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COMPLETE COMPUTER SYSTEM FOR PROCESSING CHROMATOGRAPHIC DATA

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SUMMARY

A complete data processing system for complex chromatogram analysis is described. Although this system is essentially designed to treat high-performance liquid chromatographic results, it can be applied to several other separation techniques. Original methods are indicated for chromatogram peak integration and evaluation of peak areas, even when separation of compounds is incomplete. For gradient analyses, techniques for filtering and correction of baseline are described. The ability to treat radioactive counting data enables the system to be applied to the processing of metabolic arrays. The use of even derivatives to obtain a criterion of peak purity and the problem of information losses during sampling are also discussed.

INTRODUCTION

The analysis of complex metabolic pathways and their regulation calls for many separations of various extracts, and a useful way of doing this is to study the variation with time of the label incorporated from a single external labelled precursor. This permits evaluations of the amount, radioactivity and consequently the specific activity of the different molecules concerned for different labelling times. Hence the kinetic parameters for the reactions involved can be calculated by fitting a metabolic model to these results. The idea is to obtain the largest number of evaluations in the shortest time, as well as precise quantification of the amounts and incorporated radioactivity for each product.

High-performance liquid chromatography (HPLC) is a convenient, sensitive and rapid method for separating and analysing compounds of the same family in a single step. The consequent accumulation of measurements and the need to obtain results in a processed form require automatic techniques of data acquisition and treatment. Chromatographic analyses such as peak separation and integration have been performed with many systems for many years. However, methods for integration, baseline calculation and identification of overlapping peaks are satisfactory only with very clean chromatograms, and cannot be applied to radioactive counting.

The power of current microcomputers allows a more sophisticated and precise

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treatment with the same computing time. In this paper, we describe some of the techniques used based on numerical filtration and Gaussian identification for baseline correction and peak integration. We have also evolved original computation methods to solve specific or difficult problems. Systems for automatic acquisition and analyses of absorbance and of its associated label are also described. Although the examples given to illustrate our techniques are confined to nucleotide and RNA metabolism, they can be used to analyse any type of metabolic pathway, provided a suitable HPLC separation method is applied.

EXPERIMENTAL

Hardware requirements

Fig. 1 is a schematic representation of the hardware configuration used for data analysis. A powerful home-made microcomputer constitutes the treatment unit (sold as Themis 2 by EFCIS, France). This computer is equipped with 64K bytes of RAM, a four-disc controller and a complete central unit in the form of one PC board, and also includes a floppy disc controller for up to four disc drives (two sided, double density and 2M bytes). Asynchronous lines and complete industrial bus connections are also provided. To this central unit we connected a microprocessor interface (8K bytes of RAM) used on-line with experiments for data acquisition. This system was coupled to the 10-V output of a Waters M440 UV detector and to a Kontron MR300 spectrometer for radioactivity determinations. Analogue to digital and digital to analogue conversions were obtained by 12-bit fast convertors. This interface can drive an *X–Y* plotter and scope visualization equipment. Data were acquired by precise timing (a programmable timer is incorporated to the interface) at a sampling speed of up to 50 kHz.

The equipment for this system is simple and does not greatly increase the total cost of a standard HPLC system. Several extensions such as those required for high-resolution video display or a rapid arithmetic processor can be added to the central unit.

Methods

Nucleotides were separated by one of two chromatographic systems: an isocratic one (KH_2PO_4, 0.05 *M*, pH 3) for the RNA hydrolysates consisting of 2'- and

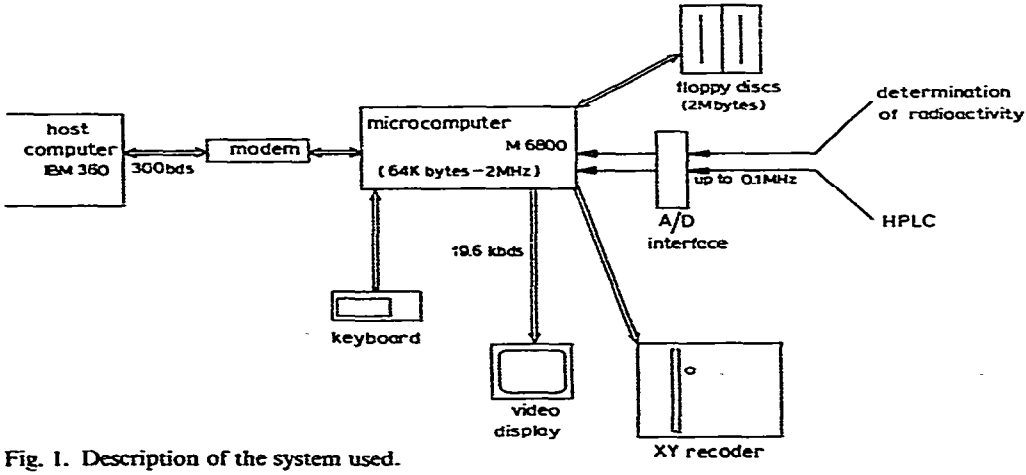


Fig. 1. Description of the system used.

