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Use of simulated liquid chromatography–diode array detection data for the definition of a guide curve in peak purity assessment by spectral comparison

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Abstract

Computing dissimilarity profiles between spectra within a chromatographic peak and comparing them to a base profile for a pure peak can be a sensitive method for detecting coelution. The power of this method is limited by noise and non-idealities. For high maximum absorbances, non-idealities prevail over noise. In current practice, either the maximum absorbances are limited, or the base profile is computed from injections of pure standards. The first approach leads to a loss in detection power, the second requires substantial extra work, since the standards have to be obtained and they have to be measured separately for each peak of interest in the chromatogram. Here, we show that an approximation to the base profile can be computed from realistic simulations of homogeneous data accounting for potential non-idealities. This is demonstrated using several graphical displays. As a result, higher maximum absorbances leading to better impurity detection can be used without extra experimentation. © 1997 Elsevier Science B.V.

Keywords: Peak purity; Computer simulation; Diode array detection; Detection, LC; Alprazolam; Triazilam; Hydrochlorothiazide

1. Introduction

Chromatography is a powerful tool for decomposing analytical samples into their components, but it is not uncommon to encounter mixtures which resist separation. Statistical modelling of the chromatographic process [1,2] has shown that overlapping peaks are a likely occurrence in complex mixtures, even with high efficiency separations. Therefore, peaks have to be checked for purity. Hyphenated chromatographic techniques, in particular liquid chromatography (LC) combined with diode array detection (DAD), increase the prospect of effective peak purity assessment. In this paper, we try to improve the way of analyzing the data generated by a LC–DAD system with the aim of peak purity assessments. Such a data set is called a spectrochromatogram and can be described as a matrix in which the rows are absorption spectra measured at regular time intervals and the columns are chromatograms measured at different wavelengths.

Currently, various mathematical procedures are available to examine a spectrochromatogram for peak purity [3–12]. These algorithms are based on principal components analysis (PCA) such as fixed size window evolving factor analysis (FSWEFA)

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[3,4,7] or the heuristic latent projections method (HELP) [5,7], on multi-component analysis (MCA) [6,7] or on spectral comparison [8–12]. As pointed out by Cuesta Sanchez et al. [9], the performance of the various techniques seem to be similar suggesting that a fundamental detection limit might be reached. We believe that all above mentioned methods are limited by the same detection barrier consisting of two components, noise and non-idealities. Progress will require reductions of noise and non-idealities and clever ways of dealing with them. Both noise (heteroscedasticity) and non-idealities (departures from Beer's law) increase with higher maximum absorbances but the non-idealities quickly prevail over noise and can generate considerable nuisance even at 0.2 AU [6,13].

Signal-to-noise ratios for detection of low concentration contaminations can be improved by using higher maximum absorbances. Note that although the noise component is heteroscedastic and roughly proportional to the maximum absorbance at levels around 1 AU, the baseline noise prevails at 0.1 AU. Thus, passing from 0.1 AU to 0.5 AU, will increase the signal produced by an impurity by a factor of 5, whereas the noise will increase by a lesser factor with the result of a higher signal-to-noise ratio. However, this approach is hampered by non-idealities. All above mentioned techniques are based on the assumption that the data follow Beer's law. Any non-ideality in the spectra exceeding the noise level will be responsible for a signal that can be difficult to distinguish from minor chemical components.

Unambiguous peak purity interpretation is still possible if the results obtained for the investigated peak are compared to those of a pure standard measured under similar experimental conditions. In spectral comparison, for instance, replicated analyses of the standard at the highest concentration expected have been used to define base profiles and purity thresholds which account for noise and non-ideal effects [8,12]. However, the need for a standard is a drawback since it is not always available. Moreover, measuring the standard several times to obtain the threshold implies extra work.

The problem could be circumvented if simulated LC–DAD data accounting for the most important non-idealities could be used instead of a standard. In a previous paper [13], we showed that, provided the specifications of the detector are known, such realistic simulations can be performed. Simulated data were successfully used in PCA and FSWEFA to anticipate the response expected for a homogeneous peak even under non-ideal conditions and hence to facilitate interpretation of otherwise ambiguous results. Here, we show that this also works for obtaining base profiles in spectral comparisons. Repeated realistic simulations at the maximum expected absorbance can be used in the same way as repeated injections to define a threshold, although we prefer to limit ourselves to qualitative comparisons. Such comparisons are shown in several different displays. These displays demonstrate the benefit of the realistic simulation approach and the considerable gain in detection power.

