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AUTOMATED QUANTITATIVE GAS—LIQUID CHROMATOGRAPHY OF INTACT LIPIDS

I. PREPARATION AND CALIBRATION OF THE COLUMN

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SUMMARY

A method for quantitative gas chromatographic determination of plasma lipids (free cholesterol, cholesteryl esters and triglycerides) in the low concentration range is described. This method permits a determination of not only the lipid classes mentioned above, but also their fractions according to molecular weight, down to 10 ng, without previous derivatization. Special attention was devoted to the preparation of columns with high efficiency and minimal losses of the test substances. The best results were obtained with a glass column 0.5 m × 2.0 mm I.D., packed with 1% OV-1 on Gas-Chrom Q (100—120 mesh). The processing of results is fully automated, using an MDS-2400 computer and includes the calculation of a non-linear calibration plot for each substance analyzed, accuracy control of the measured values, tabulation of the f_{wr} values and the calculation for analyses of biological samples. For the calibration, the pure substances were used at 15 concentrations within a range of 10—1000 ng. The coefficient of variation calculated from 20 duplicate measurements of the calibration mixture did not exceed 5% for any component in the interval from 10 to 100 ng or 3% within range from 100 to 1000 ng.

INTRODUCTION

Direct gas chromatographic (GC) analysis of plasma lipids was first described by Kuksis et al. [1] more than ten years ago. Since then, great advances have been made not only in the equipment, but also in the field of computing technique. In 1975 it was again Kuksis et al. [2] who published their experiences with fully automated GC estimation of the plasma-lipid profile. In the development of GC equipment, supports and stationary phases, as well as in the quality of standards, great progress has been made during the last decade.

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At present, the detection limit of high-molecular substances, such as lipids, is lower than 50 ng. However, quantitative analysis of such low concentrations is not carried out as a routine procedure. The main reason is the non-linear weight correction factor (f_w) vs. concentration plot for the substances analyzed [3], which was observed e.g. by Bezard and Bugaut [4, 5], even at higher concentrations. In a higher concentration range (of the order of thousands of ng), the f_w value is independent of the amount of lipid analyzed [6]; the concentration limit depends on the quality of the column and on the whole chromatographic system. With biological samples, the determination of much lower concentrations is necessary. This paper describes a computerized, fully automated procedure for the GC determination of neutral lipids down to 10 ng of each component. The sample preparation, accuracy and precision of the analysis of biological samples will be the object of the following communications.

MATERIALS

Triglycerides with carbon numbers 48, 50, 52, 54 and 60, purity 99% (tripalmitin, rac-glyceryl-1,3-palmitate-2-stearate, rac-glyceryl-1,3-stearate-2-palmitate, tristearin and triarachidin) were obtained from Supelco (Bellefonte, Pa., U.S.A.) and Sigma (St. Louis, Mo., U.S.A.), respectively. Cholesteryl esters with carbon numbers 31, 41, 43, 45 and 47 (cholesteryl butyrate, cholesteryl myristate, cholesteryl palmitate, cholesteryl stearate and cholesteryl arachidate) and free cholesterol were obtained from Applied Science Labs. (State College, Pa., U.S.A.). The OV-1 stationary phase and the Gas-Chrom Q support (100—120 mesh), were also supplied by Applied Science Labs. Isooctane (analytical grade) was provided by International Enzymes (Windsor, Great Britain). Chloroform, methanol, acetone and toluene, all analytical grade, were obtained from Lachema (Brno, Czechoslovakia). Helium of 99.99% purity was supplied by Messer (Griesheim, G.F.R.) and trimethylchlorosilan was provided by Merck (Darmstadt, G.F.R.).

Apparatus and operating conditions

All analyses were performed on a Perkin-Elmer F 30 gas chromatograph (Norwalk, Conn., U.S.A.) equipped with a dual column system with flame-ionization detection. Samples were injected by means of a Perkin-Elmer PS 4950 liquid autosampler into a 1/4 in. glass-lined injector thermostated to 300°. Oven temperature was programmed as follows: initial temperature 180°, programme rate 5°/min, final temperature 350°, detector temperature 350°. Helium flow-rate was 100 ml/min. The gas chromatograph was combined with a Perkin-Elmer Model 56 recorder, a Perkin-Elmer M-2 calculating integrator and a Teletype 33 ASR-FR (Teleprint, Frankfurt, G.F.R.) equipped with paper-tape puncher and reader. Data were processed by a computer MDS-2400 (32 K Byte core, 2 magnetic tape units, line printer, paper-tape reader) using a special programme.

