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A QUANTITATIVE DENSITOMETRIC METHOD FOR THE RAPID SEPARATION AND QUANTITATION OF THE MAJOR LIPIDS OF TISSUES AND LIPOPROTEINS BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

II* . REDUCTION OF THE DENSITOMETRIC DATA

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SUMMARY

A BASIC program is described for acquisition of data and data reduction for an automated densitometric system for quantitation of lipids separated by high-performance thin-layer chromatography. The program allows calculation of mass of samples from log/log calibration curves computed from standards. The calculated masses are reported as nmol/volume or

*For part I, see ref. 1.

nmol/mg protein. The program contains a flexible dialog system which permits its use for a variety of applications in addition to the system described for quantitation of lipids.

INTRODUCTION

The reduction of data from quantitative densitometry of high-performance thin-layer chromatography (HPTLC) can be an extremely laborious process which can detract from the many other advantages of this technique. There are two main reasons: (1) Due to improvements in the TLC plates, a large number of samples can be loaded on to each plate and the good resolution may produce a large number of components to be measured. (2) For quantitative analysis it is usually necessary to run a standard curve on each plate and it is desirable to scan each standard and sample more than once. Thus a large amount of data is generated from each analysis.

This paper describes a computer program for the reduction of data from scanning TLC systems. The program is written in BASIC for the Spectra Physics SP 4100 computing integrator which is interfaced to the TLC scanner. It was written primarily for handling quantitative analysis of lipids which we have described elsewhere [1], but it is flexibly organized so that it can be used for other analyses [2].

MATERIALS AND METHODS

The SP 4100 (Spectra Physics, Darmstadt, F.R.G.), a computing integrator programmable in BASIC, was used for data manipulation in conjunction with Minifile 4100 D (Spectra Physics), a floppy disc with 81 Kbyte memory for the automated data manipulation, reporting and storage of results.

HPTLC plates were analysed with either the semiautomated Shimadzu CS 910 TLC scanner (Shimadzu, Kyoto, Japan) or the fully automated Camag TLC scanner (Camag, Muttenz, Switzerland). When used with the Camag scanner a modified Camag interface was used to control the bidirectional

```
*****
DECIDE WHICH PROGRAM TO RUN !

"SCAN"      FOR STORING REPORTS ON DISC
OR
"CALC"      FOR RESTORING REPORTS FROM DISC
             AND POST RUN CALCULATIONS

ENTER NAME OF PROGRAM !
PROGRAM: SCAN

ENTER NAME OF SCANNER ( CAMAG OR SHIMADZU )
SCANNER: CAMAG
*****

"SCANNING" WILL BE TRANSFERRED TO SP4100
AND START AUTOMATICALLY
*****
```

Fig. 1. The printout demonstrates the dialog for the program selection (SCAN or CALC) and for the scanner used (Camag or Shimadzu).

scanning, the SP 4100 integrator and the Minifile. The program is structured in modules as described in Fig. 1. As can be seen, the two major subprograms are "SCAN" for storing scanning data on disc and "CALC", which recalls data from disc and permits a variety of subsequent data reductions. The "BOOT MOD" module is the fundamental program which is present in all subprograms and contains the necessary disc operating software. It manages and calls up the necessary subprograms.

DESCRIPTION OF PROGRAM

Data acquisition program ("SCAN")

The SCAN program starts by setting up the scan parameters (Fig. 2). The number of scans/track and the scanning parameters must be defined at the

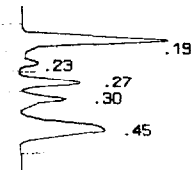
```
*****
SELECT BLOC OF FILES TO STORE REPORTS ON DISC |
SELECTED BLOC No. : 2
ENTER NUMBER OF TRACKS |
NUMBER OF TRACKS : 14
ENTER NUMBER OF SCANS/TRACK
SCANS/TRACK : 3

SCANNING PARAMETERS
ATTENUATION (AT) : 64
CHART SPEED (CS) : 4
PEAK PARAMETER (PW) : 3
              (PT) : 500
NUMBER OF TRACKS : 14
NUMBER OF SCANS/TRACK : 3

R E A D Y   T O   S C A N
*****
```

Fig. 2. The printout demonstrates the dialog for the scanning parameters in the SCAN program (number of scans, tracks, etc.) and asks for the file where the data shall be stored (BLOC-No.). The variable names of the scanning parameters, e.g. (F 7), (F 8), are given in parentheses.