2. Theoretical background

Collecting complete spectral data and mathematically comparing spectra within a peak can be an effective way to assess peak purity. The numerical evaluation of the spectral comparison can be provided by several related parameters as shown in Fig. 1 where two normalised vectors **a** and **b** are depicted [8].

The cosine of the angle between the two vectors represents the portion of **b** that is projected onto **a** and therefore is a measure of similarity. In a

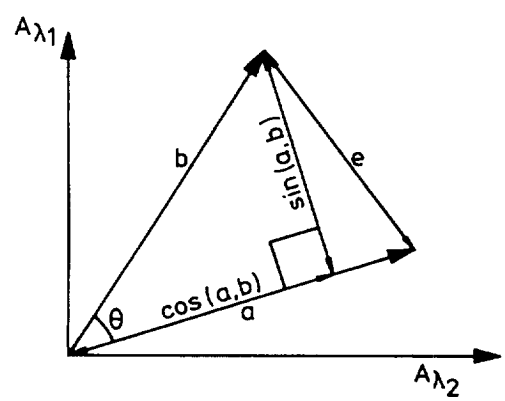

Fig. 1. Vectorial definitions of the parameters for spectral comparison.

mathematical form, a_i and b_i being the coordinates of **a** and **b**, it can be expressed as:

$$\cos(a,b) = \frac{\sum(a_i b_i)}{\sqrt{\sum(a_i^2)\sum(b_i^2)}} \quad (1)$$

The cosine of two normalised, mean centred spectra is equivalent to the correlation coefficient r defined as:

$$r = \frac{\sum[(a_i - \bar{a})(b_i - \bar{b})]}{\sqrt{\sum(a_i - \bar{a})^2 \sum(b_i - \bar{b})^2}} \quad (2)$$

The sine on the other hand describes that portion of **b** that is orthogonal to **a** and can therefore be viewed as a measure of spectral dissimilarity. The sine is related to the cosine by the relationship:

$$\sin(a,b) = \sqrt{1 - \cos^2(a,b)} \quad (3)$$

The Euclidean distance e between the tips of the two vectors in Fig. 1 is an another possible measure of spectral dissimilarity and can also be related to the cosine:

$$e = \sqrt{2 - 2\cos(a,b)} \quad (4)$$

Clearly, all these equations provide the same information, presented in different ways, and can be converted to each other. However, even though they are mathematically related, these various expressions differ in the rate of change observed for spectral differences. Sievert and Drouen [8] showed that the greatest rate of change can be observed for the sine which is therefore the first choice expression for differentiation among spectra with strong similarity.

3. Experimental

3.1. Apparatus

The data evaluated for this article were acquired using a Beckman Gold LC–DAD system (Beckman Instruments, Fullerton, CA, USA) equipped with a fixed 4 nm optical slit. The spectra covering a wavelength range from 200 to 350 nm were recorded at time intervals of 1 s with a digital resolution of 1 nm. The scan rate of the array sensor chip was 32 Hz. After collection, the data files were converted to the ASCII format using the Beckman Array View software.

3.2. Reagents and samples

HPLC-grade acetonitrile was purchased from Labscan (Dublin, Ireland) and water was obtained from an in-laboratory water purification system (Milli-Q, Millipore, Bedford, MA, USA). The samples consisted of alprazolam (Upjohn, Puurs, Belgium) with triazolam (Upjohn) as impurity (Fig. 1a), or hydrochlorothiazide (Ciba-Geigy, Brussels, Belgium) with an actual impurity (with an apparent level of approximately 1%) as available in the laboratory (Fig. 1b), dissolved in the corresponding mobile phase.

3.3. Procedure

Chromatographic runs were carried out under isocratic conditions, at ambient temperature and with a flow-rate of 1 ml min^{-1}, on a 250×4 mm I.D. RP-18 column (Merck, LiChrospher RP-select B, 5 μm). For the alprazolam–triazolam pair the mobile phase consisted of 45–60% (v/v) acetonitrile–55-40% (v/v) water and permitted us to select the chromatographic resolution between the two analytes as desired in the range of $R_s = 0.1$ to 1.0. A complete separation of hydrochlorothiazide and its impurity was realised with a mobile phase consisting of acetonitrile–water (25:75, v/v), whereas a coelution with a resolution of approximately 0.7 was obtained with an acetonitrile–water (35:65, v/v) mobile phase.