METHODS

Preparation of the column

A glass column 0.5 m × 2.0 mm I.D. was rinsed with 150-ml volumes of chloroform, methanol and acetone and dried. Then the column was filled with a 10% solution of trimethylchlorosilane in anhydrous toluene, allowed to stand for 10 min and rinsed with toluene. The column was then treated for 5 min with methanol and rinsed with the same solvent to the neutral reaction. After drying in an oven, the column was packed immediately with 1% OV-1 on Gas-Chrom Q (100—120 mesh), using the combined effect of suction and vibration. The packing was prepared by the evaporation technique and pre-stabilized in a stream of helium, initially at a temperature programme 80—350°, 1°/min, then for 3 h at 350°. After testing the separation ability (by means of a standard mixture of triglycerides and cholesteryl esters), the column was further stabilized by 5 injections of 10,000-ng amounts of triolein. The reproducibility of the recovery was then checked by 5 analyses of the calibration mixture containing equal amounts of all substances analyzed (500 ng in 2 μl of injected sample). The respective f_w values calculated from these analyses did not exceed a 2% interval.

Calibration of the column

The column was calibrated by a mixture of equal amounts of cholesterol, cholesteryl esters with 41, 43, 45 and 47 carbon atoms and triglycerides with carbon numbers 48, 50, 52, 54 and 60. The calibration mixture was prepared from stock solutions containing 1 mg/ml of individual substances in a mixture isooctane—chloroform 80:20 (v/v). After drying, the mixture was dissolved in the internal standard solution (cholesteryl butyrate 200 ng/μl) and diluted standards were prepared according to Table I. All solutions were injected in duplicate by means of the PS-4950 autosampler; the volume of the injected samples was 2 μl.

Mathematical processing of the calibration curve

The f_w plot vs. the amounts of the individual components serves as a basis for mathematical processing of the calibration curves, with special consideration of the analytical use in the non-linear region of this plot. As the plot of f_w vs. the amount of the test substance is convex, a multilinear approximation of n—1 linear sections was used in order to simplify the problem of the calibration curve (n = 15, i.e. 14 sections). In Practice, the scatter of the measured values may cause oscillations of the calibration curve. To solve this problem the values measured were replaced mathematically by another set of values which meets the condition of a minimum scatter between these two sets, together with that of the convex character of the calculated plot. The f_w values were calculated from eqn. 1 [3].

$$f_w = \frac{\text{weight (\%)}}{\text{area (\%)}} \qquad (1)$$

The f_{wr} values can be calculated from the formula

$$f_{wr} = \frac{f_w}{f_{wis}} \qquad (2)$$

where f_{wis} is the weight correction factor for the internal standard. From eqn. 1 and 2 we get the formula for calculation of f_{wr} values for individual components

$$f_{wri} = \frac{m_i A_{is}}{m_{is} A_i} \qquad (3)$$

where f_{wri}, m_i and A_i are the weight correction factor, the weight and the peak area for component i, respectively. Analogously, m_{is} and A_{is} are the weight and the peak area for the internal standard.

Indicating the measured peak areas as x_i and the corresponding calculated f_{wr} values as y_i, the problem can be formulated as follows: for the set of points y_i, a corresponding set of points y'_i should be found under the following conditions: The value of the expression $z = (y_i - y'_i)^2$ is minimal and the system is described by n−2 inequalities

$$\frac{y_{i+1} - y_i}{x_{i+1} - x_i} \leqslant \frac{y_{i+2} - y_{i+1}}{x_{i+2} - x_{i+1}}$$

By meeting these conditions, the convex shape of the calculated plot is ensured. From the mathematical point of view, it is necessary to find the extreme of a function, under certain limitations. In this case the minimum quadratic function with n variables should be found with simultaneous validity of n−2 linear limitations. In practice, the problem can be solved by application of the saddle point theorem proved in 1951 by Kuhn and Tucker [7]. A modified algorithm

TABLE I

PREPARATION OF DILUTED STANDARDS

Sample No.	Injected amount (ng)
1	1000
2	760
3	578
4	439
5	334
6	254
7	193
8	146
9	111
10	85
11	64
12	49
13	28
14	16
15	10

of the simplex linear-programming method, described first by Wolfe [8], was used.

