Journal of Chromatography, 282 (1983) 123–140
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 142

NOVEL TECHNIQUES FOR PEAK RECOGNITION AND DECONVOLUTION BY COMPUTER-AIDED PHOTODIODE ARRAY DETECTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Digital algorithms for manipulating spectrochromatographic data, obtained by means of linear photodiode array detectors, have been examined as tests for peak purity in high-performance liquid chromatography and for their quantitative performance. Two model systems, noscapine and papaverine, and the red pigments R112 and R3, served as examples of closely overlapping and slightly overlapping systems, respectively. The absorbance ratio plot as a function of time performed less well in recognising peak overlap than the second derivative of the elution profile (d^2A/dt^2). A novel approach has been developed for optimising the spectral suppression technique, where a difference absorbance function deconvolutes a component of known spectral properties to permit detection of 1% noscapine in papaverine, for which the spectra differ substantially. Spectral suppression was less successful in deconvoluting trace amounts of R112 and R3, the spectra of which are closely similar. Spectral deconvolution with a "dummy" linear absorbance standard, and a non-absorbing reference wavelength, permitted the elution profiles of both model systems to be completely resolved. This method revealed the elution profile of an unknown, closely overlapping peak in a forensic sample containing papaverine and noscapine.

INTRODUCTION

Rapid-scanning detectors, based on the linear photodiode array device, have transformed the practice of UV–visible detection in high-performance liquid chromatography (HPLC)[1–4]. The spectral information acquired during elution enables flexible selection of wavelengths for optimum detection sensitivity of each component. Coupled with a microcomputer for digital processing, the extra dimension of spectral information can be used to improve selectivity, so that eluting components can be more readily characterised[2,5,6]. Moreover, the spectral data can be manipulated by a variety of algorithms to ascertain the purity of an eluting peak.

0021-9673/83/$03.00

The detection of multiple components eluted together has long been a classical problem area in chromatography, addressed by numerous groups of workers[7–14]. Among the several approaches explored are the techniques of curve fitting on the basis of ideal, skewed, or modified Gaussian functions[7–9], the use of statistical moments as sensitive criteria for peak shape[10] and the use of fast Fourier transform (FFT) self-deconvolution for band-sharpening purposes[11–13]. More recently FFT has seen elegant application to the study of Gaussian and Chesler–Cram peaks, which strongly overlap in the time-domain elution profile, and which are detected and resolved in the frequency domain[14].

Transformation of the elution profile to its first, second, or higher derivative has also received attention in the literature[15–25]. The first derivative chromatogram, proposed many years ago by Boeke[15], has proved useful in detecting overlapping components[16,17] and for measuring physicochemical parameters in chromatography[18,19]. This method forms the basis for peak detection in automatic chromatographic methods[20]. It transpires, however, that the second and higher even derivatives of the detector response, R, in the time domain (d^2R/dt^2, ...) give a sharpened representation of overlapping components in the elution profile, a feature which has been exploited by several groups working in gas and liquid chromatography[5,21–23]. Although the resolution enhancement attainable is limited by interference from the satellite artefacts generated[5,24], the second derivative chromatogram has been found useful as a criterion for multiple peak recognition in automated method development[23]. Indeed, the relative amplitude ratio of the second derivative on the leading and trailing edges of a peak has been proposed as a criterion for peak purity[25].

Other approaches to recognition of composite peaks in HPLC, proposed by Berg *et al.*[26], are based on a modified Vierordt procedure or on the use of isoabsorptive points. However, this method implies significant knowledge of the spectral properties of the entities that are eluted together. An analogous approach, based on dual-wavelength detection in HPLC, has also been reported for the quantitation of partially resolved components[27]. Dual-wavelength UV detection is widely used to generate absorbance ratios as a relatively non-specific method for characterising peak purity in HPLC[28,29]. The sensitivity of the absorbance ratio criterion depends, *inter alia*, on the magnitude of the difference in molar absorptivities for analyte and simultaneously eluted impurity at the wavelengths selected. However, if several wavelengths, pairs are selected, as is possible when the multichannel LDA detector is used, the selectivity of the absorbance ratio method and its sensitivity to the interfering impurity can be increased[6,29]. An extension of the principle of dual-wavelength detection is the technique of spectral suppression, proposed by Carter *et al.*[30]. This technique can be used to suppress a known component in the chromatogram and is readily implemented for any wavelength pair by using the LDA detector. Any residual peak, which may be observed as positive or negative excursions about the baseline, as discussed below, gives an indication of the presence of another component, provided only that its spectral characteristics differ from those of the component to be suppressed.

The total spectral information can also be employed in a least-squares matrix-based routine for spectral deconvolution[2,31] at sequential points in the chromatogram, but this again presupposes that the spectra of the overlapping components are well-defined and available in digital archive.

In the present paper, the conditions for optimal application of the method of spectral suppression are examined. This method is compared with the method of spectral deconvolution and the method of differentiation in the time domain with respect to two model overlapping systems of biomedical and forensic interest: pigments and the alkaloids noscapine and papaverine.

EXPERIMENTAL

Reagents and materials

Analytical-grade toluene, dichlorobenzene, and dichloroethane (BDH, Poole, U.K.), HPLC-grade dichloromethane and HPLC-grade S acetonitrile (Rathburn Chemicals, Walkerburn, U.K.) were used as received. All eluents were filtered through Millipore® 0.45-μm filters in all-glass apparatus. Aqueous acetonitrile was degassed under reduced pressure in an ultrasonic bath for 10 min. Tetrabutylammonium phosphate (PIC A reagent, Waters Assoc., Milford, MA, U.S.A.) was diluted to 0.005 *M* with glass-distilled water. Sulphuric acid (BDH) was of reagent grade. The pigments R3 and R112 (Ciba-Geigy, Horsham, U.K.), the alkaloids noscapine and papaverine, and small amounts of forensic samples came from a collection of the Central Research Establishment, Home Office Forensic Science Serivce, Aldermaston, U.K. Standard solutions of the pigments were prepared in eluents as used for HPLC (see below) or in dichloromethane. Solutions of noscapine (NOS) and papaverine (PAP) were prepared in the appropriate eluent or in acetonitrile – 0.02 *M* sulphuric acid (4:1). This latter solvent was used for the forensic samples.

Equipment

A constant-flow LC pump with integral pulse damping (Gilson Model 302; Villiers-le-Bel, France) and a Rheodyne injection valve (Model 7125, Berkley, CA, U.S.A.) with a 20-μl loop were used to assemble the modular liquid chromatograph. A 250 × 5 mm I.D. stainless-steel column (Shandon Southern Instruments, Cheshire, U.K.) was slurry-packed with 5-μm Hypersil by upward displacement with methanol, followed by hexane. A 100 × 5 mm I.D. stainless-steel column (Shandon Southern Instruments) was slurry-packed with aminopropyl-bonded silica (5-μm APS Hypersil, Shandon Southern Instruments) as recommended by the manufacturers.

Two optical multichannel detector systems were employed. System I included a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model HP-8450A UV-visible spectrophotometer, equipped with an 8-μl quartz flow-cell (Model 178.32 QS; Hellma, Mühlheim-Baden, F.R.G.). The spectrophotometer was configured via the RS 232C port (9600 baud) with a Model HP-85 microcomputer, equipped with 16-kbyte additional RAM, input/output, plotter/printer, mass storage and matrix ROMs and RS 232C and HP-IB IEEE-488 interfaces. A graphics plotter (HP 7225B) and a dual 8-in. flexible-disk drive (HP 9895A) were employed. The configuration of these units was as previously described (configuration B)[33] which permitted the acquisition and storage on disk of sequential spectra at 2.5-sec intervals during elution, under control of the HP-85 microcomputer. Alternatively, a smaller number of spectra could be measured and stored at 1-sec intervals within the HP 8450A spectrophotometer itself and subsequently transferred to disk.

