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REDUCTION OF DATA FROM THE AUTOMATED GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF COMPLEX EXTRACTS FROM HUMAN BIOLOGICAL FLUIDS USING A DIGITAL ELECTRONIC INTEGRATOR AND AN OFF-LINE COMPUTER PROGRAMME

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SUMMARY

A system is described for handling data from an automated gas chromatograph equipped with a digital electronic integrator and punched tape facilities. An off-line computer programme gives quantitation in micrograms by internal standardisation, and calculation of relative retention data. The system is used for the analysis of complex extracts of acidic metabolites from biological fluids and is easily adapted for use with other analyses. The computer processing time is 3 sec per sample of thirty to forty components and it provides a very economical data-handling system for chromatographers with access to an off-line computer.

INTRODUCTION

We have recently developed methods for studying in detail the pattern of acidic metabolites in biological fluids[1–4]. The gas chromatography (GC) has now been fully automated and the volume of data produced necessitates assistance with the data handling problems encountered. This communication describes the system developed for handling data from an automated gas chromatograph using a digital electronic integrator and an off-line computer programme with quantitation by internal standardisation and calculation of relative retention data as a primary aid to identification (gas chromatography–mass spectrometry is used for absolute identification).

EXPERIMENTAL

Gas chromatographic system

The GC system consists of a Hewlett-Packard (Avondale, Pa., U.S.A.) Model

5750B gas chromatograph equipped with linear temperature programming, dual glass columns and dual flame ionisation detectors, automatic on-column liquid injection by conventional glass microlitre syringe using a Hewlett-Packard Model 7670 automatic liquid sampler and an on-line Hewlett-Packard Model 3370B digital electronic integrator. This apparatus is used for the analysis of extracts of acidic metabolites from urine, blood plasma, and cerebrospinal fluid as their trimethylsilyl (TMS) and ethoxime derivatives on 10% OV-101 on Chromosorb W using temperature programming from 110 to 285° at 4°/min with an initial 5-min isothermal delay. GC internal standards are tetracosane ($C_{24}H_{50}$) and hexacosane ($C_{26}H_{54}$), added at the same concentration at the time of trimethylsilylation. An acidic internal standard, undecanedioic acid, is also incorporated, being added to the biological fluid prior to initial extraction. Full details of this extraction and analytical procedure have been published elsewhere[1-4]. Neutral and basic metabolites can also be analysed using similar procedures.

Data handling system

Basic system. The integrator is a Hewlett-Packard Model 3370B digital integrator equipped with a code converter board and an ASR 33 teletype output with punched tape facilities. The integration cycle is initiated by the automatic sampler on injection of the sample and is stopped by the sampler after a preset time interval. The integrator converts the analogue gas chromatograph signal into digital form and when a peak is detected will print out a retention time at the peak apex in minutes or seconds. The area is measured by a voltage to frequency converter which develops an output pulse rate directly proportional to the input signal, the pulses being counted to give the area in terms of μV sec. The integrator has a linear range of 1 to 10^6 and is equipped with up and down sensitivity controls that determine peak detection, a baseline reset device that determines whether integration is by tangent measurement or perpendicular drop, front and rear shoulder controls and noise suppression and area threshold limits. The chart recorder is controlled by the integrator which superimposes event markings on the charts. Full details are given in the Hewlett-Packard Operating Manual[5].

The integrator produces a 5-digit binary coded decimal output to the code converter board which transmits this information to the teletype in 6-bit parallel, digit serial format. The teleprinter produces a listing and corresponding punched paper tape. Each row contains a 4-digit number followed by a 1-digit code. The first row for each sample contains the sample number (from the autosampler turntable). Next, pairs of rows contain peak retention times in hundredths of minutes (code 8) and peak areas (codes 0 to 4, denoting the number of omitted zeroes after the printed figures). The end of the data on a sample is signalled by a row with code 6 and this is followed by a new sample number (code 0) automatically incremented by 1 from the previous one. Other code numbers are treated as aberrant and cause an error print out.

At the beginning of a run the teleprinter keyboard is used to punch on the tape a run serial number, the number of the last sample to be presented and the weight of the primary internal standard (tetracosane) in the amount of sample injected (usually 4 μl). The first sample number (usually 0001, 0) is punched on the next row, and the run is then begun. After the final sample, a further sample number is punched and the autosampler is then stopped by a microswitch activated by a plug in the sample tray.

