THE USE OF A COMPUTER IN THE DETERMINATION BY GAS-LIQUID CHROMATOGRAPHY OF THE CONCENTRATION AND IDENTIFICATION OF INDIVIDUAL FATTY ACIDS PRESENT AS FREE FATTY ACIDS, TRIGLYCERIDES AND CHOLESTERYL ESTERS

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(Received February 14th, 1967)

The analysis of fatty acids by gas-liquid chromatography (GLC) requires many repetitive calculations which could be readily carried out by a computer. Such calculations assist in the identification of the fatty acids and allow them to be quantitatively determined. Tentative identification of the fatty and methyl esters is made from the relative apparent retention times or from the carbon numbers. The calculation of the proportion of fatty acids from the relative areas of the peaks on the chromatogram can be extended to determine the absolute concentration of fatty acids by the use of an internal standard. The fatty acid chosen as an internal standard, heptadecanoic acid, occurs only in low concentrations in avian and mammalian plasma. The internal standard is added to the sample before analysis in the form of the free acid, triglyceride and cholesteryl ester prior to the separation of the various lipids present in plasma by thin-layer chromatography (TLC). Since the proportion of material lost in subsequent operations is similar for the sample and for the internal standard, the ratio of the concentration of the standard and sample lipids remains unchanged. The concentrations of the plasma free fatty acid (FFA), triglyceride and cholesteryl ester are then obtained from the relative amounts of methyl heptadecanoate present in the methyl esters of the fatty acids of each isolated fraction, measured by GLC. The complete computer programme used to calculate the results has been written in general terms to give it the widest possible use in the calculation of results from the GLC of fatty acids.

MATERIALS AND METHODS

Extraction of plasma lipid

Plasma was extracted by the method of FOLCH, LEES AND SLOANE STANLEY[1], using 0.1 M phosphate buffer, pH 6.0 instead of water. The internal standard substances, *i.e.* heptadecanoic acid, glyceryl triheptadecanoate and cholesteryl heptadecanoate, were added to the chloroform-methanol extract in the proportions 0.05, 0.2 and 0.5 mg/ml plasma, respectively. The weights of the various standards were sometimes varied if the concentrations of the lipids expected were vastly different from the average. The heptadecanoic acid was obtained from Eastman Organic Chemicals, Distillation Products Industries, Rochester 3, N.Y., U.S.A. and the glyceryl triheptadecanoate and cholesteryl heptadecanoate were synthesised via the

acyl chloride and purified by the TLC procedure described below. The Folch extract was taken to dryness under nitrogen at 30–40° and dissolved in the minimum volume of chloroform–methanol (2:1, v/v) before application to the TLC plate.

The solvent used for the extraction of lipids contained the antioxidant 2,6-di-*tert.*-butyl-*p*-cresol (butylated hydroxytoluene or BHT) at a concentration of 0.005 %[2]. It has been shown that this antioxidant interferes with certain lipid analyses[3]. Under the conditions used it has a slightly lower R_F than cholesteryl ester and is incompletely resolved from it during TLC. It has a carbon number of 13.85 and 14.20 during GLC on polyethylene glycol adipate (PEGA) at 167° and on Apiezon L at 180°, respectively. When the fatty acids are methylated by the methanol–sulphuric acid method, the BHT present remains unchanged and can be identified during subsequent GLC.

Thin-layer chromatography

The thin layers (400 μ) of Silica Gel G (E. Merck, Darmstadt, Germany) were prepared on 20 × 20 cm glass plates and activated for 40 min at 105° before use. A marker solution containing approximately 50 μg each of cholesteryl oleate, cholesterol, tripalmitin, 1,3-dipalmitin, monopalmitin, palmitic acid and lecithin purified by TLC before use was applied to two outside lanes, each of 3 cm. The plasma extract was applied quantitatively to the centre lane. The plates were then developed in tanks fitted with ground glass lids previously saturated with the solvent (diethyl ether–hexane–acetic acid, 30:70:2, v/v/v). When the solvent had reached the top of the plate, which took approximately 40 min, the plate was removed and air-dried for 10 to 15 min. The plate was then sprayed with an aqueous solution (0.1 % w/v) of Ultraphor (Badische Anilin und Soda Fabrik, A.G., Ludwigshafen-am-Rhein, Germany), dried at 40° and examined under U.V. light. The presence of Ultraphor at this concentration has no adverse effect on subsequent operations.

The lipids were separated as shown in Fig. 1. The separation of the partial glycerides was poor but these materials occur only in very small amounts in plasma. Three fractions were considered, *viz.* (1) FFA, (2) triglyceride, and (3) cholesteryl ester (containing some BHT). The fractions were transferred to 5 cm sintered glass funnels, porosity 3, fitted with B19 glass cones. The funnels were seated on 25 ml glass stoppered test tubes with side arms and the silicic acid packed down under reduced pressure. The lipids were eluted with 15 ml of diethyl ether–petroleum ether (b.p. 40–60°)–formic acid (50:50:1, v/v/v). Methyl esters of the fatty acids were prepared by the methanol–sulphuric acid method.

Methylation of fatty acids by the methanol–sulphuric acid method

The sample to be methylated was transferred to a 15 cm B19 test tube and the solvent removed by evaporation under nitrogen. Redistilled methanol–redistilled benzene–conc. sulphuric acid (20:10:1, v/v/v), 2 ml, was added and the mixture heated under reflux for 90 min. A sand bath is used for this operation since the use of a water bath results in condensed steam running down inside the condensers. After cooling to room temperature, sodium carbonate, 10 % w/v, 5 ml, and petroleum ether, b.p. 40–60°, 5 ml, were added, the tube shaken for one minute and the lower phase removed by aspiration. The petroleum ether phase was washed twice with sodium carbonate (5 ml) and once with water (5 ml), the petroleum ether transferred to a fresh tube and dried down with anhydrous sodium sulphate. There is no evidence of

preferential adsorption of the shorter chain fatty acid methyl ester, methyl myristate, compared with methyl stearate or methyl oleate.

Gas–liquid chromatography

The proportion of the individual long chain fatty acid methyl esters was determined using a Pye Model 104 gas chromatograph. The instrument was fitted with a flame-ionisation detector and a coiled glass column (120 cm long, 4 mm I.D.).