INJECT TIME 10:25:57



TRACK 1		SCANS/TRACK 1	
SCAN No.			
1	0.19	23451	02
2	0.23	1923	03
3	0.27	8812	02
4	0.30	10080	02
5	0.45	28287	03

SAVED ON DISC FILE No. 201

Fig. 3. The printout demonstrates the report format of the scanned track. Each scan is reported in terms of retention time, peak area and the code number for baseline correction (01-65).

```
*****
ALL TRACKS HAVE BEEN SCANNED AND THE REPORTS
ARE STORED ON DISC IN BLOC No. 2
*****
```

R E T E N T I O N T I M E S

SCAN No.	PEAK RETENTION TIMES				
# 1	0.19	0.23	0.27	0.30	0.45
# 2	0.18	0.23	0.26	0.29	0.45
# 3	0.19	0.24	0.27	0.31	0.46
# 41	0.18	0.23	0.28	0.31	0.46
# 42	0.19	0.24	0.27	0.30	0.45

```
FOR NEW MEASUREMENT OR CALCULATION
DEPRESS THE KEYS "SHIFT" AND "SLOPE"
"BOOT MOD" WILL THEN BE TRANSFERRED TO SP4100
ENTER "RUN" WHEN TRANSFER IS FINISHED
*****
```

Fig. 4. The printout demonstrates the list of all peak retention times encountered during scanning. Up to six retention times per scan can be listed. This list allows selection of the peak of interest for further calculation according to the retention times.

beginning of the program. Up to three are possible; the number of tracks is dependent on the plate used and the spot size. The program prints a listing of all scan parameters and responds "Ready to scan".

The analog signal from the densitometer is integrated by the SP 4100 using a 50-Hz data sampling rate, permitting high resolution of signal from noise for the very fast peaks produced by the scanner. Each scan is recorded on printer plotter. Peaks are automatically baseline-corrected and reported in terms of retention times and the area under the peak (Fig. 3). At the end of each scan, the data are automatically stored in files on discette, defined by its track-number and number of scans for each track.

```
*****
DATE OF MEASUREMENT : *12.02.82
DATE OF EXPERIMENT  : *10.02.82
EXPERIMENT           : PERI/PREINCUBATION

SELECT BLOC OF FILES TO RESTORE REPORTS FROM DISC !
SELECTED BLOC No. :2

ENTER NUMBER OF TRACKS !
TRACKS : 9

ENTER NUMBER OF SCANS/TRACK !
SCANS/TRACK : 3
*****

PEAK IDENTIFICATION

NUMBER OF PEAKS : 3

RETENTIONTIME : .51
MOLECULAR WEIGHT : 647.1
NAME : CHOL'ESTER

RETENTIONTIME : .57
MOLECULAR WEIGHT :
NAME : INT. STD.

RETENTIONTIME : 1.57
MOLECULAR WEIGHT : 386.6
NAME : CHOL

PEAK IDENTIFICATION WINDOW :
*****
```

Fig. 5. The printout demonstrates the dialog of the CALC program to define the experiment (up to 35 characters are available), to address the relevant peaks as demonstrated in Fig. 4 and to name the components by means of the substance and molecular weight.

```

*****
EXTERNAL STANDARDS
NUMBER OF EXTERNAL STANDARDS : 5
EXT. STD. CONC. LEVEL DATA
CHOL'ESTER
LEVEL 1 : 1
LEVEL 2 : .8
LEVEL 3 : .5
LEVEL 4 : .3
LEVEL 5 : .2
INT. STD.
LEVEL 1 : 1
LEVEL 2 : .8
LEVEL 3 : .5
LEVEL 4 : .3
LEVEL 5 : .2
CHOL
LEVEL 1 : 1
LEVEL 2 : .8
LEVEL 3 : .5
LEVEL 4 : .3
LEVEL 5 : .2
*****

