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QUANTITATIVE DETERMINATION OF THE FATTY ACID COMPOSITION OF HUMAN SERUM LIPIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the analysis of the fatty acid composition of human serum lipids with fluorescence detection was examined. Both free and total fatty acids extracted from serum were derivatized with 9-anthryldiazomethane and were analysed using methanol—water (94.7:5.3) as mobile phase. Twelve kinds of fatty acid were detected, both in the free and total fatty acids, and were well separated. Concentrations of individual fatty acids of serum lipids were estimated from an internal standard, heptadecanoic acid. The results correlated well with those from two other quantitative analyses. These results indicate that the high-performance liquid chromatographic analysis of fatty acids is a reliable method for determining individual fatty acids of human serum lipids. The compositions of free fatty acids and total fatty acids of serum lipids were analysed and compared in 27 normal subjects, 27 diabetics, and 20 angina pectoris patients by this method.

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INTRODUCTION

Recent advances concerning the arachidonate cascade reaction revealed that the alteration of the fatty acids composition is an important factor in the development of the various pathological conditions. Thus, thromboxane (TX) A_2 synthesized from arachidonic acid has a potent vasoconstrictive and platelet-aggregation effect and TXA_3 synthesized from eicosapentaenoic acid has a weak activity of platelet aggregation [1]. Dyerberg et al. [2] suggested that the decrease in the ratio of eicosapentaenoic acid to arachidonic acid in blood lipids is one of the risk factors in coronary heart diseases. Accordingly, a non-laborious, unambiguous and rapid analysis for fatty acids is important from the clinical point of view. Gas chromatography (GC) has been used for the analysis of fatty acids [3]. However, it is necessary to esterify the fatty acids before GC analysis, and it is possible that some artifact such as methoxy acid might be formed from mineral acids during esterification [4]. Esterification with boron fluoride–methanol minimized the side-reactions [5], and the use of a capillary column made it possible to separate *cis* and *trans* isomers of fatty acid methyl esters in a single GC run [6]. However, the limited sensitivity of GC means that a large amount of serum (4—10 ml) is required for the analysis of free fatty acids, so it is unsuitable for clinical usage. Advances in high-performance liquid chromatography (HPLC) permit the quantitative analysis of the composition of fatty acids in serum using a fluorescence reagent that labels carboxyl groups [7, 8]. In this study, we have examined the optimal conditions for the analysis of the fatty acid composition of human serum lipids by HPLC with fluorescence detection. We also used the method to determine fatty acid compositions in serum samples from patients with diabetes or angina pectoris.

A preliminary report has already appeared [9].

EXPERIMENTAL

Materials

Serum was obtained from 27 normal persons (age 30—60 years), 27 patients with diabetes mellitus* (age 40—70 years) and 20 with angina pectoris** (age 40—70 years) after overnight fasting. The control subjects were checked in a physical examination and were considered to be in good health. The diabetic subjects had a blood sugar concentration of 150—284 mg per 100 ml under overnight fasting conditions. The authentic fatty acids were purchased from Wako (Osaka, Japan) and Sigma (St. Louis, MO, U.S.A.). 9-Anthryldiazomethane (ADAM) was the product of Funakoshi (Tokyo, Japan). Cholic acid (CA), deoxycholic acid (DCA), litocholic acid (LCA), glycolitocholic acid (GLCA) and L-α-dimyristoylphosphatidylcholine were obtained from Sigma,

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*Diabetic subjects were diagnosed as diabetes mellitus by the definition of the National Institutes of Health (National Diabetes Data Group, Classification and Diagnosis of Diabetes Mellitus and Other Categories of Glucose Intolerance, Diabetes 28 (1979) 1039—1057).

**Angina subjects were diagnosed by treadmill test using standard Bruce Protocol (R.A. Bruce, J.R. Blackmon, J.W. Jones and G. Strait, Pediatrics 32 (1963) 742—745).

methanol for HPLC from Wako, and NEFA kit-k for enzymatic analyses of fatty acids, based on the method of Mizuno et al. [10], from Nippon Shoji Kaisha (Osaka, Japan). Other chemicals used were of reagent grade.