3.4. Simulated data

Simulation of realistic homogeneous data sets for our LC–DAD system was performed according to the following procedure [13]:

(i) The effect of the optical slit was simulated by extracting the apex spectrum and the apex chromatogram from the data, deconvolving the spectrum using Burger and van Cittert's method [14], recombining the deconvoluted spectrum and the chromatogram into an artificial spectrochromatogram and finally reconvolving each spectrum by transmittance averaging. In this procedure, a deconvoluted apex spectrum

is obtained by subtracting the effect of the convolution function to the original data set. In this case, the differences δ_λ between the measured and real data:

$$\delta_\lambda = T_\lambda - T^0_\lambda = \left(\frac{1}{\Delta} \sum_{j=\lambda-\left(\frac{\Delta-1}{2}\right)}^{\lambda+\left(\frac{\Delta-1}{2}\right)} T^0_j \right) - T^0_\lambda \quad (5)$$

can be approximated by the differences d_λ between the reconvoluted and the measured data:

$$d_\lambda = \left(\frac{1}{\Delta} \sum_{j=\lambda-\left(\frac{\Delta-1}{2}\right)}^{\lambda+\left(\frac{\Delta-1}{2}\right)} T_j \right) - T_\lambda = T'_\lambda - T_\lambda \quad (6)$$

Thus an adjustment of T_λ towards T^0_λ can be performed by subtracting d_λ from T_λ:

$$A^0_\lambda = -\log(T_\lambda - d_\lambda) \quad (7)$$

where A^0_λ is the corrected absorbance at the nominal wavelength λ. In practice, the difference between observed and convoluted spectra should be smoothed prior subtraction in order to avoid noise amplification. The operation can be repeated until the standard deviation of the differences between the deconvoluted spectra obtained for two successive applications is comparable to the estimated noise level. A bandpass (Δ) involving seven wavelengths was found to give a good agreement between actual spectrochromatograms and data simulated according to the deconvolution–reconvolution procedure.

(ii) Proportional heteroscedastic noise was superimposed to the simulated spectrochromatogram by the following relation:

$$s_{t,\lambda} = s_0(1 + \alpha A_{t,\lambda})\xi_{t,\lambda} \quad (8)$$

with $s_{t,\lambda}$ = standard deviation of the noise at time t and wavelength λ, s_0 = standard deviation of the baseline noise, $A_{t,\lambda}$ = signal measured at time t and wavelength λ, α = proportionality factor and $\xi_{t,\lambda}$ = standard normal random number.

(iii) The scan time effect was introduced according to the following equation:

$$A^*_{t,\lambda} = A_{t,\lambda} - (A_{t,\lambda} - A_{t-\Delta t,\lambda}) \cdot \left(\frac{p-1}{n-1}\right)\left(\frac{t_{\text{scan}}}{\Delta t}\right) \quad (9)$$

with $A^*_{t,\lambda}$ = the distorted absorbance at time t and wavelength λ, p = the position of the corresponding wavelength in the spectral range investigated, n = the number of wavelengths covered by the array sensor chip, t_{scan} = the scan time and Δt = the sampling interval.

The array sensor chip of the DAD system used in this study consisted in 512 diodes covering the range 190 to 600 nm so that n equals 410. The spectra were recorded from 200 to 350 nm so that p is in the range 1 to 151. The scan time was 31.25 ms and the sampling interval was 1 s.

(iv) Finally, the error created by transmittance averaging over time was added according to:

$$A^*_{\frac{1}{r}\sum_{i=1}^{r} ti,\lambda} = -\log\left(\frac{\sum_{i=1}^{r} T_{ti,\lambda}}{r} \right) \quad (10)$$

with $T_{ti,\lambda}$ = transmittance at time ti and wavelength λ, r = the scan rate in Hz divided by 16.

The number of data points was first multiplied by r through linear interpolation between the measured data and the basic noise level was accordingly multiplied by $\sqrt{r}$.