3′-nucleotide mixtures and a gradient (A, KH_2PO_4, 0.05 *M*, pH 3; B, KH_2PO_4, 0.3 *M*, pH 6; 15 to 100% of B in 85 min) system[1,2] for nucleotides extracted from acid-soluble pools. Separations were performed through a Varian AX5-15 column at 2000 p.s.i. for 85 min. Acid-soluble pools were extracted by homogenization in 0.6 *N* perchloric acid (PCA) or 5% trichloroacetic acid (TCA) of tissues labelled for 3 h *in vitro* with 20 μCi/ml of [^{3}H]uridine. After centrifugation of insoluble material (Sorvall SS34, 7500 rpm for 10 min) the supernatants were neutralized. Addition of 1 *N* potassium hydroxide solution to PCA extracts resulted in the formation of insoluble potassium perchlorate, which was removed by centrifugation and discarded. TCA was eliminated by two or three diethyl ether extractions, a method which caused smaller nucleotide losses than the PCA method.

RNA was hydrolysed in 0.6 *N* potassium hydroxide solution for 16 h at 37°C. The extracts were neutralized by mixing the incubated preparation with Dowex 50W-X8 previously equilibrated to its acidic form. This method allowed complete nucleotide recovery without any sample dilution.

Software

Two sets of programs were used (Table I). To improve their performances, acquisition programs for HPLC and for the radioactivity counter were written in the 6800 microprocessor assembly language. Data were acquired separately from the UV detector and radioactivity counter because of the low capacity of the interface memory and two different programs, ANALOG and PBETA, were written for HPLC and radioactivity, respectively.

ANALOG contains everything required for working out analogic interfaces, *i.e.*, timer programing, sampling, data storage and vectors tracing on an *X*–*Y* plotter

TABLE I

DESCRIPTION OF PROGRAMS

Language	*Program name*	*Subroutine name*	*Function*
FORTRAN	LISS	PARAM	Determination of events
		DERIV	Computation of the first derivative
		PEAK	Determination of the peak parameters for Gaussian integration
		AREA	Computation of peak area
		EVMARK	Printing of an event mark on chromatogram plot
		PLOT	Plotting of chromatograms
		LBASE	Correction of baseline
	CPMIMP		Plotting of data for radioactivity determinations
	DOIMP		Plotting of data for absorbance results for the interpolated label
	SPLINCPM		Spline interpolation of radioactivity data
	DER4		Computation and plotting of fourth derivative spectra
M6800 ASSEMBLER	ANALOG	Acquisition program for absorbance data. Driving of *X*–*Y* plotter and UV monitor in connection with DOACQ on the central unit	
	PBETA	Acquisition program for radioactivity data. Used in connection with CPMACQ on central unit	

by the Bresenham algorithm[3]. This program necessitates 1K memory bytes and much data storage space (4K bytes to store 2000 points).

PBETA acquires radioactive counting data from the MR300 β-spectrometer, stores results in a buffer memory and provides facilities for dialogue with the central unit. It necessitates 0.7K bytes of program memory.

The other programs executed with the system are run on the central unit and are written in FORTRAN, to ensure their portability. Of these, DOACQ and CPMACQ acquire data from the peripheral interface and store it on floppy disc files. DOACQ allows the user to define the point density and the data acquisition rate from the UV detector. Acquisition can be automated by the use of a synchronization line, whose setting starts the process.

LISS performs the main treatments involved in chromatogram analysis and is the most complex of the programs reported here. It is capable of processing data stored in floppy disc files, with the absorbance format. The main calculations performed by LISS are indicated in the flow chart in Fig. 2. We developed original techniques for peak position detection and for correction of background and baseline before integration. Briefly, the computer calculates the retention time of each peak, the abscissas of the inflection points and the first and last points of the peak area and

