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AUTOMATED QUANTITATIVE GAS–LIQUID CHROMATOGRAPHY OF INTACT LIPIDS

II. ACCURACY, PRECISION AND REPRODUCIBILITY OF RESULTS

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SUMMARY

The effect of various factors on the precision and accuracy of the gas chromatographic determination of neutral lipids was studied in the concentration range where the correction factors are dependent on the amount analyzed. The mutual effect of individual components of the neutral lipid spectrum on the recovery was examined. A method is described which provides the stable recovery of the components present at low concentrations, using the addition of high-molecular-weight triglyceride (triarachidin) which does not interfere in the determination of the usual triglycerides. The validity of the correction factors measured with pure compounds was verified by hydrogenation of biological samples of various compositions. Hydrogenation of the sample also solves the problem of the determination of the triglyceride fraction of carbon number 46, which interferes under normal conditions with the determination of the cholesteryl ester fraction of carbon number 47. A method for the standardization of the gas chromatographic determination of neutral lipids is given, using pure compounds instead of lyophilized biological samples.

Long-term quality control was carried out using synthetic control samples. The results show sufficiently low values of the variation coefficients over the whole period. The values of the variation coefficients measured over an interval of 25 weeks are about 4% for the main components of the neutral lipid spectrum and 6.3% for the components present at concentrations up to 5%. The within-day variation for the most neutral lipid fractions and for lipid classes attains a value of 40—75% of the day-to-day variation. The most satisfactory values are obtained for the variation within a single series which amounts to less than 2% for all substances except for triglyceride fractions 48 and 54. The correlation of the determination of total cholesterol and triglycerides by gas chromatography and by enzymatic methods shows a very good agreement between the results obtained by the two methods. Using quality control, it is possible to follow the accuracy of the calibration and to demonstrate objectively the necessity for system recalibration.

INTRODUCTION

The precision and accuracy of the results of gas chromatographic analyses have been discussed in a number of papers [1—6]. However, in all cases the concentration of the compounds was in the region where the weight correction factor is independent of the amount injected. The possibility of using quantitative gas chromatography for the determination of amounts in the nanogram range was first discussed by Kuksis et al. [4]. The effect of a larger peak preceding a smaller one on the recovery of the smaller peak in triglyceride analysis was also discussed [4]. The conditions for quantitative analysis of neutral lipids in the concentration range where the weight correction factor is a function of the amount analysed were studied and discussed in the previous part of this study [7].

The precision, accuracy and reproducibility of the results were studied especially with respect to the accuracy and stability of the correction factors, to the relation of the recovery of individual components and their concentration ratios and to the possibility of using the weight correction factors measured using saturated compounds for the analysis of biological samples.

The accuracy of the results was checked using model samples prepared from pure compounds; a comparison of the results measured gas chromatographically and by enzymatic methods [8, 9] using commercial Boehringer-Mannheim sets was evaluated statistically. The time dependence of the analytical results due to changes of the correction factor was studied using internal quality control by means of the model samples (the same as those used for the accuracy control). The accuracy of the results obtained with individual fractions of the lipid profile was also studied with respect to the determination of some minor triglyceride components, which are not separated from cholesteryl esters under the conditions used.

MATERIALS

All standards, solvents, stationary phases and supports were the same as those described in the previous paper [7]. The hydrogenation catalyst, PtO_2, was obtained from Merck (Darmstadt, G.F.R.). Reagent sets for the enzymatic determination of total cholesterol and triglycerides were supplied by Boehringer, Mannheim, G.F.R. (cat. Nos. 172626 and 126012, respectively). Hexane, p.a., obtained from Lachema (Brno, Czechoslovakia), was distilled before use.

METHODS

Apparatus and operating conditions

In addition to the instruments described in the previous paper [7], a Perkin-Elmer F-17 gas chromatograph with a Perkin-Elmer Sigma 10 Laboratory Data System which was equipped with a Teletype 33 ASR-FR Terminal (Teleprint, Frankfurt, G.F.R.) was used for the chromatographic analyses. Samples were injected manually using an 85-N Hamilton microsyringe. The gas chromatograph was equipped with glass-lined stainless-steel columns (Supelco,

Bellefonte, Pa., U.S.A.), 0.6 m × 1.8 mm I.D. Other analytical conditions were the same as described previously [7].

Preparation of the control samples

Model mixtures were prepared from the stock solutions of the individual compounds used for calibration. The concentration of all stock solutions was 1 mg/ml in a solvent mixture of isooctane—chloroform (80:20, v/v). Such solutions are stable for several months when stored at 4°.

After all components had been pipetted the samples were dried, redissolved in a corresponding volume of the internal standard solution, divided into 1-ml aliquots and dried again. The control samples were stored at —20° and redissolved in the isooctane—chloroform solvent mixture before analysis. The biological samples were hydrogenated as described in the literature [10].