Detector system II included a Hewlett-Packard Model HP-1040A LC detector,

equipped with a 4.5-μl quartz flow-cell. This detector requires an HP-85 microcomputer, equipped with input/output, plotter/printer, mass storage and advanced programming ROMs, 16-kbyte additional memory and an HP-IB IEEE-488 interface. A graphics plotter (HP-7470A) and a dual 5¼ in. flexible-disk drive (HP-82901M) were connected to the HP-85 microcomputer via the HP-IB interface.

To enable system I to be used efficiently for LC detection, a suite of programs for the HP-85 microcomputer was written. These programs handle control of spectrophotometer, acquisition of data, and post-analytical manipulation and presentation of chromatograms and spectra. A method for optimising the spectral suppression technique[30] was developed. Sequential UV–visible spectra, acquired during elution, were processed by a graphics routine for plotting three-dimensional spectrochromatograms of (absorbance, wavelength, time) at 25° in forward or reverse projection, as previously described[33].

For detector system II the software on disk supplied by the manufacturers was employed. The capability of this detector was extended by developing a routine for differentiation in the time domain, based on the Savitzky–Golay[32] quadratic-cubic algorithm (n = 5 points). This "stand-alone" program was used to provide the second (and, if necessary, higher even order) derivative chromatogram, from the data held on disk, with a small degree of smoothing.

HPLC methods and procedures

Pigments were separated by adsorption HPLC on Hypersil (5-μm silica) with toluene–dichlorobenzene–dichloroethane (1:1:1) as eluent. The flow-rate was 1.5 ml/min. The column efficiency for the pigments employed was typically 16,000–24,000 plates/m. Noscapine and papaverine were separated in the reversed-phase system proposed by Baker and Gough[34], somewhat modified as previously described[33]; the eluent was acetonitrile–0.005 M tetrabutylammonium phosphate (85:15), the column performance being typically 35,000 plates/m.

In both detector systems the signal-to-noise ratio (SNR) was significantly improved by referencing the "analytical" detection wavelength or wavelengths of interest against a remote wavelength where no analyte absorption occurred. The SNR was further improved by 'bunching' diodes, symmetrically disposed about the nominal analytical or reference wavelengths[35].

Spectral suppression in HPLC

For a pure compound with a well-defined absorption spectrum, the molar absorptivity (ε) at wavelength λ_1 is directly proportional to that at any other wavelength, λ_2:

$$\varepsilon_{\lambda 1} = K_{1,2} \cdot \varepsilon_{\lambda 2} \tag{1}$$

where the proportionality constant, $K_{1,2}$, is characteristic of the compound at the wavelengths selected. In particular, this constant is independent of concentration, within the limits of obedience of the Beer–Lambert law. By rearrangement of eqn. 1, a null relationship is obtained:

$$\Delta\varepsilon_{1,2} = \varepsilon_{\lambda 1} - K_{1,2} \cdot \varepsilon_{\lambda 2} = 0 \tag{2}$$

Thus, at any concentration within the linear range, the absorbance contribution of a pure compound can be suppressed by computing the difference absorbance function, $\Delta A_{1,2}$:

$$\Delta A_{1,2} = A_{\lambda 1} - K_{1,2} \cdot A_{\lambda 2} = 0 \tag{3}$$

In the simplest application, any two equiabsorptive wavelengths (for which $K_{1,2}$ is unity) in the absorption spectrum of the compound will permit the compound to be suppressed.

Eqn. 3 also applies in cases where the concentration varies as a function of time, as in elution chromatography, provided only that the sampling time of absorbance measurement is small relative to the gradient of the elution profile. This latter condition can be achieved by using a rapid-scanning, multichannel detector, as proposed by Carter *et al.*[30].

In the general case of two components eluted together, such as, for example, an analyte, X, and a minor impurity, Z, the total absorbance at any wavelength λ, A_λ^T, at any elution time, t, is the linear sum of the individual absorptive contributions:

$$A_\lambda^T = A_\lambda^X + A_\lambda^Z \tag{4}$$

By analogy, the difference absorbances at λ_1 and λ_2 are similarly additive:

$$\Delta A_{1,2}^T = \Delta A_{1,2}^X + \Delta A_{1,2}^Z \tag{5}$$

If for analyte X the value of $K_{1,2}^X$ is defined at two appropriate wavelengths, then according to eqn. 3 the difference function $\Delta A_{1,2}^X$ becomes zero. Provided that the condition

$$K_{1,2}^X \neq K_{1,2}^Z \tag{6}$$

is fulfilled at the two wavelengths selected, the difference function for impurity Z, $\Delta A_{1,2}^Z$, assumes a finite value, positive or negative, so that at any elution time, t,

$$\Delta A_{1,2}^T \equiv \Delta A_{1,2}^Z = A_{\lambda 1}^Z - K_{1,2}^X \cdot A_{\lambda 2}^Z \tag{7}$$

The difference function, $\Delta A_{1,2}^T$, recorded as elution proceeds, yields a chromatogram where impurity Z is detected, whereas analyte X is spectrally suppressed. Conversely, if in eqns. 3 and 5 the corresponding value of $K_{1,2}^Z$ is used to compute the difference function, $\Delta A_{1,2}^T$, then impurity Z will be suppressed and the elution profile will feature component X alone. In practice, it is unlikely that the spectral characteristics of such an impurity will be as well-defined as those of the analyte itself. Thus, the spectral suppression method is more readily applicable to the detection of uncharacterised impurity or impurities eluted together with an analyte, as originally proposed[30].

Eqn. 7 may be rearranged by using the relation

$$A_{\lambda 1}^Z = K_{1,2}^Z \cdot A_{\lambda 2}^Z \tag{8}$$

so that

$$\Delta A^{T}_{1,2} = (K^{Z}_{1,2} - K^{X}_{1,2})A^{Z}_{\lambda 2} \tag{9}$$

It follows from eqn. 9 that discrimination between X and Z is only observed if the two wavelengths are selected so that the condition in eqn. 6 is fulfilled. Moreover, the detection sensitivity for impurity Z is seen to depend upon two factors:

$$\Delta A^{T}_{1,2} \propto (K^{Z}_{1,2} - K^{X}_{1,2}) \tag{10}$$

and

$$\Delta A^{T}_{1,2} \propto A^{Z}_{\lambda 2} \propto \varepsilon^{Z}_{\lambda 2} \tag{11}$$

The question arises as to how best to select the two wavelengths for optimum sensitivity. One approach to optimising the condition expressed in eqn. 10, when both components are well-defined, is to compute the ratio of the two spectra. A plot of this ratio as a function of wavelength, λ_n, yields maximum and minimum points, which correspond to the wavelengths where the absorbance ratios differ most:

$$A^{Z}_{\lambda n}/A^{X}_{\lambda n} = (K^{Z}_{n,0}\ A^{Z}_{\lambda 0})/(K^{X}_{n,0}\ A^{X}_{\lambda 0}) \tag{12}$$

$$= \alpha^{X,Z}_{\lambda 0}\ (K^{Z}_{n,0}/K^{X}_{n,0}) \tag{13}$$

The term λ_0 represents any defined 'reference' wavelength in the spectrum. Its value changes the value of the absorbance ratio, $\alpha^{X,Z}_{\lambda 0}$, which scales the amplitude but does not change the shape of the graph of absorbance ratio *vs.* λ_n.