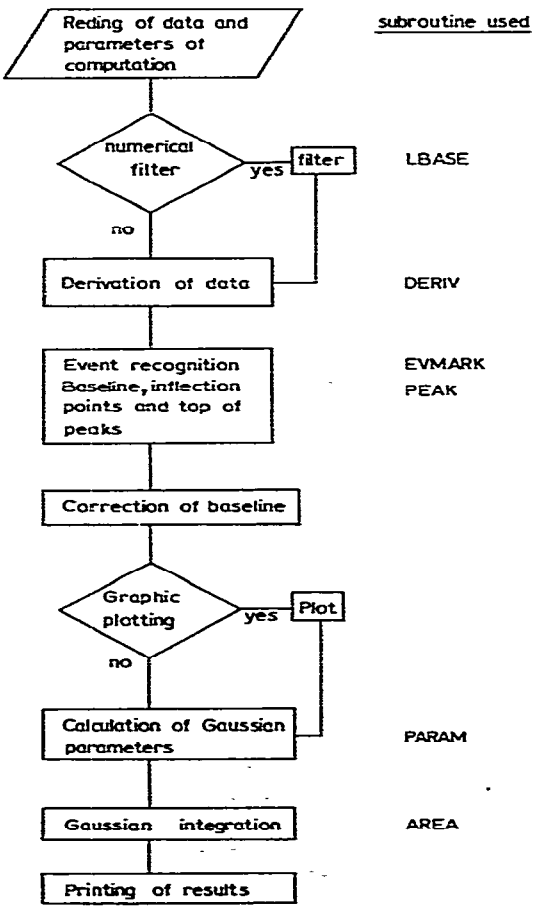


Fig. 2. Flow chart of the LISS program.

then performs Gaussian identification to obtain the area of each peak. This method has the advantage of being more accurate than direct integration, particularly for overlapping peaks.

Derivation method

The five main chromatographic events retained are the respective identifications of the initial and final points of the integration area, the inflection points, the position of the peaks and asymmetry. Determination of these events necessitates computation of the first derivative of the initial data. To eliminate noise and rapid baseline variations, the derivation interval of the chromatogram can be adapted to any experimental conditions. A long interval attenuates and smoothes a noisy baseline. This technique is equivalent to working with the mean initial function over the derivation interval. Thus $\frac{df(x)}{dt}$ is approximated by $\Delta f(x)$. If

$$\frac{\Delta f(x)}{s} = \frac{1}{s}\int_{x-s/2}^{x+s/2} \frac{df(\tau)}{dt}\cdot d\tau = \frac{1}{s}[f(x + s/2) - f(x - s/2)]$$

then

$$\frac{f(x)}{s} = \frac{1}{s}\cdot\frac{d}{dx}\int_{x-s/2}^{x+s/2} f(\tau)d\tau = \frac{\overline{df(x)}}{dx}$$

If we define $\overline{df}(x)/dx$ as the exact derivative of $S(x)$, the smoothed function corresponding to $f(x)$, then $S(x) = f(x)_s$. $S(x)$ is the average of $f(x)$ along the s interval. The application of Bienaime–Tchebycheff inequality shows that for $h > 5$, the probability that a 1-dB signal differs from the background is 95%. For $h = 1$, this probability is only 18%; with these conditions, the signal-to-noise ratio increases by 2.6 dB.

Numerical filter

A first numerical filter is applied to the signal in order to correct the slow variations in the baseline. Taking $u(t)$ as the input function, we define the corrected output function as $y(t) = u(t) - v(t)$ where $v(t)$ is a solution of

$$u(t) = a\cdot\frac{dv}{dt} + bv$$

(a and b being real positive parameters). Knowing u_i, v_i and the integration interval h, v_{i+1} is given by the equation

$$v_{i+1} = hu_i/a + (1 - bh/a)v_i$$

This filter is analogous to an electrical integrator. The operations involved and the obtained results are illustrated in Fig. 3. The transformed chromatogram is derived with an appropriate derivation interval (see derivation method above).

Correction of baseline (LBASE)

In contrast to the above filter, LBASE performs geometric operations. These

operations are required when the baseline is too high or shifted; accurate integration results can be obtained even with a high or variable baseline (*i.e.*, without previous correct zeroing, and with shifting due to gradient mode). Unduly drastic correction of overlapping peaks is avoided by slope conditions, as shown in Fig. 4. LBASE acts with the algorithm shown in the Appendix. The start (d) and end (f) points are determined for a peak by the following criteria:

$|p(i)| < sx$ and $t(i) < 2000$ (chromatograms are normalized to 4096) defines $d = i$, and after completion of this first condition, $p(j) < sx$ defines $f = j$. These parameters define a straight line between $t(d)$, d and $t(f)$, f. Correction at the abscissa point l is simply given by