Mutual effect of components on the recovery of individual compounds of the neutral lipid spectrum

A 20-μl volume of the stock solution of the component being studied and 1000 μl of the internal standard solution were pipetted into a glass microvial. The sample was dried under nitrogen (temperature of the water-bath about 60°) and redissolved in 1000 μl of the isooctane—chloroform (80:20, v/v) mixture. After 1 μl of the sample containing 20 ng of the component under study and 200 ng of the internal standard had been injected into the gas chromatograph, 200 μl of a solution of the interfering component were added to the sample solution. The sample was dried, redissolved in the solvent mixture and analysed as described before. For the mathematical evaluation, the recovery of the component studied in the first analysis was calculated as 100%.

Recovery study of rac-glycerol-1,3-stearate-2-palmitate

Five samples containing 20 μl of the stock solution of the triglyceride and 1000 μl of the internal standard solution were dried under nitrogen, redissolved in the isooctane–chloroform (80:20, v/v) mixture and analysed. Then 200 μl of the triarachidin stock solution were added, the sample was dried and redissolved in the same way, and analysed again.

Quality control and statistical evaluation

The quality control was carried out using synthetic samples described before. In each run the first and the tenth samples were control samples. The reproducibility of the repeated analyses was calculated from six samples analysed in one run. Within-day and day-to-day variations were calculated on the basis of 28 duplicate control samples analysed over 14 weeks according to the formulae generally used [11]. Long-term reproducibility was evaluated from all the control samples analysed over a period of 25 weeks.

RESULTS AND DISCUSSION

The precision and accuracy of the results of neutral lipid analysis are dependent on the accurate and reproducible measurement of the weight correction factors (f_w) for individual components. The greatest reproducibility of

the correction factor measurement is required when f_w depends on the amount of the component analysed. It is known that especially in such cases the recovery of individual components is dependent on their concentration ratios [4]. Theoretically, the correction factors measured during calibration should be valid under the conditions of measurement. In practice, each measurement is made under different conditions, because of the different compositions of the biological samples. The selection of the optimum compounds and conditions for the calibration is very important. For the measurement of the correction factor of triglycerides, a number of different compounds, synthetic and natural, were used [3–5]. Natural compounds are chemically insufficiently stable; from an analytical point of view they are not exactly defined. However, calibration mixtures of natural origin are chemically more similar to the compounds being analysed in the biological samples. Using natural triglyceride mixtures it is possible to study neither the interactions between individual components of the sample and the chromatographic system, nor the dependence of the recovery of individual components on their concentration ratios. These relationships were studied using pure compounds — standards for the column calibration. It was found that a larger amount of one component increased the recovery of a second component present only in a low concentration, irrespective of the elution order. Some results are given in Fig. 1.

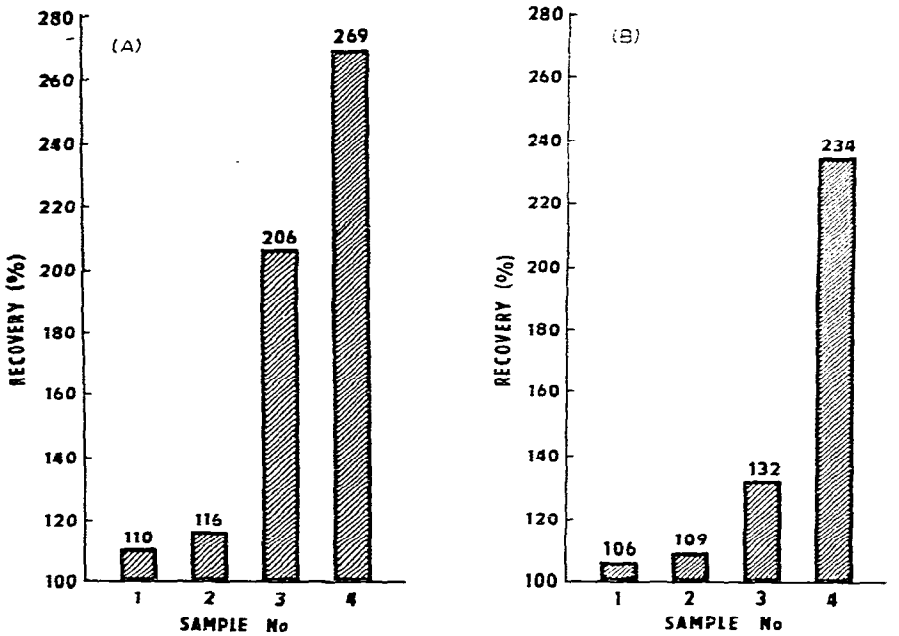


Fig. 1. Effect of some triglycerides on the recovery of tristearin (A) and *rac*-glycerol-1,3-stearate-2-palmitate (B). Composition of the samples injected: component studied (A) tristearin (20 ng), (B) *rac*-glycerol-1,3-stearate-2-palmitate (20 ng); internal standard, cholesteryl butyrate (200 ng); interfering components (200 ng). (A) tripalmitin (1), *rac*-glycerol-1,2-palmitate-3-stearate (2), *rac*-glycerol-1,3-stearate-2-palmitate (3), triarachidin (4); (B) tripalmitin (1), *rac*-glycerol-1,2-palmitate-3-stearate (2), tristearin (3), triarachidin (4). The recovery obtained with the sample without interfering components (20 ng of the component studied and 200 ng of the internal standard) was taken as 100%.

