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ANALYSIS OF TRIACYLGLYCEROLS BY COMBINED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the analysis of the triacylglycerols from animal and human adipose tissue has been developed by combining high-performance liquid chromatography and gas–liquid chromatography (HPLC) and (GLC). Following isolation by thin-layer chromatography, the triacylglycerols are chromatographed by HPLC on a 5-μm Spherisorb ODS column with a gradient of 45 to 60% 2-propanol in acetonitrile as the mobile phase. Fractions eluted from the column are collected at intervals ranging from 0.2 to 1.0 min, and the triacylglycerols present are then transesterified prior to analysis of the resulting fatty acid methyl esters by an automated packed column GLC system. Triacylglycerols eluted from the column may be identified by a combination of their elution volume in HPLC and their fatty acid content. From the GLC data, profiles can be constructed for the distribution of several fatty acids amongst the triacylglycerols present in the adipose tissue sample. The method can be used with solvents such as acetonitrile, 2-propanol and acetone in the mobile phase, and there is no baseline drift during gradient elution. The adipose tissue triacylglycerols of normal and streptozotocin induced diabetic rats have been compared. No differences in the distribution of the fatty acids in the two groups of rats were observed under the conditions of our study.

INTRODUCTION

The triacylglycerol composition of human adipose tissue has been studied by several workers. A common analysis procedure is the hydrolysis of the triacylglycerols, followed by analysis of the component fatty acids or their derivatives by gas–liquid chromatography (GLC)[1,2]. In some cases, a stereospecific hydrolysis has been used[3].

We wish to determine the distribution of saturated and unsaturated fatty acids amongst the different triacylglycerols in adipose tissue and how this distribution may be affected by diet and by diabetes. In order to separate the complex mixture of triacylglycerols present in adipose tissue, we have used non-aqueous reversed-phase high-performance liquid chromatography (HPLC) after a preliminary isolation of the triacylglycerol fraction by thin-layer chromatography (TLC). We have then an-

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alysed the fatty acids in the triacylglycerol fractions eluted from the HPLC by a transmethylation procedure, followed by GLC. Using regular sample collection in HPLC, we can then construct a quantitative profile for the distribution of each fatty acid in the tissue.

Non-aqueous HPLC has been used extensively for the separation of natural mixtures of triacylglycerols[4–20]. Reviews on the use of HPLC for triacylglycerol analysis have been presented by Aitzetmüller[21] and Plattner[22]. The major difficulty in the HPLC of triacylglycerols is that of detection[16,21,22]. Traditional modes of detection, such as refractive index and ultraviolet absorbance offer only low sensitivity towards triacylglycerols[16,21]. It is not normally possible to enhance sensitivity towards these molecules by derivatisation without disruption of their original structure. Applications of the use of ultraviolet detection at low wavelengths (190–237 nm) have been reported[12,16]. Detection is based upon the absorbance caused by the ester carbonyl group and isolated double bonds. Baseline drift may occur during the operation of gradients, and solvents which absorb strongly at these wavelengths, such as acetone, cannot be used in the mobile phase. Refractive-index detection has been most popular for triacylglycerol analysis by HPLC[4–15], but this mode of detection is much less sensitive to triacylglycerols than UV detection and is incompatible with the use of gradient elution. The infra-red detector gives similar sensitivity to that of the refractive-index detector, but is compatible with gradient elution[22]. The major constraint is finding a suitable absorption window for monitoring. Triacylglycerols are normally detected at 5.75 μm. Quantitation of peaks by these modes of detection is difficult, since in natural samples each peak may contain more than one species of triacylglycerol, and there may be a difference in detector response to saturated and unsaturated triacylglycerols. Flame ionisation[18–20] and mass detection[17,22] have been shown to be suitable for monitoring triacylglycerols when a pure, volatile solvent is used as the mobile phase. Several applications of the use of liquid chromatography–mass spectrometry (LC–MS) to the separation of triacylglycerols have been reported[23–26]. Some workers[4,8] have collected peaks in HPLC for analysis of the fatty acids of the triacylglycerols by GLC.

To solve problems of detection and to aid the identification of peaks, we have collected the material eluted from the HPLC column at intervals ranging from 0.2 to 1.0 min with a fraction collector. The triacylglycerols present in each fraction are then transesterified to fatty acid methyl esters prior to analysis on an automated, packed-column GLC system. Identification of a triacylglycerol peak is based upon a combination of its fatty acid composition and its elution time in HPLC.

