Journal of Chromatography, 297 (1984) 203–214
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 360

ANALYSIS AND CHARACTERISATION OF AROMATIC AMINO ACIDS, METABOLITES AND PEPTIDES BY RAPID-SCANNING PHOTODIODE ARRAY DETECTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

ANTHONY F. FELL*, BRIAN J. CLARK and HUGH P. SCOTT
Department of Pharmacy, Heriot-Watt University, 79 Grassmarket, Edinburgh EH1 2HJ (U.K.)

SUMMARY

The role of rapid-scanning UV detectors based on the linear photodiode array has been examined in studies on the chromatography of aromatic amino acids, some metabolites and peptides. Multichannel detectors can generate characteristic qualitative information presented as individual spectra, as three-dimensional spectrochromatograms or as contour maps in the (λ, t) plane. The use of such contour plots for mapping amino acids and their metabolites, and for peptide mapping Tyr–Gly, Gly–Tyr, Phe–Gly, Tyr–Tyr and Phe–Val, is examined. The application of computer-aided methods for validating peak homogeneity by spectral suppression, second derivative in the time domain and by other methods is discussed with respect to Tyr and its metabolite dopamine, which are not resolved by the system described, and which have closely similar spectral characteristics. The use of colour graphics with the IBM Personal Computer is demonstrated for simplifying the presentation of contour maps of amino acids, metabolites and dipeptides.

INTRODUCTION

The analysis of amino acids and peptides has long attracted considerable interest in food science, in agricultural chemistry and in the pharmaceutical and biomedical sciences. In recent years the impact of high-performance liquid chromatography (HPLC) on this area of analysis has been remarkable for the speed, selectivity and sensitivity attained, as compared with classical chromatographic procedures[1]. In particular, reversed-phase HPLC has emerged as a technique of considerable importance for the analysis of polar, ionogenic solutes.

Of the several methods proposed for the analysis of amino acids and peptides in biological materials, most rely on pre- or post-column derivatisation to generate the phenylthiohydantoin[2], dansyl[3], *o*-phthalaldehyde[4] or other derivatives which can be detected by conventional UV or fluorescence spectrometers with high sensitivity. Only the aromatic amino acids and cysteine display appreciable natural absorption above 220 nm, which can be used both for characterisation and for added selectivity. This can be achieved using a rapid-scanning, linear photodiode array UV detector,

0021-9673/84/$03.00

which is capable of acquiring spectral information at sequential time intervals under computer control during elution[5–7]. Moreover, the recent development of a number of digital algorithms for the spectral recognition and deconvolution of multiple overlapping peaks in the elution profile could significantly extend the selectivity of detection[7–9], in addition to characterising the individual peaks through their UV spectra[7,10,11]. Computer graphics packages have recently been developed for presenting the spectra (A, λ) acquired during elution (t) as isometric three-dimensional plots of (A, λ, t) viewed from any convenient angle[8,10]. These "spectrochromatograms" may suffer from the disadvantage that small peaks tend to be overshadowed by larger peaks, unless the viewpoint is rotated around the data plane under computer control. A novel concept recently proposed for HPLC by the authors is to present the data matrix as a two-dimensional contour plot[11], in which isoabsorptive contour lines are mapped in the (λ, t) plane. This technique has the advantage that all peaks, major and minor, can be visualised simultaneously. Furthermore, the optimum strategy for selecting detection wavelength can be developed. Although this mode of data presentation may be unfamiliar in HPLC, it is well-known in NMR and spectrofluorimetry[12].

The present work reports the use of rapid-scanning UV detection as a means for characterising aromatic amino acids, some of their metabolites and some dipeptides separated by HPLC. This work has been extended to preliminary studies on the use of spectral suppression as a digital technique for resolving strongly-overlapping peaks in the chromatogram. Computer-aided graphics routines have been developed to present these components either as a three-dimensional plot or as a two-dimensional contour map. The use of eight-colour graphics to simplify the presentation of such contour maps has also been examined.

EXPERIMENTAL

Reagents and materials

Methanol and acetonitrile (HPLC grade, Rathburn Chemicals, Walkerburn, U.K.) were used as received. A 0.05 M solution of potassium dihydrogen phosphate (Analar BDH, Poole, U.K.) was prepared with glass-distilled water and filtered by Millipore 0.45-μm filters using an all-glass apparatus. All eluents were degassed for 10 min in an ultrasonic bath under reduced pressure.

The amino acids tyrosine (Tyr, 1), phenylalanine (Phe, 3), tryptophan (Trp, 4), and the internal reference standard β-2-thienyl-DL-alanine (IS, 2), cystine, cysteine and glutamine were obtained from Sigma (St. Louis, MO, U.S.A.), as were the dipeptides Tyr–Gly (7), Gly–Tyr (8), Phe–Gly (9), Tyr–Tyr (10), Phe–Val (11). The metabolites dopamine (DOPA, 5), 5-hydroxytryptamine (5HT, 6), phenylpyruvic acid, phenylacetic acid and phenyl-lactic acid were obtained from Sigma.

Standard solutions

Stock solutions of metabolites (*ca.* 200 μM), phenylalanine (2000 μM), tyrosine (500 μM) were prepared in distilled water. Serum standards were prepared by spiked dilutions in horse serum (Horse Serum 3, Wellcome Reagents, Beckenham, U.K.).