$$c = sl \cdot l + m \text{ and } t(l) = t(l) - c$$

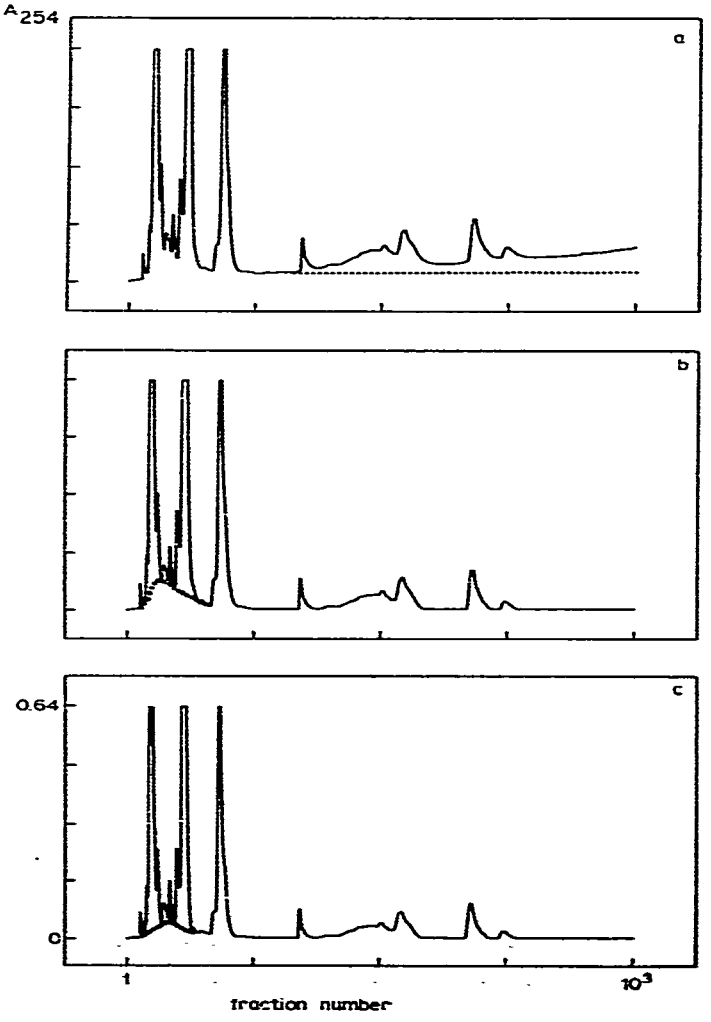


Fig. 3. Effect of the numerical filter I and of the execution of LBASE. (a) Starting chromatogram; (b) filtered chromatogram; (c) chromatogram after baseline correction.

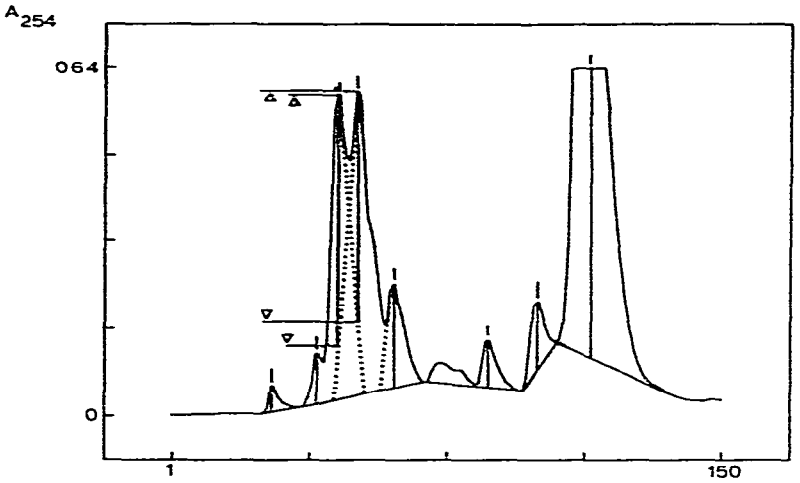


Fig. 4. Geometric correction for overlapping peaks by mutual rejection.

Determination of main events

The routine PEAK is applied after these preliminary corrections and the derivatives are calculated with an interval depending on the noise of the chromatogram. This program computes main events such as the start and end of a peak, inflection points or top position. We shall describe below only unconventional computations in chromatogram analyses such as the determination of inflection points, necessary for Gaussian identification.

Conditions for the start of a peak are $p(i) > s$, $t(i) > = sx$ and $ipic = 0$; $t(i)$ and $p(i)$ are respectively the value of the chromatogram and the value of its derivative at the abscissa point i. We introduced two variables, *ipic* and *inf*, which indicate whether the program is looking for the start of a peak ($ipic = 0$) or whether, having found this start, it is seeking an inflection point. This method simplifies the tests performed when complex overlapping peaks occur. Fig. 5 shows its application to three overlapping peaks. Conditions for peak termination are either (1) $ipic = 0$; $|p(i)| + |p(i-1)| = 0$ and $p(i+1) \geqslant p(i)$ or (2) $p(i+1) \geqslant p(i)$ and $2(|p(i)| - |p(i-1)|)/(|p(i)| + |p(i-1)|) \leqslant sgm$, $p(i) \geqslant 0$ and $p(i-1) < 0$. s, sx and sgm are threshold parameters fixed by the user. As Fig. 4 shows, the different situations encountered in conventional chromatography are solved by these tests. After *ipic* and *inf* are set to 1, the program seeks an inflection point by testing $t(i) < 4095$ (for special treatment of saturated peaks) and $|p(i)| < |p(i-1)|$. *Inf* is then set to 0 and the program performs the tests described above, to find the top of the peak.