As shown in Fig. 1, the recovery is affected not only of the components eluted immediately one after another, but also of those with greater differences in elution times. The degree of such interference depends on the molecular weights of both components, also on their concentration ratio and on the amount of each injected. In the 20—200-ng range of the compound injected, the higher the molecular weights of both compounds (studied and interfering) and their concentration ratio, the greater is the effect on the recovery. This dependence decreases with increasing amounts of the test compound. For example, with amounts over 400 ng of tristearin or *rac*-glycerol-1,3-stearate 2-palmitate, the change in the recovery caused by any interfering compound did not exceed 5% at concentration ratios of the component studied to the interfering one of 1:10, 1:5, 1:2 and 1:1.

According to these experiments, the equilibrium state of the chromatographic system, which determines the recovery of the compounds, is influenced by the composition of the sample being analysed. The reason is probably a competitive saturation of the system by compounds with increasing molecular weight. The compound added in excess probably affects the physical and chemical properties of the stationary phase; the shift of the sorption equilibrium can lead to a higher recovery of the compound being analysed. This hypothesis was also supported by additional experiments in which the effect of triarachidin on the recovery of *rac*-glycerol-1,3-stearate-2-palmitate in repeated analyses was studied. The results are given in Fig. 2. Taking into account the results of further analyses of other neutral lipid components and their mutual influence, control samples of different compositions were prepared for the quality control. Using triarachidin, which is not usually present in biological samples, the mutual effect of individual cholesteryl esters and triglycerides on the recovery was practically eliminated. Under such conditions, the calibration data measured with pure compounds with constant concentration ratios are valid for the biological samples of variable composition.

A further problem studied was to check the usefulness of the correction factors measured with saturated compounds for the determination of biological

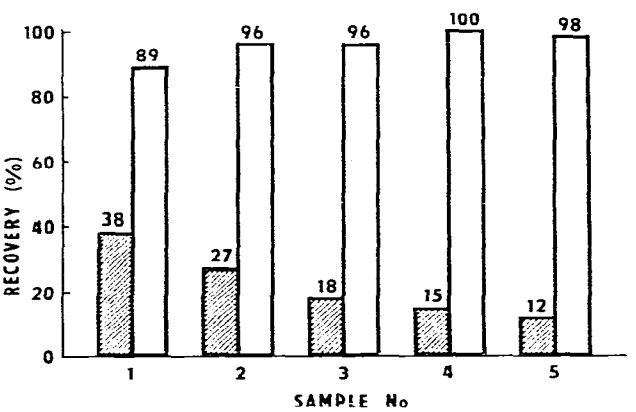


Fig. 2. Effect of triarachidin on the recovery of *rac*-glycerol-1,3-stearate-2-palmitate. Composition of the samples injected: ▨, *rac*-glycerol-1,3-stearate-2-palmitate, 20 ng, internal standard (cholesteryl butyrate), 200 ng; □, the same samples after the addition of 200 ng of triarachidin.

samples containing both saturated and unsaturated compounds. As known from the literature, the f_w values for compounds with an identical number of carbon atoms but with a different number of double bonds are different under various conditions [2, 12, 13]. Bezard and Bugaut [14] studied the dependence of f_w on the number of the double bonds and the amount analysed for the triglycerides with carbon number 54 (tristearin, triolein, trilinolein and trilinolenin). It was found that for all the triglycerides studied the f_w values increase with increasing number of double bonds and with decreasing amounts of the compound analysed. The course of the plot of f_w versus the amount analysed was similar for all triglycerides studied. In the case of the f_w values for tristearin and triolein, the smallest differences were observed in the whole range under investigation.

The f_w values are dependent not only on the chemical structure and amount of the compounds analysed, but also on the properties of the entire chromatographic system (especially on the column, construction of the injection port and the sampling technique). The validity of the correction factors calculated from the calibration data was checked over a wide range of plasma neutral lipid concentration using hydrogenation of the biological samples. The results obtained in the measurement of the correction factors for both saturated and unsaturated individual triglycerides and cholesteryl esters were also confirmed by the hydrogenation experiments.

The chromatograms of two typical samples with normal and elevated triglyceride levels before and after hydrogenation are shown in Fig. 3. The respective results are given in Table I.