In cases where the spectra of analyte X and impurity Z are dissimilar, the maximum of the ratio $(A^{Z}_{\lambda n}/A^{X}_{\lambda n})$ would be expected to occur close to the maximum absorptivity of the impurity, thus fulfilling the condition for high sensitivity implied in eqn. 11. Although the relationship between $\Delta A^{T}_{n,0}$, molar absorptivity $\varepsilon^{Z}_{\lambda 0}$, and $(K^{Z}_{n,0} - K^{X}_{n,0})$ is rather complex, suitable initial conditions can be established by selecting as one of the wavelengths the maximum absorptivity of the impurity. The second wavelength is then taken as the point in the absorbance ratio plot where the greatest difference in $K_{n,0}$ is observed.

The spectral ratio graph has the further property that it can be used to detect wavelength pairs where the absorbance ratios are identical. At these wavelengths, both analyte X and a known contaminant, Z, can be deconvoluted from the chromatogram. This technique then permits any further, undefined impurity or impurities to be detected, albeit at reduced sensitivity.

RESULTS AND DISCUSSION

The two model analytical systems selected for this study represent two different types of band overlap situation in chromatography. The alkaloids noscapine (NOS) and papaverine (PAP) are eluted at closely similar retention times (42 and 45 sec,

respectively) under the HPLC conditions employed[34]. So strongly overlapping are these peaks (resolution, $R_s \approx 0.32$) that only a slight shoulder is apparent in the elution profile (Fig. 1). By contrast, the red pigments Red 112 (R112) and Red 3 (R3) are better resolved ($R_s \approx 1.6$) by the adsorption system described (Fig. 2). Of relevance to the spectroscopic basis of some of the techniques examined is the fact that the pigment spectra are closely similar (Fig. 3), whereas the alkaloids differ considerably in their spectral properties (Fig. 4).

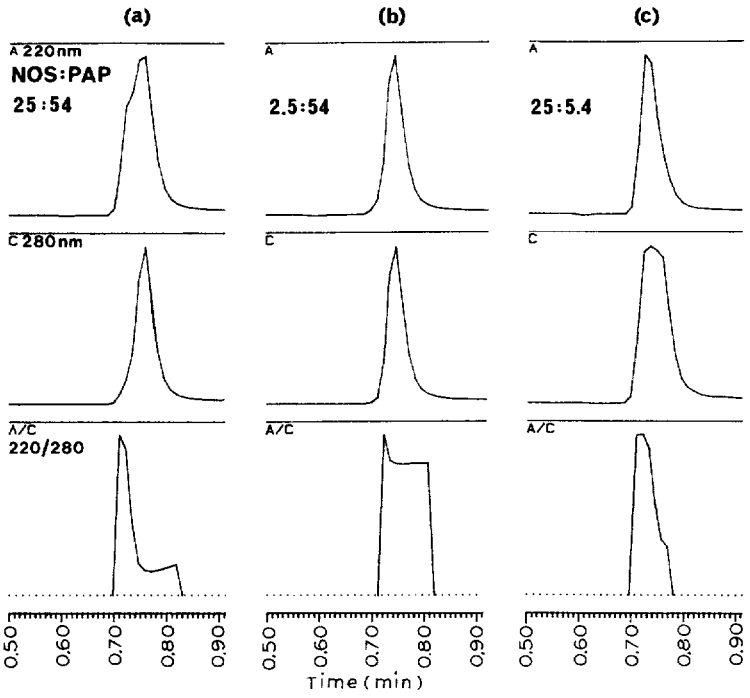

Fig. 1. Elution profiles at 220 nm (A) and 280 nm (C), and the corresponding absorbance ratios, for mixtures of noscapine and papaverine. The ratios of noscapine to papaverine were: (a) 25:54 μg/ml; (b) 2.5:54 μg/ml; (c) 25:5.4 μg/ml. Detection System II was used; for chromatographic conditions, see text; reference wavelength, 550 nm; bandwidht, 10 nm; bandwidth at 220 and 280 nm, 4 nm.

The classical method of plotting the absorbance ratio at two characteristic wavelengths (220 and 280 nm) as a function of time can be seen to give a sensitive indication of the presence of small amounts of noscapine in papaverine down to the 5% level (Fig. 1b). Although an equiabsorptive concentration of papaverine can be readily detected (Fig. 1a), the absorbance ratio does not give a satisfactory indication of papaverine, even at the 20% level (Fig. 1c). The absorbance ratio criterion performs less well for papaverine as 'impurity' than for noscapine, reflecting the differing spectral characteristics of the two alkaloids.

The inverted, sharpened profile observed in the second derivative chromatogram of the red pigments, detected at 500 nm (Fig. 2), displays resolution enhancement, accompanied by a degradation in SNR. In the presence of a constant amount

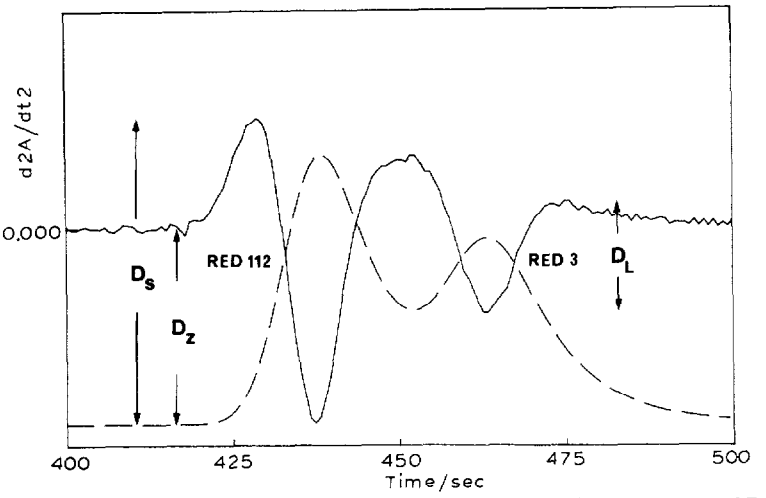

Fig. 2. Normal (---) and second-derivative (——) (d^2A/dt^2) chromatograms of Red 112 and Red 3, each at 100 μg/ml (injection volume 10 μl), separated by adsorption HPLC (see text). Detector wavelength, 500 nm; reference wavelength, 600 nm, bandwidth, 10 nm; Detector System II. Derivative amplitude measures: D_s, amplitude of R112 minimum with respect to satellite eluted early; D_Z, amplitude of R112 minimum with respect to derivative zero; D_L, amplitude of R3 minimum to satellite eluted late.