3.5. Data analysis

Background correction was carried out prior to analysis of the actual data sets by subtracting a linear interpolation between the baseline average spectra calculated before and after the peak from the measured spectra.

Simulation and data analysis were performed using routines written in-laboratory in the Windows version of S-PLUS (Statistical Sciences, Seattle, WA, USA). All calculations were done on a 486-DX4 based IBM-compatible personal computer.

4. Results and discussion

The spectra of the compounds used as experimental models are displayed in Fig. 2. The spectral similarity, expressed by the correlation coefficient (Eq. (2)), is 0.991 for the alprazolam–triazolam pair (Fig. 2a) and 0.908 for hydrochlorothiazide and its impurity (Fig. 2b).

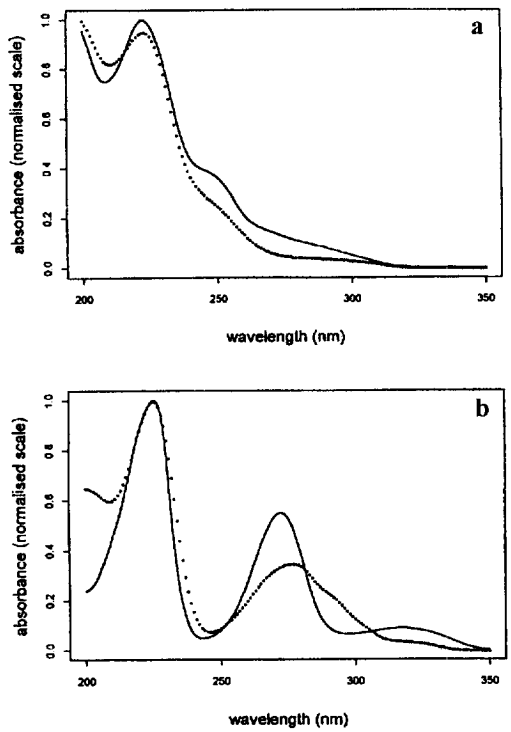

Fig. 2. Normalised spectra of the investigated compounds: (a) alprazolam (solid line) and triazolam (dotted ine), $r=0.991$; (b) hydrochlorothiazide (solid line) and its impurity (dotted line), $r=0.908$.

4.1. Ideal bilinear data

The techniques for peak purity assessment rely on the assumption that the data measured with a LC–DAD system follow a bilinear structure, meaning that each compound present in the sample can be expressed as the outer product of a chromatogram and a pure spectrum. In practice, however, a non-ideal response behaviour is observed for UV detectors causing deviations from the hypothetical bilinear structure which can seriously affect the results of a peak purity analysis. Non-idealities may be due to different phenomena, such as a non-zero or sloping baseline, polychromatic radiation, the DAD scan rate, the transmittance averaging error and heteroscedastic noise [13,15]. Since both noise and deviations from linearity are greater at high absorbance, the occurrence of artefacts is a function of the magnitude of the signal measured and its likelihood can be minimised by limiting the maximum absorbance of the peak within the spectral range of the measurements. The absorbance ceiling required to avoid a non-ideal behaviour will depend on the detector characteristics and on the shape of the spectrum of the analyte in the spectral range investigated [16]. For the instrument used in the present study and a drug compound showing a relatively smooth spectrum such as alprazolam (Fig. 2a), an ideal bilinear behaviour could be observed for a maximum absorbance of about 100 mAU.

Fig. 3a shows the results of the spectral comparison analysis obtained from a homogeneous peak of alprazolam measured under these conditions. A dissimilarity curve is drawn by plotting the sine computed by comparing each spectrum measured along the elution of a peak with one selected spectrum (the base spectrum) against retention time. The base spectrum corresponds here to the apex spectrum of the investigated peak but alternatives can be imagined such as the average spectrum [8,10]. One could even compare each spectrum to all others [8]. For a homogeneous peak, the ideal dissimilarity curve is a horizontal line with a value of zero indicating the absence of both impurity and noise. However, the measurement noise causes all the absorbance spectra to have different shapes at some level so that non-zero values are observed in practice. Actually, one zero value is computed for the apex spectrum since the latter is used as base spectrum which explains the negative spike under the apex of the peak in Fig. 3a. In addition to a zero offset, a dissimilarity curve tends to rise at the leading and trailing edges of the peak since the signal intensity decreases and a larger proportion of the spectral response is due to noise. Consequently, the continuous dissimilarity plot of a pure peak yields a bathtub shaped curve. Such a graphical format hinders straightforward interpretation since a departure from pure behaviour is not easy to identify. A base profile of the pure peak is therefore required for objective peak purity assignment. The technique is illustrated in Figs. 4 and 5 for impure samples containing 1% of a closely related impurity with a chromatographic resolution (R_s) of 0.7 and 0.3. Note that besides a base profile, an absorbance threshold screening out the spectra near the signal baseline is