As shown in Table I, there are no significant differences in the results when the correction factors measured with saturated compounds were used for the determination of unsaturated compounds. This finding is in agreement with the results of correction factor measurements for saturated and unsaturated compounds published by other authors [12—14]. The differences that appear with fractions of higher carbon number, especially 56 and 58, could be caused by the lower chemical stability of these compounds which contain a relatively high percentage of unsaturated fatty acids [15]. Part of the highly unsaturated triglycerides could be decomposed during the analysis [6]. However, the differences observed have only a small influence on the recovery of the total triglycerides. After hydrogenation, the individual fractions are more homogeneous, resulting in a narrower peak width and a better separation of the compounds. This can also influence the comparison of analyses of the same sample in hydrogenated and non-hydrogenated form. According to our results, columns with high efficiency (ΔC_{48-54} = 1.3—1.8) are suitable for the separation of non-hydrogenated samples [7]. When the column does not have sufficient efficiency, the result of integrating the non-hydrogenated sample can be different from that of the hydrogenated sample, because of insufficient separation. A final solution to the problem for triglycerides of carbon numbers 56 and 58 is possible only on the basis of analytical data measured with pure compounds. These measurements cannot be performed at present because these compounds are not commercially available. The experiences with the analysis of plasma neutral lipids cannot be generalized for the triglycerides with a high number of the double bonds without previous confirmation based on a detailed study using pure synthetic standards.

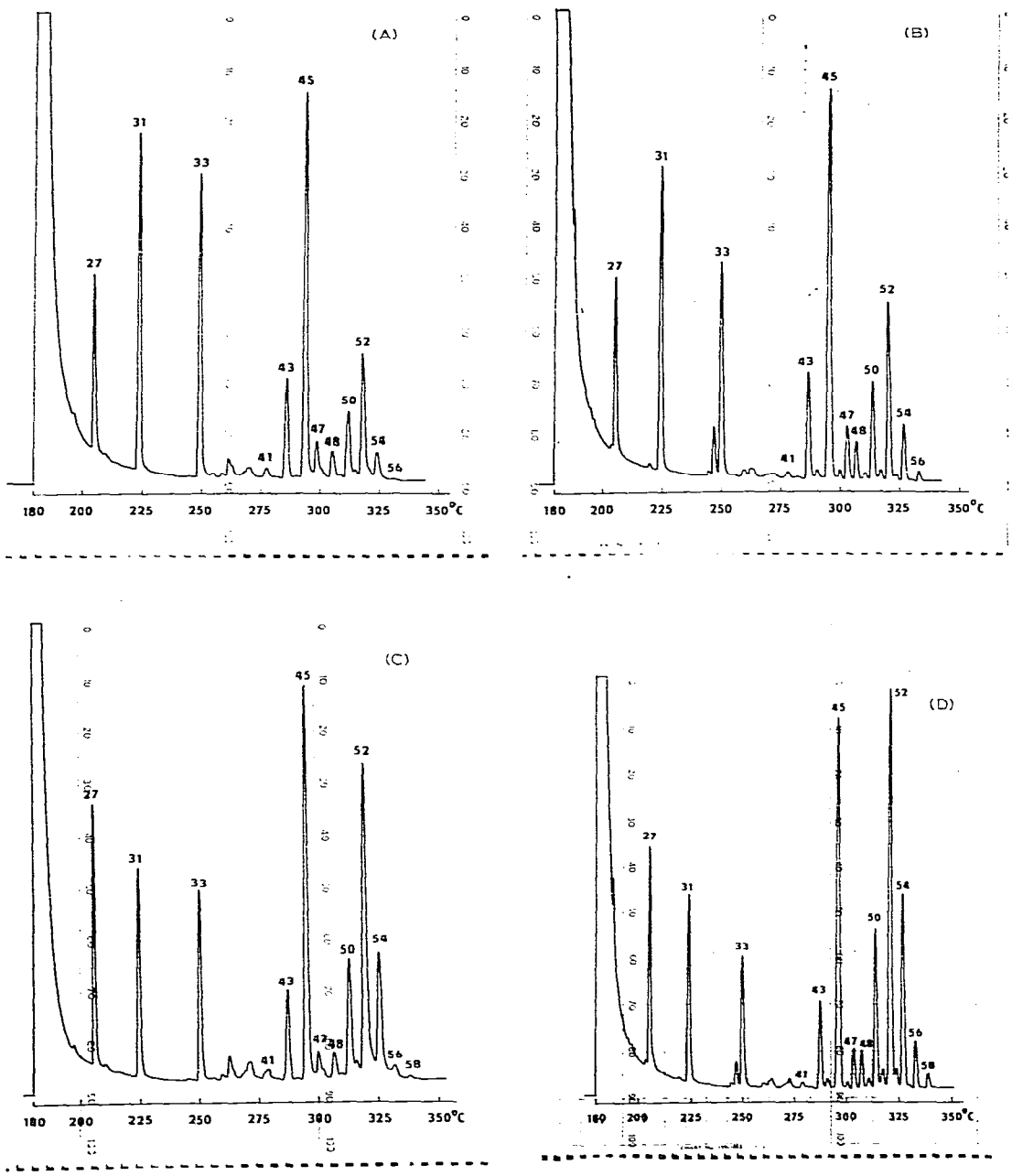


Fig. 3. Gas chromatograms of normal plasma before (A) and after (B) hydrogenation, and of hyperlipidemic plasma before (C) and after (D) hydrogenation. 27 = free cholesterol, 31 = cholesteryl butyrate (internal standard), 33 = cholesteryl benzoate (standard for laboratory control), 41—47 = cholesteryl esters, 48—58 = triglycerides. Column, 0.6 m × 1.8 mm I.D. glass-lined stainless-steel packed with 1% OV-1 on Gas-Chrom Q (100—120 mesh); carrier gas, helium 100 ml/min; temperatures, injector 300°, oven 180°, rate 5°/min, detector FID 350°, sample volume, 2 μl; solvent, isooctane—chloroform (80:20, v/v); sensitivity, 1/64; chart speed, 5 mm/min.