Equipment and methods

Two liquid chromatographic systems were employed. The first consisted of an LKB 2150 pump (LKB-Produkter AB, Bromma, Sweden) with a Rheodyne injection valve (Model 7125, Berkeley, CA, U.S.A.) provided with a 20-μl or 100-μl loop, together with the LKB 2140 rapid scanning spectral detector (spectral resolution nominally 1 nm per diode in the range (190–370 nm). The optical and data units of the LKB 2140 system were interfaced via modified RS232C to the IBM Personal Computer, equipped with dual 5.25 in. diskette drives and a Canon A-1210 Ink-Jet eight-colour printer (Canon, New York, U.S.A.).

The second system employed a constant-flow LC pump with integral pulse damping (Gilson Model 302; Villiers-le-Bel, France) and a Rheodyne injection valve (Model 7125), coupled to a Hewlett-Packard Model 1040A linear photodiode array detector (Waldbronn, F.R.G.), supplied with a 4.5-μl flow cell. Nominal spectral resolution was 2 nm per diode in the range 200–600 nm. The HP1040A system consists of an optical unit connected via HP-IB interface bus (IEEE 488) to an HP-85 microcomputer equipped with a dual 5.25 in. HP 82901 M flexible disk drive unit.

The column system for amino acids was 250 × 4.6 mm I.D. stainless steel with an integral guard column, slurry-packed with 5-μm ODS-Hypersil (Shandon Southern Instruments, Cheshire, U.K.). For peptide separations a 100 × 3.2 mm I.D. stainless-steel column was slurry-packed with 5-μm C_{18}-Nucleosil.

The amino acids, metabolites and peptides were weighed on a Cahn Model 29 electromicrobalance (Cahn, CA, U.S.A.). The optimised solvent system developed for amino acids and metabolites was methanol–0.05 *M* potassium dihydrogen phosphate (13:87, v/v) adjusted to pH 4.0, at 1.2 ml/min[13]. The chromatographic efficiency was typically 40,000 plates/m. For peptide separations the solvent system employed was methanol–0.05 *M* potassium dihydrogen phosphate (pH 3) (15:85, v/v) at 1.0 ml/min.

A rapid deproteinisation procedure was used, based on the Somogyi method[14], where zinc hydroxide is generated *in situ* by addition of excess zinc sulphate (10% w/v; 100 μl) and sodium hydroxide (0.5 *M*; 50 μl) to 50 μl of serum, followed by spiral mixing (Spiramix 5, Denley, Sussex, U.K.) for 2 min and centrifugation at 120 *g* for 5 min (Centaur 1, MSE, Sussex, U.K.), as previously described for studies on phenylketonuria[13].

Graphics routines

Software developed by LKB for the IBM Personal Computer was used with the rapid spectral detector to generate the contour maps ("isograms") and to select the individual slices in the time domain (elution profile) and in the wavelength domain (spectra). For the HP1040A system, software developed by the manufacturers was used to generate contour maps, while the authors' software was used for pseudo-isometric plots in forward and reversed projection, and for spectral suppression and differentiation in the time domain, as previously reported[9,11]. Data capture and the selection of elution profiles and the relevant spectra were performed using software developed by Hewlett-Packard.

RESULTS AND DISCUSSION

In screening serum samples for endogenous analytes, it is often helpful to have additional information of a qualitative nature in order to confirm the peak assignations assumed. Early work relied on stop-flow UV-scanning, a relatively slow technique where the quantitative relationships of the elution profile are lost[15]. In the present work, the utility of rapid-scanning linear photodiode array (LDA) detectors has been examined in this context. Sequential scans of the UV spectrum within a