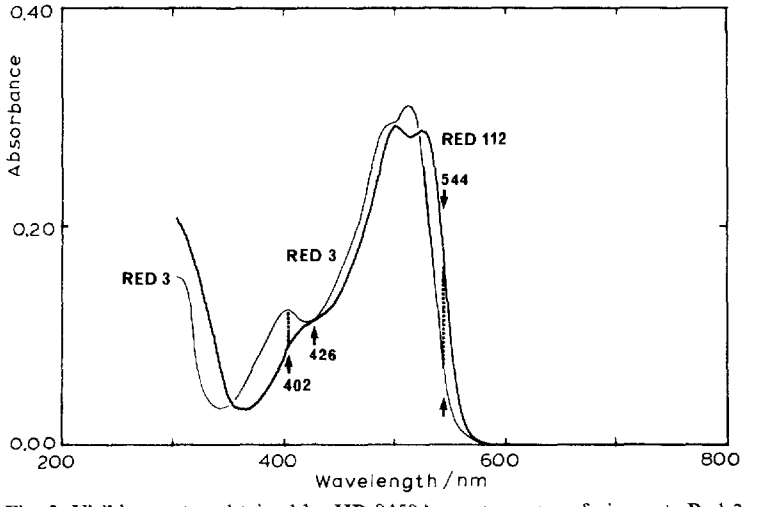

Fig. 3. Visible spectra obtained by HP-8450A spectrometer of pigments Red 3 and Red 112 (100 μg/ml each in dichloromethane), showing the wavelengths employed for spectral suppression chromatography.

of R3 (100 μg/ml in dichloromethane), the R112 can be readily quantitated in the derivative chromatogram by measuring the peak amplitude of the minimum with respect to either the satellite eluted earlier (D_s) or the derivative baseline zero (D_z). In the range 5-100 μg/ml, the regression data for the D_s measure of duplicate injections of R112 were:

$$y = 1.02x + 2.10 \ (n = 14)$$

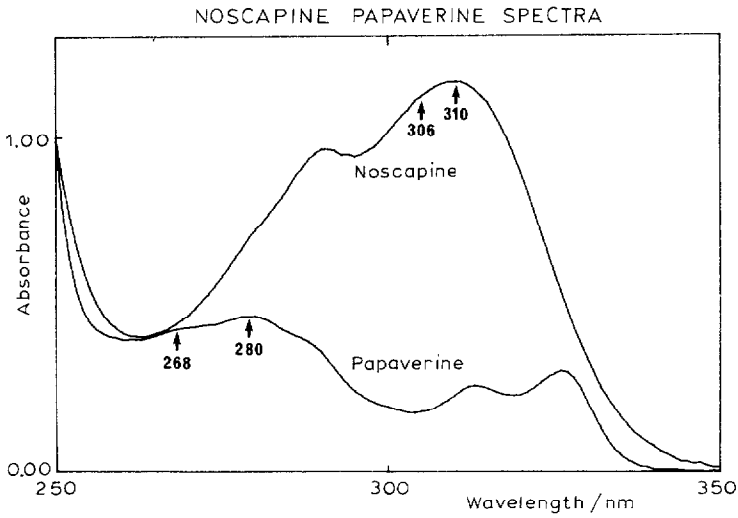

Fig. 4. UV spectra obtained by HP 8450A spectrometer of noscapine and papaverine in acetonitrile–0.02 *M* sulphuric acid (4:1), normalised at 250 nm. Wavelength maxima: papaverine, 280 nm; noscapine, 310 nm; wavelengths for spectral suppression chromatogram: 268 nm and 306 nm.

The relative confidence limits ($p = 0.95$) at 40 μg/ml were ±3.2%. The amplitude of the corresponding graphic measure for R3 (derivative peak minimum to satellite) was independent of R112 over this concentration range, whereas the direct absorbance measure of R3 increased by 10% at 100 μg/ml of R112. Care should be taken in selecting the graphic measure for quantitation: if the amplitude of the R3 derivative peak minimum is measured with respect to the satellite maximum located between the peaks, the R3 derivative amplitude is not independent of R112 concentration, due to the complex interaction of the individual overlapping satellites at this position. Similar considerations apply to the measurement of the R112 derivative peak with respect to this central satellite. In general, it is preferable to measure each of the peak amplitudes in the second derivative chromatogram with respect to satellites on the outer wings of the composite peak[5,24].

The elution profile of noscapine and papaverine at 220 nm gives little indication of there being more than one component (Fig. 5a), since in this case $R_s \approx 0.32$. However, the second derivative chromatogram partially resolves the two alkaloids (Fig. 5b), giving a clear indication of peak in-homogeneity. The individual contributions of each inverted, sharpened alkaloid derivative peak (Fig. 5c) can be seen to overlap strongly, so that quantitative assay of each component is impossible in this case, although the resolution enhancement obtained does permit recognition of peak overlap over a wide range of concentration ratios of noscapine to papaverine (*ca.* 5:95 to 90:10). In fact, the wavelength flexibility of the LDA spectrophotometer can be exploited in this case, since the second-derivative chromatogram at 280 nm discriminates strongly in favour of papaverine (Fig. 5d) to give more sensitive detection of low papaverine levels in the presence of noscapine. The combination of flexible detection wavelength and transformation of the elution profile to the second or higher

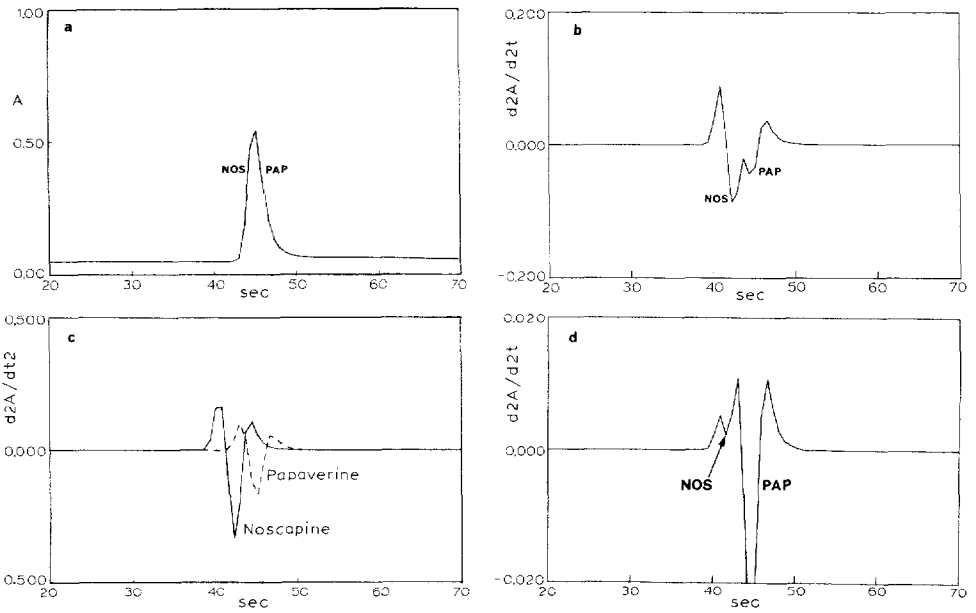

Fig. 5. Normal and second-derivative chromatograms of noscapine-papaverine mixture (100 μg/ml each) by reversed-phase HPLC (see text); detector system II at 220 nm (except d: 280 nm).

even derivative for multiple peak recognition offers a new, more powerful tool for the optimisation of HPLC separations by simplex procedures[23].