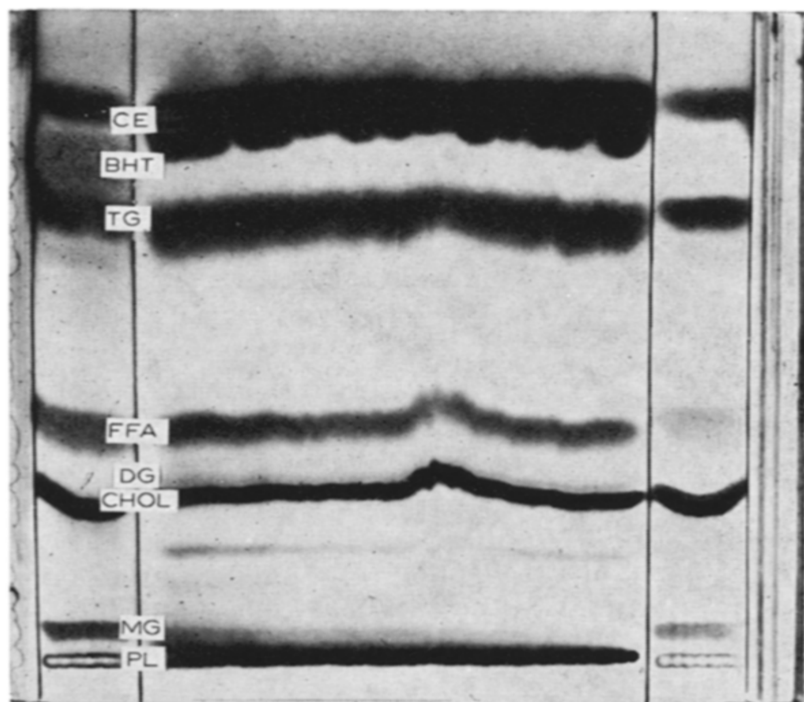

Fig. 1. A chromatoplate illustrating lipid class separation using a TLC plate of silicic acid 400 μ thick with the developing solvent diethyl ether–hexane–acetic acid (30:70:2, v/v/v). Detection by charring after spraying with a saturated solution of $K_2Cr_2O_7$ in 80 % (w/w) H_2SO_4. Phospholipids (PL) remained at the origin; monoglycerides (MG) moved just above the origin; cholesterol (CHOL) and diglyceride (DG); the free fatty acids (FFA) were removed as fraction 1; the triglycerides (TG) were removed as fraction 2; butylated hydroxytoluene (BHT); and cholesteryl ester (CE) were removed as fraction 3. A mixture of standards was run in lanes 1 and 3. BHT was included in lane 1. A sample from goat plasma containing the C-17 markers was run in lane 2.

The column support material used was kieselguhr (Chromosorb W, 60–80 mesh). For most purposes, a polar-stationary phase, 10 % polyethylene glycol adipate, was used at 167°. When BHT was present, *e.g.* in the cholesteryl ester fraction, a non-polar stationary phase, 10 % Apiezon L, was used at 180°. The areas of the peaks on the chromatograms were calculated by "triangulation". Tangents were drawn to the frontal and distal slopes of the peaks at the points of inflection and the base line constructed. The area equivalent to twice the actual area was calculated from the product of the distance between the intercepts of the tangents on the base line and the perpendicular distance between the intercepts of the tangents and the base line[4]. The area thus calculated was not halved since all the areas calculated were twice the actual area.

The distance corresponding to the uncorrected retention time was measured

from the time of the injection of the sample to the half base width determined by triangulation[5].

Identification of peaks

Relative retention times can be used to identify various fatty acids as their methyl esters. However the relative time of a given fatty acid methyl ester is not constant and depends on many factors, *e.g.* the dead spaces between the column packing and the site of injection and between the packing and the detector, the temperature, the flow rate, etc. This variability can be reduced by the use of relative apparent retention times[5] or "carbon numbers"[6] otherwise known as "equivalent chain length" (or ECL) values[7]. Both relative apparent retention times and carbon numbers depend on the linear relationship between the carbon number and the logarithm of the retention time of the straight chain saturated acids. A linear relationship also exists between the carbon number and the logarithm of the retention time of unsaturated fatty acids with the same number of double bonds and the same position of the latter relative to the terminal methyl group[5]. The number of carbons after the centre of the ultimate double bond from the carboxyl end is referred to as the "end carbon chain". This means that the difference between the "carbon number" of fatty acids within such a series and the carbon number of their straight chain analogues is a constant.

It is not generally desirable to publish retention data by carbon number without the original adjusted retention times or relative retention times, since this usually lends to a compounding of errors in subsequent calculations. However the use of carbon number systems enables tentative identification of fatty acid methyl esters. Hence the data of ACKMAN[5] are published in Table I together with carbon numbers calculated by means of the computer programme.

Fitting of relationship between retention time and carbon number

The assumed linear relationship between the log of apparent retention time and carbon number may be written as follows:

$$\log_e (t + k) = \alpha + \beta x$$

where t = observed retention time, x = carbon number, and α, β and k are constants.

To fit this linear relationship to a set of points, one can determine those values of α, β and k which minimise the residual sum of squares (RSS) of a regression of $\log_e (t + k)$ on x, *i.e.* determine the Least Squares estimates.