Computer programme. The computer programme first reads in the initial data and hunts for a sample number row on the tape (code 0). The retention times and peak areas, distinguished by their code numbers, are read from the tape and stored, ignoring peaks with a retention time less than 5.2 min or areas less than 1000 units. The programme then obtains approximate peak heights as the ratios of peak areas to corresponding increments in retention time. The highest two peaks later than 39 min are taken to be the two standards, and a check is applied that their positions and area ratio lie between the predetermined limits —if this check fails, a warning message is printed. Finally the areas are converted to absolute units by reference to the known quantity of tetracosane, and the results are printed out. Each peak is shown with its retention time and area (both absolute and relative to the primary standard) and its amount in micrograms, assuming a unit response factor relative to tetracosane. The programme then proceeds to the next sample.

A listing of the programme is given in Appendix 1 and a flow diagram of the peak-reading section in Fig .1. The latter shows the checks which are applied and exemplifies the complexity of a routinely useful programme for such a relatively simple task; as many as possible of the forseeable errors and exception conditions must be coped with in such a way as to permit the continued analysis of uncorrupted data.

EVALUATION OF THE METHOD IN THE PRESENCE OF OVERLAPPING PEAKS

A typical chromatogram of derivatives of organic acid metabolites from the urine of a patient with phenylketonuria and the corresponding computer printout are shown in Figs. 2 and 3, respectively.

The analytical chemist manually compares the chromatograms and computer printout, where necessary, for a final evaluation of the samples. Where peak identifications are known and overlaps are minimised by the integrator and chromatography, and relative response factors to tetracosane have been determined, the components may be quantitated in terms of micrograms simply by dividing the relative peak area by the corresponding relative response factor. In the present analyses, in the case of urinary specimens the amount of sample injected into the chromatograph is equivalent to 12 μg creatinine and hence results may be finally expressed as μg per μg creatinine.

It can be seen from the chromatogram (Fig. 2) of urinary organic acids from a patient with phenylketonuria that the major phenylalanine metabolites of interest elute close together and that the peaks due to the derivatives of 2-hydroxyphenylacetic acid and 3-phenyllactic acid partially overlap. Peak area measurement by the Hewlett-Packard integrator is either by perpendicular drop or by tangential separation, which is determined by the setting of the baseline reset delay control. For perpendicular drop integration the baseline is reset after a finite time ($>$0.1 min) and for tangential integration the baseline reset control is set at zero and a new baseline is set up immediately integration of a peak has stopped. With overlapping peaks, therefore, and using perpendicular drop integration, the baseline to which peaks are referred is that of the start of the first peak of any overlapping series. A new baseline is not set up until the set finite time has elapsed before another peak elutes, and thus all the peaks are integrated with respect to the first established baseline of the group. In the analysis of complex mixtures of compounds extracted from biological fluids, compounds are continually eluting from the column and almost continuous small