A necessary condition for the even derivative recognition of overlapping peaks through enhancement of resolution is that the peaks be displaced in the elution profile. In cases where bands are eluted at the same retention position, or with resolution less than *ca.* 0.3[14], the derivative method breaks down. However, the multi-wavelength capability of the LDA detector, coupled with the rapid, computer-aided manipulation of data, permits the relatively new technique of spectral suppression[30] to be employed, even in cases of coincident peak elution, provided that the spectrum of at least one component is well characterised. In the case of the strongly overlapping alkaloid system, where both components are known, the optimum pair of wavelengths can be assessed, as discussed above. The difference absorbance function at these optimised wavelengths should generate the most sensitive parameter for measuring either papaverine (suppressing noscapine) or noscapine (suppressing papaverine). The ratio of the normalised papaverine to noscapine spectra reveals a maximum absorbance ratio near 268 nm and a minimum near 306 nm (Fig. 6). Taking these wavelengths as initial values, corresponding to the maximum *difference* in absorbance ratio (*cf.* eqn. 9), it can be seen (Fig. 4) that they approximate, respectively, to the maximum absorptivity of papaverine (280 nm) and of noscapine (310 nm) in this spectral range, thus satisfying the second criterion for maximum sensitivity (eqn. 11). The requisite equations for monitoring the elution profile of the one component, while suppressing the other, can be expressed as:

$$\Delta A^{\mathrm{NOS}}_{268,306} = A_{268} - 2.39A_{306} \text{ for monitoring noscapine} \quad (14)$$

$$\Delta A^{\mathrm{PAP}}_{306,268} = A_{306} - 2.58A_{268} \text{ for monitoring papaverine} \quad (15)$$

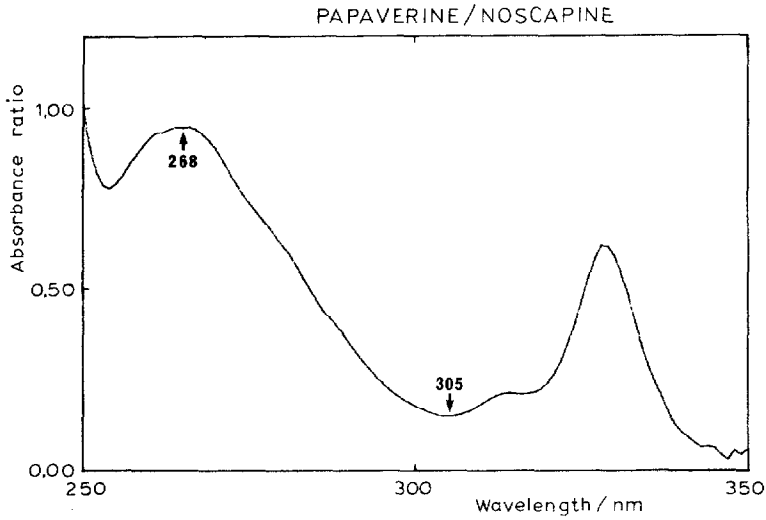

Fig. 6. Ratio of papaverine to noscapine normalised spectra (by HP 8450A) in acetonitrile–0.02 *M* sulphuric acid (4:1), illustrating the optimum wavelengths for spectral suppression chromatography.

The elution of a mixture, containing 100 μg/ml of each of the two alkaloids, monitored by each of these difference absorbance algorithms, yields the individual peaks for noscapine and papaverine in two separate profiles, obtained from the same matrix of spectral data acquired during the chromatogram (Fig. 7). The chromatogram at 306 nm, and the absorbance ratio A_{268}:A_{306}, clearly indicate the inhomogeneity of the peak in this case. The choice of wavelength in using the absorbance

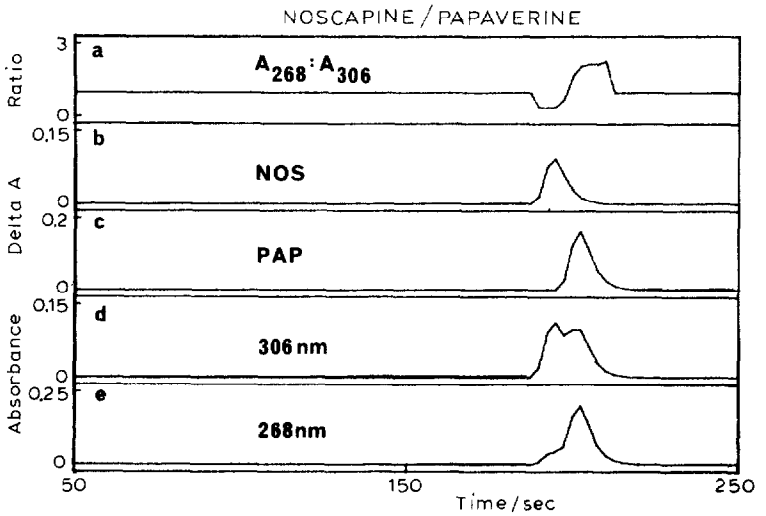

Fig. 7. Normal, spectral suppression and absorbance ratio chromatograms of noscapine and papaverine (100 μg/ml each in HPLC eluent, see text). (a) Absorbance ratio test for peak homogeneity; (b) spectral suppression of papaverine, monitoring noscapine; (c) spectral suppression of noscapine, monitoring papaverine; (d) and (e) elution profiles at 306 and 268 nm, respectively (detector system I).

ratio is critical to performance: in this example the wavelength pair aids discrimination by generating a negative absorbance ratio for noscapine and a positive ratio for papaverine. When noscapine is reduced to 10 μg/ml, while papaverine is constant at 100 μg/ml, the absorbance ratio still indicates the peak inhomogeneity. At the 1 μg/ml noscapine level, however, only the spectral suppression method still reports the presence of noscapine, albeit with increased baseline noise (Fig. 8). This represents the detection limit for noscapine (SNR = 2) under the wavelength conditions adopted. The sensitivity of the method is only slightly increased (less than 5%) for papaverine by using its wavelength maximum (280 nm) together with the wavelength for optimum difference in absorbance ratio (306 nm); no significant sensitivity increase is observed for noscapine when its wavelength maximum (310 nm) is employed with the corresponding wavelength for optimum difference in absorbance ratio (268 nm). However, sensitivity for both could probably be increased by examining the spectral properties in the 210–240 nm region, where both alkaloids have appreciable absorption. The present example points to a substantial increase in sensitivity for the optimised spectral suppression technique, compared with the second derivative or absorbance ratio methods.

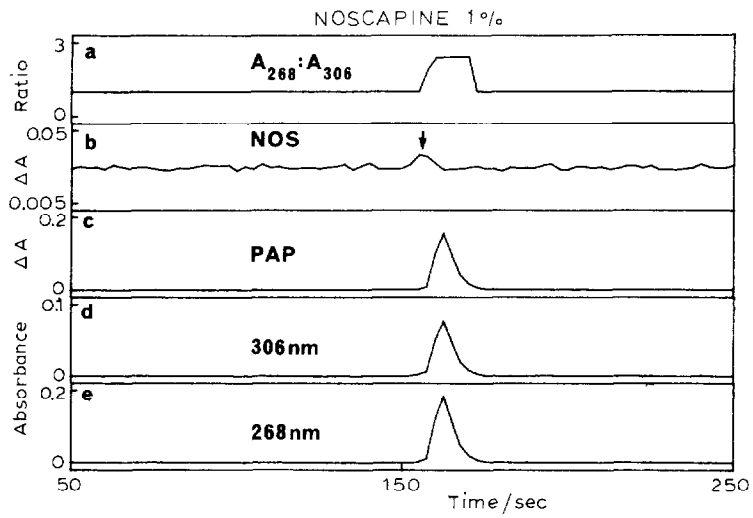

Fig. 8. Normal, spectral suppression and absorbance ratio chromatograms of noscapine (1 μg/ml) and papaverine (100 μg/ml), dissolved in HPLC eluent (see text). (a) Absorbance ratio test for peak homogeneity; (b) spectral suppression of papaverine, monitoring noscapine; (c) spectral suppression of noscapine, monitoring papaverine; (d) and (e) elution profiles at 306 and 268 nm, respectively (detector system I).