For a given value of k, the minimum RSS is

$$\text{RSS}^* = C_{yy} - C^2_{xy}/C_{xx} \qquad (1)$$

where, with the usual notation (and writing y for $\log_e (t + k)$):

$$C_{xx} = \sum_1^p x^2 - \left(\sum_1^p x\right)^2 / p \qquad (2)$$

TABLE I

STRUCTURES OF VARIOUS FATTY ACIDS WITH RELATIVE APPARENT RETENTION TIMES AND CARBON NUMBERS[a]

Group	*Fatty acid*[b]		*End carbon chain*	*Relative apparent retention times*	*Carbon number*
S	16:0	—	—	0.550	16
	18:0	—	—	1.00	18
	20:0	—	—	1.82	20
	22:0	—	—	3.30	22
I	18:1	9	9	1.12	18.379
	20:1	11	9	2.02	20.354
	22:1	13	9	3.68	22.362
II	16:2	6,9	7	0.740	16.992
	18:2	8,11	7	1.33	18.955
III	16:2	9,12	4	0.810	17.294
	18:2	11,14	4	1.45	19.244
IV	16:3	7,10,13	3	0.950	17.828
	18:3	9,12,15	3	1.72	19.815
V	18:4	6,9,12,15	3	1.97	20.270
	20:4	8,11,14,17	3	3.51	22.203
VI	18:2	6,9	9	1.29	18.852
	20:2	8,11	9	2.32	20.817
VII	18:2	9,12	6	1.34	18.980
	20:2	11,14	6	2.45	21.000
	22:2	13,16	6	4.38	22.945
VIII	18:3	6,9,12	6	1.54	19.445
	20:3	8,11,14	6	2.76	21.399
IX	20:4	5,8,11,14	6	3.04	21.722
	22:4	7,10,13,16	6	5.50	23.707
X	20:5	5,8,11,14,17	3	3.85	22.513
	22:5	7,10,13,16,19	3	7.00	24.514
XI	16:1	7	9	0.625	16.426
	16:1	9	7	0.634	16.474
	16:2	7,10	6	0.745	17.014
	16:3	4,7,10	6	0.86	17.495
	16:3	6,9,12	4	0.904	17.662
	16:4	4,7,10,13	3	1.08	18.258
	16:4	6,9,12,15	1	1.11	18.349
	18:2	10,13	5	1.37	19.054
	18:?	?	?	2.18	20.609
	22:2	5,13	7	3.90	22.556
	22:5	4,7,10,13,16	6	6.09	24.048
	22:5	?	4–5	6.60	24.317
	22:6	4,7,10,13,16,19	3	7.75	24.855

[a] Table derived from ACKMAN[5] with carbon numbers calculated for fatty acids other than the saturated acids by means of the computer.
[b] Notation after FARQUHAR *et al.*[8]

$$C_{yy} = \sum_1^p y^2 - \left(\sum_1^p y\right)^2 / p \tag{3}$$

and

$$C_{xy} = \sum_1^p xy - \left(\sum_1^p x\right)\left(\sum_1^p y\right) / p \tag{4}$$

(p = number of points).
The estimates of β and α corresponding to this minimum RSS are:

$$\hat{\beta} = C_{xy}/C_{xx} \tag{5}$$

and

$$\hat{\alpha} = \left(\sum_1^p y - \hat{\beta} \sum_1^p x\right) / p \tag{6}$$

Hence, to fit the required relationship, it is necessary to minimise expression (1) with respect to k, and to use the value $k = \hat{k}$ thus obtained, to estimate α and β using the above equations. $\hat{k}$ must be obtained using an iterative technique, and the method used in the computer programme is as follows:

Expression (1) is evaluated for two "initial" values, k_1 and k_2, of k. The poorer value of k—that is, the one which corresponds to the larger value of expression (1)—is *replaced* by the "reflection" of that value in the other k. Expression (1) is then evaluated again for the k which has been altered, and the comparison of k_1 and k_2 is repeated.

This process is carried out repetitively, but with the proviso that whenever the same k is to be altered twice in succession, half-reflection, not full-reflection, is used.

The iterations are continued, with a maximum of 200 iterations (in the programme), until the minimum is located with sufficient accuracy—namely, until

$$|k_1 - k_2| \leqslant \varepsilon K$$

and

$$|\text{RSS*}_{k=k_1} - \text{RSS*}_{k=k_2}| \leqslant \varepsilon R$$

where

$$K = \text{Max}\,(|k_1|, |k_2|, 0.1)$$

$$R = \text{Max}\,(|\text{RSS*}_{k=k_1}|, |\text{RSS*}_{k=k_2}|, 0.0001)$$

The estimate of k is then $\hat{k} = \frac{1}{2}(k_1 + k_2)$. ($\varepsilon$ is some small value, taken as 0.00001 in the computer programme. The starting values for k are $k_1 = 0$ and $k_2 = 1/10 \min(t)$).

Having determined $\hat{k}$, $\hat{\alpha}$ and $\hat{\beta}$ may be computed from the expressions (2) to (6) above. The computer programme has been written to print out these three parameters and, in addition, the standard deviation ($= \sqrt{(RSS^*_{k=\hat{k}}/(p-2))}$) about the fitted regression line.

For the p points (corresponding to the saturated straight-chain compounds), the computer prints the observed, and fitted, log $(t + \hat{k})$ (for comparison); and for the remaining compounds, estimated carbon numbers, computed using the fitted relationship, are printed.

Apparent retention times (*i.e.* $t + \hat{k}$) are also printed for all acids relative to each named acid.

The proportion of individual fatty acids

The area of the chromatogram peaks was determined by triangulation as described earlier. Other methods could have been used to calculate the area of the chromatogram peaks for input into the computer, *e.g.* the product of the height of the peak and the width at half height. The computer programme can use data to represent the area of the individual peaks either as two numbers when multiplied together give a value proportional to the area or as the area calculated by other means (*e.g.* using an integrator) which could be multiplied by unity to give a value proportional to the area.

When flame-ionisation detectors are used to measure the amount of fatty acid methyl esters from C_{10} to C_{20} the response is proportional to the weight of methyl ester within the limits of accuracy of the method. Since the response of the detector to the carbonyl carbon is not as great as that of the other carbons, correction is necessary with shorter chain acids[9]. Correction factors for methyl esters of the fatty acids of given carbon number, based on the results of ACKMAN AND SIPOS[9] are as follows: C-8 and above, 1.00; C-6, 1.156; C-4, 1.435; C-3, 1.765; C-2, 2.354. These factors are not constants and should be determined for each detector used since they vary with the geometry of the detector. Correction factors are also necessary to estimate the amount of fatty acids of all chain lengths with a β-ionisation detector[10]. Provision has been made in the program for the use of such weighting factors.

Since the response of the detector is proportional to the weight of a derivative and not the free acid, provision has been made in the computer programme for the calculation of the molecular weight of the derivative. The molecular weight of the free acid is added to the difference between the molecular weight of the free acid and the derivative. This difference is designated as the "methyl group molecular weight" and can have a positive, zero or negative value. The value will be zero when fatty acids are chromatographed as the free acids and 14.03 when chromatographed as the methyl esters.