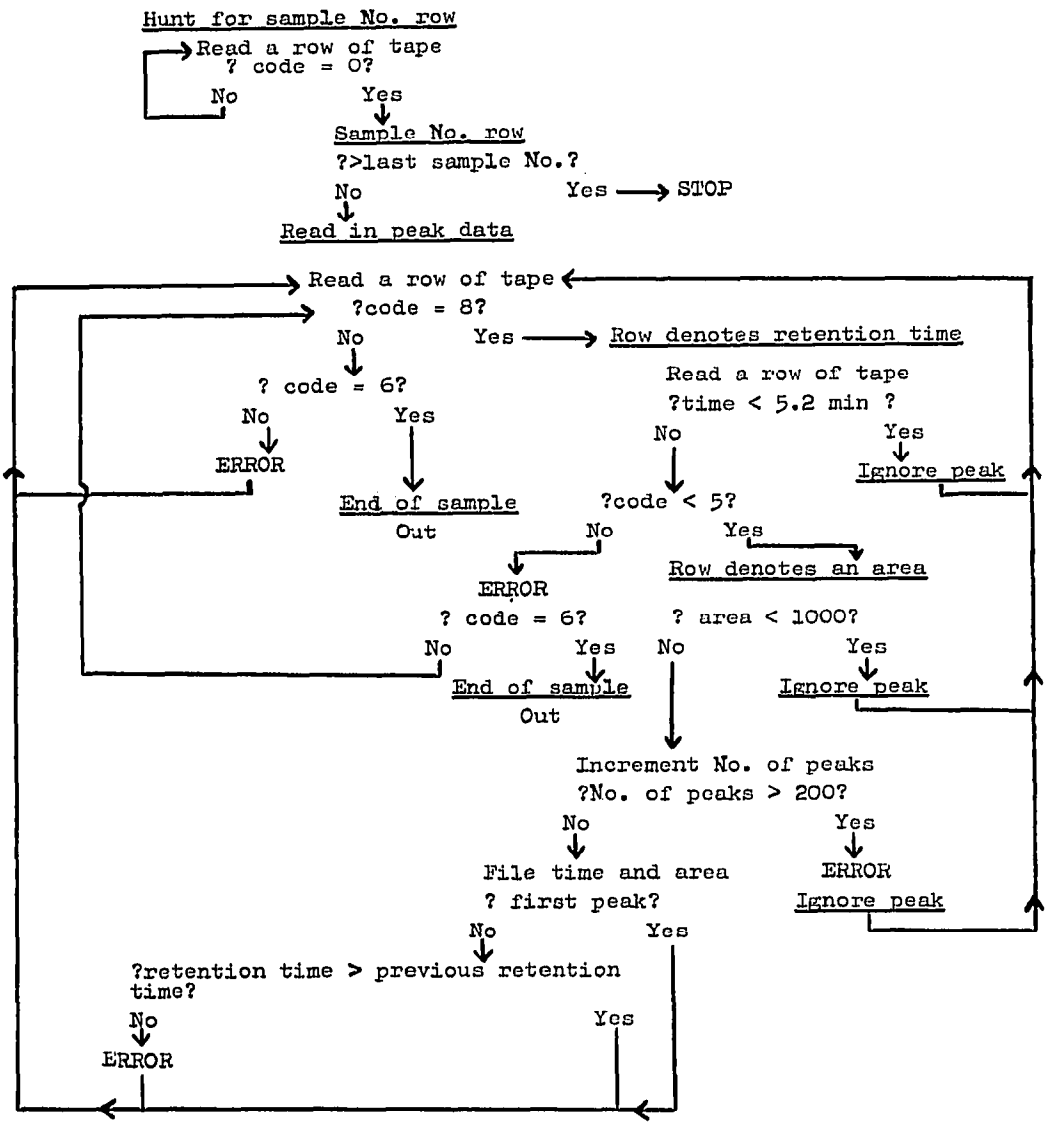

Fig. 1. Flow diagram of the peak reading section of the computer programme.

overlaps occur at base (see Fig. 2). New baselines are thus set up at only a few places in the chromatogram using perpendicular drop integration. With tangential integration a new baseline is set after each peak throughout the analysis, this baseline being on a tangent to the down slope of the preceding peak where overlap occurs.

In order to examine which of the two integration methods is best for such complex chromatograms, five standard mixtures containing varying proportions of the three major phenylalanine metabolites were prepared as their TMS derivatives and in the case of 3-phenylpyruvic acid as its TMS-ethoxime derivative and analysed