The alkaloid system represents a favourable case for spectral suppression, where the individual spectra differ quite considerably. However, the red pigments display closely similar spectra (Fig. 3), and therefore it would be anticipated that the selection of wavelengths for optimum discrimination may be more difficult. The ratio spectrum (Fig. 9) features a sharp maximum at 544 nm, with a practical minimum at 402 nm (corresponding to the lowest wavelength detected by the LDA element used

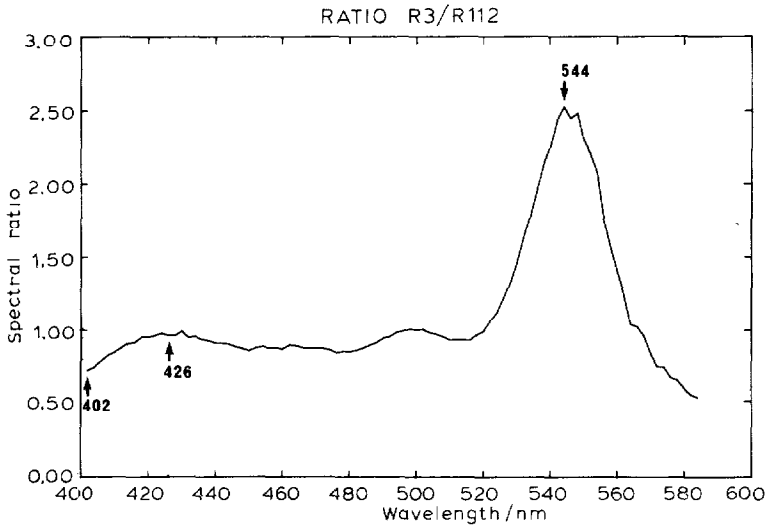

Fig. 9. Ratio of Red 3:Red 112 normalised spectra (by HP-8450A) in HPLC eluent (see text), illustrating the optimum wavelengths for spectral suppression chromatography.

for visible detection); 426 nm gives slightly greater sensitivity. The corresponding difference absorbance algorithms become:

$$\Delta A^{R112}_{544,426} = A_{544} - 0.542\, A_{426} \text{ for monitoring R112} \quad (16)$$

$$\Delta A^{R3}_{402,544} = A_{402} - 0.526\, A_{544} \text{ for monitoring R3} \quad (17)$$

Application of the spectral suppression method to the same pool of data for the pigment system (Fig. 2) yielded a satisfactory elution profile for R112, whereas the deconvoluted R3 peak displayed a negative excursion (Fig. 10). In both spectral

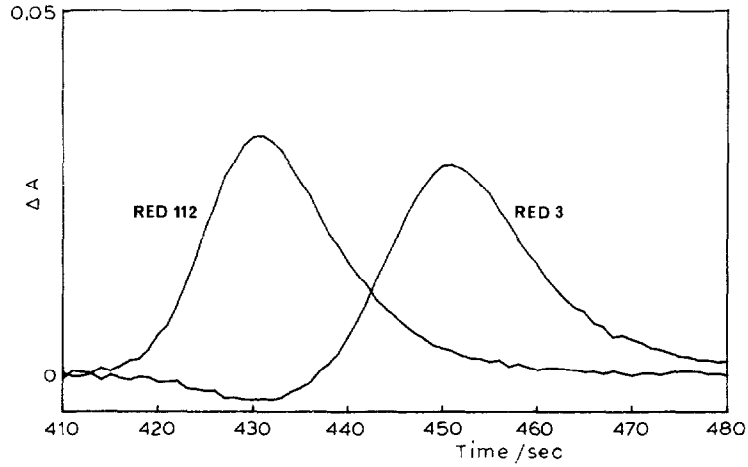

Fig. 10. Spectral suppression chromatograms, monitoring R112 and R3, respectively (concentrations, 100 μg/ml; detector system I, see text).

suppression chromatograms the SNR was clearly degraded. Moreover, sensitivity to R112 was at least a factor of two worse than in the second derivative chromatogram. Thus, it would appear that for overlapping peaks with closely similar spectra, the spectral suppression technique requires careful optimisation and that sensitivity is compromised by a degradation in SNR. Nevertheless, the method can be used quantitatively for the assay of either defined component; the regression data for R112 in the range 5–100 μg/ml are:

$$y = 3.36x + 0.23 \ (n = 14)$$

the relative confidence limits ($p = 0.95$) at 40 μg/ml being ±3.6%.

A very powerful technique, available as a firm-ware routine as part of detector system I, is the least-squares spectral deconvolution method. Unlike the spectral suppression technique, where only one spectrum needs to be defined, spectral deconvolution requires all sample spectra to be available in digital archive, preferably acquired under HPLC eluent conditions in order to standardise solvent-induce artefacts. When this routine was applied to the matrix of spectral data for the pigment chromatogram, R112 and R3 yielded noisy deconvolution chromatograms with a negative excursion in R3, similar to those by spectral suppression (Fig. 10). However, by the simple strategem of inserting a "dummy" standard (actually a constant 'spectrum' of 0.10 AU over the visible wavelength range), the SNR and peak symmetry were substantially improved (Fig. 11). Moreover, the sensitivity of the method was such that R112 could be easily detected at 5 μg/ml, with R3 at 100 μg/ml (Fig. 12), whereas in the two-component spectral deconvolution method, R112 could not be recovered at this concentration due to the high noise level. It would seem that the "dummy" standard permits the linear additive disturbances in the spectrochromatogram to be deconvoluted and, therefore, suppressed. The close similarity of the

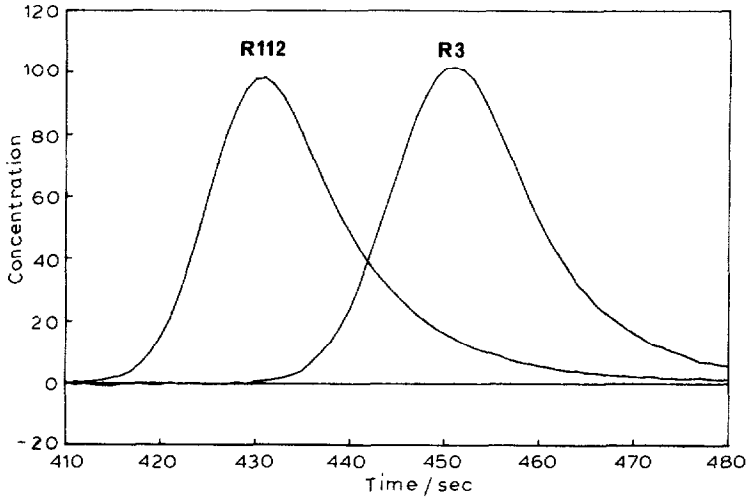

Fig. 11. Spectral deconvolution chromatograms, monitoring R112 and R3, respectively (concentrations 100 μg/ml) based on use of a "dummy" linear absorbance standard (detector system I; conditions, see text; concentration axis calibrated in μg/ml).