TABLE I

EFFECT OF HYDROGENATION ON THE ANALYTICAL RESULTS OF BIOLOGICAL SAMPLES OF VARIOUS COMPOSITIONS

All results represent a mean ± S.D. of five measurements obtained within two days. The statistical significance of the differences between the results measured with hydrogenated and non-hydrogenated samples was tested by the *t*-test ($P<0.01$). Instrument: Perkin-Elmer F-17.

Compound* (carbon number)	Sample A			Sample B		
	Before hydrogenation (mg/dl)	After hydrogenation (mg/dl)	SS**	Before hydrogenation (mg/dl)	After hydrogenation (mg/dl)	SS**
41	4.91 ± 0.22	4.57 ± 0.27	NS	7.01 ± 0.36	6.32 ± 0.44	NS
43	36.93 ± 0.97	37.55 ± 0.78	NS	56.12 ± 1.52	53.24 ± 1.38	NS
45	146.78 ± 2.39	151.54 ± 2.24	NS	247.75 ± 4.55	252.01 ± 3.72	NS
47	24.95 ± 0.98	23.02 ± 0.91	NS	36.73 ± 1.81	37.07 ± 1.60	NS
48	18.34 ± 0.68	17.81 ± 0.50	NS	28.67 ± 1.11	29.76 ± 0.96	NS
50	40.32 ± 0.92	41.99 ± 0.73	NS	113.10 ± 1.64	115.85 ± 1.76	NS
52	76.96 ± 1.52	77.63 ± 1.27	NS	325.03 ± 4.65	325.50 ± 3.60	NS
54	28.98 ± 1.08	30.90 ± 0.85	NS	154.96 ± 4.78	160.71 ± 3.42	NS
56	7.70 ± 0.71	10.71 ± 1.19	S	40.84 ± 1.78	53.73 ± 1.46	S
58	—	—	—	17.69 ± 1.35	25.89 ± 1.05	S
FC	41.97 ± 1.80	43.12 ± 1.95	NS	93.41 ± 1.27	91.20 ± 1.08	NS
CE	213.44 ± 2.99	216.57 ± 2.43	NS	348.49 ± 6.86	348.57 ± 5.27	NS
TC	169.53 ± 3.01	172.60 ± 1.99	NS	301.07 ± 4.83	299.34 ± 3.52	NS
TG	172.12 ± 4.45	178.96 ± 3.86	NS	680.06 ± 13.75	711.28 ± 9.33	S

*FC = free cholesterol; CE = cholesteryl esters; TC = total cholesterol; TG = triglycerides.
**SS = statistical significance; S = significant; NS = not significant.

Analysis of some minor and interfering components of the neutral lipid spectrum

In parallel with the problem of the accuracy of the results in neutral lipid analysis by gas chromatography, the problem of the minor and interfering components was also studied. These components can be divided into two groups:

(1) Components of the neutral lipid spectrum that are present in minor concentrations but which do not interfere in the determination of other fractions. This group includes, in particular, triglycerides of carbon number 56, 58 and 60. The problems connected with the analysis of these compounds were discussed in our previous paper [7].

(2) Components that are present in minor concentrations but which do interfere in the determination of other lipid fractions. This group contains, in particular, triglycerides of carbon number 46 and 44, or lower, which interfere in the determination of cholesteryl esters with carbon numbers 47, 45, or 43. In most cases the concentration of these compounds is sufficiently low that the effect on the determination of cholesteryl esters is negligible. In samples with higher triglyceride levels or in those with an atypical triglyceride composition, the concentration of the fractions with carbon numbers 46, 44, or lower is increased. The effect of fraction 46 on the determination of cholesteryl ester of carbon number 47 can be significant, even if the effect on the determination of the total cholesteryl ester level is not significant, as shown in Table II. In most cases this problem can be solved by hydrogenating the sample, which enables the separation of fractions 46 and 47, as shown in Fig. 4.

The decision as to whether correction is necessary depends on the accuracy required for the determination of cholesteryl ester fraction 47. For orientation,

the amount of interfering fraction 46 can be estimated from the ratio of fractions 48 and 50. With extremely high triglyceride levels or atypical compositions, separation of the triglycerides and two separate analyses of the cholesteryl esters and triglycerides are necessary.