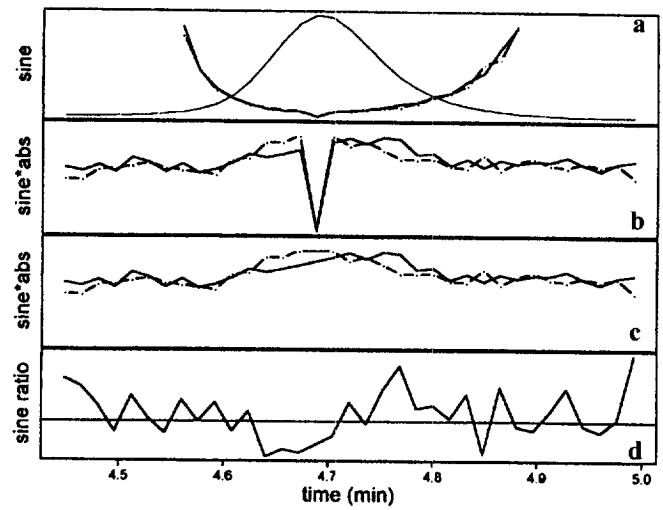

Fig. 3. Dissimilarity plots computed for a homogeneous peak of alprazolam with a maximal absorbance of about 100 mAU: (a) sine (solid line) and guide (dotted line) curves with an absorbance threshold equal to 3% of the maximal absorbance value; (b) weighted sine display; (c) idem but with the value computed for the apex spectrum omitted; (d) sine-ratio display, the horizontal line corresponds to 1.

required in order to improve the sensitivity of the method (compare Fig. 4a and Fig. 4b).

In an alternative to the classic dissimilarity plot, the rising of the dissimilarity curve at the edges of the peak can be eliminated by multiplying the dissimilarity curve by the chromatographic profile of the investigated peak averaged over the spectral range used, as shown in Fig. 3b [6]. By doing this,

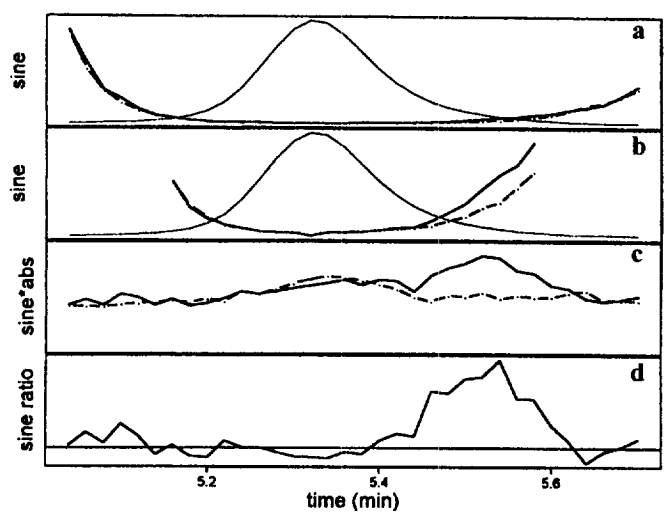

Fig. 4. Dissimilarity plots computed for a heterogeneous peak of alprazolam containing 1% of impurity with a resolution of 0.7 and with a maximal absorbance of about 100 mAU: (a) sine (solid line) and guide (dotted line) curves plotted with the chromatogram without absorbance threshold; (b) idem but with an absorbance threshold equal to 3% of the maximal absorbance value; (c) sine-ratio display.