Plattner *et al.*[4] observed that under isocratic conditions the logarithm of the elution volume of a triacylglycerol is directly proportional to the total number of carbon atoms (CN) and inversely proportional to the total number of double bonds (DB) in the three fatty acyl chains. This elution behavior is controlled by the partition number (PN) of a triacylglycerol[15], which may be defined as $PN = CN - nDB$, where n is the factor for double-bond contribution and is normally close to 2. No distinction is made here between triacylglycerols which are positional isomers. We have used this behavior to predict the approximate elution volume of a mixed triacylglycerol (one which contains two or three different fatty acids) from the elution volumes of unmixed triacylglycerols, used as standards. Identification of an eluted triacylglycerol, therefore, is based upon a combination of its elution volume in HPLC and its

fatty acid content, determined by GLC. We have applied these methods to the analysis of adipose tissue triacylglycerols from normal and diabetic rats.

EXPERIMENTAL

Streptozotocin, triacylglycerol standards, and reference samples of fatty acid methyl esters were obtained from Sigma (Poole, U.K.). All solvents were of AnalaR or HPLC grade.

Rats

The fourteen rats used were male Sprague-Dawley, weighing *ca.* 300–400 g. To induce diabetes, eight of these rats were given a single tail-vein injection of streptozotocin (50 mg/kg body weight) in 0.1 ml citrate-buffered isotonic saline (pH 4.5). After 4–6 days, the rats were killed by cervical dislocation and the adipose tissue was removed from an epididymal pad.

HPLC

An Altex 100A dual-piston pump and Altex 110A pump were controlled by an Altex 420 programmer (Altex Scientific, Berkeley, CA, U.S.A.), and the solvents from the pumps were passed through an Altex solvent-mixing chamber. A Rheodyne 7126 injection valve (Rheodyne I, Cotati, CA, U.S.A.) with a 20-μl loop and a column (25 cm × 4.6 mm I.D.) packed with 5-μm Spherisorb ODS (Phase Separations, Queensferry, U.K.) were used. The mobile-phase composition was 2-propanol–acetonitrile (45:55) for 30 min, then to 2-propanol–acetonitrile (60:40) in 15 min, at a flow-rate of 1.0 ml/min. Ultraviolet detection was carried out with a Kratos Spectroflow 757 detector (Kratos Analytical Instruments, Ramsey, NJ, U.S.A.), having a cell volume of 12 μl, at a wavelength of 213 nm. A LKB 7000 UltroRac fraction collector (LKB, Bromma, Sweden) was used to collect material emerging from the detector.

GLC

GLC of fatty acid methyl esters was carried out with a Hewlett-Packard 5710A gas chromatograph, equipped with a flame-ionisation detector and Hewlett-Packard 7672A autosampler. A 1-μ volume of each methyl ester solution was analysed isothermally on a glass column (6 ft. × 2 mm I.D.), packed with 10% SP-2330 on 100–120 Chromosorb W AW (Supelchem, Sawbridgeworth, U.K.), using nitrogen as the carrier gas at a flow-rate of 25 ml/min and an oven temperature of 155°C. Integration and autosampler control were performed by a Hewlett-Packard 3388A integrator.

Methods

A sample of adipose tissue from an epididymal fat pad (approximately 50 mg) was removed from each rat and homogenised in 15 ml chloroform–methanol (2:1). The homogenate was filtered through a 0.45-μm pore-size PTFE filter (Millipore SA, Molsheim, France) and, after removal of the solvent under a stream of nitrogen, the residue was dissolved in 2 ml chloroform. Approximately 400 μl of this was chromatographed on a Silica Gel 60 TLC plate (BDH Chemicals, Poole, U.K.), using

hexane–diethyl ether–acetic acid (85:25:1) as the developing solvent. The triacylglycerol fraction was removed from the plate with chloroform–methanol (2:1). Approximately 1 mg triacylglycerol, dissolved in 20 μl acetone, was injected into the HPLC apparatus. Ten min after injection, fractions were collected from the HPLC column at intervals of 0.2 min. After 20 min, this interval was increased in stages to a final value of 1.0 min, to take into account band-broadening. Triheptadecanoin (16 μg) in acetone was then added to each fraction to act as an internal standard for the quantitation of the GLC peaks. The triacylglycerols were redissolved in diethyl ether and transesterified to fatty acid methyl esters by sodium methoxide, according to the method of Christie[28]. After removal of the diethyl ether by a stream of nitrogen, the fatty acid methyl esters were taken up in 200 μl *n*-dodecane, and 1 μl of this was analysed by packed-column gas–liquid chromatography. The fatty acids analysed were palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids.