The accuracy of the analytical results was evaluated using model samples prepared from pure compounds. The composition was chosen to be as close as possible to that of an average biological sample. The compositions of the model samples and the results of their analysis are surveyed in Table III, from which a very good agreement between the analytical values and the real composition

TABLE II

EFFECT OF THE TRIGLYCERIDE LEVEL AND COMPOSITION ON THE DETERMINATION OF CHOLESTERYL ESTER FRACTION 47, TOTAL CHOLESTEROL AND TRIGLYCERIDES

Each value represents a mean of five measurements obtained within one week. The statistical significance of the differences between the corrected and non-corrected values was checked by the *t*-test ($P<0.01$). Instrument: Perkin-Elmer F-17. For abbreviations see Table I.

Sample No.	46 (mg/dl)	47 (mg/dl)	47_{corr}* (mg/dl)	SS	TC (mg/dl)	TC_{corr}* (mg/dl)	SS	TG (mg/dl)	TG_{corr}* (mg/dl)	SS
948	17.1	42.9	29.6	S	303.5	290.6	S	373.1	390.2	S
1053	16.0	35.2	21.0	S	258.0	244.7	S	810.6	826.6	NS
949	14.5	20.6	14.9	S	251.2	245.3	NS	366.2	380.7	NS
977	8.7	21.3	14.7	S	171.8	164.4	NS	217.8	226.5	NS
967	7.6	40.3	36.4	S	270.9	266.9	NS	175.2	182.8	NS
980	3.9	17.3	15.2	S	149.1	147.2	NS	122.7	126.6	NS
975	2.4	27.4	26.3	NS	171.1	170.5	NS	301.7	304.1	NS
964	1.6	37.4	36.8	NS	313.4	312.6	NS	547.4	549.0	NS

*A correction was made according to the determination of the triglyceride fraction 46 after the isolation of triglycerides using column chromatography with Florisil. The purity of the fraction isolated was checked by thin-layer chromatography.

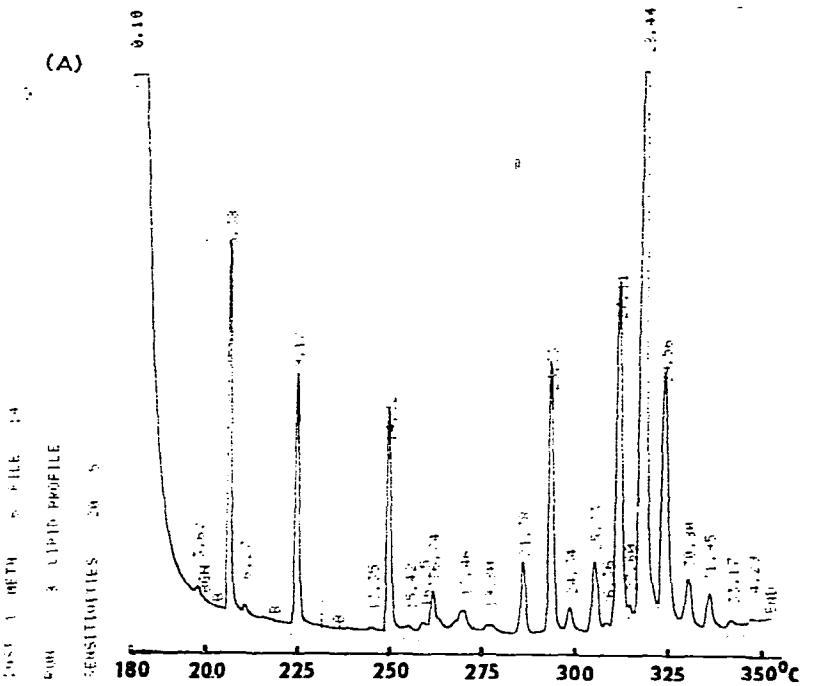
TABLE III

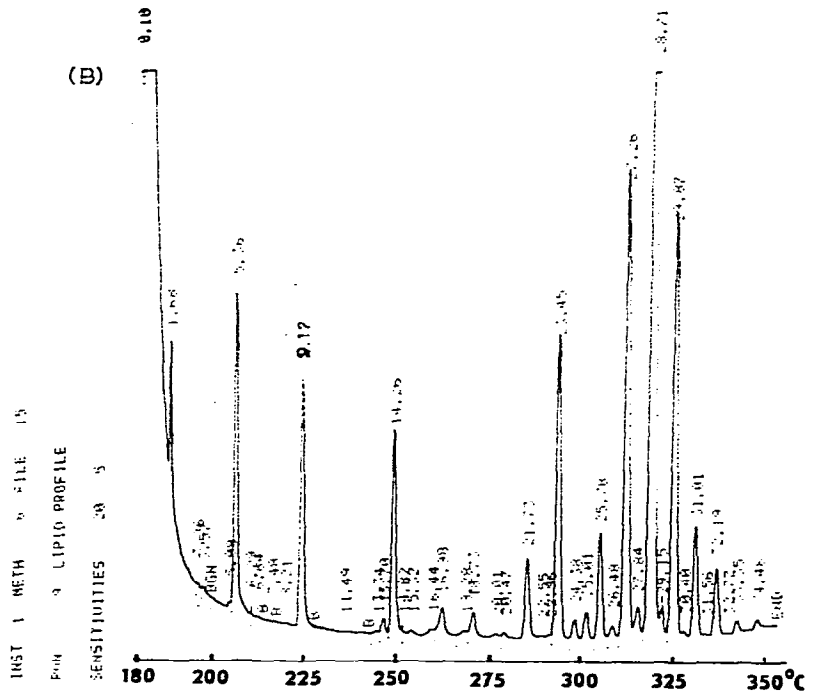
A SURVEY OF THE ANALYTICAL RESULTS OBTAINED WITH MODEL SYNTHETIC SAMPLES

Volume injected = 2 μl. The results are expressed in mg/dl. Instrument: Perkin-Elmer F-30.