```

```

INTERNAL STANDARDS
NUMBER OF INTERNAL STANDARDS : 2
PEAK No. OF INT. STD. : 2
INT. STD. CONC. LEVEL DATA
INT. STD.
LEVEL 1 : .30
LEVEL 2 : .60
*****

```

Fig. 6. The printout demonstrates the dialog for the applied masses of the external standard calibration curves and the internal standards.

```

*****
SAMPLE DATA
SPECIFY THE KIND OF INPUTS YOU NEED !
ENTER "YES" OR "NO"
REDISSOLVED SAMPLE VOLUME : YES
APPLICATED SAMPLE VOLUME : YES
ALIQOT VOLUME : YES
DISH VOLUME : YES
ALIQOT PROTEIN : YES
INT. STD. AMOUNT : YES

NUMBER OF SAMPLES : 2

SAMPLE No. 1
SAMPLE NAME: TEST A
REDISSOLVED SAMPLE VOLUME : 60
APPLICATED SAMPLE VOLUME : 1
ALIQOT VOLUME : 1000
DISH VOLUME : 500
ALIQOT PROTEIN : 70.4
INT. STD. AMOUNT : .3

SAMPLE No. 2
SAMPLE NAME: TEST B
REDISSOLVED SAMPLE VOLUME :
APPLICATED SAMPLE VOLUME :
ALIQOT VOLUME :
DISH VOLUME :
ALIQOT PROTEIN : 70.38
INT. STD. AMOUNT :
*****
END OF DIALOG

*EXT.CALC* WILL BE TRANSFERRED TO SP4100
AND START AUTOMATICALLY
*****

```

Fig. 7. The printout demonstrates the dialog for the sample data as indicated (e.g. volumes, protein, etc.). There is a major "YES" or "NO" decision to select only the inputs which are necessary. The program asks in the following only for the YES input parameters. For the first sample all variables must be entered. For the following samples the default value for any input is the last valid input that was made.

When all scans are done, the program lists the retention times of all components encountered when scanning (Fig. 4). This enables the user to identify each component and to give it a name by which it is identified in subsequent calculations.

Calculation program ("CALC")

Definition of the calculation program ("DIALOG"). The calculation program starts with the subprogram DIALOG (Figs. 5-7). This is necessary to: (1) define the experiment (Fig. 5); (2) address the relevant sample peaks (Fig. 5); (3) address the external and internal standards (Fig. 6); (4) label the sample and enter sample variables (Fig. 7).

Calibration curve calculation ("EXT. CALC"). At the end of the dialog the

```
*****
EXTERNAL STANDARD AREA

TRACK 1      SCAN 1      SCAN 2      SCAN 3      REJECT ?
CHOL'ESTER  71780      73889      73890
INT. STD.   83737      96009      96021
CHOL        92049      94616      94598

TRACK 2      SCAN 1      SCAN 2      SCAN 3      REJECT ?
CHOL'ESTER  157089     68396      69853      1
INT. STD.   0      65905      86214      1
CHOL        78755      79830      80798

TRACK 3      SCAN 1      SCAN 2      SCAN 3      REJECT ?
CHOL'ESTER  58411      57440      57517
INT. STD.   69055      71042      71321
CHOL        73450      70005      70180

TRACK 4      SCAN 1      SCAN 2      SCAN 3      REJECT ?
CHOL'ESTER  45192      44847      45447
INT. STD.   54291      55042      55537
CHOL        54943      54460      54987

TRACK 5      SCAN 1      SCAN 2      SCAN 3      REJECT ?
CHOL'ESTER  37758      34910      34832
INT. STD.   43503      42952      43327
CHOL        45315      45184      45084
*****

MEAN STANDARD DEVIATION OF AREAS

CHOL'ESTER  1.88%
INT. STD.   1.1 %
CHOL        1.28%
*****

CALIBRATION CURVES

Y=mX+b FOR Y=LOG(AREA) AND X=LOG(CONC)

CHOL'ESTER  m *SLOPE*      b *INTERCEPT*      CORRELATION
INT. STD.   0.445          4.879                0.989
CHOL        0.483          4.984                0.996
CHOL        0.435          4.965                0.987
*****
END OF EXTERNAL STANDARD CALCULATIONS
THE NEXT SUBPROGRAM WILL BE TRANSFERRED
TO SP4100 AND START AUTOMATICALLY
*****
```