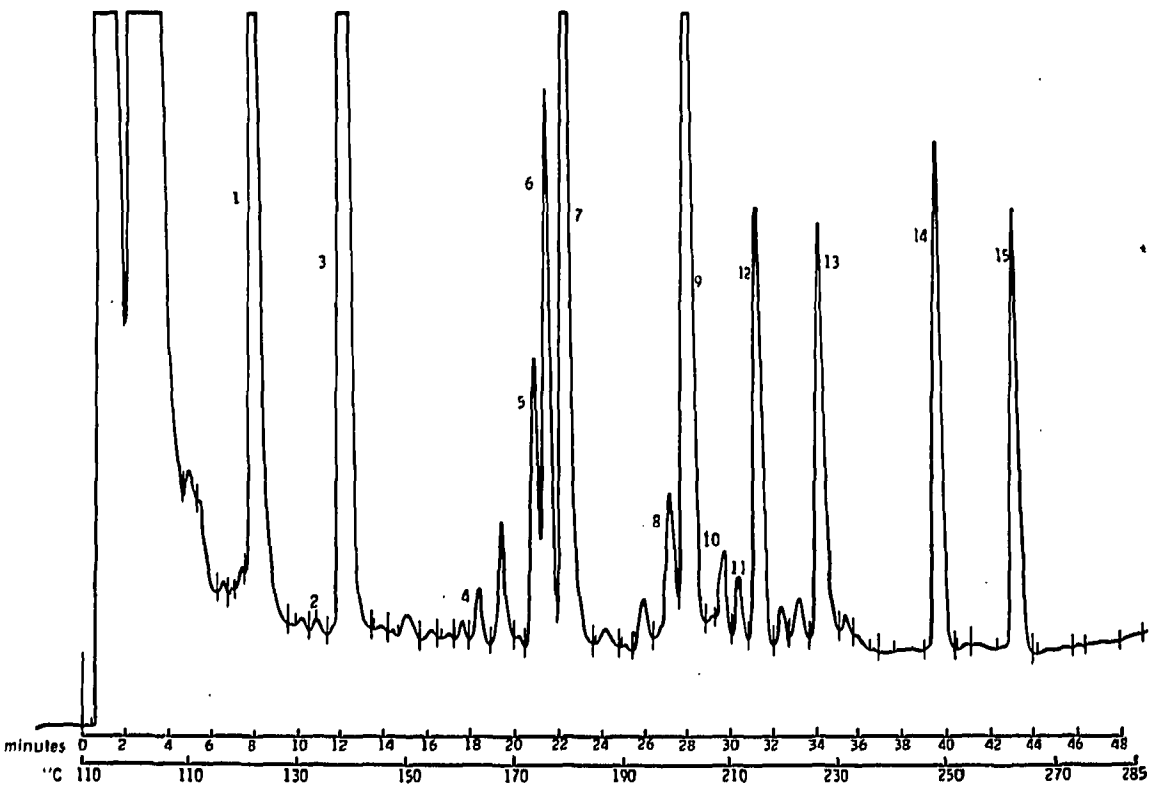

Fig. 2. Chromatogram of TMS and TMS-ethoxime derivatives of acidic metabolites extracted from an aliquot of urine (equivalent to 12 μg creatinine) of a patient with phenylketonuria. Chromatography was on 10% OV-101 on HP Chromosorb W (AW-DMCS), 80–100 mesh, using temperature programming from 110 to 285° at 4°/min with an initial 5-min isothermal delay. Identities of the peaks are as follows: 1 = sulphate; 2 = benzoate; 3 = phosphate; 4 = mandelate; 5 = 2-hydroxyphenylacetate; 6 = 3-phenyllactate; 7 = 3-phenylpyruvate; 8 = hippurate; 9 = citrate; 10 = 4-hydroxy-3-phenyllactate; 11 = 4-hydroxy-3-phenylpyruvate; 12 = undecanedioate (internal standard); 13 = urate; 14 = tetracosane (internal standard); 15 = hexacosane (internal standard).

in duplicate using both methods of integration. Recovery figures for 2-hydroxyphenylacetic acid, 3-phenyllactic acid, and 3-phenylpyruvic acid by tangential integration were 148.1 ± 3.4%, 77.0 ± 3.2%, and 88.0 ± 4.8%, respectively (means ± standard error). By perpendicular drop integration recoveries were 150.9 ± 3.0%, 104.6 ± 3.0%, and 95.5 ± 3.1%, respectively. 2-Hydroxyphenylacetic acid appears overestimated by both methods, this being due to overlap with 3-phenyllactic acid. Combining the recoveries of these two acids, and expressing the total as 3-phenyllactic acid, gives recoveries of 104.0 ± 1.6% for tangential integration and 122.5 ± 2.5% for perpendicular drop integration. This indicates that tangential integration gives more accurate results with such chromatograms, and this was further confirmed by calculating the recovery of the undecanedioic acid internal standard by both methods of integration for 14 separate urine samples. Tangential integration gave recoveries of 108 ± 1.7% and perpendicular drop integration gave recoveries of 134.0 ± 2.2%.