The programme output is in the form of a magnetic tape containing the coordinates of the optimized points and the slopes of their connecting lines, as well as a printed table which renders possible the control of the scatter of the measured points. The output further contains a table of the f_{wr} values for all the substances analyzed, which permits a check of the plot of f_{wr} vs. the peak area (or alternatively peak height) and manual calculation if necessary.

For triglycerides with carbon number of 56 and 58, the computerized calibration based on the linear interpolation of f_{wr} values between C-54 and C-60 is performed.

Evaluation of the analyses of biological samples

For the quantitative analyses of biological samples, an internal standard method was used. For calculation of the results, a special programme was prepared, based on the following formula

$$\text{mg/dl}_i = C \cdot f_{wri} \cdot A_i; \qquad C = \frac{10^{-4} V_t m_{is}}{V_p V_a A_{is}} \tag{4}$$

where C is the constant for each sample analyzed, V_t (μl) is the volume of internal standard solution, which was used for dissolving the sample before analysis, V_p (ml) is the corresponding volume of plasma, V_a (μl) is the volume of injected sample and m_{is} (ng) is the amount of internal standard in the injected sample.

RESULTS AND DISCUSSION

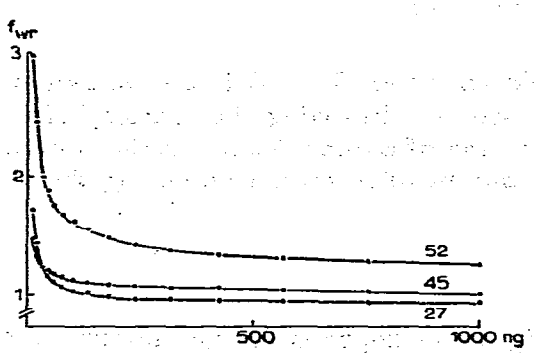
As observed by other authors, the f_w value depends on the amount and molecular weight of the substance analyzed [2, 5, 9]. However, different f_w values have been published for the same substances [4, 10] obtained under comparable conditions, but with different columns and apparatus. According to our present knowledge, a loss of the substances separated is caused by pyrolytic decomposition and irreversible sorption. It is also known that under strictly constant conditions, f_w values are highly reproducible [2]. From the chromatographic aspect, the f_w value increases with increased loading with stationary phase and with increased column length [3, 11]. The percentage of loading with stationary phase also influences the efficiency of the column. It is known from the literature, that columns with a higher percentage of loading need a longer time for stabilization than those with a lower one. A similar effect is also exerted by the column length. With such columns, relatively high f_w values are reached even after stabilization. For preparation of the column with the highest recovery of the test substances, lower loading with the stationary phase is necessary. The support should be highly inert. Chromosorb W HP, Chromosorb 750, Gas-Chrom Q and Supelcoport coated with 1—3% OV-1 or JXR were tested. Best results were obtained with glass column 0.5 m × 2.0 mm I.D., packed with 1% OV-1 on Gas-Chrom Q (100—120 mesh).

TABLE II

REPRODUCIBILITY OF THE GC DETERMINATION OF THE STANDARD MIXTURE

Each value represents average of three analyses; 2 μl of the standard mixture containing 100 ng/μl of each component were injected.

