, *Anal. Chim. Acta*, 267 (1992) 63.
20 S. Wold, K. Esbensen and P. Geladi, *Chemometr. Intell. Lab. Syst.*, 2 (1987) 37.
21 B.G.M. Vandeginste, C. Sielhorst and M. Gerritsen, *Trends Anal. Chem.*, 7 (1988) 286.
22 H.R. Keller and D.L. Massart, *Anal. Chim. Acta*, 263 (1992) 21.
23 E.V. Dose and G. Guiochon, *Anal. Chem.*, 61 (1989) 2571.
24 H.R. Keller, D.L. Massart, P. Kiechle and F. Erni, *Anal. Chim. Acta*, 256 (1992) 125.