The degree of overlap of the peaks due to 2-hydroxyphenylacetic acid and 3-

phenyllactic acid is constant using set analytical conditions and thus correction factors may be applied to obtain accurate results. These factors were calculated from the standard data as 0.675 for 2-hydroxyphenylacetic acid and 1.299 for 3-phenyllactic acid. As an example of applying these factors to recovery figures for these acids, where original results were 144.6% and 86.9%, respectively, final corrected results of 97.7% and 112.8%, respectively, were obtained. Accurate results from partially overlapping peaks may be obtained therefore by running the standard materials involved and calculating and applying the appropriate correction factors. The use of correction factors in the calculation of overlapping chromatographic peaks has been discussed in detail by Novák *et al.*[6].

SAMPLE NO 1

CAUTION - STANDARDS NOT SURELY IDENTIFIED!

	TIME	AREA	RRT		RPA
1	6.42	4990.	0.161	0.015	0.06
2	7.02	2678.	0.176	0.008	0.03
3	7.36	15720.	0.185	0.046	0.19
4	7.93	916700.	0.199	2.693	10.89
5	10.08	3075.	0.253	0.009	0.04
6	10.80	5487.	0.271	0.016	0.07
7	12.08	1231000.	0.304	3.616	14.62
8	13.96	1019.	0.351	0.003	0.01
9	14.97	15500.	0.376	0.046	0.18
10	16.02	6075.	0.403	0.018	0.07
11	16.95	1868.	0.426	0.005	0.02
12	17.52	7680.	0.440	0.023	0.09
13	18.30	30130.	0.460	0.089	0.36
14	19.38	86880.	0.487	0.255	1.03
15	20.88	190500.	0.525	0.560	2.26
16	21.52	269400.	0.541	0.791	3.20
17	22.34	675000.	0.562	1.983	8.02

Fig. 3. The computer printout corresponding to the chromatogram shown in Fig. 2.

DISCUSSION

Use of the system over several months and for the analysis of more than 500 specimens has indicated its wide applicability and usefulness for the data handling of complex gas chromatographic analyses. With minor adaptations in the set limits appropriate to the analytical conditions being used, the system may be used in other analyses including liquid chromatographic systems, and is currently being adapted for use in the analysis of the neutral metabolites. Most of the computer systems and programmes that are commercially available or are described in the literature involve the use of dedicated on-line computers and are suitable only for relatively simple

18	24.27	13580.	0.610	0.040	0.16	
19	25.95	37690.	0.652	0.111	0.45	
20	26.85	5877.	0.675	0.017	0.07	
21	27.21	104300.	0.684	0.306	1.24	
22	28.08	714900.	0.706	2.100	8.49	
23	29.30	2895.	0.737	0.009	0.03	
24	29.67	44790.	0.746	0.132	0.53	
25	30.37	36490.	0.763	0.107	0.43	
26	31.33	284800.	0.788	0.837	3.38	
27	32.45	25740.	0.816	0.076	0.31	
28	33.26	30700.	0.836	0.090	0.36	
29	34.22	342000.	0.860	1.005	4.06	
30	35.48	8733.	0.892	0.026	0.10	
31	39.08	1092.	0.982	0.003	0.01	
32	39.78	340400.	1.000	1.000	4.04	TETRACOSANE STANDARD
33	41.02	2852.	1.031	0.008	0.03	
34	43.33	295700.	1.089	0.869	3.51	HEXACOSANE STANDARD
35	45.77	11980.	1.151	0.035	0.14	
36	46.45	9573.	1.168	0.028	0.11	
37	47.96	29740.	1.206	0.087	0.35	
38	49.03	31500.	1.233	0.093	0.37	

analyses with quantitation by a normalisation procedure and expression of results as percentages of the total peak area[7-19]. On-line systems are of greatest use where more than ten gas chromatographs are being run simultaneously and where the analytical conditions are very similar on each instrument with relatively simple mixtures being analysed, as in a process control laboratory. The greater the variation between analyses and hence programmes required and the more complex the mixture being analysed, the fewer the number of chromatographs that can be accommodated by one computer. An off-line system has the additional advantage that when a variety of analyses of different type are being made, not all of which are amenable to computer processing, the integrator still produces listings of peak retention times and areas in the real time of the analysis.

The processing time with the present system is about 60 sec for one full punched paper tape of 20 samples each containing approximately 30–40 components. It therefore provides a relatively inexpensive method provided that the analyst has access to an off-line computer.

