Journal of Chromatography, 619 (1993) 21–28
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7016

Combined enzymic and chromatographic techniques to determine specific radioactivity in free and triglyceride fatty acid plasma fractions

Isabel Lladó, Andreu Palou* and Antoni Pons

Bioquímica i Biologia Molecular, Departament de Biologia Fonamental i Ciències de la Salut, Universitat de les Illes Balears i Institut d'Estudis Avançats (UIBCSIC), Cra Valldemossa km. 7.5, 07071 Palma de Mallorca (Spain)

(First received March 23rd, 1993; revised manuscript received May 28th, 1993)

ABSTRACT

A reliable method to determine both free and triglyceride fatty acids and simultaneously to determine the specific radioactivity in each fraction has been developed. The procedure can be used to analyse a large number of samples. Lipoprotein lipase was used to hydrolyse triglyceride fatty acids, and a Carbopack B column was used to isolate free fatty acids. The radiolabeled fatty acids were determined by liquid scintillation counting, and individual fatty acid levels in each fraction were determined by gas chromatography. Free and triglyceride fatty acids were eluted in different fractions from the Carbopack B column. No interferences from other compounds were significant.

INTRODUCTION

There are methods combining solvent extraction, chemical derivatization and chromatography to determine free fatty acids in biological samples. However, there are no methods to determine both individual free fatty acids (FFA) and triglyceride fatty acids (EFA) and simultaneously to determine their specific radioactivity in these fractions. This possibility seems especially useful in metabolic studies involving radiolabeled precursors.

We have previously developed methods in which enzymic and chromatographic techniques are combined to determine both specific radioactivity and levels of compounds of metabolic interest, such as alanine [1], lactate [2], pyruvate [3] and hydroxybutyrate [4]. The general basis of these methods is a specific, generally enzymic, conversion of the hydrophilic analyte into a hydrophobic product, which can be isolatead by eluting the sample (in a single chromatographic step) using hydrophobic porous resins such as Amberlite.

Recently, Carbopack B has been successfully used as an adsorbing medium to isolate FFA [5] and other acidic compounds [6–10] from blood serum. In this paper, we propose a method to determine simultaneously total FFA and total EFA and their radioactivity using lipoprotein lipase (LPL) to hydrolyse EFA, Carbopack B to isolate FFA, and scintillation counting to determine the radioactivity. In the samples treated as described here, there is no problem in carrying out a determination of the different fatty acid levels by gas chromatography (GC), separately in the FFA and EFA fractions.

* Corresponding author.

0378-4347/93/$06.00 © 1993 Elsevier Science Publishers B.V. All rights reserved

EXPERIMENTAL

Apparatus

A Hitachi U-2000 spectrophotometer for absorbance measurements and a Beckman LS-3801 liquid scintillation counter for radioactivity measurements were used.

A Hewlett Packard 5890A gas–liquid chromatograph equipped with a flame-ionization detector was used. The SP-2330 fused-silica capillary column, 30 m × 0.25 mm I.D. and 0.20 μm df, was from Supelco (Bellefonte, PA, USA). Both injector and detector temperatures were maintained at 220°C. Helium was used both as the column carrier and the detector make-up gas, at flow-rates of 2 and 18 ml/min, respectively. The capillary injection port was used in the splitless mode. For analysis, 1 μl of the final sample was injected into the column using a Hewlett Packard 7630A automatic injector. The oven temperature was programmed at 70°C for 0.5 min, was then increased to 190°C at 10°C/min, and was then held at 190°C for 13.5 min.

Reagents

Solvents of analytical grade were from Fluka (Buchs, Switzerland). [1-^{14}C]Oleic acid (104 Bq/nmol), tri[1,2,3-^{14}C]oleate (25 Bq/nmol), [^{14}C]-phenylalanine (33 Bq/nmol), [^{14}C]lysine (7 Bq/nmol), [^{14}C]alanine (7 Bq/nmol), [^{14}C]glycine (17 Bq/nmol), [^{14}C]leucine (7 Bq/nmol), [^{14}C]-glutamine (4 Bq/nmol), [^{14}C]glutamate (33 Bq/nmol) and [^{14}C]saccharose (0.66 Bq/nmol) were obtained from Amersham Ibérica S.A. (Madrid, Spain).

Triglyceride [INT] reagent for the enzymic determination of triglycerides and the lipase inhibitor (*p*-toluenesulphonyl fluoride, TSF) were from Sigma (St. Louis, MO, USA). The Triglyceride [INT] reagent, when reconstituted according to the directions, contains the following concentrations of active ingredients: ATP 2 m*M*, NAD^+ 2 m*M*, Mg^{2+} 3 m*M*, 2-(*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT) 1 m*M*, glycerol kinase (microbial) 200 U/l, glycerol-1-phosphate dehydrogenase (rabbit muscle) 4000 U/l, lipase (microbial) as required for the complete hydrolysis, diaphorase (microbial) 455 U/l, buffer pH 7.8 ± 0.1.