Compound (carbon number	Day to day variations		After 200 analyses	After 600 analyses
	1st day	2nd day		
27	202.1	198.7	201.9	213.7
41	204.0	196.8	203.0	208.7
43	203.9	196.5	203.3	206.9
45	203.4	197.6	202.6	209.0
47	202.9	195.9	198.5	215.9
48	201.8	199.7	200.9	210.2
50	200.9	198.9	197.9	213.7
52	200.2	199.5	201.9	216.5
54	200.7	201.2	204.8	216.8
60	202.5	201.5	205.9	223.2



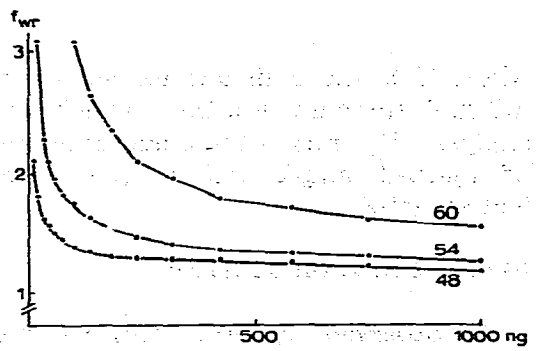


Fig. 1. The plot of f_{wr} vs. amount of the substance analyzed, for main components of the lipid profile. 27 = Free cholesterol; 45 = cholesteryl stearate; 52 = 1,3-stearate-2-palmitate.

Fig. 2. The plot of f_{wr} vs. amount of the substance analyzed, for triglycerides with different molecular weights. 48 = Tripalmitin; 54 = tristearin; 60 = triarachidin.

The efficiency of the column, expressed as $\Delta\ C_{48-54}$ [3], was sufficient. The column needed a relatively short time for stabilization.

The reproducibility of the calibration data plays an important role in the lipid analysis when measured in the region where f_w is independent of the amount of substance analyzed. This requirement is much more important in the non-linear region. The repeatability of the calibration data with time should also be checked as an important criterion of the reproducibility. Table II shows the results for repeated analyses of the calibration mixture (200 ng of each component) obtained with the column mentioned above. Similarly, the reproducibility of calibration was tested over the whole concentration range. It was confirmed that the quantitative lipid analysis in the non-linear

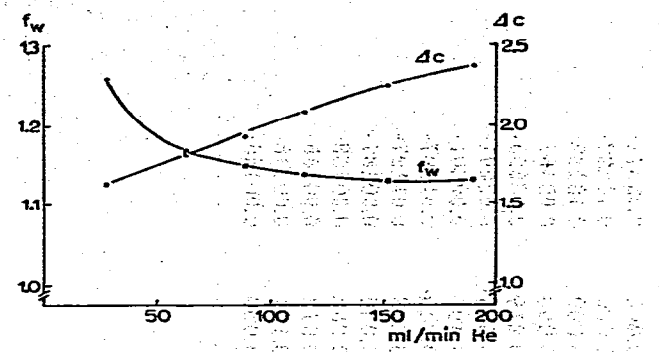


Fig. 3. The plot of f_w and ΔC vs. carrier-gas flow-rate.