Previously described systems are not suitable for the analysis of complicated mixtures of compounds extracted from biological fluids in which the components have widely differing relative responses and where not all the peaks are of interest. Many of the components may be unidentified and quantitation of identified peaks by internal standardisation is the only suitable method available. The highly complex chromatograms produced require a series of closely interrelated subjective decisions for their interpretation; these cannot, as yet, be made by a computer without extensive programming. Hence, the computer cannot be used to identify peaks and to quantitate them directly in terms of micrograms, and manual interpretation is still required. This is particularly important when a series of analyses is carried out over several months and minor variations in column material and exact analytical conditions such as temperatures and carrier flow rates occur. The early eluting peaks tend to be more greatly affected than the later peaks. In the case of urinary specimens, the positions of major peaks that always occur (*e.g.*, sulphate, phosphate and the undecanedioic acid internal standard) are used for identification purposes. Further improvements in the chromatography will be necessary before the computer can be programmed to undertake full evaluation of a complex chromatogram.

APPENDIX

Full listing of the computer programme (Fig. 4).

```
0010          C     MASTER METAR1
0011          C
0012          C  REDUCTION OF GAS CHROMATOGRAPH DATA
0013          C  NEAR-STANDARD FORTRAN IV VERSION
0014          C
0015                INTEGER TIME(200)
0016                REAL AREA(200),HT(200)
0017                COMMON/LINEHP/LHP(30)
0018          C  SET UP BUFFER (CHANNEL 10) FOR INPUT ROUTINE
0019                CALL DEFBUF(10,120,LHP)
0020          C  READ IN NO OF SAMPLES AND WT OF STANDARD
0021          9     CALL HPLINE
0022                IF(ICRCHAR1(LHP(1),'    '))78,9,78
0023          C  ICRCHAR1(I,J)=0 IFF I=J.  OVERFLOW CANNOT BE SET
0024          78    READ(10,100)ISERL,NRUNS,WTTET
0025          C  FREE FORMAT - ITEMS SEPARATED BY BLANKS
0026          100   FORMAT(2I0,F0.0)
0027                WRITE(6,208)ISERL
0028          208   FORMAT('0  RUN SERIAL NO.',I10/)
0029          C  HUNT FOR SAMPLE NO ROW
0030          10    CALL INP3(K,L)
0031                IF(L)99,99,10
0032          99    IF(K-NRUNS)22,22,98
0033          98    STOP
0034          22    WRITE(6,200)K
0035          200   FORMAT('1 SAMPLE NO',I4)
0036                NP=0
0037          C  READ IN PEAKS
0038          14    CALL INP3(K,L)
0039          13    IF(L-8)97,11,12
0040          97    IF(L-6)12,13,12
0041          12    WRITE(6,206)K,L
0042          206   FORMAT('0 ABERRANT ROW - ',I4,',',I1)
0043                GOTO14
0044          11    CALL INP3(M,N)
0045          C  REJECT TIMES BEFORE 5.2 MINUTES
0046                IF(K-520)14,96,96
```

Fig. 4

(Continued on p. 374/375)