Fig. 8. This printout provides the opportunity to reject a maximum of two scanned areas for a substance in a track. At the end of each line the system asks for this rejection of areas. Input can either be nothing (then the program calculates the mean area and the standard deviation for all areas of this line), or the numbers of the areas to be rejected. If more than one area is to be rejected, the numbers must be separated by a point. The program will then calculate a mean area and standard deviation of the remaining areas. The system then prints the mean standard deviations of areas for each substance and the coefficients of the calibration curve.

calibration curve subprogram (EXT. CALC) is automatically transferred from the disc storage to the SP 4100. The peak areas of the external standard tracks identified by their retention times are transferred from disc storage into the RAM of the computing integrator. The transferred data are then printed for all tracks, scans and substances as can be seen from Fig. 8. This listing provides the opportunity for the user to control the values of the areas and if necessary to reject a maximum of two scanned areas for a substance in a track. From the peak areas the mean value and the relative standard deviation for each substance are calculated and printed out (Fig. 8). From these values log/log calibration curves are calculated and slope (m), intercept (b) and correlation coefficient of the function $\log y = m \log x + b$ are printed as described in Fig. 8.

Internal standard calculation ("INT. CALC"). At the end of the EXT. CALC the internal standard subprogram is automatically transferred from disc storage to the SP 4100. The peak areas of the internal standard are transferred and reported as described for EXT. CALC. The concentration of the internal standard is calculated from the mean of the internal standard integral = INT. STD. CALC and compared to the actual internal standard concentration (see Fig. 9). This comparison offers the opportunity to the user to carry out an "intercept correction of internal standard calibration curve".

```

*****
INTERNAL STANDARD AREA
TRACK 6      SCAN 1      SCAN 2      SCAN 3      REJECT ?
INT. STD.    59637      53360      54012      1
TRACK 7      SCAN 1      SCAN 2      SCAN 3      REJECT ?
INT. STD.    88717      78664      0          1.3
*****
MEAN STANDARD DEVIATION OF AREAS
INT. STD.    0.86%
*****
INTERNAL STANDARDS
          APPLICATED      CALCULATED      DIFF. %
          CONCENTRATION    CONCENTRATION
INT. STD. No. 1      0.3             0.298          -.75
INT. STD. No. 2      0.6             0.657          9.44
INTERCEPT CORRECTION OF INTERNAL STANDARD CALIBRATION CURVE
ACCORDING TO INT. STD. No. 1
          APPLICATED      CALCULATED      DIFF %
          CONCENTRATION    CONCENTRATION
INT. STD. No. 1      0.3             0.3            0
INT. STD. No. 2      0.6             0.662         10.28
INTERCEPT CORRECTION OF INTERNAL STANDARD CALIBRATION CURVE
ACCORDING TO INT. STD. No.
*****
"SP4.CALC" WILL BE TRANSFERRED TO SP4100
AND START AUTOMATICALLY
*****

```

Fig. 9. This printout demonstrates the rejection sequence similar to the one of the external standards. The program compares the calculated concentrations of internal standard in the internal standard tracks to the applied concentrations and offers the opportunity to correct the internal standard calibration curve according to one of the used internal standards. Input is either the number of internal standard, then the intercept correction is carried out, or 0, then the original calibration curve is reestablished, or nothing, then the program will continue.

```

*****
SPECIFY FORMAT OF SUMMARY REPORT I
*MOL/PROTEIN* FORMATS THE RESULTS AS FOLLOWS:
  "nmol /mg PROTEIN   %       nmol /DISH"
*MG/DL*        FORMATS THE RESULTS AS FOLLOWS:
  "mg/dl   %       nmol /dl "
REPORT FORMAT : MOL/PROTEIN
*****