RESULTS

Figs. 1 and 2 compare the distribution of the most abundant fatty acids in the triacylglycerols obtained by GLC detection following HPLC fractionation of the triacylglycerols. The system was previously calibrated for these fatty acids.

The majority of the triacylglycerols of rat adipose tissue were found to be eluted at retention times between 10 and 50 min. Fig. 3 shows the elution pattern of the triacylglycerols between 23 and 34 min obtained by measuring the amounts of the individual fatty acids in the eluted triacylglycerols by GLC. We have chosen to show this particular portion of the chromatogram, since it gives a clear demonstration of the way in which GLC analysis of eluted components may be used for the identification of eluted triacylglycerols. The amounts of oleic and palmitic acids in the triacylglycerols eluted from the column were plotted with respect to retention time. These two acids are the major constituents of the triacylglycerols eluted at this time. The triacylglycerols containing these fatty acids can be identified by a combination of elution volume and fatty acid profile. For example, on the basis of the fatty acid data alone, there are various possible explanations for the peak labelled POP in Fig. 3, in which palmitic and oleic acids are eluted in the ratio 2:1. One possibility is that the peak is oleoyldipalmitoyl glycerol (POP). It is also possible that the peak is a mixture of tripalmitin (PPP) and triolein (OOO). Yet another possibility is a mixture of POO and PPP. All of these possibilities, with the exception of POP, may be eliminated on the basis of their HPLC elution volumes. Of the oleate- and palmitate-containing triacylglycerols, only POP would be expected to be eluted at 27 min. Although the retention times of the various triacylglycerols may vary slightly from one analysis to the other, their elution volumes relative to each other remain constant. This approach will not, however, distinguish between positional isomers, since these are eluted together under these conditions.

DISCUSSION

Our work demonstrates the advantages of HPLC–GLC for the analysis of triacylglycerols over methods which employ traditional modes of detection. Our method gives information about the nature of the eluted peaks, including those which