Fig. 4. *(continued)*

```
0047          C  FOR PEAK AREA, N SHOULD BE LT 5
0048       96    IF(N-5)15,95,95
0049       95    AK=FLOAT(K)*0.01
0050             WRITE(6,211)AK
0051       211   FORMAT('0 NO PEAK AREA AT TIME',F7.2)
0052          C  BUT IT MAY BE AN END OF SAMPLE ROW (N=6)
0053             IF(N-6)94,13,94
0054       94    K=M
0055             L=N
0056             GOTO16
0057       15    A=FLOAT(M)*10.0**N
0058          C  REJECT AREAS LESS THEN 1000
0059             IF(A-1000.0)14,93,93
0060       93    NP=NP+1
0061             IF(NP-200)28,28,92
0062       92    WRITE(6,207)
0063       207   FORMAT('0 MORE THAN 200 PEAKS IN THIS RUN')
0064             GOTO10
0065       28    TIME(NP)=K
0066             AREA(NP)=A
0067             IF(NP-1)14,14,91
0068       91    IF(TIME(NP)-TIME(NP-1))90,90,14
0069       90    WRITE(6,212)NP
0070       212   FORMAT('0 TIME SEQUENCE ERROR AT PEAK NO',I4)
0071             GOTO14
0072          C
0073          C  END OF SAMPLE - PROVIDED 'TIME' GT 5.2 MINS
0074       13    IF(K-520)11,77,77
0075          C  GET FIRST TIME GT THAN 39 MINUTES
0076       77    DO 20 I=1,NP
0077             IF(TIME(I)-3900)20,20,21
0078       20    CONTINUE
0079             T=FLOAT(TIME(NP))*0.01
0080             WRITE(6,202)T
0081       202   FORMAT('0 LONGEST TIME IS ONLY',F6.2)
0082             GOTO10
0083          C  GET APPROX PEAK HEIGHTS
0084       21    DO32 J=I,NP
0085       32    HT(J)=AREA(J)/FLOAT(TIME(J)-TIME(J-1))
```

```
0086          C  GET BIGGEST SUBSEQUENT PEAK
0087                P=0.0
0088                DO23 J=I,NP
0089                IF(HT(J)-P)23,23,89
0090           89   P=HT(J)
0091                JM=J
0092           23   CONTINUE
0093          C  AND THE NEXT BIGGEST
0094                P=0.0
0095                DO24 J=I,NP
0096                IF(J-JM)88,24,88
0097           88   IF(HT(J)-P)24,24,87
0098           87   P=HT(J)
0099                JN=J
0100           24   CONTINUE
0101          C  MAKE JM < JN
0102                IF(JM-JN)25,25,86
0103           86   J=JM
0104                JM=JN
0105                JN=J
0106          C  CHECK POSITIONS AND RELATIVE AREAS OF STANDARD PEAKS
0107           25   IF(TIME(JM)-4120)26,85,85
0108           85   IF(TIME(JM)-4420)84,84,26
0109           84   IF(TIME(JN)-4500)26,83,83
0110           83   IF(TIME(JN)-4760)82,82,26
0111           82   R=HT(JN)/HT(JM)
0112                IF(R-0.75)26,81,81
0113           81   IF(R-1.30)27,27,26
0114          C  STANDARDS QUESTIONABLE
0115           26   WRITE(6,203)
0116           203  FORMAT('0 CAUTION - STANDARDS NOT SURELY IDENTIFIED!')
0117          C
0118          C  NOW STANDARDISE AND PRINT OUT
0119           27   AMG=AREA(JM)/WTTET
0120                RETT=TIME(JM)*0.01
0121                WRITE(6,204)
0122           204  FORMAT(///'          TIME      AREA      RRT      RPCT/)
0123                DO30 I=1,NP
0124                T=FLOAT(TIME(I))*0.01
```

Fig. 4.

(Continued on p. 376)

Fig. 4. *(continued)*

```
0125                RRT=T/RETT
0126                RPA1=AREA(I)/AREA(JM)
0127                RPA2=RPA1*WTTET
0128                WRITE(6,205)I,T,AREA(I),RRT,RPA1,RPA2
0129          205   FORMAT('0',I3,F10.2,F10.0,2F10.5,F10.2)
0130                IF(I-JM)50,80,51
0131          80    WRITE(6,209)
0132          209   FORMAT('+',56X,'TETRACOSANE STANDARD')
0133                GO TO 50
0134          51    IF(I-JN)30,79,30
0135          79    WRITE(6,210)
0136          210   FORMAT('+',56X,'HEXACOSANE STANDARD')
0137          30    CONTINUE
0138                GOTO10
0139                END

END OF SEGMENT, LENGTH   444, NAME  META81

0140                SUBROUTINE INP3(K,L)
0141          C  PAPER TAPE INPUT ROUTINE
0142                COMMON/LINEHP/LL(30)
0143          10    CALL HPLINE
0144          C  HPLINE IS ROUTINE TO READ PAPER TAPE IN NON-STANDARD CODE
0145          200   FORMAT(2X,2A4)
0146          C  IGNORE BLANK LINE
0147                IF(ICRCHAR1(LL(1),'    '))11,10,11
0148          11    READ(10,100)K,L
0149          100   FORMAT(I4,1X,I1)
0150                RETURN
0151                END

END OF SEGMENT, LENGTH    43, NAME  INP3
```

Fig. 4. Full listing of the computer programme.

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