```

Fig. 10. This printout illustrates the output for one of the two available summary reports (MOL/PROTEIN or MG/DL).

```

*****
          S U M M A R Y   R E P O R T
          -----
DATE OF MEASUREMENT: 08.03.82
DATE OF EXPERIMENT  : 08.03.82
EXPERIMENT          : PWS

      mg/dl      %      nmol/dl
SERUM A
TOTAL      117.03275
PC          100.52451      85.89      128.87758
SPM         16.50824      14.11      21.164417

      mg/dl      %      nmol/dl
SERUM B
TOTAL      106.17328
PC          88.157841      83.03      113.02287
SPM         18.015437      16.97      23.096714

*****
FOR PRINTING ANOTHER REPORT, ENTER "RUN"
FOR NEW MEASUREMENT OR CALCULATIONS
DEPRESS THE KEYS "SHIFT" AND "SLOPE"
"BOOT MOD" WILL THEN BE TRANSFERRED TO SP4100
ENTER "RUN" WHEN TRANSFER IS FINISHED
*****

```

```

*****
          S U M M A R Y   R E P O R T
          -----
DATE OF MEASUREMENT: 12.02.82
DATE OF EXPERIMENT  : 12.02.82
EXPERIMENT          : PERI/PREINCUBATION

      nmol /mg PROTEIN   %       nmol /DISH
TEST A
TOTAL      57.840295
CHOL'ESTER 2.9240131      5.06      1.0292526
CHOL       54.916282      94.94      19.330531

      nmol/mg PROTEIN   %       nmol/DISH
TEST B
TOTAL      58.487073
CHOL'ESTER 1.1855367      2.03      0.4349734
CHOL       57.301536      97.97      21.023934

*****
FOR PRINTING ANOTHER REPORT, ENTER "RUN"
FOR NEW MEASUREMENT OR CALCULATIONS
DEPRESS THE KEYS "SHIFT" AND "SLOPE"
"BOOT MOD" WILL THEN BE TRANSFERRED TO SP4100
ENTER "RUN" WHEN TRANSFER IS FINISHED
*****

```

Fig. 11. This figure gives an example of both of the available summary reports (MOL/PROTEIN or MG/DL).

Sample calculation sequence ("SPL. CALC"). At the end of INT. CALC the sample calculation subprogram is automatically transferred from the disc storage to SP 4100. The peak areas of the samples are transferred as described for the EXT. CALC program. For the mean areas and the compound calibration curves the mass of compound in $\mu\text{g/ml}$ applied volume is calculated. For each sample a correction factor resulting from the comparison of the calculated samples internal standard concentration and the INT. STD. CALC is computed. At the end of SPL. CALC one of two alternative summary reports can be selected (Fig. 10).

One of the summary reports offers the results as nmol/mg protein, percentage mass of total mass, nmol/dish or nmol/vol. The other formats are according mg/dl, percentage submass of total mass/dl, nmol/dl. An example for each report format is given in Fig. 11.

DISCUSSION

Automatic computation of data from an HPTLC scanner requires the calculation of an appropriate regression equation relating the integrated fluorescence or absorption signal to the mass of material. In the BASIC program described here a linear regression between the log of the integrated signal and the log of the mass is used. This technique gives the best fit for most of the HPTLC calibration curves [3, 4]. As with any computed regression equation, it is not important that values for unknowns are calculated by interpolation of the curve and that any data which lie beyond the range of the appropriate calibration curves are detected and can then be rejected. As is shown in Fig. 8, the calculated correlation coefficient (r) may be used as an indicator of the quality of the calibration curve. Thus the user has the opportunity to reject curves of poor quality where, for example, r is less than 0.96. The program allows the use of an internal standard so that the analysis is independent of the exact volume of sample applied to the plate.

The use of a BASIC programmable computing integrator with a log/log linear curve fitting enables the large quantities of data to be reduced with a high degree of precision and accuracy and reported in an acceptable form, in vastly improved turn-around time.

NOTE TO READERS

The BASIC program is available from the authors free of charge when a 5.25 inch standard discette is sent, together with a prepaid envelope, to our laboratory.

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