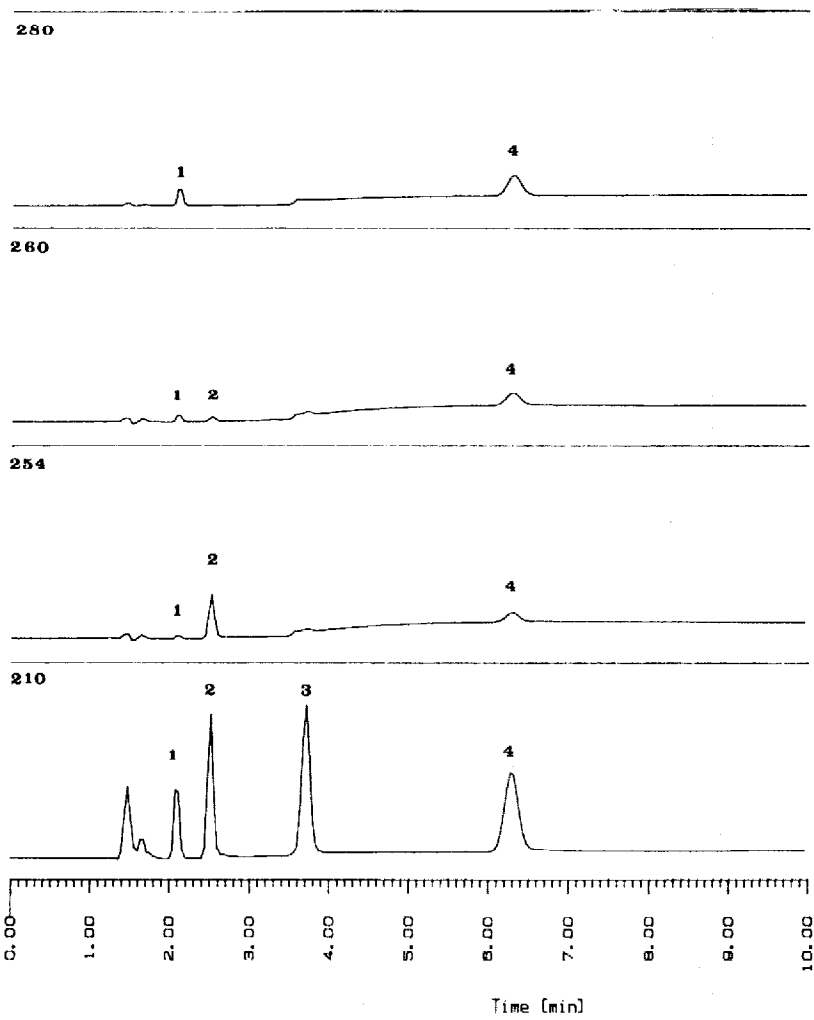

Fig. 1. Chromatogram of spiked serum sample from phenylketonuric patient, presented at several wavelengths after detection by photodiode array detector. For chromatographic conditions, see text; injection volume, 20 μl. Parameters for HP 1040A detector: sensitivity, 0.1 a.u.f.s.; repetition rate, 160 msec; reference wavelength, 550 nm; bandwidth, 100 nm; analytical detection wavelengths, 210, 254, 260 and 280 nm, bandwidth 5 nm. Peaks: 1 = Tyr (200 μM); 2 = internal standard (500 μM); 3 = Phe (250 μM); 4 = Trp (100 μM).

defined range can be acquired at defined intervals for storage as a matrix of (A, λ, t). This can be readily interrogated post-run to obtain the elution profiles of a serum sample at any wavelength within the range (Fig. 1) or to present the spectrum of each component for qualitative comparison with standards. The individual chromatograms for the serum sample confirm the choice of 210 nm as the optimum detection wavelength for sensitive quantitation.

Some of the metabolites of Tyr and Trp were also examined including 5-hydroxytryptamine (5-HT), a metabolite of Trp, and dopamine (DOPA), a metabolite of Tyr, which was eluted with Tyr itself. The individual spectra for each peak, and for the composite DOPA and Tyr peak, can be readily examined, as shown in Fig. 2.

The homogeneity of a chromatographic peak is of course a central question in the separation of analytes from biological matrices, and a number of techniques have been proposed to address this problem. These include spectral suppression[16], differentiation of the elution profile in the time domain[5,9,11], absorbance ratios and spectral deconvolution[9].

In the spectral suppression method, the absorbance values at two carefully-selected wavelengths are manipulated in such a way that the known peak of interest is suppressed by recording the difference absorbance as a function of elution time.

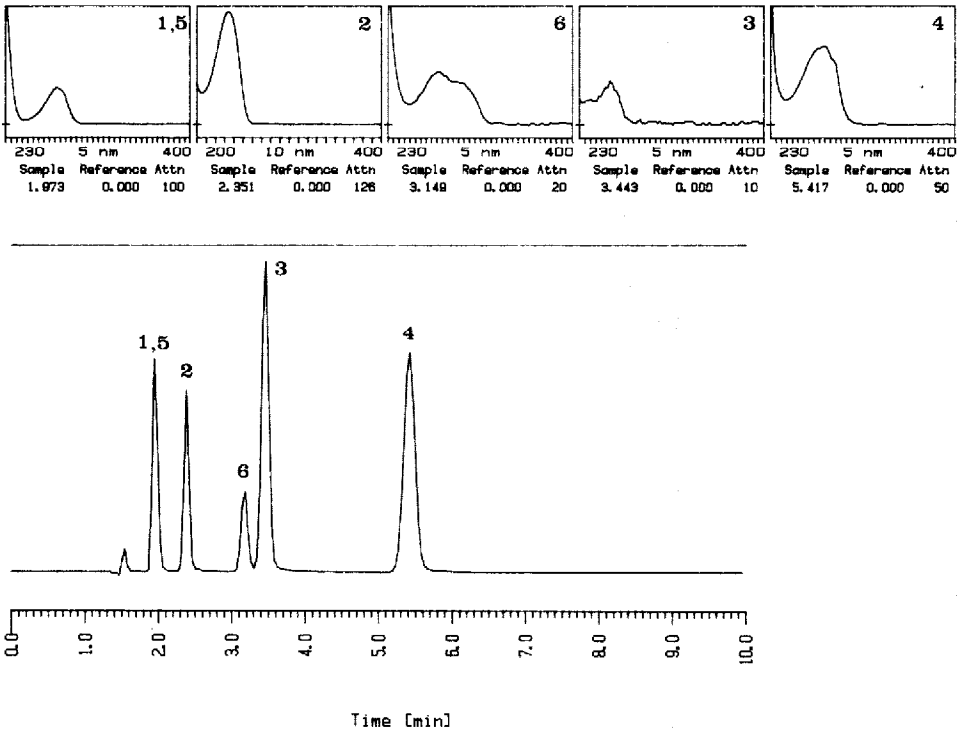

Fig. 2. Chromatogram of aromatic amino acids and metabolites at 210 nm, with normalised spectra captured at peak maxima. For chromatographic conditions, see text. For parameters for HP 1040A detector: see Fig. 1. Peaks: 1 = Tyr (808 μM); 2 = internal standard (500 μM); 3 = Phe (808 μM); 4 = Trp (560 μM); 5 = DOPA (205 μM); 6 = 5-HT(313 μM).

Thus any residual peak observed in the spectral suppression chromatogram represents a simultaneously eluted component whose spectral characteristics differ at the two difference wavelengths selected[9]. The technique relies on there being a sufficient difference between the spectra of the species which may be eluted together under the peak of the known solute.