The molecular weight of a fatty acid is calculated from (1) the number of carbon atoms, (2) the number of double bonds, and (3) the number of substituents of a given molecular weight. The molecular weight of the substituent is expressed as the "molecular weight change for the substituent", which is the difference between the molecular weight of an unsubstituted fatty acid and a fatty acid with one substituent group, *e.g.* the molecular weight change for the keto group is 13.98. This parameter can also be positive, zero or negative. The proportion of free acids is then calculated firstly in moles by dividing the area of each peak by the molecular weight of the methyl ester

and expressing each value as a percentage of the total; and secondly by weight, by multiplying the first calculated values by the molecular weight of the free acids and expressing each value as a percentage of the total.

The absolute amount of individual fatty acids

The absolute amount of each fatty acid and their total is then calculated. The results are expressed in moles and the weight of fatty acid per ml of plasma. The area of the fatty acid internal standard peak and the amount of internal standard fatty acid added to the sample in μg fatty acid/ml of plasma is used for this calculation. The amount of marker is not taken into account in the proportion or total amounts of the fatty acids. Since the calculation of the proportion of the fatty acids is independent of the internal standard, its use is optional when the method is used to determine the relative proportions of fatty acids in a sample.

The proportion and weight of glycerides in esters containing the fatty acids

Since fatty acids occur naturally not only free but as esters such as glyceryl and cholesteryl esters, the computer has been programmed to calculate the proportion of fatty acid esters on a weight basis and the individual and total weight of fatty acid esters when the internal standard is used. The "molecular weight of the esterifying alcohol" is expressed in terms of the weight of alcohol associated with each mole of fatty acid, *e.g.* monoglyceride, 74.01; diglyceride, 28.00; triglyceride, 12.70; and cholesteryl ester, 376.28.

The computer programme

The programme has been written in Fortran IV suitable for an IBM 360 digital computer. A description of the various operations is described in the preceding sections and the program is presented in Table II.

Results obtained using the methods described: the identification and concentration of fatty acids present as free fatty acids and triglycerides in the plasma of fed goats

In this laboratory we are studying the metabolism of FFA and triglycerides in relation to milk lipid synthesis in collaboration with Dr. J. L. LINZELL of the A.R.C. Institute of Animal Physiology, Babraham, Cambridge. Results of work in which this method has been used have already been published[11] and further papers are in preparation for the *Biochemical Journal*. Examples of the results of the identification and concentration of fatty acids in FFA and triglycerides in arterial plasma of fed goats are shown in Table III. The values for the relative apparent retention times and carbon numbers are shown as the mean and standard deviation of the mean of the number of observations shown while individual typical results are shown for the concentration of FFA and triglyceride fatty acids. The values for the relative apparent retention times and carbon numbers are similar to those of ACKMAN (see Table I).

There is no change in the concentration of plasma FFA when blood passes through the mammary gland (Table III). However studies using ^{14}C-labelled fatty acids have shown that FFA are taken up by the gland. This uptake is accompanied by a concomitant release of FFA[11,12], some of which has been shown to come from the plasma triglyceride taken up by the gland[11]. The concentration of plasma triglyceride falls across the gland. This uptake of triglyceride has been shown to be confined to the chylomicron and low-density β-lipoprotein fractions of plasma[13].