Determination of parameters for Gaussian integration

Knowledge of the three parameters A_i, a_i, and l_i completely defines the Gaussian function $y = A_i \exp[-(t - a_i)/2l_i]$. a_i is given directly by the peak position computed by PEAK. Since peak tops are not always enclosed by inflection points, l_i may be computed by different methods, indicated in Table II.

When PEAK fails to find the correct inflection points, l_i is calculated assuming that peak boundaries are given by the second derivative minima. For the Gaussian functions, these points have the abscissa $a_i \pm l_i\sqrt{3}$. This mostly leads to underevalu-

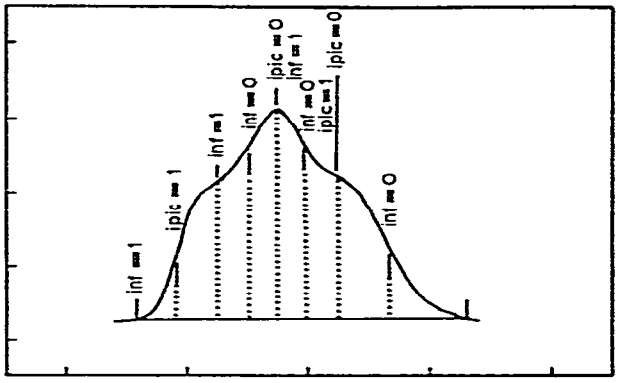


Fig. 5. Determination of the main events for overlapping peaks.

ation of peak area, and a better method is to consider the baseline as reached when the value of the Gaussian function is lower than $A_i/100$.

The abscissa points corresponding to peak boundaries are given by $a_i + \sqrt{2\log 100} = a_i + 3.0348 l_i$. These values do not greatly differ from the abscissa of the most external roots of the fifth derivative ($a_i + l_i\sqrt{5 + \sqrt{10}} \approx a_i + 2.857 l_i$). After determination of these values, the program corrects the maxima (A_i) for overlapping peaks. Simple geometric interpolation of the mutual rejection was found to be the best correction method. The technique is illustrated in Fig. 4. When corrections are so drastic that they completely erase a peak, they are not made but an error message is given for this peak area.

In addition, the dissymetry of each peak due to tailing is computed in order to give the user a quality index for their chromatogram (used in conjunction with theoretical plate determination). This asymmetry factor d_i is also used to calculate peak area.

Once the parameters A_i and l_i have been ascertained, this area is calculated by integrating two half-Gaussian functions, whose parameters are defined by A_i and $s_1^- = 2l_i d_i/d_{i+1}$, $s_1^+ = 2l_i/d_{i+1}$, where s_1^- and s_1^+ are the halfwidths at $A_i/2$ for backward and forward half-Gaussians curves, respectively.

Determination of peak area

The upper section in Table III shows the output of an alaysed chromatogram of PCA-soluble nucleotides. The area computed gives the respective amounts of each separated compound. The corresponding chromatogram is shown in Fig. 3. Areas calculated by triangulation or direct determination by peak weighing exhibit very similar values.

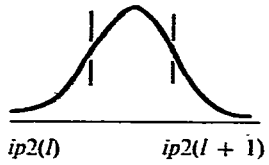
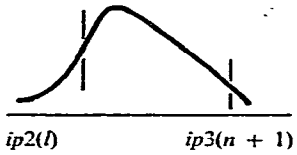
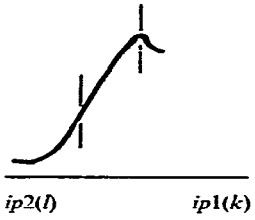
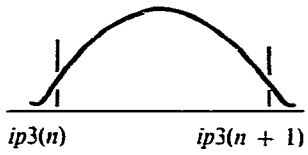
Radioactivity data

The use of radioactive labels makes HPLC a very powerful tool in metabolic analyses. The need to take account of labelling experiments and to determine accurately the radioactivity in each peak led us to investigate methods of labelled peak integration. Specific methods are required as the sampling rate is usually lower for data relating to the label than for the absorbance data. The results cannot be treated directly by a program such as LISS, which is designed to work with numerous experimental points. The small number of points necessitates interpolation methods. We chose a technique of spline interpolation, because spline functions have the same

TABLE II

DIFFERENT METHODS OF EVALUATING PEAK WIDTH

The figures only show examples of difficult determinations. The program may fail to detect the inflection points for numerous other reasons than those reported here (*e.g.*, unduly small variation of derivatives, peak end encountered before an inflection point. *ip*1, *ip*2 and *ip*3 represent peak maxima, inflection points and peak start or end, respectively.