A particular problem therefore arises in selecting appropriate wavelengths for closely-related components, such as drugs and their metabolites[11] or, as in the present case, an endogenous component Tyr, and its biotransformation product, DOPA, whose spectra are, in fact, very closely similar. It turns out that the optimum wavelength pair can be selected by using a technique proposed recently[9], and based essentially on a plot of the *ratio* of the individual spectra for the components known to be eluted simultaneously. In this case, the two wavelengths for optimum sensitivity and discrimination were found to be 228 nm and 240 nm.

The optimised equations corresponding to efficient suppression of Tyr and of DOPA, respectively, were predicted to be:

$$\Delta A_{\mathrm{DOPA}} = A_{228} - 15.08\, A_{240} \tag{1}$$

$$\Delta A_{\mathrm{Tyr}} = A_{228} - 4.24\, A_{240} \tag{2}$$

Application of eqn. 1 suppresses Tyr completely (Fig. 3) and yields a negative-going response to the single DOPA standard, whereas application of eqn. 2 can be seen to suppress DOPA, while detecting the Tyr standard as a positive response (Fig. 3). In a mixture of Tyr and DOPA, the difference absorbance function reports each component quantitatively in the presence of the other (Fig. 3). Thus the spectral suppression method would appear to have significant potential for testing peak homogeneity and for deconvoluting overlapping peaks, even where the peaks concerned have closely similar spectra, as in the present case.

If peaks are not coincident in the elution profile, the second derivative ($\mathrm{d}^2A/\mathrm{d}t^2$) has been found to enhance the perceived resolution[9,11]. In the present case, however, it was not found possible to resolve DOPA and Tyr in the second derivative chromatogram, because they were eluted very closely in the system employed. Other methods for testing peak homogeneity include spectral deconvolution, and the absorbance ratio plot as a function of elution time. Compared with the spectral suppression technique, where the absorbance *difference* is calculated, the absorbance *ratio* method has been found to be relatively insensitive, and does not yield a response which can be readily related to analyte concentration[9,11]. As with the spectral suppression method, the absorbance ratio plot depends critically on the pair of wavelengths selected and also relies on there being sufficient difference in the spectra of the solutes which may overlap in the chromatogram.

The total (A, λ, t) data presented as a three-dimensional pseudoisometric plot (Fig. 4) permits the spectral characterisation of all the resolved amino acid and metabolite peaks to be examined. However, minor peaks adjacent to more intense peaks may be partially or entirely obscured at the isometric projection angle of 25° selected for the present study[8], unless the viewpoint is rotated. A novel method recently proposed for studies on drugs and metabolites involves presenting the (A, λ, t) data as a contour map[11]. In this method, the spectral information is plotted as a

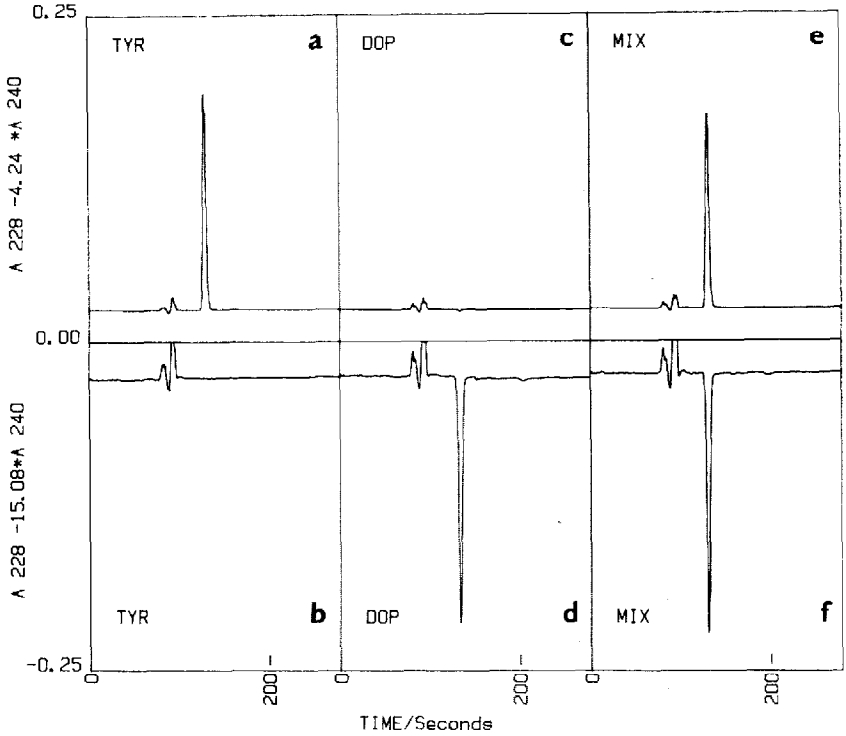

Fig. 3. Spectral suppression chromatograms of unresolved Tyr (211 μM) (1) and DOPA (114 μM) (5). For chromatographic conditions, see text. Parameters for HP 1040A detector as in Fig. 1. Spectral suppression wavelengths: 228 nm and 240 nm. Spectral difference function to suppress Tyr: $\Delta A_{DOPA} = A_{228} - 15.08A_{240}$; spectral difference function to suppress DOPA: $\Delta A_{Tyr} = A_{228} - 4.24A_{240}$. (a, b) Detector response to Tyr alone; (c, d) response to DOPA alone; (e, f) detector response to a mixture of Tyr (211 μM) and DOPA (114 μM).