TABLE II

THE COMPUTER PROGRAM LISTING FOR THE DETERMINATION BY GAS–LIQUID CHROMATOGRAPHY OF THE CONCENTRATION AND IDENTIFICATION OF INDIVIDUAL FATTY ACIDS

```
/DATA                                                                           02/01/67
C      PROGRAM CH0450                                                  4501   1
C      ANALYSIS OF GAS CHROMATOGRAPHY DATA                             4501   2
      DIMENSION TTLE(20),NMAL(3,2),XMWEA(3),WTFA(50),HT(50),WD(50),WT(504501   3
     1),XMWFA(50),NDBIS(50),INDN(6),PM(50),PW(50),T(6),WTAL(3),WTALF(3),4501   4
     2XX(3),YY(3)                                                      4501   5
      COMMON RT(50),INDX(50),NCAT(50),NX                               4501   6
C      NUMERICAL EQUIVALENTS OF CH.STRINGS OF LENGTH 4                 .4501   7
      IPL=1312868033                                                   4501   8
      IFT=-471603136                                                   4501   9
      IAC=-1044133436                                                  4501  10
      IWT=-421311680                                                   4501  11
      IFIN=-959851200                                                  4501  12
      INUM=-185208768                                                  4501  13
      IX=-415219648                                                    4501  14
      IN=-717209536                                                    4501  15
      IM=-733986752                                                    4501  16
      IBLNK=1077952576                                                 4501  17
      READ(5,101)ITST                                                  4502   1
  101 FORMAT(A3)                                                       4502   2
   57 IF(ITST-IFIN)1,2,1                                               4502   3
    2 STOP                                                             4502   4
    1 IF(ITST-INUM)4,3,4                                               4502   5
    4 WRITE(6,102)ITST,IFIN,INUM                                       4502   6
  102 FORMAT(///10X,14HPROGRAM CH0450,5X,31HA HEADER/TRAILER CARD CONTAI4502   7
     1NS ,A3,10H (AND NOT ,A3,4H OR ,A3,14H ) IN COLS 1-3)             4502   8
      STOP                                                             4502   9
    3 READ(5,103)(TTLE(I),I=1,20)                                      4502  10
  103 FORMAT(20A4)                                                     4502  11
      WRITE(6,104)(TTLE(I),I=1,20)                                     4502  12
  104 FORMAT(1H1,10X,42HANALYSIS OF GAS LIQUID CHROMATOGRAPHY DATA,10X,14502  13
     14HPROGRAM CH0450,//// ,10X,20A4)                                 4502  14
      READ(5,105)XMWS,XMGMW,AWM,((NMAL(I,J),J=1,2),XMWEA(I),I=1,3)     4502  15
  105 FORMAT(3F8.0,3(2A4,F8.0))                                        4502 .16
      SUMA=0.0                                                         4502  17
      SUMB=0.0                                                         4502  18
      NPT=0                                                            4503   1
      NX=0                                                             4503   2
      NN=0                                                             4503   3
      MARK=0                                                           4503   4
      RTMIN=100000.0                                                   4503   5
   24 NPT=NPT+1                                                        4503   6
      READ(5,110)ITST,NCAT(NPT),NDB,NISM,NSTAT,JX,N,M,RT(NPT),HT(NPT),WD4503   7
     1(NPT),WT(NPT)                                                    4503   8
  110 FORMAT(A3,I2,3I1,3A1,4F8.0)                                      4503   9
      IF(ITST-IFIN)10,11,10                                            4503  10
   10 IF(ITST-INUM)12,11,12                                            4503  11
   12 XMWFA(NPT)=12.011*NCAT(NPT)+2.016*(NCAT(NPT)-NDB)+31.999+        4503  12
     1NSTAT*XMWS                                                       4503 121
      NDBIS(NPT)=   10*(10*NDB+NISM)+NSTAT                             4503  13
      IF(NN-5)333,13,13                                                4503  14
  333 IF(N-IN)13,14,13                                                 4503  15
   14 NN=NN+1                                                          4503  16
      INDN(NN)=NPT                                                     4503  17
   13 IF(JX-IX)15,16,15                                                4503  18
   16 NX=NX+1                                                          4503  19
      INDX(NX)=NPT                                                     4503  20
   15 PM(NPT)=HT(NPT)*WD(NPT)/(XMWFA(NPT)+XMGMW)                       4504   1
      IF(WT(NPT))17,18,17                                              4504   2
   17 PM(NPT)=PM(NPT)*WT(NPT)                                          4504   3
   18 PW(NPT)=PM(NPT)*XMWFA(NPT)                                       4504   4
      IF(M-IM)19,20,19                                                 4504   5
   20 MARK=NPT                                                         4504   6
      GOTO 21                                                          4504   7
   19 SUMA=SUMA+PM(NPT)                                                4504   8
      SUMB=SUMB+PW(NPT)                                                4504   9
   21 IF(RTMIN-ABS(RT(NPT)))24,24,23                                   4504  10
   23 RTMIN=ABS(RT(NPT))                                               4504  11
      GOTO 24                                                          4504  12
C       END OF CODING ON DATA INPUT                                    4504  13
   11 NPT=NPT-1                                                        4504  14.
      IF(NX-2)25,881,881                                               4504 144
  881 IF(RTMIN)801,801,802                                             4504 145
  801 WRITE(6,803)RTMIN                                                4504 146
  803 FORMAT(//10X,'NEG.OR ZERO RET.TIME - RTMIN= ',E11.4//)           4504 147
      GO TO 57                                                         4504 148
  802 IF(NX-3)95,26,26                                                 4504 149
   95 XKX=0.0                                                          4504 153
      SD=0.0                                                           4504 155
      CALL FNX(XKX,RSS,0,A,B)                                          4504 156
      GO TO 97                                                         4504 157
   26 XKX=0.0                                                          4504  16
      CALL FNMIN(XKX,0.1*RTMIN,0.00001,200,NCI)                        4504  17
      IF(200-NCI)27,27,28                                              4504  18
   27 WRITE(6,111)NCI,XKX                                              4504  19
  111 FORMAT(////10X,5HAFTER,I5,58H ITERATIONS THE MIN.RSS HAS NOT BEEN 4504  20
     1LOCATED -  PRESENT K=,E11.4)                                     4505   1
   28 CALL FNX(XKX,RSS,0,A,B)                                          4505   2
      IF(RSS)201,202,202                                               4505   3
```

(continued on p. 71)