Compound* (carbon number)	Sample** K-3A		K-3B		K-4B	
	Given	Found ± S.D.***	Given	Found ± S.D.***	Given	Found ± S.D.***
41	4.25	5.01 ± 0.32	5.50	5.82 ± 0.31	1.25	1.54 ± 0.13
43	40.98	41.75 ± 0.97	41.30	43.21 ± 1.13	12.50	13.28 ± 0.54
45	166.75	168.65 ± 3.78	165.00	166.28 ± 2.69	62.50	60.70 ± 1.73
47	25.00	26.40 ± 0.92	18.30	18.84 ± 0.76	8.75	9.20 ± 0.32
48	4.25	5.03 ± 0.28	5.50	5.50 ± 0.26	1.87	2.11 ± 0.19
50	33.25	32.30 ± 0.89	43.80	44.52 ± 0.65	4.52	4.48 ± 0.21
52	83.25	82.85 ± 1.75	82.50	84.99 ± 1.52	12.50	12.70 ± 0.44
54	41.75	42.02 ± 1.71	48.80	47.66 ± 1.53	6.25	6.48 ± 0.26
FC	62.50	63.40 ± 0.72	68.80	67.01 ± 1.01	21.00	21.88 ± 0.58
CE	237.75	241.03 ± 4.22	230.10	233.17 ± 4.17	85.00	84.72 ± 1.72
TC	204.65	207.48 ± 3.05	206.58	206.42 ± 2.77	71.62	72.50 ± 1.37
TG	162.50	162.18 ± 2.59	180.60	182.07 ± 2.47	24.75	25.76 ± 0.74

*For abbreviations see Table I.
**All samples contained triarachidin (200 ng/μl).
***Each value represents the mean ± S.D. of five measurements obtained within one week.


(A)
180
200
225
250
275
300
325
350°C


(B)
9.10
9.17
180
200
225
250
275
300
325
350°C

of the sample can be seen. The positive differences found with low carbon numbers, especially 41 and 48, are probably caused by the residual effect of the other components because these fractions were lowest in concentration. However, the differences did not exceed the limit of ±3 S.D. as shown by the recovery of the individual fractions and lipid classes.

Quality control

Model samples were also used for quality control. The results obtained over 25 weeks using one column are given in Table IV, and include within-day and day-to-day variation. As shown in the table, the coefficient of variation never

TABLE IV

STATISTICAL EVALUATION OF THE CONTROL SAMPLE K-3B

Instrument: Perkin—Elmer F-30.

Compound* (carbon number)	Actual content (mg/dl)	Variation**							
		Within series		Within-day		Day-to-day		Long-term	
		Mean (mg/dl)	C.V. (%)	Mean (mg/dl)	C.V. (%)	Mean (mg/dl)	C.V. (%)	Mean (mg/dl)	C.V. (%)
41	5.50	5.82	1.24	5.70	3.58	5.70	5.21	5.73	6.13
43	41.30	43.21	1.46	43.37	2.22	43.37	2.79	43.41	3.92
45	165.00	166.28	1.33	166.93	1.61	166.93	4.02	167.19	4.25
47	18.30	18.84	1.64	18.62	2.29	18.62	3.47	18.71	4.00
48	5.50	5.50	2.05	5.54	3.68	5.54	4.95	5.60	6.31
50	43.80	44.52	0.83	44.35	1.90	44.35	2.10	44.22	2.79
52	82.50	84.99	1.05	85.14	1.80	85.14	2.74	84.94	2.95
54	48.80	47.66	2.72	47.96	2.74	47.96	4.06	48.09	4.29
FC	68.80	67.01	0.51	66.93	0.80	66.93	1.33	66.91	1.53
CE	230.10	233.17	1.31	233.56	1.61	233.56	2.75	232.13	3.68
TC	206.58	206.42	0.96	206.65	1.16	206.65	2.26	205.73	2.73
TG	180.60	182.07	0.89	183.11	1.88	183.11	2.73	183.44	3.54

*For abbreviations see Table I.

**The series variation was calculated on the basis of six analyses of the same sample in one run; the within-day and the day-to-day variations were calculated from 28 duplicate analyses of the control sample measured over a period of 14 weeks; the long-term variation was calculated from 75 analyses of the control sample measured over a period of 25 weeks. C.V. = coefficient of variation.