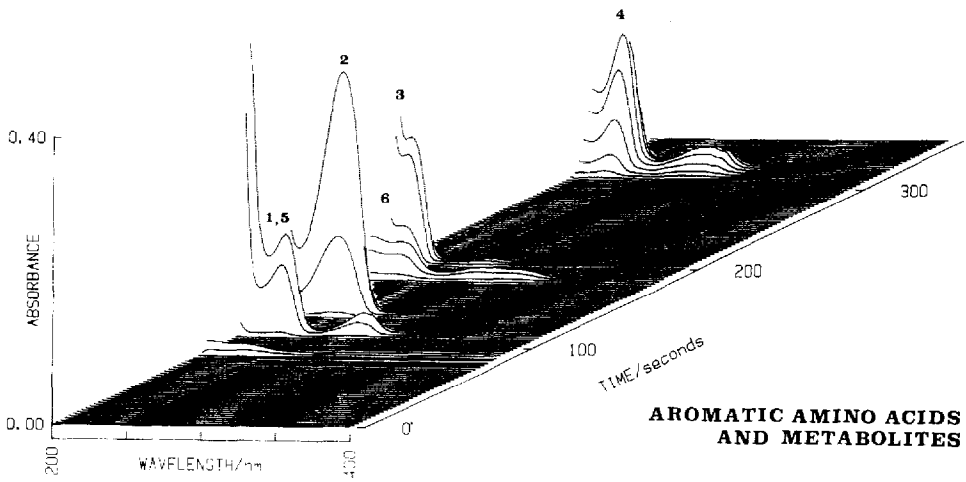

Fig. 4. Pseudoisometric spectrochromatogram of aromatic amino acids and metabolites, showing spectra acquired at 2.5 sec intervals during elution. For chromatographic conditions, see text. For HP 1040A parameters, see Fig. 1. Peaks: 1 = Tyr; 2 = internal standard; 3 = Phe; 4 = Trp; 5 = DOPA; 6 = 5-HT.

series of isoabsorptive, concentric lines in the (λ, t) plane (Fig. 5), thus permitting all the spectral information to be presented and inspected simultaneously. The presence of any resolved component can be established without the problem of selecting the appropriate viewing angle. Moreover, the optimum wavelength for detection can also be established for each separated peak.

Indeed, the presence of non-homogeneity can also be detected by examining the symmetry of the contour lines in the wavelength domain; nonhomogeneity is seen as skewed contours representing the spectral characteristics of each simultaneously eluted component, provided that there is sufficient difference in the corresponding spectra themselves. In the contour map (Fig. 5) of amino acids and metabolites, the characteristic aromatic absorption bands of Tyr, Trp, 5-HT and of Phe are evident.

The overall spectrochromatographic picture can be deduced from the contour map, which is seen as an efficient presentation of the large amount of spectral and chromatographic data acquired, and should be especially useful in method development. It was of interest in the present work to examine the applications potential of rapid-scanning detectors in studies on peptides. The separation of five dipeptides is illustrated in Figs. 6 and 7, where the three-dimensional spectrochromatogram and its contour plot equivalent illustrate the potential use of rapid-scanning spectral detection for peptide mapping as an investigative tool. In this preliminary examination of the dipeptides, each can be clearly discriminated in the isoabsorptive contours plotted in the (λ, t) plane (Fig. 7). The high-resolution contours confirm the symmetry of each resolved peak, indicating the chromatographic purity of each dipeptide examined. It is probably fair to suggest, however, that a certain degree of familiarity is

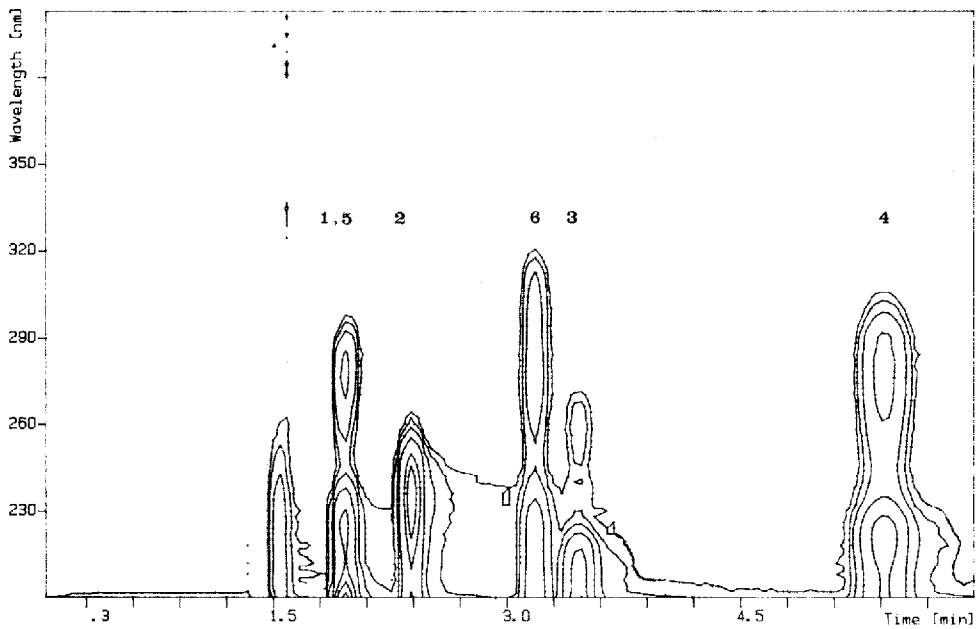

Fig. 5. Contour map of aromatic amino acids and metabolites. For chromatographic conditions, see text. Parameters for HP 1040A, see Fig. 1; contour intervals: 1, 2, 5, 20, 100, 200, 400, 600 mAU. Peaks: 1 = Tyr; 2 = internal standard; 3 = Phe; 4 = Trp; 5 = DOPA; 6 = 5-HT.