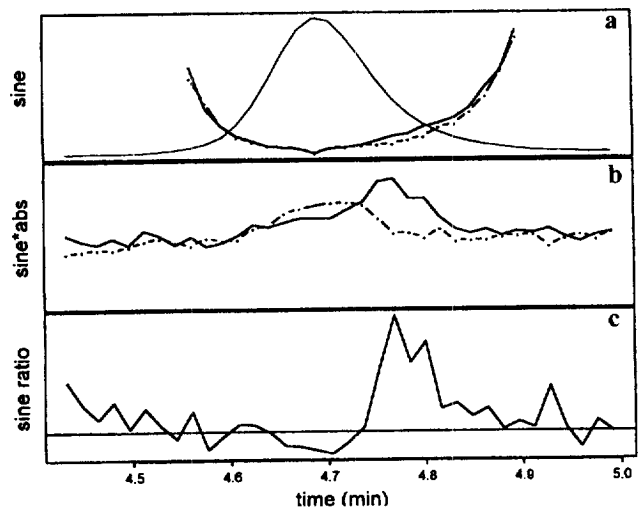

Fig. 5. Dissimilarity plots computed for a heterogeneous peak of alprazolam containing 1% of impurity with a resolution of 0.3 and with a maximal absorbance of about 100 mAU: (a) sine (solid line) and guide (dotted line) curves with an absorbance threshold equal to 3% of the maximal absorbance value; (b) weighted sine display; (c) sine-ratio display.

the dissimilarity factor values are weighted by the intensity of the measured signal so that the dissimilarity curve is reset at the baseline where the absorbance is close to zero. Moreover, the response intensity is not significantly affected under the regions of the peak where the dissimilarity factor is close to zero (selective regions). By erasing the rising edges of the curve, the transformation constricts the range of the results and therefore leads to a magnification effect. As a first consequence, the negative spike under the apex of the peak now becomes especially visible. For convenience, the corresponding dissimilarity value can be excluded from the weighted sine plot without loss of information as shown in Fig. 3c.

For a homogeneous peak, one then obtains a more or less flat profile, a graphical format that enhances the visual interpretation of the results. Any bulge in the dissimilarity plot can effectively be interpreted as a contamination and in principle a base profile is no longer necessary (Fig. 4c and 5b). In addition, owing to the magnification effect, one perceives a slightly increased sensitivity particularly when the coelution is more severe (compare Fig. 5b and Fig. 5c). Finally, the application of an absorbance threshold becomes superfluous which permits the detection of impurities coeluting under the extreme edges of the peak.

4.2. Guide curves

In practice, always limiting the maximal intensities to avoid artefacts is neither possible, practical, nor desirable. As mentioned in Section 1, imposing an absorbance ceiling is a drawback since it results in a lower signal-to-noise ratio (S/N) and hence lowers the capabilities to sensitive detection of impurity. The user has therefore to cope with the possible occurrence of artefacts, and the superimposition of some base profile is still desirable in order to prevent misinterpretation.

Such a base profile can be obtained from the peak of a pure standard. A detection threshold can be added using information about the reproducibility of the spectral comparison results computed for a standard [8,12]. For instance, the threshold can be based on the mean response plus some confidence interval that accounts for noise and non-ideal effects. A significant coelution is therefore detected when the dissimilarity curve intersects the threshold curve. Such a procedure however requires the availability of a standard and must be carried out as a separate

exercise for each peak of interest in the chromatogram.

Here we suggest constructing the base profile directly from the investigated data matrix. Such a guide curve corresponds to the dissimilarity curve computed for a realistic simulation of the investigated peak as described in Section 3.4. In Fig. 3, the agreement between the experimental and guide curves demonstrates the capability of this procedure in the case of bilinear data. Of course, comparisons of the measured curve with the guide curve are qualitative. The peak will be assigned as impure if the user sees "sufficient" discrepancy between the shapes of the two curves (Figs. 4 and 5). At present, we prefer limiting our work to guide curves since we believe that thresholds obtained from "pure" standards under "similar" experimental conditions may give a false sense of security. Users of the realistic simulation method who wish to use a threshold curve can simply repeat the realistic simulation procedure several times and obtain the threshold in the same way as when standards are used. More conservative thresholds can be obtained by using an inflated maximum absorbance in the realistic simulations.