TABLE II (*continued*)

```
201 SD=0.0                                                               4505  31
    IF(RSS+0.0001*(NX-2))210,97,97                                       4505  33
210 CALL PDUMP(RSS,RSS,5)                                                4505  35
    GO TO 97                                                             4505  36
202 SD=SQRT(RSS/(NX-2))                                                  4505  38
 97 WRITE(6,112)A,B,XKX,SD                                               4505  4
112 FORMAT(////10X,14HLOG(T+K)=A+B*C,//10X,22HWHERE T=RETENTION TIME,/4505  5
   116X,15HC=CARBON NUMBER,//10X,13HAND ESTIMATED,6X,2HA=,F9.4,/29X,2H4505  6
   2B=,F9.4,/29X,2HK=,F9.4,//14X,17HS.D.(ABOUT LINE)=,F9.4)            4505  7
    WRITE(6,113)                                                         4505  8
113 FORMAT(/10X,20H(LOGS ARE TO BASE E))                                 4505  9
    WRITE(6,118)                                                         4505 10
    DO 32 I=1,NPT                                                        4505 11
    IF(RT(I))203,32,203                                                  4505 115
203 CRT=RT(I)+XKX                                                        4505 12
    TLOG=ALOG(CRT)                                                       4505 13
    FTLG=A+B*NCAT(I)                                                     4505 14
    ESTC=(TLOG-A)/B                                                      4505 15
    M=IBLNK                                                              4505 16
    IF(I-MARK)30,31,30                                                   4505 17
 31 M=IM                                                                 4505 18
 30 JX=IX                                                                4505 19
    DO 34 J=1,NX                                                         4506  1
    IF(I-INDX(J))34,33,34                                                4506  2
 34 CONTINUE                                                             4506  3
    JX=IBLNK                                                             4506  4
 33 IF(NN)35,36,35                                                       4506  5
 35 DO 37 J=1,NN                                                         4506  6
    JJ=INDN(J)                                                           4506  7
 37 T(J)=CRT/(RT(JJ)+XKX)                                                4506  8
    IF(JX-IX)38,39,38                                                    4506  9
 39 WRITE(6,120)NCAT(I),NDBIS(I),M,JX,RT(I),CRT,TLOG,FTLG,(T(J),J=1,NN4506 10
   1)                                                                    4506 11
    GO TO 32                                                             4506 12
 38 WRITE(6,129)NCAT(I),NDBIS(I),M,JX,RT(I),CRT,TLOG,ESTC,(T(J),J=1,NN4506 13
   1)                                                                    4506 14
    GO TO 32                                                             4506 15
 36 IF(JX-IX)138,139,138                                                 4506 16
139 WRITE(6,120)NCAT(I),NDBIS(I),M,JX,RT(I),CRT,TLOG,FTLG                4506 17
    GO TO 32                                                             4506 18
138 WRITE(6,129)NCAT(I),NDBIS(I),M,JX,RT(I),CRT,TLOG,ESTC                4506 19
 32 CONTINUE                                                             4506 20
 25 IF(SUMA)57,57,804                                                    4507  1
804 WRITE(6,115)                                                         4507  15
    SWM=0.0                                                              4507  2
    SWW=0.0                                                              4507  3
    DO 50 I=1,NPT                                                        4507  4
    XMWME=XMWFA(I)+XMGMW                                                 4507  5
    PMP=100.0*PM(I)/SUMA                                                 4507  6
    PWP=100.0*PW(I)/SUMB                                                 4507  7
    IF(MARK)52,51,52                                                     4507  8
 52 IF(AWM)53,51,53                                                      4507  9
 53 WM=PM(I)*AWM/(PM(MARK)*XMWFA(MARK))                                  4507 10
    WW=PW(I)*AWM/PW(MARK)                                                4507 11
    IF(I-MARK)204,55,204                                                 4507 115
204 SWM=SWM+WM                                                           4507 12
    SWW=SWW+WW                                                           4507 13
    M=IBLNK                                                              4508  1
    GO TO 54                                                             4508  2
 55 M=IM                                                                 4508  3
 54 IF(WT(I))154,155,154                                                 4508  4
155 WT(I)=1.0                                                            4508  5
154 WRITE(6,122)NCAT(I),NDBIS(I),M,HT(I),WD(I),WT(I),XMWME,XMWFA(I),     4508  6
   1PMP,M,WM,M,PWP,M,WW,M                                                4508  7
    WTFA(I)=WW                                                           4508  8
    GOTO 50                                                              4508  9
C    NO MARKER                                                            4508 10
 51 WRITE(6,121)NCAT(I),NDBIS(I),HT(I),WD(I),WT(I),XMWME,XMWFA(I),PMP,4508 11
   1PWP                                                                  4508 12
 50 CONTINUE                                                             4508 13
    IF(MARK)56,57,56                                                     4508 14
 56 WRITE(6,192)SWM,SWW                                                  4508 15
.192 FORMAT(/86X,F7.4,17X,F7.2)                                          4508 16
    IF(XMWEA(1))60,57,60                                                 4509  1
C    PROCESSING FOR ESTERIFYING ALCOHOLS OPTION                           4509  2
 60 NAL=0                                                                4509  3
 63 NAL=NAL+1                                                            4509  35
    IF(XMWEA(NAL))61,805,61                                              4509  4
 61 WTAL(NAL)=0.0                                                        4509  5
    WTALF(NAL)=0.0                                                       4509  6
    IF(NAL-3)63,62,62                                                    4509  8
805 NAL=NAL-1                                                            4509  85
 62 WRITE(6,124)(IBLNK,IBLNK,IBLNK,I=1,NAL),(IWT,(NMAL(K,J),J=1,2),K=14509  9
   .1,NAL)                                                               4509 10
    WRITE(6,125)(IWT,(NMAL(K,J),J=1,2),K=1,NAL),(IPL,IFT,IAC,I=1,NAL) 4509 11
    DO 64 I=1,NPT                                                        4509 12
    IF(MARK-I)65,64,65                                                   4509 13
 65 DO 66 J=1,NAL                                                        4509 14
    XX(J)=WTFA(I)*XMWEA(J)/XMWFA(I)                                      4509 15
    YY(J)=XX(J)+WTFA(I)                                                  4509 16
```

(*continued on p. 72*)