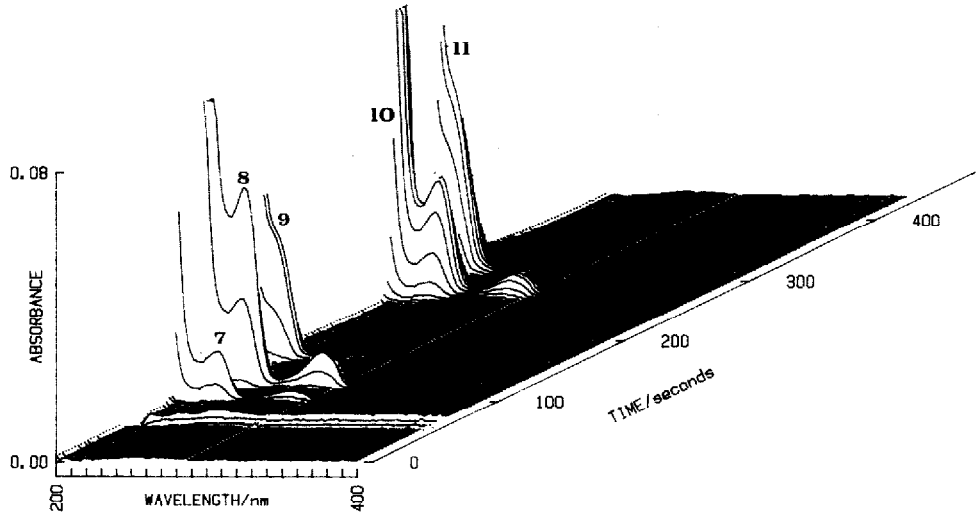

Fig. 6. Pseudoisometric spectrochromatogram of dipeptides, showing spectra acquired at 2.5 sec intervals. For chromatographic conditions, see text; injection volume, 20 μl. Parameters for HP 1040A: sensitivity, 0.08 a.u.f.s.; repetition rate, 160 msec; reference wavelength, 550 nm and bandwidth, 100 nm. Peaks: 7 = Tyr-Gly (17 μM); 8 = Gly-Tyr (70 μM); 9 = Phe-Gly (48 μM); 10 = Tyr-Tyr (28 μM); 11 = Phe-Val (77 μM).

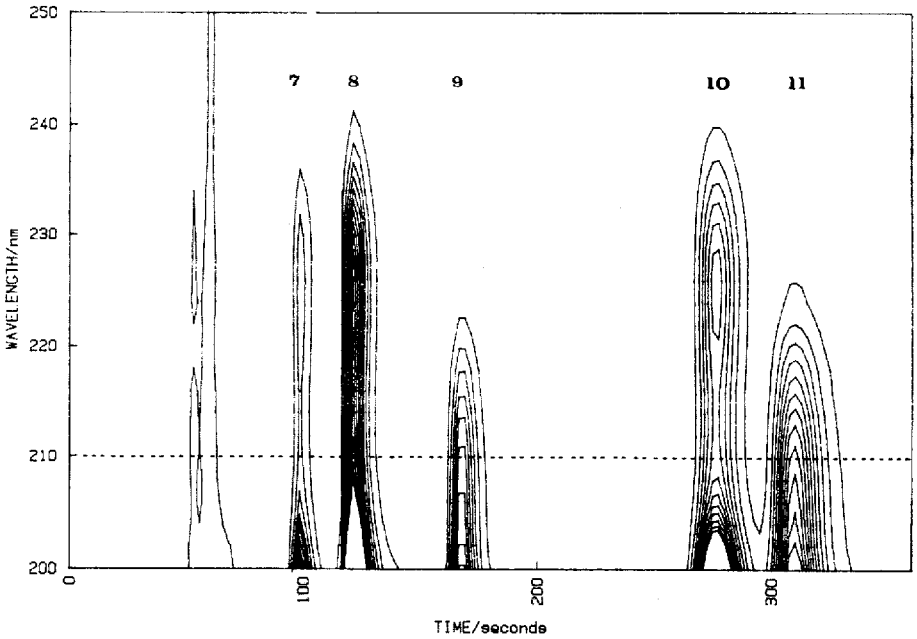

Fig. 7. Dipeptide contour map. For chromatographic conditions, see text. Parameters for HP 1040A, see Fig. 6. Contour interval constant at 0.01 AU.