$2\sigma = [ip2\ (l + 1) - ip2\ (l)]$	
$2\sigma = 2[ip3\ (n + 1) - ip2\ (l)]/2.0348$	
$2\sigma = 2[ip1\ (k) - ip2\ (l)]$	
$2\sigma = [ip3\ (n + 1) - ip3\ (n)]/3.0348$	

derivatives at experimental points the starting chromatogram. This similarity is essential as LISS is based on derivative calculations. A second-order spline function is used to construct a set of 1000 interpolated points from the radioactivity results; the LISS program is then applied to these points in order to perform the same operations as for the absorbance analyses. Fig. 6 shows a comparison between a plot of the initial radioactivity data obtained by the direct plot program CPMIMP and the plot of a spline function derived from these data. This figure shows that interpolation gives accurate and reliable results. The lower section in Table III shows the results obtained by this method with the data replotted on Fig. 6. These data correspond to the absorbance analysis shown in Fig. 3 and allow the calculation of specific activity in all compounds of the uridylate family.

Use of even derivatives

We undertook a large number of experiments to discriminate better between overlapping peaks, and to obtain a reliable criterion of peak purity. Such a criterion is

TABLE III

TYPICAL PRINTOUT OF RESULTS FOR ACID-SOLUBLE NUCLEOTIDES

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PAGE 001  RESULT  .SA:1

 *** HPLC ANALYSIS ***


 DONNEES DANS FICHIER PCA4DO:1
 1000 POINTS
 ATTENUATION: 2
 DETECTION THRESHOLD:    50.00          BASELINE:     5.00
 DIFF. THRESHOLD:         5.00          MINIMAL DER. SLOPE:     5.00

 NR. OF THEORETICAL PLATES: 8162

  PEAK NR      MAXIMUM     RETENTION TIME  WIDTH       TAILING      AREA       COMMENT

      1        259.24          29.00         2.00         0.50       647.55
      2        665.86          41.00         5.00         0.50      4168.45
      3       3776.62          46.00         3.00         1.00     14167.30
      4       3619.93          51.99         3.00         0.28     13600.02                 Ur
      5        583.07          74.00         5.00         0.75      3642.73
      6        821.24          86.99         3.00         0.37      3078.88
      7        543.60         100.00         3.00         0.80      2030.34                 UMP
      8       4095.00         112.99        10.00         0.23     51176.62   FALSE
      9        406.24         173.99        12.99         0.15      6603.13
     10       4095.00         185.00         5.00         0.52     25738.95                 UDPG
     11        431.04         344.00         4.00         0.18      2126.33                 UDP
     12        426.39         544.00        10.99         0.28      5879.89
     13        538.54         681.99        10.00         0.33      6686.66                 UTP

*** HPLC ANALYSIS ***


DONNEES DANS FICHIER DATA
1000 POINTS
ATTENUATION: 2
DETECTION THRESHOLD:     5.00          BASELINE:     5.00
DIFF. THRESHOLD:         2.00          MINIMAL DER. SLOPE:     2.00