TABLE II (*continued*)

```
      WTAL(J)=WTAL(J)+XX(J)                                              4509 17
   66 WTALF(J)=WTALF(J)+YY(J)                                            4509 18
      WRITE(6,123)NCAT(I),NDBIS(I),WTFA(I),(XX(J),J=1,NAL),(YY(J),J=1,   4509 19
     1NAL)                                                               4510  1
   64 CONTINUE                                                           4510  2
      WRITE(6,126)SWW,(WTAL(J),J=1,NAL),(WTALF(J),J=1,NAL)               4510  3
      GO TO 57                                                           4510  4
  118 FORMAT(///15X,8HOBSERVED,3X,9HCORRECTED,/11X,2(3X,9HRETENTION),/9X4511  1
     1,2(8X,4HTIME),6X,8HOBSERVED,5X,6HFITTED,4X,9HESTIMATED,5X,23HRETEN4511  2
     2TION TIME RELATIVE,/5X,4HACID,8X,3H(T),8X,5H(T+K),5X,9HLOG (T+K),34511  3
     3X,9HLOG (T+K),3X,9HCARBON NO,5X,20HTO NAMED ACIDS .....,//)        4511  4
  115 FORMAT(///50X,9HMOLECULAR,15X,10HPERCENTAGE,14X,10HPERCENTAGE,/50X4511  5
     1,9HWEIGHT OF,16X,8HOF FATTY,3X,9HWEIGHT OF,4X,8HOF FATTY,3X,9HWEIG4511  6
     2HT OF,/50X,10HFATTY ACID,2X,9HMOLECULAR,4X,7HACID ON,4X,10HFATTY A4511  7
     3CID,3X,7HACID ON,4X,10HFATTY ACID,/17X,4HPEAK,8X,4HPEAK,5X,9HWEIGH4511  8
     4TING,5X,6HMETHYL,4X,9HWEIGHT OF,5X,5HMOLAR,6X,8HON MOLAR,5X,6HWEIG4511  9
     5HT,4X,9HON WEIGHT,/5X,4HACID,7X,6HHEIGHT,7X,5HWIDTH,4X,6HFACTOR,8X4511 10
     6,5HESTER,5X,9HFREE ACID,5X,5HBASIS,3(7X,5HBASIS),//)               4511 11
  120 FORMAT(3X,I2,1H.,I3,1X,2A1,2X,F7.2,5X,F7.2,7X,F7.3,5X,F7.3,15X,5(24511 12
     1X,F7.3))                                                           4511 13
  129 FORMAT(3X,I2,1H.,I3,1X,2A1,2X,F7.2,5X,F7.2,7X,F7.3,17X,F7.3,3X,5(24511 14
     1X,F7.3))                                                           4511 15
  122 FORMAT(3X,I2,1H.,I3,1X,A1,3X,F7.2,5X,F7.2,4(5X,F7.2),1X,A1,3X,F7.44511 16
     1,1X,A1,2(3X,F7.2,1X,A1))                                           4511 17
  121 FORMAT(3X,I2,1H.,I3,6(5X,F7.2),17X,F7.2)                           4512  1
  123 FORMAT(3X,I2,'.',I3,2X,F9.4,6(5X,F9.4))                            4512  2
  124 FORMAT(////,14X,6HWEIGHT,2X,6(2X,3A4))                             4512  3
  125 FORMAT(4X,4HACID,4X,10HFATTY ACID,6(2X,3A4))                       4512  4
  126 FORMAT(/,6X,7(5X,F9.4))                                            4512  5
      END                                                                4512  6
      SUBROUTINE FNX(XK,RSS,NCD,A,B)                                     4513  1
      COMMON RT(50),INDX(50),NCAT(50),NX                                 4513  2
      SX=0.0                                                             4513  3
      CXX=0.0                                                            4513  4
      SY=0.0                                                             4513  5
      CYY=0.0                                                            4513  6
      CXY=0.0                                                            4513  7
      DO 1 I=1,NX                                                        4513  8
      IX=INDX(I)                                                         4513  9
      Y=ALOG(RT(IX)+XK)                                                  4513 10
      SX=SX+NCAT(IX)                                                     4513 11
    1 SY=SY+Y                                                            4513 12
      SX=SX/NX                                                           4513 13
      SY=SY/NX                                                           4513 14
      DO 4 I=1,NX                                                        4513 15
      IX=INDX(I)                                                         4514  1
      Y=ALOG(RT(IX)+XK)-SY                                               4514 15
      X=NCAT(IX)-SX                                                      4514  2
      CXX=CXX+X*X                                                        4514 25
      CYY=CYY+Y*Y                                                        4514  3
    4 CXY=CXY+X*Y                                                        4514 35
      RSS=CYY-CXY*CXY/CXX                                                4514  4
      IF(NCD)2,3,2                                                       4514  5
    3 B=CXY/CXX                                                          4514  6
      A=SY-B*SX                                                          4514  7
    2 RETURN                                                             4514  8
      END                                                                4514  9
      SUBROUTINE FNMIN(C,D,EPS,MAXNI,NCI)                                FMIN1 10
      DIMENSION X(2),Y(2)                                                FMIN1 15
      COMMON RT(50),INDX(50),NCAT(50),NX                                 FMIN1 16
      INTEGER SW                                                         FMIN1 20
      X(1)=C                                                             FMIN1 25
      X(2)=D                                                             FMIN1 26
      NCI=0                                                              FMIN1 30
      SW = 0                                                             FMIN1 40
      ILAST = 0                                                          FMIN1 50
      CALL FNX(X(1),Y(1),1,A,B)                                          FMIN1 60
      CALL FNX(X(2),Y(2),1,A,B)                                          FMIN1 70
      IP=1                                                               FMIN1 80
  120 IF(Y(1)-Y(2))5,5,6                                                 FMIN1 90
    5 IP = 2                                                             FMIN1100
    6 IF (ILAST - IP) 10,20,10                                           FMIN1110
   10 ILAST = IP                                                         FMIN1120
      SW = 0                                                             FMIN1130
      GO TO 30                                                           FMIN1140
   20 SW = 1                                                             FMIN1150
   30 IF (SW) 40,40,50                                                   FMIN1160
   40 F = 1.0                                                            FMIN1170
      GO TO 60                                                           FMIN1180
   50 F = 0.5                                                            FMIN1190
   60 IF (IP - 1) 70,70,80                                               FMIN1200
   70 IOPP = 2                                                           FMIN1210
      GO TO 90                                                           FMIN2 10
   80 IOPP = 1                                                           FMIN2 20
   90 X(IP)=X(IOPP)+(X(IOPP)-X(IP))*F                                    FMIN2 30
      CALL FNX(X(IP),Y(IP),1,A,B)                                        FMIN2 4
      NCI = NCI + 1                                                      FMIN2 50
      WX=ABS(X(IP))                                                      FMIN2 52
      WX=ABS(Y(IP))                                                      FMIN2 55
      IF(WX-0.1)201,202,202                                              FMIN2 57
```

(*continued on p. 73*)

TABLE II (*continued*)

```
201 WX=0.1                                              FMIN2 60
202 IF(WY-0.0001)203,204,204                            FMIN2 62
203 WY=0.0001                                           FMIN2 64
204 IF(ABS(Y(1)-Y(2))-EPS*WY)101,101,110                FMIN2 66
101 IF(ABS(X(1)-X(2))-EPS*WX)100,100,120                FMIN2 68
110 IF (MAXNI - NCI) 100,100,120                        FMIN2 70
100 C=0.5*(X(1)+X(2))                                   FMIN2 80
    D=X(2)                                              FMIN2 81
    RETURN                                              FMIN2 85
    END                                                 FMIN2 90
```

DISCUSSION

Since the services of a computer are readily available to many laboratories, their use in processing the output from analytical instruments is increasing. The advantages compared with manual calculation of the data are the increased speed and accuracy which usually results in lowering the unit cost of the work output. Before a computer is used for a certain task a program has to be written and proven. This only becomes worthwhile if this initial cost and the unit cost of processing the data are less than the cost of calculating the results by hand using a desk calculator. The use of a computer to calculate the GLC data seemed to be warranted in our laboratory. The operations with the time involved for each sample for manual and computer processing of the data are set out below.

Operations common to both methods:

(1) Construction of triangles and measurement of peak base and height —30 min.

(2) Measurement of retention times —5 min.

(3) Recording of measurements —5 min. When the computer method is used the results are written on data forms which are given to the punch card operator.

Operations for manual calculation:

(1) Calculation of proportion and concentration of the individual and total fatty acids —30 min. If the results are expressed both in terms of weight and in moles these calculations may take 15 min longer.

(2) Calculation of relative apparent retention times and carbon numbers; graphically —45 min; algebraically —over 4 h. In practice, however, because of its tedious nature, this operation is carried out less frequently and less accurately than is desirable.