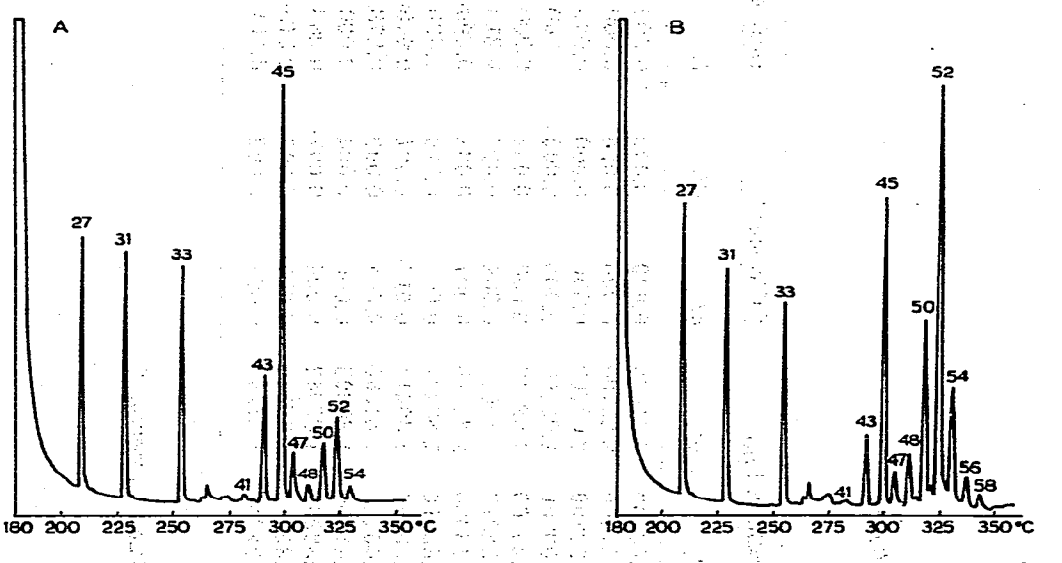


Fig. 4. Gas chromatograms of: (A) normal and (B) hyperlipidemic plasma neutral lipid. 27 = Free cholesterol; 31 = cholesteryl butyrate (internal standard); 33 = cholesteryl benzoate (standard for laboratory control); 41—47 = cholesteryl esters; 48—58 = triglycerides; sample volume = 2 μl; solvent = isooctane—chloroform (80:20, v/v); sensitivity = 1/64; chart speed = 5 mm/min; other analytical conditions are given in the text.

range of f_w values is sufficiently precise. The method described differs from that of the internal standard in that the f_w value is obtained from the f_w vs. peak-area calibration plot for the test substance. Figs. 1 and 2 shows these calibration plots for some individual substances.

As shown in Figs. 1 and 2, the higher the molecular weight of the substance, the more curved is the calibration plot. Furthermore, the parameters discussed previously, i.e. the quality of the column and the operating conditions also influence the shape of the calibration plot. It is known that the shape of the plot of f_w vs. the carrier gas flow-rate is the reverse of that for the separation ability vs. the flow-rate [3]. In our investigation, these conclusions were confirmed. Fig. 3 shows the optimization of the carrier gas flow-rate for the column described.

TABLE III

CALIBRATION OF THE COLUMN NO. 33

Date: 11-11-77; Carbon number: 54

Peak areas*		f_{wr}**		Optimised f_{wr}		Difference***		Difference (%)		Slope
A_1	A_2	f_1	f_2	of_1	of_2	df_1	df_2	f_1	f_2	
0.4	1.1	4.484	2.919	4.48400	2.91900	0.000	0.000	0.000	0.000	2.23571-
1.1	2.5	2.919	2.297	2.91900	2.29700	0.000	0.000	0.000	0.000	0.44429-
2.5	3.6	2.297	2.097	2.29700	2.09700	0.000	0.000	0.000	0.000	0.18182-
3.6	5.1	2.097	1.955	2.09700	1.95500	0.000	0.000	0.000	0.000	0.09467-
5.1	7.0	1.955	1.825	1.95500	1.82917	0.000	0.004	0.000	0.219	0.06623-
7.0	9.8	1.825	1.763	1.82917	1.75635	0.004	0.007-	0.219	0.399-	0.02601-
9.8	14.5	1.763	1.626	1.75635	1.63410	0.007-	0.008	0.399-	0.490	0.02601-
14.5	20.5	1.626	1.572	1.63410	1.56227	0.008	0.010-	0.490	0.640-	0.01197-
20.5	28.7	1.572	1.460	1.56227	1.46411	0.010-	0.004	0.640-	0.273	0.01197-
28.7	39.2	1.460	1.413	1.46411	1.41300	0.004	0.000	0.273	0.000	0.00487-
39.2	53.9	1.413	1.385	1.41300	1.38905	0.000	0.004	0.000	0.288	0.00163-
53.9	71.6	1.385	1.370	1.38905	1.36343	0.004	0.007-	0.288	0.513-	0.00145-
71.6	99.2	1.370	1.321	1.36343	1.32346	0.007-	0.002	0.513-	0.151-	0.00145-
99.2	134.7	1.321	1.272	1.32346	1.27206	0.002	0.000	0.151	0.000	0.00145-

*Integrator output divided by 5000.
**Calculated from measured values.
***Difference between f_{wr} optimized and f_{wr} measured.

TABLE IV

CALIBRATION OF THE COLUMN No. 33

Date: 11-11-77.