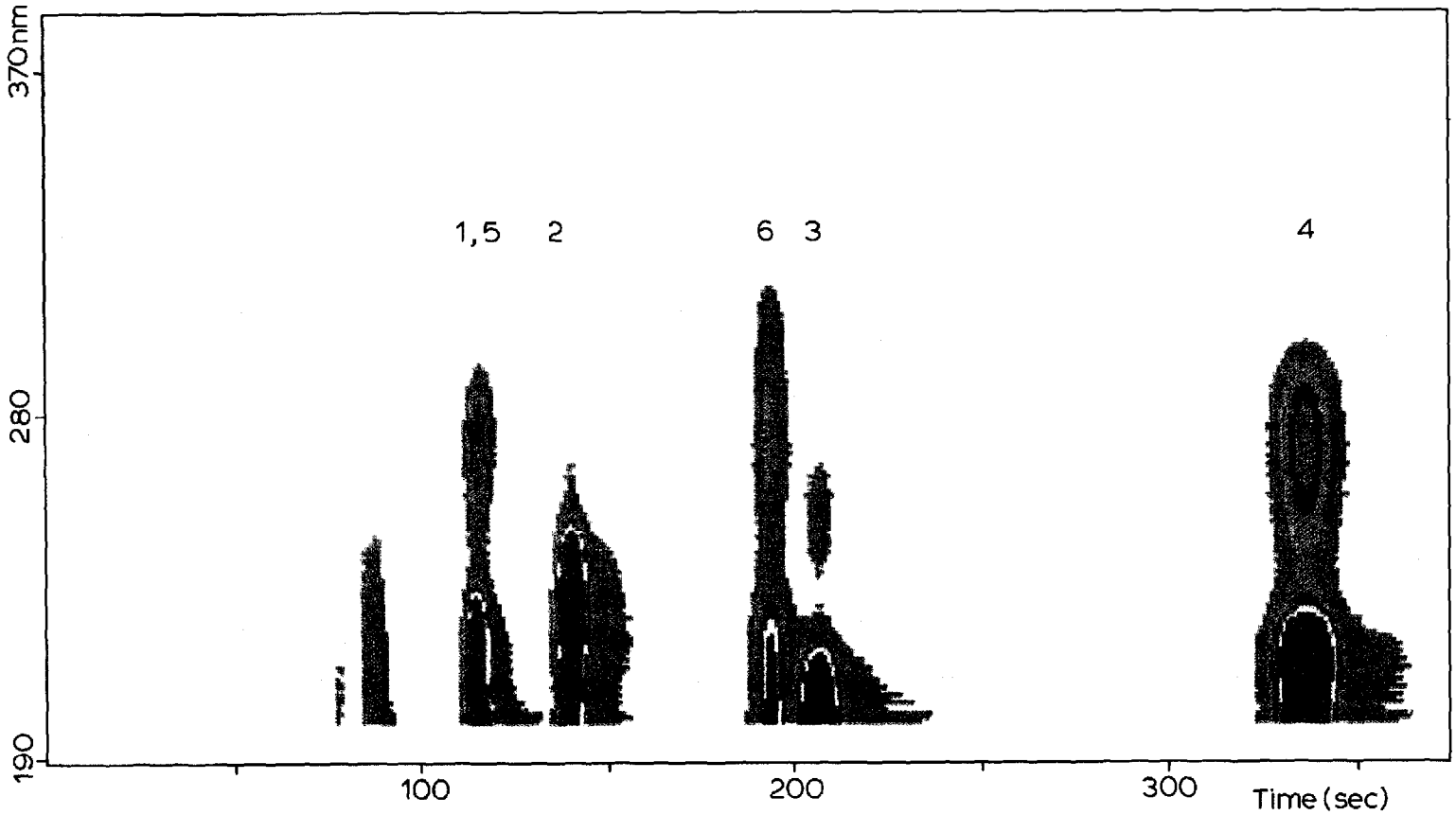

Fig. 8. Colour-coded contour map of aromatic amino acids and metabolites. For chromatographic conditions, see text. Parameters for LKB 2140 detector and IBM-PC microcomputer: sensitivity, 0.026 a.u.f.s.; repetition rate, 1 sec. Contour coding: black, 8%; cyan, 23%; blue, 38%; green, 54%; yellow, 69%; red, 85%; magenta, 100%. Peaks: 1 = Tyr; 2 = internal standard; 3 = Phe; 4 = Trp; 5 = DOPA; 6 = 5-HT.