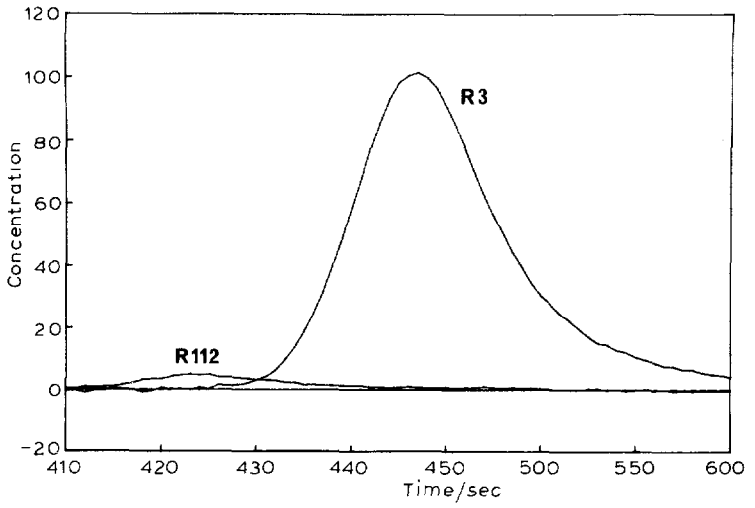

Fig. 12. Spectral deconvolution chromatograms, monitoring R112 (5 μg/ml) and R3 (100 μg/ml), respectively, based on the "dummy" standard routine (detector system I; conditions, see text; concentration axis calibrated in μg/ml).

pigment spectra would be expected to lead to errors in the recovery of each component, and this may partly account for the distortion observed in the two-component deconvolution chromatogram.

Finally, the spectrochromatogram of a forensic sample of heroin (coded H12) was examined for the presence of papaverine and related alkaloids (Fig. 13). By visual comparison with spectrochromatograms of noscapine and papaverine standards (Fig. 14), it was clear that a third component (or group of components) was eluted at the leading edge of H12. A spectrum was acquired on the leading edge of the sample, making the assumption that this should reasonably represent the third unknown component. This was then used with the standard papaverine and noscapine spectra to deconvolute each spectral file acquired at sequential time intervals during the chromatogram. Three overlapping elution profiles were obtained (Fig. 15), the first representing the impurity, scaled in arbitrary fashion due to the manner of its acquisition.

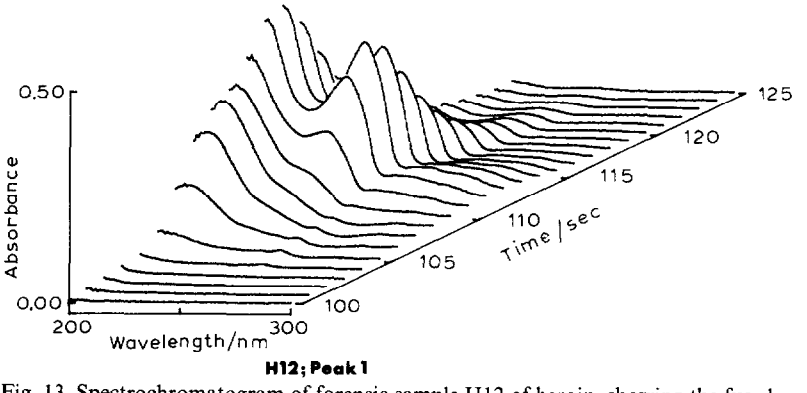

Fig. 13. Spectrochromatogram of forensic sample H12 of heroin, showing the fused, early-eluted alkaloid peaks (detector system I; conditions, see text).

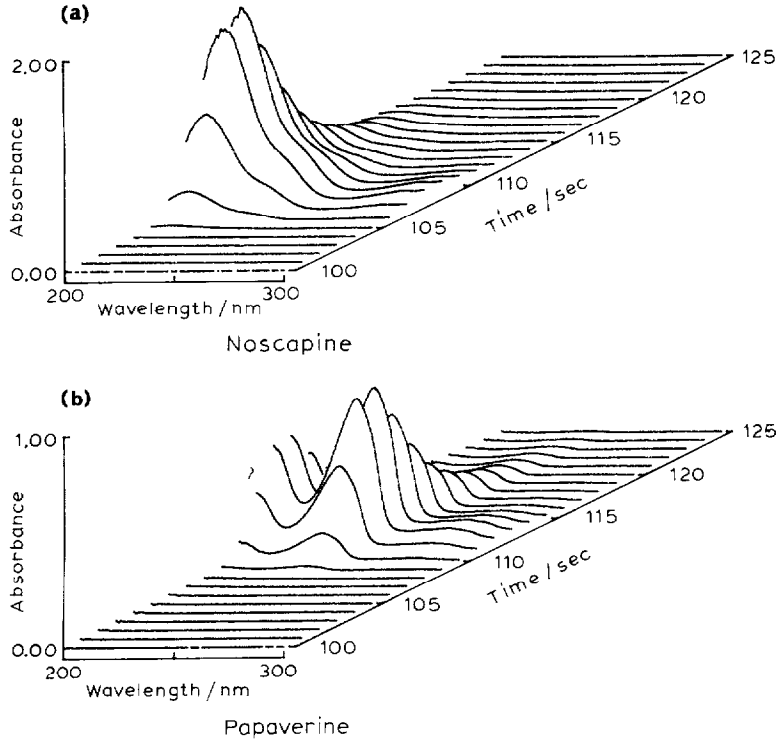

Fig. 14. Spectrochromatograms of (a) noscapine and (b) papaverine reference standards (detector system I; conditions, see text).

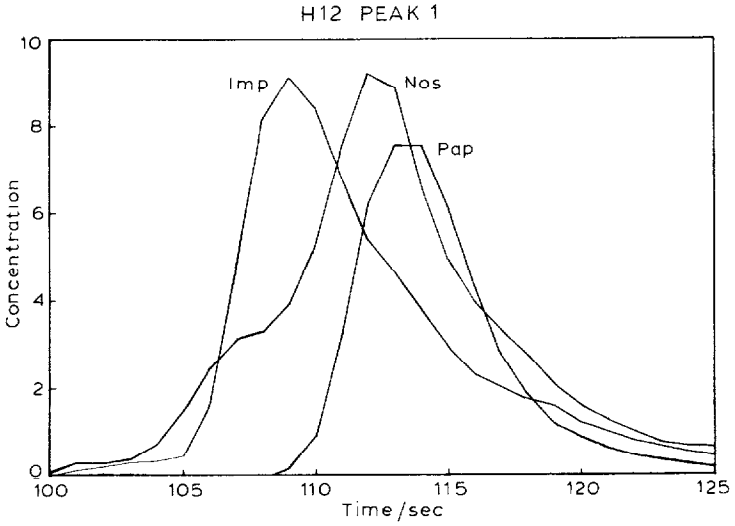

Fig. 15. Spectral deconvolution chromatogram of forensic sample H12 of heroin, using a spectrum captured on the leading edge as a third "impurity standard" with noscapine and papaverine for the deconvolution routine (detector system I; chromatographic conditions, see text; concentration axis in arbitrary units).

Spectral deconvolution can also be used quantitatively for the assay of each defined component, when the regression data for R112 in the range 5–100 μg/ml were

$$y = 0.962x - 0.966 \ (n = 14)$$

by direct deconvolution using two standards, the relative confidence limits ($p = 0.95$) at 40 μg/ml being $\pm 3.8\%$. However, if a "dummy" standard is employed as described, the regression statistics for the same set of spectrochromatographic data become:

$$y = 0.995x - 0.050 \ (n = 14)$$

with relative 95% confidence limits of $\pm 2.0\%$ at 40μg/ml.