Equipments and conditions for HPLC

Equipment used comprised the following units, obtained from Shimadzu (Kyoto, Japan). The liquid chromatograph system was a Model LC-3A, equipped with a guard column ODS (5 × 0.21 cm I.D.) and two reversed-phase columns of Zorbax ODS (DuPont, Wilmington, DE, U.S.A.) (particle size 5 μm, 15 × 0.46 cm I.D.) linked in series. The column temperature was set at 60°C by a column oven (Model CTO-2A). The separation of an ADAM-derivatized fatty acid mixture was carried out using methanol—water (94.7:5.3) as a mobile phase at a flow-rate of 1.0 ml/min. The eluate was monitored by a fluorescence spectrophotometer (Model RF-500LCA) with a 14-μl flow-cell. The excitation wavelength was set at 365 nm and the emission wavelength at 412 nm [8]. The results were displayed on a data processor, Chromatopac C-R1A.

Extraction of fatty acids from serum

Free fatty acids (FFAs) were extracted from serum according to the method of Itaya and Ui [11]: 0.3 ml of serum were mixed in a stoppered tube with 1 ml of 0.033 mol/l phosphate buffer (pH 6.4) and 6 ml of chloroform containing 56.6 nmol of heptadecanoic acid as an internal standard. FFAs were extracted into chloroform by shaking vigorously for 90 s. After centrifugation (1500 *g*, 5 min), 4 ml of chloroform were taken into the other tube. The solution was flushed with nitrogen and evaporated on a rotary evaporator under reduced pressure. FFAs in the tube were immediately redissolved with 0.4 ml of methanol and mixed with 1.2 ml of an ADAM solution (0.5 mg/ml of methanol) for derivatization.

Total fatty acids (TFAs) of serum lipids were obtained as follows. The total lipids in 0.1 ml of serum were extracted with 8 ml of a Folch solution containing 226 nmol of heptadecanoic acid as internal standard by shaking vigorously for 1 h. After centrifugation (1500 *g*, 5 min), 4 ml of the solution were taken into the other tube and were evaporated under reduced pressure. The lipids were saponified with 10 ml of an alkaline ethanol solution (1 mol/l potassium hydroxide in 90% ethanol) at 100°C for 3 h. After addition of 10 ml of water, the alkaline solution was washed three times with 10 ml of *n*-hexane and acidified to pH 1—2 with 2.5 *M* sulphuric acid. Fatty acids in the solution were extracted three times with 10 ml of *n*-hexane. The *n*-hexane solution was washed with 10 ml of water, flushed with nitrogen, and evaporated on a rotary evaporator under reduced pressure. Fatty acids obtained were immediately redissolved in 0.5 ml of methanol and mixed with 1.5 ml of the ADAM solution for derivatization.

RESULTS

Derivatization of fatty acids with ADAM

Authentic myristic and arachidonic acids were mixed with ADAM for deriva-

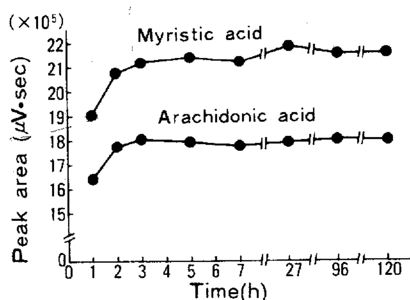


Fig. 1. Time dependence of the binding of fatty acids to ADAM. A 1-ml volume of myristic acid solution (10.0 μg/ml) or arachidonic acid solution (11.3 μg/ml) in methanol was mixed with 3 ml of ADAM solution (0.5 mg/ml of methanol). The mixture was shielded from light and incubated at room temperature (25°C), and 10 μl of each mixture were taken for HPLC analysis at the time shown in the figure. The abscissa is the time after mixing of each fatty acid and ADAM, and the ordinate is the peak area of the derivatized fatty acid printed out by the data processor.

tization. The mixtures were shielded from light and were incubated at room temperature (25° C). The binding of both fatty acids to ADAM was time-depended (Fig. 1). Approximately 90% of the fatty acids were bound to ADAM after 1 h incubation, and the reaction reached a plateau after 3 h. Therefore, fatty acids were incubated with ADAM for over 3 h at room temperature in subsequent experiments.