Note that one can also display the ratio of the dissimilarity curve and the base profile [11]. In the case of a homogeneous sample the sine-ratio shows a more or less random fluctuation around 1 (Fig. 3d).

4.3. Non-ideal behaviour

Figs. 6 and 7 show the spectral comparison results obtained from homogeneous peaks of alprazolam and hydrochlorothiazide both with a maximal absorbance of about 500 mAU. As expected for such high signal intensities, non-idealities introduce distortions in the dissimilarity profiles which could be misinterpreted as coelution. Owing to the magnification effect, the artefacts pattern is especially visible for the weighted sine format and leads to a bimodal shape since the similarity with the base spectrum increases near the apex of the peak. In the case of the hydrochlorothiazide peak, the deformation of the bathtub profile of the dissimilarity curve is directly visible (Fig. 7a). This is not surprising since typical non-idealities are more marked for a compound whose spectrum shows high slope/absorbance ratio.

In both cases, however, the guide curve derived from the corresponding realistic simulation matches the observed dissimilarity profile. This demonstrates that our simulation procedure mimics non-idealities

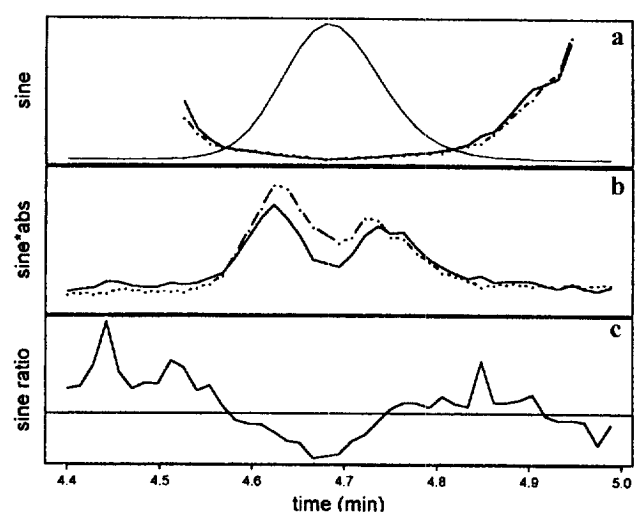

Fig. 6. Dissimilarity plots computed for a homogeneous peak of alprazolam with a maximal absorbance of about 500 mAU: (a) sine (solid line) and guide (dotted line) curves with an absorbance threshold equal to 0.5% of the maximal absorbance value; (b) weighted sine display; (c) sine-ratio display.

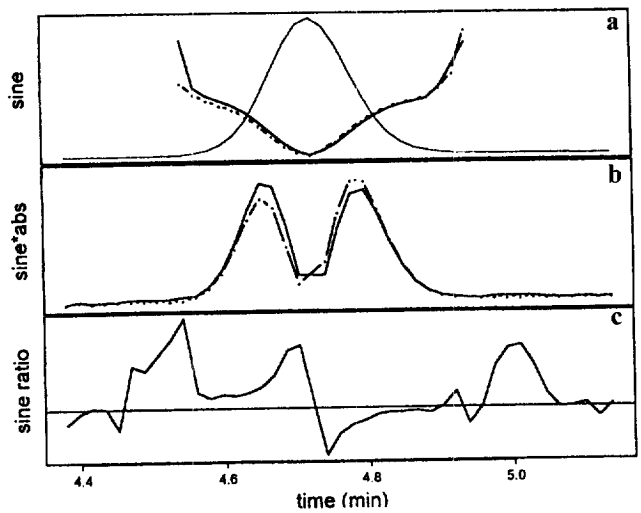

Fig. 7. Dissimilarity plots computed for a homogeneous peak of hydrochlorothiazide with a maximal absorbance of about 500 mAU: (a) sine (solid line) and guide (dotted line) curves with an absorbance threshold equal to 0.5% of the maximal absorbance value; (b) weighted sine display; (c) sine-ratio display.

quite well. We showed elsewhere that such realistic simulations could be obtained throughout the absorbance range generally used, i.e., 0.0 to 0.8 AU [13].