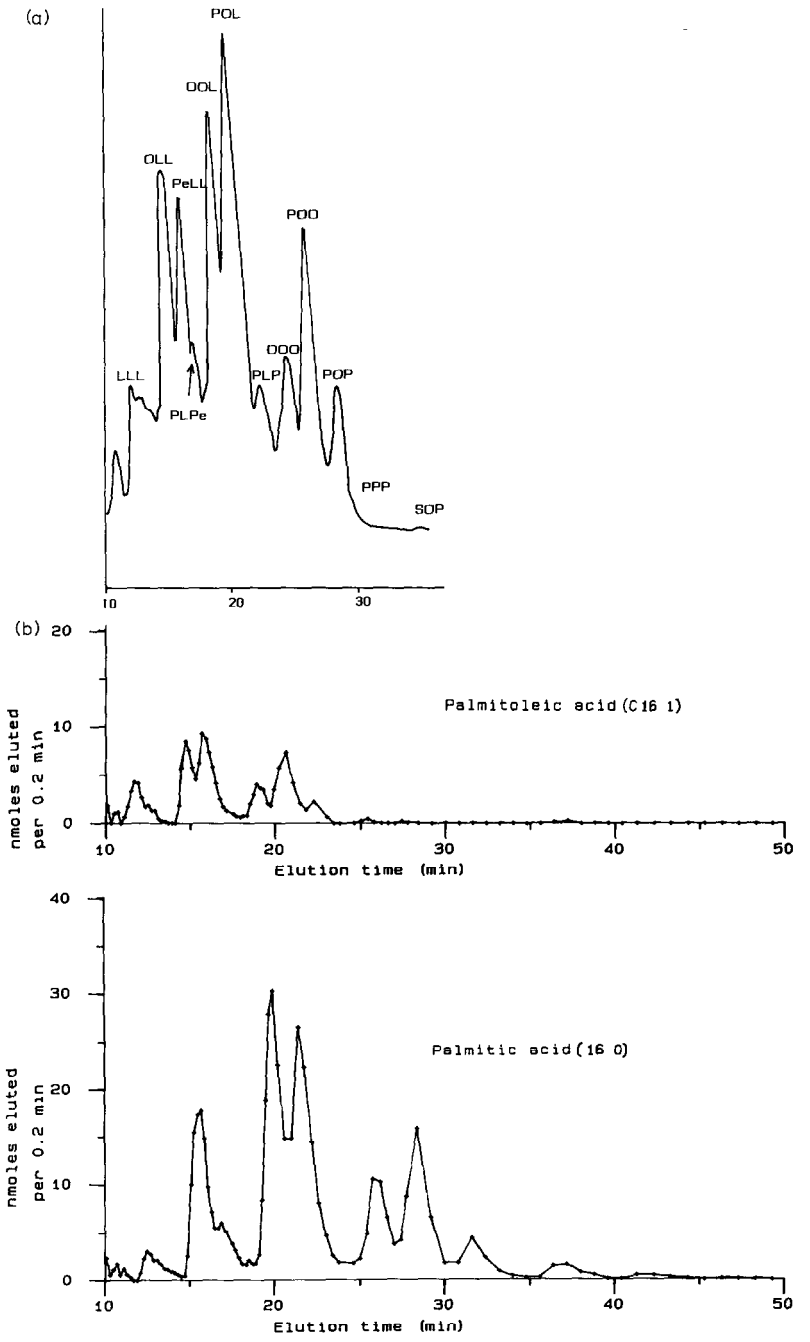

Fig. 1. (a) Chromatogram of rat adipose tissue triacylglycerols obtained by HPLC and UV detection at 213 nm. For details, see text. (b) Distribution of palmitic and palmitoleic acids in the triacylglycerols, eluted from the HPLC column. P = palmitic acid; Pe = palmitoleic acid; S = stearic acid; O = oleic acid; L = linoleic acid. For conditions see text.