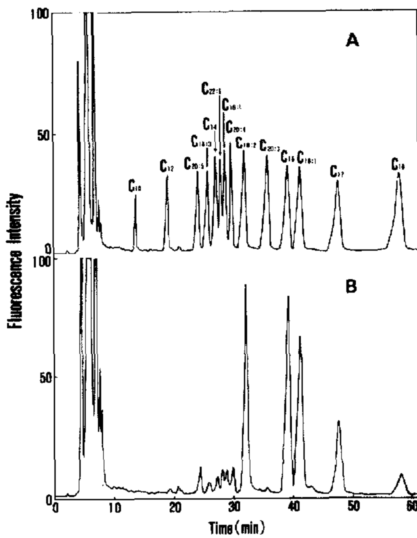
Separation of ADAM-derivatized fatty acids

From the results of examination of HPLC conditions for separating ADAM-derivatized fatty acids of serum lipids, it was concluded that methanol—water (94.7:5.3) was the best mobile phase. The column temperature was set at 60°C in order to decrease the operating pressure. Chromatograms of the derivatized authentic fatty acids and of the derivatized TFAs of serum lipids are shown in Fig. 2. ADAM-derivatized fatty acids of serum lipids were well separated in ca. 60 min under the conditions, and twelve fatty acids (lauric, eicosapentaenoic, linolenic, myristic, docosahexaenoic, palmitoleic, arachidonic, linoleic, eicosatrienoic, palmitic, oleic and stearic acids) were detected in the serum lipids. Heptadecanoic acid, which was used as the internal standard, was detected between oleic acid and stearic acid. Fig. 3 shows the relationship between the retention time and the properties of fatty acids. The retention time of the derivatives increased with an increase in the number of the carbon atoms in the fatty acids and decreased with an increase in the number of double bonds.

Bile acids in serum are also extracted by organic solvent and react with ADAM. Therefore, the retention times of ADAM-derivatized CA, DCA, LCA and GLCA were examined. Derivatized CA, DCA and GLCA were eluted within 10 min, and derivatized LCA at 17 min. All the derivatized bile acids were eluted earlier than the derivatized fatty acids of serum lipids.

Quantitative analyses of fatty acids by HPLC

Since heptadecanoic acid was used as the internal standard, a calibration curve of the fatty acid was linear from 5 pmol up to 125 nmol. The peak area



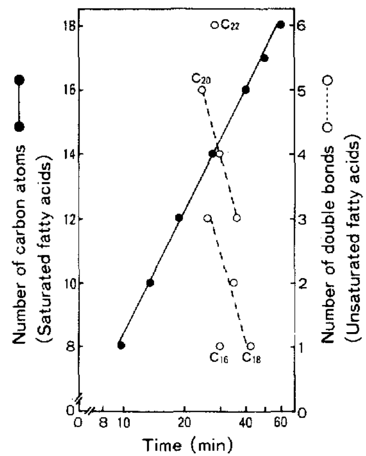


Fig. 2. Chromatograms of ADAM derivatives of authentic fatty acids and total fatty acids of serum lipids. Authentic fatty acids and total fatty acids extracted from serum lipids of a normal subject were derivatized with ADAM and analysed by HPLC. (A) Chromatogram for authentic fatty acids; (B) chromatogram for total fatty acids of serum lipids. Peaks: C_{10} = decanoic acid; C_{12} = lauric acid; $C_{20:5}$ = eicosapentaenoic acid; $C_{18:3}$ = linolenic acid; C_{14} = myristic acid; $C_{22:6}$ = docosahexaenoic acid; $C_{20:4}$ = arachidonic acid; $C_{16:1}$ = palmitoleic acid; $C_{18:2}$ = linoleic acid; $C_{20:3}$ = eicosatrienoic acid; C_{16} = palmitic acid; $C_{18:1}$ = oleic acid; C_{17} = heptadecanoic acid; C_{18} = stearic acid.

Fig. 3. Relationship between the retention time of ADAM-derivatized fatty acids and the number of carbon atoms of fatty acids, and that between the retention time and the number of double bonds of fatty acids.

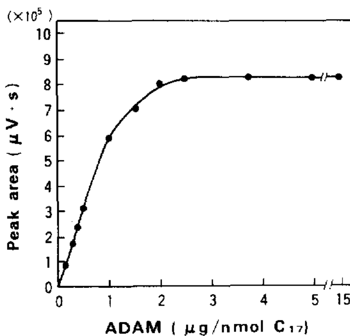


Fig. 4. The amount of ADAM required for derivatization of a fatty acid. A 2-ml volume of 100 μmol/l heptadecanoic acid solution in methanol was mixed with 0.05—6 ml of ADAM solution (0.5 mg/ml of methanol). The mixture was shielded from light and incubated at room temperature overnight. Then 10 μl of the mixture were analysed by HPLC. The abscissa is the amount of ADAM per nmol of heptadecanoic acid (C_{17}), and the ordinate is the peak area of the ADAM derivative per nmol of the fatty acid.

of the derivatized fatty acid increased linearly with increasing concentration of the fatty acid. Thus, with this method, fatty acids could be determined in the range from 1 nmol to ca. 5 pmol, with a detection limit of ca. 10 fmol.