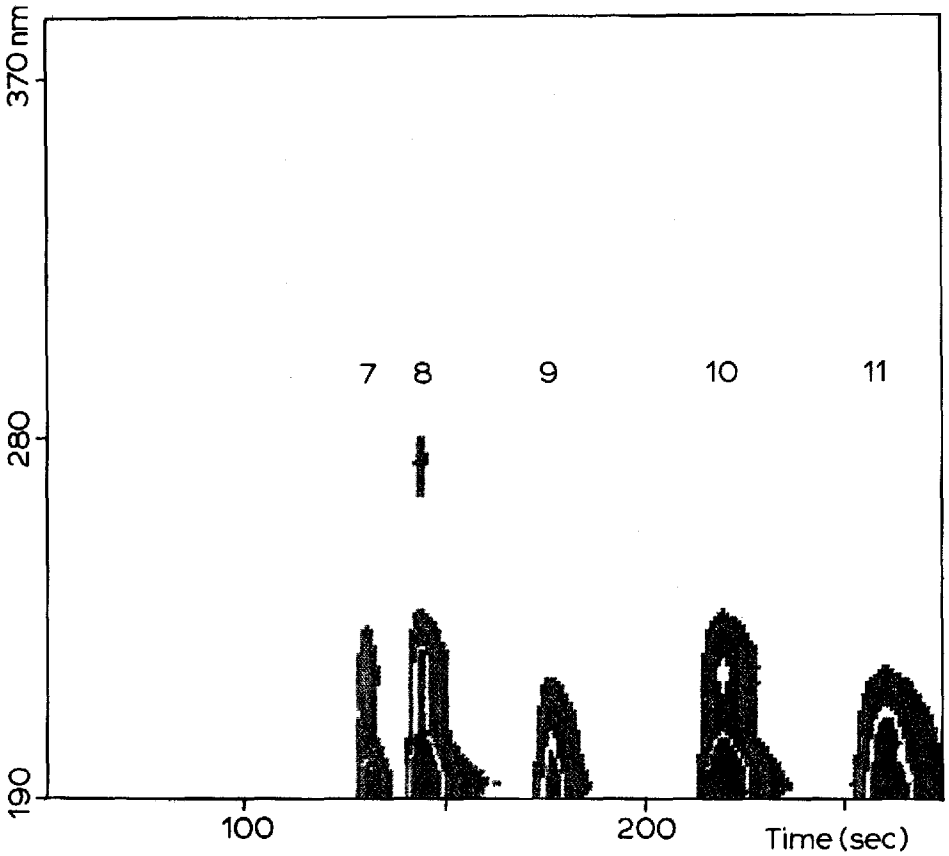

Fig. 9. Colour-coded dipeptide contour map. For chromatographic conditions, see text. For parameters for LKB 2140 detector and IBM-PC microcomputer, see Fig. 8; sensitivity, 0.03 a.u.f.s. Contour coding as in Fig. 8. Peaks: 7 = Tyr-Gly; 8 = Gly-Tyr; 9 = Phe-Gly; 10 = Tyr-Tyr; 11 = Phe-Val.

required to perceive clearly the three-dimensional nature of the contour plot. In this respect, the use of simplified contour intervals coded by colour graphics by means of the IBM-PC represents a novel approach in presenting spectrochromatographic data, as illustrated by the amino acid and metabolite map (Fig. 8) and the dipeptide map (Fig. 9). The complexity of the data is reduced, while at the same time enabling the three-dimensionality of the map surface to be interpreted in terms of the high-intensity "hot spots" (coded red and magenta), and the less intense "slopes" of the peaks (coded black, cyan, blue, green and yellow, in ascending order of absorbance (Figs. 8 and 9). By expanding the plot sensitivity, minor components can be detected and a rapid survey analysis of the total data acquired by the IBM-PC can be performed, before examining more closely the elution profile at any specified wavelength, or the spectrum at any elution time. Although useful in examining the spectra of individual components both during chromatography and post-run, the isometric plot is arguably less useful than the contour map in permitting the data to be evaluated systematically.

CONCLUSIONS

The present work suggests that rapid-scanning LDA detectors have considerable potential in the biomedical sciences. When such detectors are incorporated with studies on the amino acids and their metabolites, the UV spectra can be used to characterise each peak. The data can be presented either as a three-dimensional isometric plot, or as a contour plot which maps the distribution of the components in the (λ, t) plane. The visual accessibility of the contour map can be increased by the use of simplified contour intervals combined with eight-colour graphics. The exploitation of these approaches for generating peptide maps in the wavelength and time domains indicates the potential of this new detector technology in the biomedical sciences in general, and in the field of amino acid, peptide and protein studies in particular.

This preliminary examination of the use of colour graphics in HPLC, coupled with techniques for validating peak homogeneity based on computer-aided transformation of the spectrochromatographic data, points to a number of new approaches which could be explored in the area of biomedical analysis. In this respect the application of principal component analysis[17] for the prediction of the maximum probable number of components in the (A, λ, t) matrix will offer significant assistance in developing and optimising HPLC separations of complex biological molecules.

ACKNOWLEDGEMENTS

The authors thank Hewlett-Packard GmbH, Waldbronn, F.R.G., for the use of a Model HP 1040A photodiode array LC detector and peripherals. Thanks are also due to LKB Produkter AB, Bromma, Sweden for kindly providing a Model 2140 rapid spectral detector and IBM Personal Computer for use in this work. Dr. J. C. Eilbeck, Department of Mathematics, Heriot-Watt University, Edinburgh, is also thanked for his advice on the design of the contour mapping program.

REFERENCES

1 M. T. W. Hearn, F. E. Regnier and C. T. Wehr, *Int. Lab.*, 14 (1983) 16–35.
2 C. C. Zimmermann, E. Appella and J. J. Pisano, *Anal. Biochem.*, 77 (1977) 569.
3 T. Yamabe, N. Takai and H. Nakamura, *J. Chromatogr.*, 104 (1975) 359.
4 L. M. Neckers, L. E. Delisi and R. J. Wyatt, *Clin. Chem.*, 27 (1981) 146–148.
5 A. F. Fell, *Anal. Proc.*, 17 (1980) 512–519.
6 A. F. Fell, *Anal. Proc.*, 20 (1983) 356–359.
7 A. F. Fell, H. P. Scott, R. Gill and A. C. Moffat, *Chromatographia*, 16 (1982) 69–78.
8 A. F. Fell, H. P. Scott, R. Gill and A. C. Moffat, *J. Chromatogr.*, 273 (1983) 3–17.
9 A. F. Fell, H. P. Scott, R. Gill and A. C. Moffat, *J. Chromatogr.*, 282 (1983) 123–140.
10 R. T. Ghijsen, B. F. H. Drenth, F. Overzet and R. A. de Zeeuw, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 5 (1982) 192–198.
11 B. J. Clark, A. F. Fell, H. P. Scott and D. Westerlund, *J. Chromatogr.*, 286 (1984) 271–283.
12 I. Soutar, *Anal. Proc.*, 20 (1983) 19–21.
13 B. J. Clark, A. F. Fell and J. M. Rattenbury, *J. Chromatogr.*, submitted for publication.
14 M. Somogyi, *J. Biol. Chem.*, 86 (1930) 655–663.
15 R. Schuster, *Anal. Chem.*, 52 (1980) 617–620.
16 G. T. Carter, R. E. Schiesswohl, H. Burke and R. Yang, *J. Pharm. Sci.*, 71 (1982) 317–321.
17 E. R. Malinowski and D. G. Howery, *Factor Analysis in Chemistry*, Wiley, New York, 1981.