This approach to the resolution of chromatographic peaks in the time domain, using rapidly scanned spectral data in the wavelength domain, would seem to have significant potential for the resolution of strongly overlapping and coincident chromatographic peaks. The method can be readily extended by transforming the spectral data to the first or higher wavelength derivative ($dA/d\lambda$, $d^2A/d\lambda^2$, ...), thereby reducing the effect of low-frequency additive factors to give improved selectivity.

CONCLUSIONS

Several algorithms for peak recognition and deconvolution are currently available for use with rapid-scanning multichannel detectors in HPLC. The absorbance ratio method for peak recognition is simple and can be readily optimised for each eluted component, but lacks sensitivity, especially for components with similar spectra. A complementary method involves capturing spectra at the two turning points and at the apex of a peak, followed by overlay presentation of the normalised spectra, to give a clear visual indication of peak inhomogeneity. Differentiation in the time domain can also give a useful qualitative indication of peak inhomogeneity and is capable of quantitative performance, except for closely overlapping peaks.

When spectrally active components are eluted together with little or no resolution, spectral suppression and the new technique for its optimisation would appear to have considerable merit for the recognition of overlapping peaks, provided that the identity of the principal constituent is defined. Alternatively, the technique can be employed to suppress the contribution of a well-characterised minor component, to permit a better quantitative assay of the major constituent peak. However, in cases where the spectra of overlapping components are similar, the method lacks sensitivity.

Perhaps the most powerful route for the resolution of fused chromatographic peaks is spectral deconvolution, but the spectral properties of each component must be defined and sufficiently dissimilar. This method can operate on three or more overlapping components and, when a 16-bit microprocessor (as in detector System I) is used, it is very fast. Use of a spectral standard acquired on the leading or trailing edge of the peak can give a qualitative guide to the peak profile of an unknown component in the fused composite peak. The use of first or higher even derivatives should increase the specificity of spectral deconvolution at the cost of a decrease in SNR.

The rapid development of chemometric methods[36,37] and especially principal component analysis[38–40], coupled with the increasing availability of rapid-scanning multichannel detectors, can be expected to yield powerful new approaches to the recognition and deconvolution of overlapping bands in HPLC.

ACKNOWLEDGEMENTS

Financial support for this work is acknowledged from the Home Office Forensic Science Service, U.K. The loan of a Hewlett-Packard Model 1040A diode array chromatography detector and peripherals by Hewlett-Packard GmbH, Waldbronn, is acknowledged with thanks. Dr. D. Keen-Laing is thanked for valuable discussions and for providing samples of pigments.

REFERENCES

1 M. J. Milano, S. Lam and E. Grushka, *J. Chromatogr.*, 125 (1976) 315–326.
2 M. J. Milano, S. Lam, M. Savonis, D. B. Pautler, J. W. Pav and E. Grushka, *J. Chromatogr.*, 149 (1978) 599–614.
3 R. E. Dessy, W. G. Nunn, J. Titus and W. R. Reynolds, *J. Chromatogr. Sci.*, 14 (1976) 195–200.
4 Y. Talmi, *Appl. Spectroscopy*, 36 (1982) 1–18.
5 A. F. Fell, *Anal. Proc.*, 17 (1980) 512–519.
6 A. F. Fell, H. P. Scott, R. Gill and A. C. Moffat, *Chromatographia*, 16 (1982) 69–78.
7 A. W. Westerberg, *Anal. Chem.*, 41 (1969) 1770–1777.
8 A. H. Anderson, T. C. Gibb and A. B. Littlewood, *Anal. Chem.*, 42 (1970) 434.
9 H. J. Hancock, L. A. Dahm and J. F. Muldoon, *J. Chromatogr. Sci.*, 8 (1970) 57–62.
10 E. Grushka, M. N. Myers and J. C. Giddings, *Anal. Chem.*, 42 (1970) 21–26.
11 D. W. Kirmse and A. W. Westerberg, *Anal. Chem.*, 43 (1971) 1035–1039.
12 R. Annino, *Advan. Chromatogr.*, 15 (1977) 33.
13 N. A. Wright, D. C. Villalanti and M. F. Burke, *Anal. Chem.*, 54 (1982) 1735–1738.
14 M. P. Malczewski and E. Grushka, *J. Chromatogr. Sci.*, 19 (1981) 187–194.
15 J. Boeke, in R. P. W. Scott (Editor), *Gas Chromatography*, Butterworths, London, 1960, p. 88.
16 T. Kambara and K. Saitoh, *J. Chromatogr.*, 35 (1968) 318–322.
17 J. M. Essigman and N. Catsimpoulas, *J. Chromatogr.*, 103 (1975) 7–13.
18 C. N. Reilley, G. P. Hildebrand and J. W. Ashley, *Anal. Chem.*, 34 (1962) 1198–1213.
19 P. Kucera, S. A. Moros and A. R. Mlodozieniec, *J. Chromatogr.*, 210 (1981) 373–388.
20 J. L. Excoffier and G. Guiochon, *Chromatographia*, 15 (1982) 543–545.
21 A. B. Berman, Yu. A. Frank and M. I. Yanovskii, *Zavod. Lab.*, 34 (1968) 272–274.
22 D. T. Zelt, J. A. Owen and G. S. Marks, *J. Chromatogr.*, 189 (1980) 209–216.
23 J. C. Berridge, *Chromatographia*, 16 (1982) 172–174.
24 A. F. Fell, *UV Spectrom. Group Bull.*, 8 (1980) 5–31.
25 E. Grushka and G. C. Monacelli, *Anal. Chem.*, 44 (1972) 484–489.
26 R. C. Berg, C. Y. Ko, J. M. Clemons and H. M. McNair, *Anal. Chem.*, 47 (1975) 2480–2482.
27 R. N. Smith and M. Zetlein, *J. Chromatogr.*, 130 (1977) 314–317.
28 P. C. White, *J. Chromatogr.*, 200 (1980) 271–276.
29 R. Yost, W. MacLean and J. Stoveken, *Chromatogr. Newsl.*, 4 (1976) 1–4.
30 G. T. Carter, R. E. Schiesswohl, H. Burke and R. Yang, *J. Pharm. Sci.*, 71 (1982) 317–321.
31 R. P. Bauman, *Appl. Spectroscopy*, 13 (1959) 156–157.
32 A. Savitzky and M. J. E. Golay, *Anal. Chem.*, 36 (1964) 1627–1639.
33 A. F. Fell, H. P. Scott, R. Gill and A. C. Moffat, *J. Chromatogr.*, 273 (1983) 3–17.
34 P. B. Baker and T. A. Gough, *J. Chromatogr. Sci.*, 19 (1981) 483–489.
35 B. J. Clark, A. F. Fell, H. P. Scott and D. Westerlund, *J. Chromatogr.*, in press.
36 B. R. Kowalski, *Trends Anal. Chem.*, 1 (1981) 71–74.
37 I. M. Warner, in D. M. Hercules, G. M. Hieftje, L. R. Snyder and M. A. Evenson (Editors), *Contemporary Topics in Analytical and Clinical Chemistry*, Vol. 4, Plenum Press, New York, 1982, p. 75.
38 J. M. Halket, *J. Chromatogr.*, 175 (1979) 229–241.
39 J. M. Halket, *J. Chromatogr.*, 186 (1979) 443–455.
40 E. R. Malinowski and D. G. Howery, *Factor Analysis in Chemistry*, Wiley, New York, 1981.