Fig. 4. Separation of triglyceride fraction 46 and cholesteryl ester fraction 47 using hydrogenation of the sample with an atypical triglyceride composition. Individual compounds are identified according to their elution times (min): (A) 5.38 = cholesterol; 9.17 = cholesteryl butyrate (internal standard); 14.32 = cholesteryl benzoate (standard for laboratory control); 15.42—18.46 = diglycerides and decomposition products of phospholipids; 19.80, 21.78, 23.33 = cholesteryl esters of carbon numbers 41, 43, 45, respectively; 24.34 = cholesteryl esters of carbon number 47 and triglycerides of carbon number 46 (not separated); 25.73, 26.36, 27.11, 27.60, 28.44, 29.56, 30.80, 31.95, 33.17, 34.29 = triglycerides of carbon numbers 48, 49, 50, 51, 52, 54, 56, 58, 60, 62, respectively. (B) 5.36 = cholesterol; 9.12 = cholesteryl butyrate (internal standard); 14.26 = cholesteryl benzoate (standard for laboratory control); 15.32—20.01 = diglycerides and decomposition products of phospholipids; 20.47, 21.73, 22.55, 23.45, 25.01 = cholesteryl esters of carbon numbers 41, 43, 44, 45, 47, respectively; 24.38, 25.78, 26.48, 27.26, 27.84, 28.71, 29.15, 29.87, 30.40, 31.01, 31.56, 32.19, 33.35, 34.48 = triglycerides of carbon numbers 46, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 60, 62, respectively. Gas chromatograms were recorded by means of a Sigma 10 Data System. Vertical lines under the baseline are the marks of the start and end of the integration. Sample volume, 2 μl; chart speed, 5 mm/min; other analytical conditions are given in the text.

exceeded 6.5%. This finding confirms the possibility of discontinuous analyses and shows good reproducibility of the entire chromatographic system. The within-day variation for the majority of the compounds analysed was 40—75% of the day-to-day variation. Comparison of the serial variation with the within-day, day-to-day and long-term variations shows that the variability of the results is given by changes in the decisive parameters of the chromatographic system. The relatively high values of the variation coefficients in the day-to-day and long-term variation for cholesteryl esters with carbon numbers 43, 45 and 47 are probably caused by the method of integration of the individual peak areas measured by the Perkin-Elmer M-2 Integrator. This integrator is capable of retaining in its memory only four incompletely separated peaks; each further peak detected in a group of incompletely separated peaks results in a horizontal baseline approximation for the first peak measured. Even with a very good separating ability of the columns, cholesteryl esters and triglycerides represent a group of eight incompletely separated peaks. The integration of the cholesteryl ester peaks is affected by the baseline detection before the group of incompletely separated peaks and by the start of the integration of each peak detected with the horizontal baseline approximation. With triglyceride fraction 54, the higher values of the variation coefficients are caused probably by recovery changes during reversible changes in the saturation of the whole system with triglycerides of higher molecular weight. However, the values of the variation coefficients show a very good reproducibility in the analytical results for neutral lipids and their fractions by gas chromatography. The values of the variation coefficients for free cholesterol, cholesteryl esters, total cholesterol and triglycerides measured over the concentration range in which the weight correction factors are dependent on the amount analysed are in full agreement with those measured over the range in which the correction factors are independent of the amount analysed [6].

In addition to the quality control, the accuracy of the results was also checked using enzymatic determination of the total cholesterol and triglyceride levels. The results obtained with 35 duplicate samples are compared in Table V. The values of the correlation coefficients show a very good agreement between the two methods.

TABLE V

COMPARISON OF THE TOTAL CHOLESTEROL AND TRIGLYCERIDE DETERMINATIONS BY GAS CHROMATOGRAPHY AND ENZYMATIC METHODS

Determination	r*	Reference method	Correlation equation	C.V.** (%)
Total cholesterol	0.986	Enzymatic	$y = 13.05 + 0.95x$	3.46
		Chromatographic	$x = 4.28 + 1.02y$	1.06
Triglycerides	0.998	Enzymatic	$y = -1.86 + 0.98x$	2.53
		Chromatographic	$x = -0.80 + 1.01y$	2.16

*The correlation coefficient.

**The coefficients of variation were calculated on the basis of duplicate analyses of identical samples measured by both methods.

The results of a few thousands analyses measured over a period of five years demonstrate that gas chromatography allows the determination of neutral lipids, even in a low concentration range, when the values of the correction factors are dependent on the amount analysed. The column packing should have a low percentage of loading with the stationary phase. The demands on the quality of the whole chromatographic system including the column are much higher for this type of analysis than for analyses in higher concentration ranges, when the correction factors are not dependent on the amount analysed. On the basis of the study of interactions between the compounds analysed and the chromatographic system, a method which enables reproducible results to be obtained was evaluated. The quality control system permits standardization of the gas chromatographic determination of neutral lipids. The quality control made with pure compounds eliminates the problems connected with the standardization by means of lyophilized biological samples. The method is sufficiently sensitive and enables precise measurement of the lipid levels especially in the lipoprotein classes.

The next part of this study will be devoted to the problem of sample preparation, isolation of neutral lipids and clinical applications of the method described.

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