Area*	f_{wr27}	f_{wr41}	f_{wr43}	f_{wr45}	f_{wr47}	f_{wr48}	f_{wr50}	f_{wr52}	f_{wr54}	f_{wr56}	f_{wr58}	f_{wr60}
0.5	1.872	1.404	1.454	1.587	1.733	2.267	2.552	3.067	4.261	4.295	5.515	12.018
1.0	1.740	1.364	1.408	1.522	1.648	2.081	2.305	2.687	3.143	3.826	4.699	6.373
1.5	1.608	1.324	1.362	1.456	1.564	1.896	2.057	2.355	2.742	3.356	3.884	5.205
2.0	1.476	1.284	1.316	1.391	1.479	1.767	1.945	2.219	2.520	2.887	3.526	4.649
2.5	1.381	1.244	1.270	1.333	1.416	1.722	1.867	2.083	2.297	2.734	3.275	4.151
3.0	1.344	1.221	1.247	1.307	1.384	1.677	1.790	1.964	2.207	2.595	3.094	3.888
3.5	1.306	1.202	1.229	1.281	1.352	1.632	1.742	1.913	2.116	2.505	2.929	3.625
4.0	1.269	1.183	1.212	1.255	1.320	1.601	1.715	1.862	2.060	2.415	2.833	3.499
4.5	1.231	1.165	1.195	1.229	1.292	1.570	1.688	1.828	2.012	2.343	2.737	3.374
5.0	1.210	1.148	1.182	1.218	1.281	1.545	1.664	1.795	1.965	2.281	2.666	3.249
5.5	1.194	1.142	1.175	1.211	1.270	1.530	1.640	1.762	1.929	2.220	2.612	3.124
6.0	1.177	1.135	1.169	1.204	1.259	1.515	1.616	1.739	1.896	2.166	2.558	3.044
6.5	1.162	1.128	1.163	1.197	1.249	1.500	1.598	1.722	1.863	2.143	2.504	2.976
7.0	1.153	1.121	1.158	1.190	1.238	1.489	1.584	1.706	1.830	2.120	2.450	2.908
.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.
244.0	0.946	0.970	0.963	0.969	0.977	1.086	1.104	1.133	1.114	1.161	1.213	1.264
244.5	0.946	0.970	0.963	0.968	0.977	1.086	1.103	1.132	1.113	1.161	1.212	1.263
245.0	0.946	0.970	0.963	0.968	0.977	1.085	1.103	1.132	1.113	1.160	1.211	1.262
245.5	0.946	0.970	0.963	0.968	0.976	1.085	1.102	1.131	1.112	1.159	1.210	1.261
246.0	0.946	0.970	0.962	0.968	0.976	1.084	1.102	1.131	1.111	1.158	1.209	1.260
246.5	0.946	0.970	0.962	0.967	0.976	1.084	1.101	1.130	1.110	1.157	1.208	1.259
247.0	0.946	0.970	0.962	0.967	0.975	1.083	1.100	1.129	1.110	1.157	1.207	1.258
247.5	0.946	0.969	0.962	0.967	0.975	1.082	1.100	1.129	1.109	1.156	1.206	1.257
248.0	0.946	0.969	0.962	0.966	0.975	1.082	1.099	1.128	1.108	1.155	1.205	1.256
248.5	0.946	0.969	0.961	0.966	0.975	1.081	1.099	1.128	1.107	1.154	1.204	1.255
249.0	0.946	0.969	0.961	0.966	0.974	1.081	1.098	1.127	1.107	1.153	1.203	1.254
249.5	0.946	0.969	0.961	0.966	0.974	1.080	1.098	1.127	1.106	1.152	1.203	1.253
250.0	0.946	0.969	0.961	0.965	0.974	1.080	1.097	1.126	1.105	1.152	1.202	1.252

*Integrator output divided by 5000.

We chose computerized processing of the calibration data, because manual processing is very laborious and lacking in precision. The basic mathematical assumptions have been discussed above. In this way, 500 f_w values were obtained as a function of the peak area of the test substance. The programme permits two versions of data processing. As confirmed by Gold and Mathew [6], both the peak heights and their areas can be used for the quantitative analysis of lipids. The f_w vs. peak height plot is very similar to that of f_w vs. peak area. As mentioned above, mathematical processing permits the control of the accuracy of the calibration data, using a special optimization programme.