Figs. 8 and 9 show the results obtained for impure peaks of alprazolam (1% of impurity at $R_s=0.3$) and hydrochlorothiazide (1% of impurity at $R_s=0.7$) both with a maximal absorbance of about 500 mAU. In each case, we can see large discrepancies between the dissimilarity and guide curves revealing the

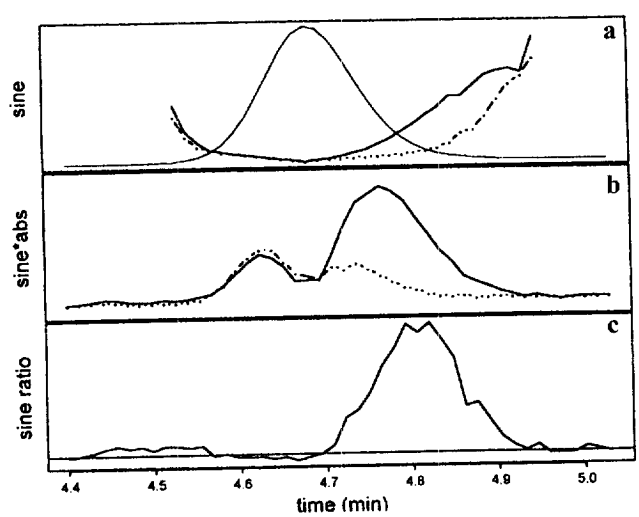

Fig. 8. Dissimilarity plots computed for a heterogeneous peak of alprazolam containing 1% of impurity with a resolution of 0.3 and with a maximal absorbance of about 500 mAU: (a) sine (solid line) and guide (dotted line) curves with an absorbance threshold equal to 0.5% of the maximal absorbance value; (b) weighted sine display; (c) sine-ratio display.

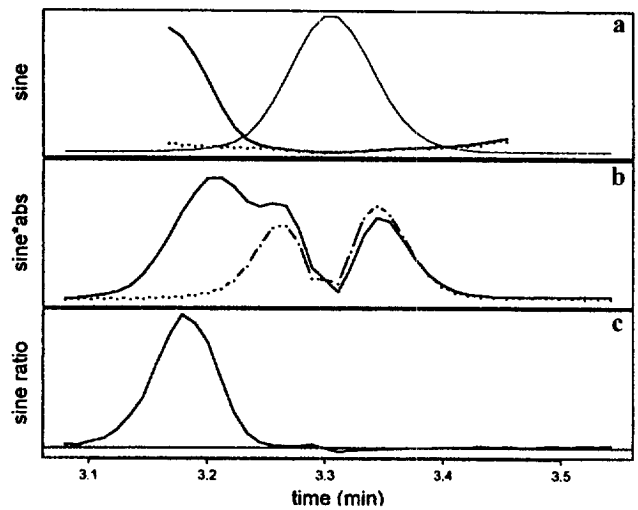

Fig. 9. Dissimilarity plots computed for a heterogeneous peak of hydrochlorothiazide containing 1% of impurity with a resolution of 0.7 and with a maximal absorbance of about 500 mAU: (a) sine (solid line) and guide (dotted line) curves with an absorbance threshold equal to 0.5% of the maximal absorbance value; (b) weighted sine display; (c) sine-ratio display.

presence of the coelution. Note that except for the concentration of the sample, the data of Fig. 5 are equivalent to those of Fig. 8. The gain in sensitivity associated with an improvement of the S/N is clearly visible when comparing these two pictures.

Even though the plot of a guide curve prevents misinterpretation of the non-ideality features, the latter are still observed in a weighted sine display and blur the picture. An even more effective format can therefore be obtained with the sine-ratio which allows an almost complete suppression of the artefacts (Fig. 8c and Fig. 9c).

5. Conclusions

Computing the dissimilarity between spectra within a peak can be a sensitive method for detecting coelution. Several displays of the dissimilarity curve are available: the direct display, the weighted sine display and the sine-ratio display. The direct display and the sine ratio display always require a base profile (guide curve), the weighted sine display can be used without guide curve if the maximum absorbance is kept small. For larger maximum absorbances, guide curves are essential. We have shown that they can be obtained by realistic simulation from the spectrochromatogram under study if some parameters governing several important non-idealities are known. This approach works even if maximum absorbances are increased substantially beyond the point where artefacts become visible. Working at such high maximum absorbances clearly increases detection power. As our comparison shows, the sine-ratio display provides the clearest impurity signal. If required, thresholds can be added by repeated simulations.

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