Operations for computer calculation:

(1) Punching of cards —5 min.

(2) Computer handling of data.

The cost of using the computer is difficult to assess. However, the unit cost of calculation is probably less than half that of the manual calculation of the results. The savings are greatest when carbon numbers and relative apparent retention times as well as the proportions and concentrations are calculated. Much greater accuracy is achieved in all the operations since manual calculation of the results is tedious.

We still have to spend a lot of time and effort measuring the areas by triangulation and the retention times of the elution peaks. These are also subject to considerable operator error. The time taken and the error involved could be reduced if we used

TABLE III

THE IDENTIFICATION AND CONCENTRATION OF FATTY ACIDS PRESENT AS FREE FATTY ACIDS AND TRIGLYCERIDES IN ARTERIAL AND MAMMARY VENOUS PLASMA OF FED GOATS

Fatty acid[a]	*Relative apparent retention time*[b]	*Carbon number*[b,c]	*Free fatty acid*				*Triglyceride*			
			Artery		*Mammary vein*		*Artery*		*Mammary vein*	
			Conc. (mequiv./l)	*Proportion (moles %)*	*Conc. (mequiv./l)*	*Proportion (moles %)*	*Conc. (mg/100 ml)*	*Proportion (moles %)*	*Conc. (mg/100 ml)*	*Proportion (moles %)*
12:0			0.0181	7.22	0.0221	8.52	0.081	0.38	—	—
14:0	0.513 ± 0.010(16)	14.016 ± 0.0922(16)	0.0123	4.89	0.0191	7.39	0.601	2.82	0.452	2.85
15:br	0.648 ± 0.008(15)	14.715 ± 0.0248(16)	0.0051	2.05	0.0057	2.18	0.447	2.10	0.296	1.86
15:0	0.715 ± 0.006(17)		0.0077	3.06	0.0074	2.86	0.455	2.14	0.356	2.24
16:br	0.863 ± 0.010(11)	15.550 ± 0.0470(11)	0.0015	0.59	0.0029	1.10	0.245	1.15	0.139	0.87
16:0	1.000		0.0545	21.77	0.0613	23.69	4.851	22.80	3.672	23.12
16:1	1.127 ± 0.008(11)	16.330 ± 0.0403(12)	0.0037	1.46	0.0017	0.67	0.633	2.98	0.326	2.05
16:2	1.201 ± 0.005(16)	16.519 ± 0.0330(16)	0.0073	2.91	0.0085	3.28	0.346	1.63	0.286	1.80
16:3	1.274 ± 0.008(10)	16.689 ± 0.0345(10)	0.0022	0.86	0.0023	0.89	0.403	1.89	0.300	1.89
18:0	1.985 ± 0.018(17)		0.0808	32.25	0.0823	31.79	7.232	33.98	5.761	36.27
18:1	2.211 ± 0.023(17)	18.306 ± 0.0480(17)	0.0405	16.17	0.0320	12.36	4.574	21.49	3.030	19.08
18:2	2.736 ± 0.035(16)	18.928 ± 0.0596(16)	0.0170	6.77	0.0136	5.26	1.413	6.64	1.265	7.96
Total[d]			0.2506		0.2589		22.28		16.62	

[a] Notation after FARQUHAR *et al.*[8].

[b] The relative apparent retention times and carbon numbers are shown with the standard deviation and the number of observations in brackets. The relative apparent retention time of heptadecanoic acid is 1.412 ± 0.008[16].

[c] Carbon numbers obtained using the computer. Regression obtained using esters of saturated, straight chain acids with 15, 16, 17 and 18 carbon atoms.

[d] Total weight of triglyceride also includes weight of glycerol moieties.

suitable electronic equipment to convert the necessary information contained in the gas–liquid chromatogram into digital information that could be processed by the computer. An integrator alone is not sufficient for this purpose since the retention time must also be measured and recorded. An appraisal of equipment which may be suitable for our purposes is at present under study. Equipment suitable for similar but more complex applications was recently described by KILBURN and his co-workers[14]. With the use of nine internal standards, the identification and concentration of over 500 compounds present in tobacco smoke could be determined. However, the cost and effort of using their techniques are prohibitive for our purposes.

The identification of fatty acids using their retention times is made easier by the computer calculation of relative apparent retention times and carbon numbers. Their use instead of relative retention times reduces the variability between determinations of parameters for individual fatty acid methyl esters making the identification of the fatty acids easier. However, it must be stressed again that it is not generally desirable to publish retention data by carbon number without the original adjusted retention times or relative retention times, since this can lead to a compounding of errors in subsequent calculations.

The absolute concentration of the fatty acids is obtained by the use of an internal standard, heptadecanoic acid. The concentrations of the fatty acids are obtained from the relative amounts of methyl heptadecanoate present in the methyl esters of the fatty acids of each isolated fraction, measured by GLC. Such a procedure was first described by TINOCO and co-workers[15] and has since been used by many workers[16,17]. The overall precision of the quantitative analysis cannot exceed that of the GLC of fatty acid methyl esters. Many of the potential errors which afflict alternative procedures, such as incomplete elution from silica gel, losses during manipulation etc., are overcome by the use of internal standards.

The use of this technique has been demonstrated in studies of the free fatty acids and triglycerides of goat arterial plasma. The apparent carbon numbers obtained in this laboratory were not substantially different to those obtained by ACKMAN (Table I).

ACKNOWLEDGEMENTS

The authors wish to thank Mrs. A. P. BEST for skilled technical assistance, Dr. E. F. ANNISON for helpful advice and discussion, and Dr. R. P. W. SCOTT for help in the preparation of the manuscript.

SUMMARY

A computer programme written in Fortran IV suitable for remote access to an IBM 360 digital computer is presented enabling the calculation, from the results of the gas–liquid chromatography of methyl esters of fatty acids, of the proportion and concentration of fatty acids using an internal standard technique. Identification of the fatty acid methyl esters is assisted by the calculation of the relative apparent retention times and carbon numbers. Thin-layer chromatography is used to separate the fatty acid containing lipids studied in plasma—the free fatty acids, triglycerides and cholesteryl esters. Some results obtained with the free fatty acids and triglycerides of arterial and mammary venous plasma from fed goats are presented and discussed.

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