Table III shows an example of computerized calibration data for one compound; from this table the differences between the measured and optimized values and also between duplicate analyses of the same sample and the slopes of the individual linear regions are apparent. This method of data processing permits rapid control of the calibration accuracy. The difference between duplicate measurements of the same sample should not exceed 5% in relation to the mean f_{wr} value for each component. Analogously, this type of computer output serves as a rapid check of recalibration. As can be seen from Table II, the interval of recalibration is longer than 200 analyses. In practice, when the chromatograph is used daily, it represents a time interval of about 1—2 months. The calibration proper is automatic and takes 32 h. The calibration data are

recorded on a magnetic tape and used throughout the period of calibration validity for the evaluation of biological samples. The calibration is repeated if any of the substances display a deviation of f_{wr} values greater than 5% in any part of the plot. The problem of calibration stability will be discussed in the following part of this paper.

During the column life, the f_{wr} values decrease in the lower concentration range, especially in the case of triglycerides with higher molecular weight. These changes are smaller when the column is properly stabilized. Table IV illustrates part of the output of tabulated f_{wr} data in relation to the peak area; similar data were obtained for peak heights. Table V shows an example of the calculation for a biological sample. Gas chromatograms of normal and hyperlipidemic plasma neutral lipid are given in Fig. 4.

The proposed method permits on-line as well as off-line coupling with a computer. From economic aspects, the off-line coupling is more effective; it employs a conversion programme, using punched or magnetic tapes containing the input data in the ASC 11 code.

The method also permits the inclusion in the computerized programme of some additional parameters suitable for clinical purposes (e.g. various sums or ratios of measured values).

TABLE V

CALCULATION OF THE LIPID PROFILE

Analysis No. 999/0/0/8; Date: 8-11-77. V_t (μl) = 1000; V_p (ml) = 0.2000; V_a (μl) = 2.0; m_{is} (ng) = 400.00; A_{is}* = 48.6: all have the same meaning as in eqn. 4.

Lipid	Carbon No.	Area*	f_{wr}	mg/dl	Chol (mg)**
Cholesterol	27	26.2	1.009	54.39	
Cholesteryl esters	41	0.6	1.396	1.72	1.11
	43	12.8	1.115	29.37	18.19
	45	62.0	1.067	136.12	80.96
	47	6.5	1.249	16.70	9.59
Total cholesteryl esters				183.91	109.85
Total cholesterol					164.24
Triglycerides	48	0.8	2.156	3.55	
	50	4.1	1.709	14.42	
	52	17.1	1.538	54.11	
	54	5.1	1.956	20.53	
	56	0.3	4.483	2.77	
	58				
	60				
Total triglycerides				95.38	

*Integrator output divided by 5000.
**Content of cholesterol in individual ester fractions.

REFERENCES

1 A. Kuksis, L. Marai and D.A. Gornall, J. Lipid Res., 8 (1967) 352.
2 A. Kuksis, J. J. Myher, L. Marai and K. Geher, J. Chromatogr. Sci., 13 (1975) 423.
3 C. Litchfield, R.D. Harlow and R. Reiser, J. Amer. Oil Chem. Soc., 42 (1965) 849.
4 J. Bezard and M. Bugaut., J. Chromatogr. Sci., 7 (1969) 639.
5 J. Bezard and M. Bugaut, J. Chromatogr. Sci., 10 (1972) 451.
6 M. Gold and G. Mathew, Anal. Chem., 47 (1975) 752.
7 H.W. Kuhn and A.W. Tucker, Non-linear Programming, Berkeley University of California Press, 1951, p. 481.
8 P. Wolfe, Econometrica 27 (1959) 382.
9 R. Watts and R. Dils, J. Lipid Res., 9 (1968) 40.
10 A. Kuksis, Fette-Seifen-Anstrichm., 73 (1971) 332.
11 A. Kuksis, J. Amer. Oil Chem. Soc., 42 (1965) 269.