TABLE I

RECOVERY OF THE INTERNAL STANDARD AND REPRODUCIBILITY OF THE DETERMINATION

	Internal standard recovered (mean ± S.D., n = 16) (%)	Reproducibility* (mean ± S.D., n = 8)
FFAs	96.8 ± 3.4	1.00 ± 0.02
TFAs	94.1 ± 4.1	1.01 ± 0.03

*Ratio of two separate determinations.

The capacity of commercially available ADAM (MW 218) to bind fatty acids was examined. The results using heptadecanoic acid are shown in Fig. 4. The peak area of the ADAM-derivatized fatty acid increased linearly until 4.6 nmol of ADAM per nmol of the fatty acid, and reached a plateau at 12 nmol of ADAM per nmol of the fatty acid, indicating that 1 nmol of the fatty acid was fully derivatized in the presence of more than 12 nmol of ADAM. The molar ratio of ADAM to fatty acids used in the following fatty acid analyses of human serum lipids was higher than 12.

Table I shows the recovery of the internal standard and the reproducibility of the determination. The recovery rates were obtained by dividing peak areas of the internal standard in the FFA and TFA analyses by that of the same amount of heptadecanoic acid derivatized without the extraction process. The recovery rates were 97% in the FFA analyses and 94% in the TFA analyses, and the determinations were well reproducible. In the analysis of TFA, TFA preparations were obtained by saponification of serum lipids; therefore the rate of saponification was also examined using authentic dimyristoylphosphatidylcholine. The rate was 100% under the conditions of saponification described in Experimental.

In order to compare the HPLC analysis with two other quantitative analyses,

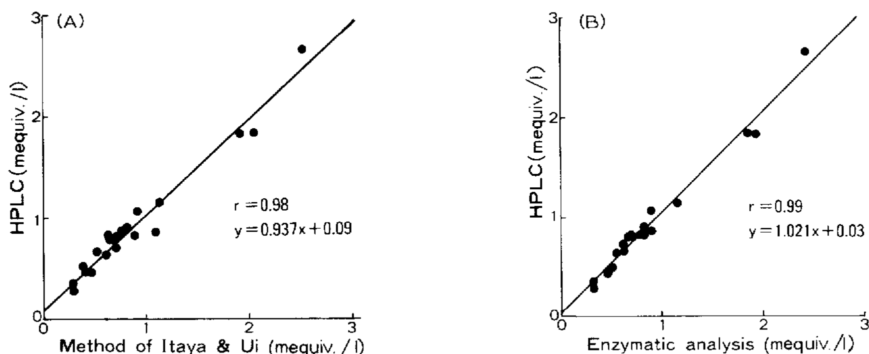


Fig. 5. Correlation between the HPLC analysis and other quantitative analyses of fatty acids. Concentrations of free fatty acids in sera of 22 subjects were determined by HPLC analysis, the method of Itaya and Ui [11], and enzymatic analysis according to Mizuno et al. [10]. (A) HPLC analysis versus method of Itaya and Ui, (B) HPLC analysis versus enzymatic analysis.

FFA concentrations in sera of 22 subjects were measured by the HPLC analysis, the method of Itaya and Ui [11], and enzymatic analysis [10]. As shown in Fig. 5A and B, the HPLC analysis correlated well with both the method of Itaya and Ui ($r = 0.98$, $y = 0.937x + 0.09$) and enzymatic analysis ($r = 0.99$, $y = 1.021x + 0.03$).

TABLE II

FREE FATTY ACID COMPOSITION OF SERUM LIPIDS OF NORMAL, DIABETIC AND ANGINA PECTORIS SUBJECTS

Fatty acids are expressed as the number of carbon atoms and that of double bonds, as shown in Fig. 2. The significance between two groups was assessed using the Student's *t*-test.