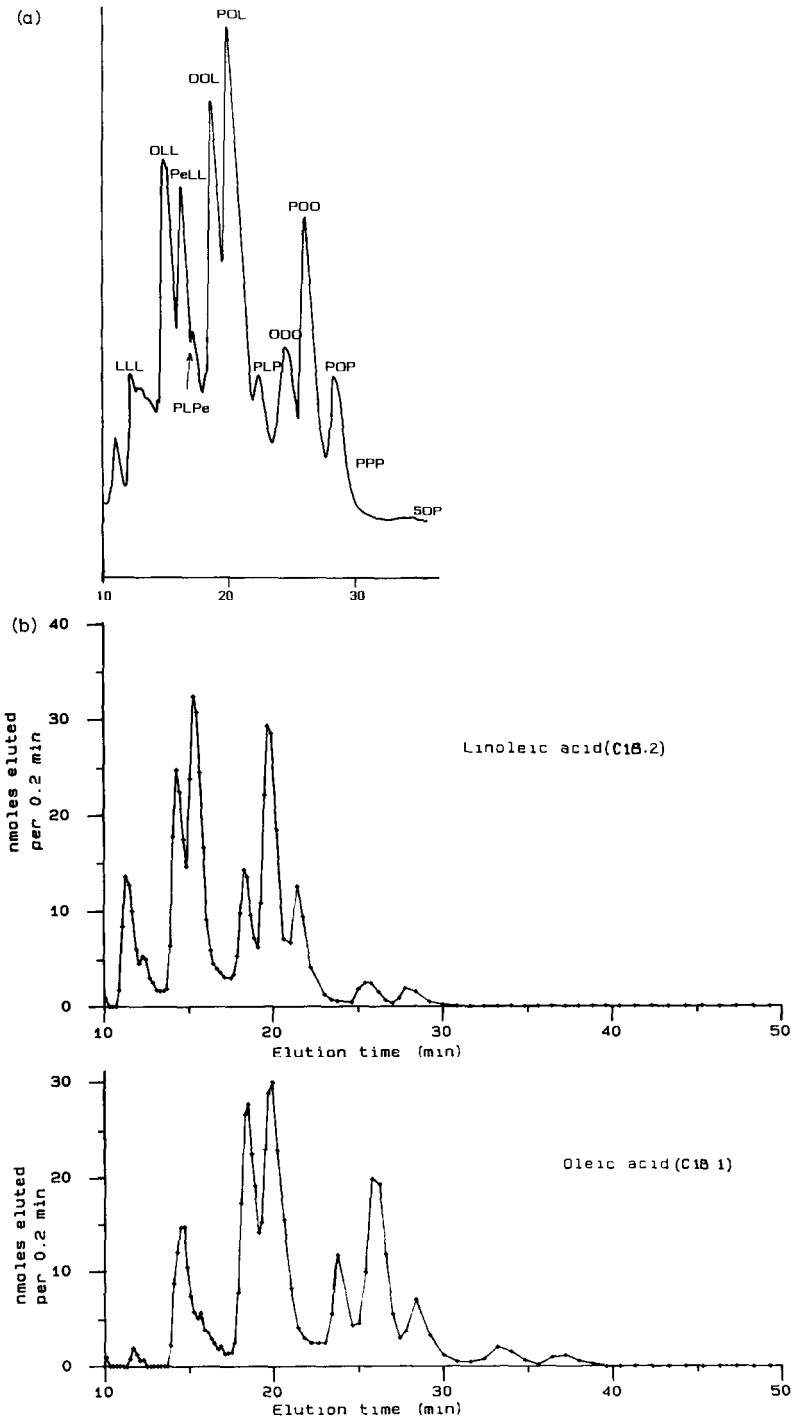

Fig. 2. (a) Chromatogram of rat adipose tissue triacylglycerols obtained by HPLC with UV detection at 213 nm. (b) Distribution of oleic and linoleic acids in the triacylglycerols eluted from the HPLC column. Conditions and identification as for Fig. 1.

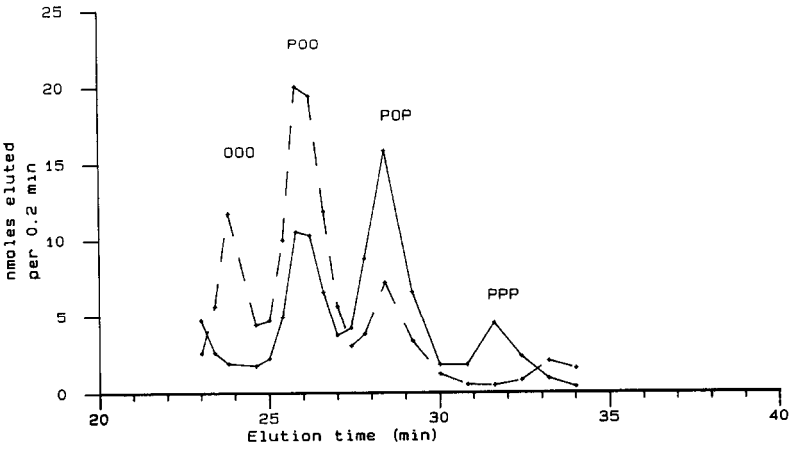

Fig. 3. Elution of palmitic and oleic acids in the triacylglycerols between 23 and 34 min. For identification, see Fig. 1. (------) Oleic acid, (——) palmitic acid.

are mixed or overlap, providing valuable clues for identification. HPLC–GLC is usable with gradient elution. The tedium of analysing the collected fractions by GLC is eliminated by the use of automated GLC analysis and data collection. This system has been used to measure palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids in the triacylglycerols. Because tripalmitolein and trilinolein are eluted closely together in this system, we are unable to separate triacylglycerols containing palmitoleoyl and linoleoyl groups completely. The sensitivity of the method is limited by the amount of triacylglycerol which may be applied to the HPLC column and by the fact that the methyl esters need to be dissolved in 200 μl of solvent for the analysis of approximately 1 μl by automated GLC. This allows us to measure only those fatty acid methyl esters which are present in the transmethylated fractions in amounts greater than 0.4 μg. At this point, we are unable to detect the less abundant fatty acids, such as arachidonic acid, which we know to be present in the plasma of rats. Quantitation of these acids may require the higher efficiency and sensitivity of capillary GLC.

Many investigators have used acetone–acetonitrile mixtures as a mobile phase for the HPLC of triacylglycerols. We have found that the separation obtained with this type of eluent is similar to that with 2-propanol–acetonitrile.

No significant differences were noted between the triacylglycerol profiles of normal and diabetic rats. This may not be surprising, since these animals were sacrificed only 4–6 days after exposure to streptozotocin. Rats may require a much longer period in a diabetic state in order to reveal any possible changes which may occur in the triacylglycerol makeup of the adipose tissue. The half-life of fatty acids in human adipose tissue is of the order of 600 days[29]. Additionally, although streptozotocin-induced diabetes raises the levels of plasma fatty acids in the rat, the relative proportions of these acids remain essentially unchanged. At present, we are unable to distinguish between positional isomers of triacylglycerols, since these compounds are eluted together from the HPLC column. The application of stereospecific analysis to collected fractions would be worthwhile.