 PEAK NR      MAXIMUM     RETENTION TIME  WIDTH       TAILING      AREA       COMMENT

      1       3973.99          56.00         6.00         0.35     29811.63                 Ur
      2         98.00          86.99         6.00         1.00       734.44
      3        240.00          99.00         6.00         0.27      1799.85                 UMP
      4         80.00         139.99         8.00         1.00       600.19
      5         83.00         190.99         8.00         0.34       629.94                 UDPG
      6         26.00         344.00         7.00         0.60       226.46                 UDP
      7         28.00         674.00        13.99         1.00       491.94                 UTP
```

necessary for working out metabolic models. A bad separation method combining different products in the same peak leads to irretrievable errors in adjusting models to experiments. It is particularly important to be sure of peak purity. We therefore adapted the work described in ref. 4 on separation of overlapping absorption bands to the problem of peak separation in chromatography. This method holds for other cases of incomplete separation in any technique used in biochemistry or chemistry; it is based on the fact that even-order derivatives exhibit reduced peak half-widths compared with the initial half-width. Such reduced peak are centred on the abscissa of the maximum. This feature allows the separation of derivative peaks even when they overlap considerably. The higher the derivative order, the better is the separation. Fig. 7 compares part of a real chromatogram (a) with its fourth derivative spectrum (b). This chromatogram corresponds to the separation of a TYMV mRNA hydrolysate, known to contain 2′- and 3′-isomers of each of the four nucleotides. 2′- and 3′-CMP almost completely overlap in the chromatogram (a), whereas their fourth derivative peaks are completely separated (b).

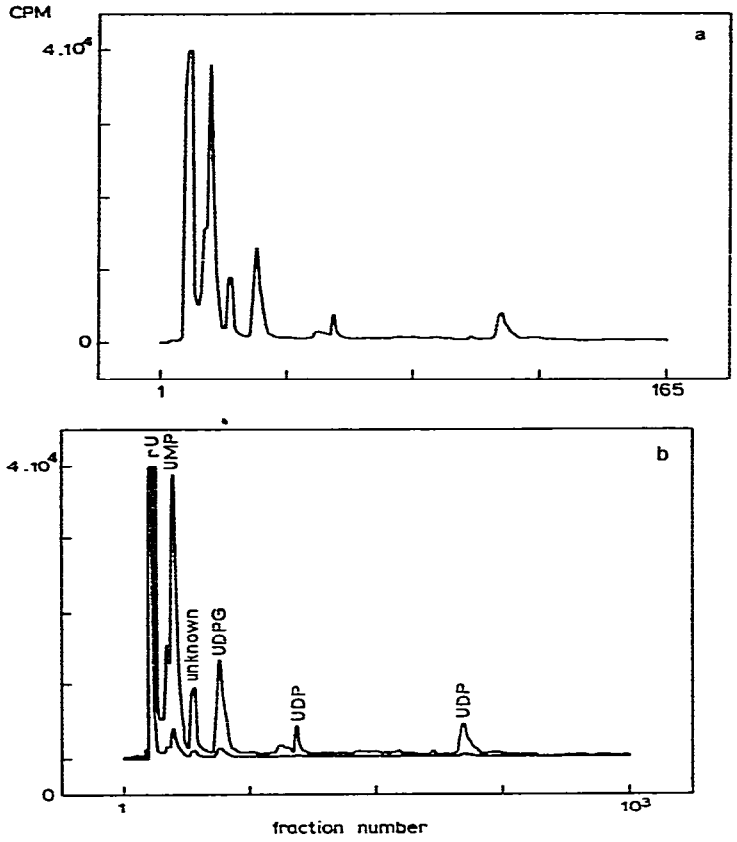

Fig. 6. Comparison between a direct radioactivity data (a) replotted by CPMIMP, and a spline-interpolated function (b) obtained from the same data.

As concluded by other workers[4,5], appropriate derivation intervals at each derivation step can greatly reduce background noise. Derivation itself is an unstable method and care should be taken to avoid introduction of noise. Fig. 8 illustrates this fact, and shows that small peaks can be extracted from noise (see arrows in Fig. 7b) by choosing suitable derivation intervals.

DISCUSSION

Problems of time

The methods described are of general interest in separation techniques, and provide original means of facilitating the treatment of radioactive data. They have never been used for processing such as the detection of main events and Gaussian integration. However, more sophisticated techniques could be applied for the same purposes. For instance, for Gaussian adjustment, non-linear least-squares fitting gives more precise results, but requires much more computation time. To execute the treatments described here on small computers, we avoided such time-consuming methods. Complete analysis by LISS takes 20–60 sec on our machine (2-MHz clock) and the computation time can be greatly reduced by the use of a wired arithmetic device. Spline interpolation is the slowest operation except for the fourth derivative

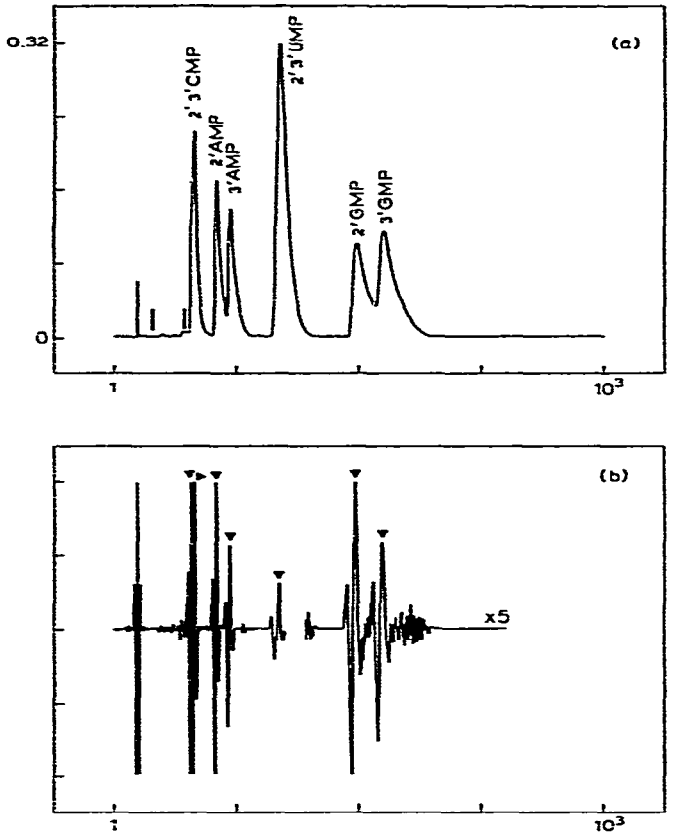

Fig. 7. A real chromatogram (a) and its fourth derivative spectrum (b). The chromatogram shows the analysis of a mixture of 2'- and 3'-nucleotides obtained by hydrolysis of TYMV mRNA.

computation, as it requires construction of 1000 interpolated points from 160 cpm points in 1.5 min. The autonomy of the microprocessor interface during data acquisition and the structure of the system allow the user to process a chromatogram while an acquisition cycle is being completed.