Fatty acid	Composition (mean ± S.D.) (%)		
	Normal (n = 27) (35.72 ± 18.87 μmol per 100 ml)	Diabetic (n = 27) (52.94 ± 20.89 μmol per 100 ml)	Angina pectoris (n = 20) (35.06 ± 23.33 μmol per 100 ml)
C_{12}	0.31 ± 0.37	0.48 ± 0.34	0.59 ± 0.51
C_{14}	2.74 ± 0.86	2.60 ± 0.76	2.30 ± 0.64
C_{16}	28.66 ± 2.79	30.19 ± 2.97	26.32 ± 3.55
$C_{16:1}$	4.96 ± 0.77	4.81 ± 1.69	4.87 ± 1.27
C_{18}	4.54 ± 1.61	5.23 ± 2.07	5.19 ± 2.46
$C_{18:1}$	28.29 ± 3.52	29.41 ± 3.61	30.59 ± 5.29
$C_{18:2}$	21.67 ± 3.49	19.60 ± 3.14 ($p < 0.05$)	20.87 ± 3.63
$C_{18:3}$	2.07 ± 0.64	2.16 ± 0.60	2.02 ± 0.50
$C_{20:3}$	0.20 ± 0.24	0.16 ± 0.23	0.17 ± 0.11
$C_{20:4}$	3.00 ± 0.83	2.21 ± 0.72 ($p < 0.01$)	3.95 ± 1.96 ($p < 0.05$)
$C_{20:5}$	0.76 ± 0.36	0.59 ± 0.57	0.36 ± 0.34 ($p < 0.01$)
$C_{22:6}$	2.80 ± 0.85	2.56 ± 1.22	2.77 ± 1.33

TABLE III

TOTAL FATTY ACID COMPOSITION OF SERUM LIPIDS OF NORMAL, DIABETIC AND ANGINA PECTORIS SUBJECTS

Fatty acids are expressed as described in Table II. The significance between two groups was assessed as described in Table II.

Fatty acid	Composition (mean ± S.D.) (%)		
	Normal (n = 27) (1.23 ± 0.26 mmol per 100 ml)	Diabetic (n = 27) (1.52 ± 0.63 mmol per 100 ml)	Angina pectoris (n = 20) (1.22 ± 0.33 mmol per 100 ml)
C_{12}	0.52 ± 0.65	0.42 ± 0.18	0.42 ± 0.30
C_{14}	1.95 ± 0.43	1.83 ± 0.47	1.78 ± 0.38
C_{16}	25.29 ± 2.44	25.24 ± 5.00	24.25 ± 2.59
$C_{16:1}$	3.04 ± 0.64	3.82 ± 1.43 ($p < 0.05$)	3.34 ± 1.02
C_{18}	8.37 ± 2.95	6.94 ± 1.15 ($p < 0.05$)	7.82 ± 1.08
$C_{18:1}$	19.90 ± 2.43	21.94 ± 5.81	19.72 ± 3.06
$C_{18:2}$	29.39 ± 3.95	27.76 ± 7.48	29.75 ± 5.16
$C_{18:3}$	0.99 ± 0.30	1.16 ± 0.43	1.12 ± 0.43
$C_{20:3}$	0.96 ± 0.16	0.99 ± 0.32	0.97 ± 0.30
$C_{20:4}$	5.74 ± 1.00	5.77 ± 1.42	6.29 ± 1.40
$C_{20:5}$	1.43 ± 0.77	1.49 ± 1.26	1.47 ± 0.91
$C_{22:6}$	2.45 ± 0.80	2.63 ± 1.17	2.98 ± 0.84

FFA and TFA compositions of serum lipids of normal healthy volunteers and patients

Table II shows the concentrations and compositions of FFAs of serum lipids in 27 normal subjects, 27 diabetics and 20 angina pectoris patients. The concentration in the diabetics increased more rapidly than that in the normal subjects. In the composition of FFAs, linoleic ($C_{18:2}$) and arachidonic ($C_{20:4}$) acids decreased significantly in the diabetics compared with normal subjects. Patients with angina pectoris show an increase in arachidonic acid ($C_{20:4}$) and a decrease in eicosapentaenoic acid ($C_{20:5}$).

Table III shows the concentrations and compositions of TFAs of serum lipids in the normal and the diabetic subjects. The concentration in the diabetics increased more rapidly than that in the normal subjects. In the composition of TFAs, palmitoleic acid ($C_{16:1}$) increased and stearic acid (C_{18}) decreased in the diabetics compared with the normal subjects. There was no significant difference between the TFA compositions of normal subjects and patients with angina pectoris.

DISCUSSION

Fatty acids extracted from human serum lipids reacted with ADAM in methanol at room temperature (25°C), and the derivatives of fatty acids were formed for HPLC analysis (Fig. 1). The procedure is simple and there is no risk of artifact formation during the reaction under such mild conditions. Tsuchiya et al. [12] used the fluorescent reagent 4-bromomethyl-7-acetoxycoumarin, which forms stable prostaglandin esters. However, this reagent needs a more complicated procedure than ADAM for the esterification, and the sensitivity of detection is comparable with that for ADAM.