We are currently investigating the use of columns packed with 3-μm particles, which have been shown to improve the separation of triacylglycerols[12]. We are applying these methods to the study of rat and human adipose tissue triacylglycerols and effects of such factors as diabetes and, particularly, diet on the incorporation of the various fatty acids into triacylglycerols.

REFERENCES

1 B. K. Jacobsen, K. Trygg, I. Hjermann, M. S. Thomassen, C. Real and K. R. Norum, *Am. J. Clin. Nutr.*, 38 (1983) 906.
2 T. Plakke, J. Berkel, A. C. Beynen, R. J. J. Hermus and M. B. Katan, *Hum. Nutr. Appl. Nutr.*, 37A (1983) 365.
3 W. Breckenridge, in A. Kuksis (Editor), *Handbook of Lipid Research, 1· Fatty Acids and Glycerides*, Plenum Press, New York, 1978, pp. 197–232.
4 R. D. Plattner, G. F. Spencer and R. Kleiman, *J. Am. Oil Chem. Soc.*, 54 (1977) 511.
5 B. Herslof, O. Podlaha and B. Toregard, *J. Am. Oil Chem. Soc.*, 56 (1979) 864.
6 J. A. Bezard and M. A. Ouedraogo, *J. Chromatogr.*, 196 (1980) 279.
7 A. H. El-Hamdy and E. G. Perkins, *J. Am. Oil Chem. Soc.*, 58 (1981) 49.
8 A. H. El-Hamdy and E. G. Perkins, *J. Am. Oil Chem. Soc.*, 58 (1981) 867.
9 B. Petersson, O. Podlaha and B. Toregard, *J. Am. Oil Chem. Soc.*, 58 (1981) 1005.
10 G. W. Jensen, *J. Chromatogr.*, 204 (1981) 407.
11 E. Geeraert and D. De Schepper, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 6 (1983) 123.
12 M. W. Dong and J. L. DiCesare, *J. Am. Oil Chem. Soc.*, 60 (1983) 788.
13 N. A. Parris, *J. Chromatogr.*, 149 (1978) 615.
14 E. G. Perkins, D. J. Hendren, N. Pelick and J. E. Bauer, *Lipids*, 17 (1982) 460.
15 K. Takahashi, T. Hirano and K. Zama, *J. Am. Oil Chem. Soc.*, 61 (1984) 1226.
16 J. A. Singleton and H. W. Patee, *J. Am. Oil Chem. Soc.*, 61 (1984) 761.
17 J. L. Robinson and R. Macrae, *J. Chromatogr.*, 303 (1984) 386.
18 F. C. Phillips, W. L. Erdahl, J. D. Nadenicek, L. J. Nutter, J. A. Schmit and O. S. Privett, *Lipids*, 19 (1984) 142.
19 F. C. Phillips, W. L. Erdahl, J. A. Schmit and O. S. Privett, *Lipids*, 19 (1984) 880.
20 O. S. Privett and W. L. Erdahl, *Anal. Biochem.*, 84 (1978) 449.
21 K. Aitzetmüller, *Prog. Lipid Res.*, 21 (1982) 171.
22 R. D. Plattner, *Methods Enzymol.*, 72 (1981) 21.
23 J. J. Myher, A. Kuksis, L. Marai and F. Manganaro, *J. Chromatogr.*, 283 (1984) 289.
24 L. Marai, J. J. Myher and A. Kuksis, *Can. J. Biochem. Cell Biol.*, 61 (1983) 840.
25 A. Kuksis, J. J. Myher and L. Marai, *J. Am. Oil Chem. Soc.*, 61 (1984) 1582.
26 A. Kuksis, J. J. Myher and L. Marai, *J. Am. Oil Chem. Soc.*, 62 (1985) 767.
27 A. Kuksis, L. Marai and J. J. Myher, *J. Chromatogr.*, 273 (1983) 43.
28 W. W. Christie, *J. Lipid Res.*, 23 (1982) 1072.
29 J. Hirsch, J. W. Farquhar, E. H. Ahrens, M. L. Peterson and W. Stoffel, *Am. J. Clin. Nutr.*, 8 (1960) 499.