Sampling problems

An important problem is to ensure that sampling does not adversely affect data or cause loss of information. These risks have been extensively discussed by system designers (for a review, see ref. 6), but are not sufficiently known by biochemists. They may be avoided by the use of Fourier transforms. Absorbance data acquisition and digitalization are obtained by sampling the initial data. Counting also involves sampling when it is not done by a continuous technique. This sampling results in a loss of information which can be calculated by applying Fourier transform to the initial data. The Shannon theorem shows that the sampling frequency must not exceed π/ω_1, where ω_1 is the largest pulsation of the signal.

Modelling of a chromatogram

The choice of Gaussian function as a model for chromatographic peaks is debatable. The use of this type of function is supported by the theory of chromatogra-

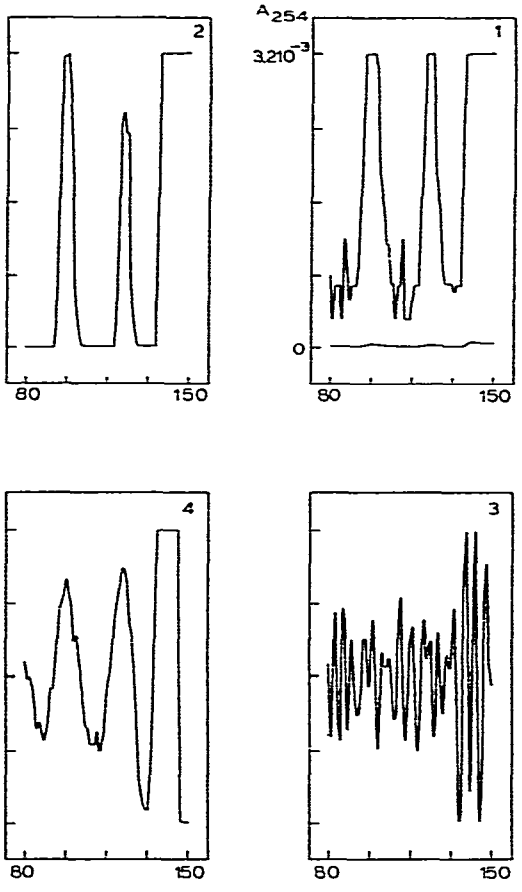

Fig. 8. Expansion of the part of the chromatogram in Fig. 7a between the two vertical bars. The expansion factor was 100. 1, Direct representation of the data; 2, data after filtering by LISS; 3, fourth derivative of the data shown in 1; derivation intervals were two points for each derivative; 4, as in 3 but with derivation intervals of 10 points.

phy; unfortunately, however, simple integration of the diffusion equation gives Gaussian function which do not take account of tailing, due to interaction with the column of the products analysed or to a non-linear absorption isotherm. Several models have been evolved to compensate empirically for the lack of a complete theory. Many functions exhibit the required characteristics, *i.e.*, cancellation at the boundaries and the possibility of width variation. Gaussian function symmetry can be corrected using two half-Gaussian curves of different width without altering continuity and derivability. Further, Gaussian functions do not vanish for $t = 0$, and this property is an essential characteristic of a chromatogram. In this way, χ^2 law provides a more realistic model; nevertheless, varying assays have demonstrated that the use of such a law for peak modelling leads to identical area calculations, so that we were able to disregard this problem.

CONCLUSION

We have described the use of a simple set of programs that can be adapted to a wide range of micro- and minicomputers for chromatographic analysis. The method

gives reliable and accurate results for a large number of chromatographic situations even in cases of incomplete separation. Commercial automatic HPLC analysis systems do not provide the facilities offered by our system for baseline correction and filtering of initial data, and are incapable of processing radioactivity results. Yet such processing is the initial condition for undertaking studies on metabolic arrays.

APPENDIX

```
    Algorithm of the LBASE subroutine

    Variables     s: derivation threshold
                 sp: slope threshold of the derivative
                 np: number of experimental points


       sx =: sp/2
       i  =:  1
d1     if |p(i)|<sx and t(i)<2000 then exit
       i =: i+1
       if i=np+1 then return
       d1
exit   d =: i
deb1   j =: i+1
deb    if |p(j)|<sx then suit
       j =: j+1
       if j≠np then deb
suit   f =: j
       i =: j
       sl =:  t(f)-t(d) /(f-d)
       s =: s/(f-d)
       if |sl|<s then cor1
       if f=np then cor
       deb1
cor    a =: t(d)-sl*d
       k =: f-1
       if f=np then k =: f
       for l=d,k do
       if t(l)<4095 then continue
       else c =: sl*l+a
       if c>t(l) then c =: t(l)
       t(l) =: t(l)-c
       do end
       if f=n return
       d =: i
       deb1
```

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