Fatty acids increase in hydrophobicity when ADAM is bound to the carboxyl group: therefore the derivatives are retained longer on a reversed-phase column than fatty acids. Good separation of individual fatty acids of serum lipids was achieved by the longer retention on the column.

Barker et al. [7], and Nimura and Kinoshita [8] used acetonitrile—water as mobile phase in HPLC analyses of ADAM-derivatized fatty acids. Since individual fatty acids of serum lipids were not separated enough by this mobile phase, we used methanol—water (94.7:5.3), resulting in successful separation of individual fatty acids of serum lipids (Fig. 2). In our analyses, twelve fatty acids were detected in human serum lipids (Fig. 2). Although eleven to fifteen fatty acids of serum lipids were reported from other studies [13—15] in which gas—liquid chromatography was used, pentadecanoic and heptadecanoic acids, which are not generally observed in serum lipids, were included in the fatty acid composition. Therefore, almost all the fatty acids of serum lipids could be detected in our analyses. Moreover, it was confirmed that ADAM derivatives of bile acids, which are extracted by organic solvents and can bind to ADAM, do not interfere with the analysis of fatty acids of serum lipids.

Since we used heptadecanoic acid as internal standard, the separated fatty acids of serum lipids were correctly estimated from the chromatogram. The fatty acids could be determined in the range from 1 nmol to ca. 5 pmol. The recovery rates of the internal standard were more than 94% in both FFA and

TFA analyses (Table I). The results from the HPLC analysis correlated well with those from other quantitative analyses of FFAs in serum (Fig. 5A and B). These results indicate that the HPLC analysis in our system is a reliable quantitative analysis of the individual fatty acids of serum lipids.

In analyses of FFA and TFA compositions of serum lipids in normal and diabetic subjects, the concentrations of the major fatty acids in FFAs and TFAs of diabetics increased more rapidly than those of normal subjects, resulting in the higher concentrations of FFAs and TFAs in diabetics than in normal subjects (Tables II and III). When percentages of the individual fatty acids in FFAs and TFAs were considered, diabetics showed less than normal levels of linoleic and arachidonic acids in FFAs and stearic acid in TFAs, but greater than normal levels of palmitoleic acid in TFAs. These results are almost consistent with those in another report [15]. We also confirmed a decrease in eicosapentaenoic acid and an increase in arachidonic acid in patients with angina pectoris, as reported by Dyerberg et al. [2].

In conclusion, the HPLC method presented here for the analysis of the fatty acid composition of serum lipids is applicable to clinical use.

REFERENCES

1 P. Needleman, A. Raz, M.S. Minkes, J.A. Ferrendelli and H. Sprecher, Proc. Natl. Acad. Sci. U.S.A., 76 (1979) 944.
2 J. Dyerberg, H.O. Bang, E. Stoffersen, S. Moncada and J.R. Vane, Lancet, ii (1978) 117.
3 R.G. Ackman, Methods Enzymol., 14 (1969) 329.
4 R.P. Hansen and J.F. Smith, Lipids, 1 (1966) 316.
5 W.R. Morrison and L.M. Smith, J. Lipid Res., 5 (1964) 600.
6 H. Jaeger, H.U. Klör and H. Ditschuneit, J. Lipid Res., 17 (1976) 185.
7 S.A. Barker, J.A. Monti, S.T. Christian, F. Benington and R.D. Morin, Anal. Biochem., 107 (1980) 116.
8 N. Nimura and T. Kinoshita, Anal. Lett., 13 (1980) 191.
9 Y. Shimomura, K. Taniguchi, T. Sugie, M. Murakami, S. Sugiyama and T. Ozawa, Clin. Chim. Acta, 143 (1984) 361.
10 K. Mizuno, M. Toyosato, S. Yabumoto, I. Tanimizu and H. Hirakawa, Anal. Biochem., 108 (1980) 6.
11 K. Itaya and M. Ui, J. Lipid Res. 6 (1965) 16.
12 H. Tsuchiya, T. Hayashi, H. Naruse and N. Takagi, J. Chromatogr., 234 (1982) 121.
13 B. Hallgren, S. Stenhagen, A. Svanborg and L. Svennerholm, J. Clin. Invest., 39 (1960) 1424.
14 W. Schrade, E. Boehle, R. Biegler and E. Harmuth, Lancet, i (1963) 285.
15 N. Tuna, S. Frankhauser and F.C. Goetz, Am. J. Med. Sci., 255 (1968) 120.