Diazomethane to methylate fatty acids was freshly synthesized from 1-methyl-3-nitro-1-nitrosoguanidine [11], obtained from Aldrich Quimica (Madrid, Spain). Carbopack B, glass columns, fatty acid methyl ester standards and *n*-heptadecanoic acid, used as the internal standard (15 m*M* and 1.5 m*M* in methanol), were supplied by Supelco.

Liquid scintillation cocktail was from Pharmacia LKB (Uppsala, Sweden) and 2-*tert.*-butyl-4-methoxyphenol (BHA), used as an antioxidant, was from Biochemica Merck (Darmstadt, Germany).

Blood samples

Blood samples used to validate the method were obtained from the hepatic vein of 320-day-old female Wistar rats under anaesthesia with Nembutal (40 mg/kg). The blood samples were collected with dry heparinized syringes, then transferred to centrifuge tubes and centrifuged at 4°C for 30 min at 1600 *g*. Plasma was divided into 100-μl and 10-μl aliquots for FFA and total fatty acid (TFA) determinations, respectively. EFA were obtained from the difference between TFA and FFA.

The aliquot for FFA determination (100 μl) was diluted with 5 ml of methanol–water (1:1, v/v) containing BHA (0.01%), 2 μl of TSF solution and 2 μl of internal standard (15 m*M*) and stored at −20°C (FFA sample). The second aliquot (10 μl) for TFA determination was stored without TSF at −70°C under nitrogen atmosphere.

Procedure

To hydrolyse fatty acids from triglycerides, 1 ml of Triglyceride [INT] reagent was added to 10 μl of plasma and incubated for 10 min at 37°C. Triglyceride plasma levels were measured from the 500-nm absorbance values, and endogenous free glycerol in plasma was corrected for its blank contribution. Then samples were diluted in 4 ml of methanol–water (5:3, v/v) containing 2 μl of *n*-heptadecanoic acid (1.5 m*M*) as internal standard (TFA sample).

Fatty acid isolation was performed using the Carbopack B column previously described [5]. The adsorbent (Carbopack B, 0.25 g) was introduced into a glass column with a piece of glass wool in the bottom. Before assaying the samples, the column needed to be washed sequentially with 5 ml of chloroform, 3 ml of methanol and 3 ml of distilled water. The sample was then percolated through the column and the resulting eluate was collected (fraction A). The remaining material in the tube containing the sample was recovered by rinsing the tube with 5 ml of 3 m*M* HCl solution, which was also passed through the column, and the eluate was collected (fraction B). Then the column was washed with 1.5 ml of methanol and this eluate was collected (fraction C). Finally, fatty acids were eluted with 10 ml of the chloroform–methanol solution (fraction D). We assayed different chloroform–methanol proportions to elute the fatty acids (see Table II). The eluate containing fatty acids (fraction D) was collected, and the solvent content was evaporated under a nitrogen stream at 40°C.

To recycle the same column, the adsorbent had to be washed sequentially with 5 ml of chloroform, 3 ml of methanol and 3 ml of water (fraction E). This washing procedure prepares the column for a second set of samples, and eliminates non-esterified fatty acid blank contribution.

To measure radioactivity levels of fatty acids, the dry residue (fraction D) was dissolved in 5 ml of the liquid scintillation cocktail, and the dpm value was registered in a liquid scintillation counter.

To measure individual fatty acid concentrations in fraction D, the dry residue was dissolved in 200 μl of methanol–diethyl ether (5:95, v/v), and fifteen drops of firstly prepared diazomethane were added to convert fatty acids into their methyl ester derivatives. After 10 min, the excess of reagents was eliminated under a nitrogen stream at room temperature, and the dry residue was dissolved in 15 μl (TFA sample) or 100 μl (FFA sample) of hexane, and 1 μl was injected into the gas–liquid chromatograph.

Calculations

EFA were calculated from the difference between TFA (sample treated with enzyme) and FFA (the same sample not treated with enzyme).

In the GC analysis, the concentration of each fatty acid was calculated from its peak area relative to that of the internal standard (*n*-heptadecanoic acid) and also corrected by a response factor (f) relative to the internal standard response, which is different for every fatty acid.

Each fatty acid was identified in the chromatogram by comparing its retention time with that of the reference standard (fatty acid methyl ester standard).

To determine the value of f for each fatty acid, 10 μl of *n*-heptadecanoic acid (15 m*M*) were derivatized and dissolved in 500 μl of hexane containing 5 μl of the mixture of fatty acid methyl ester standards. Volumes from 1–4 μl of the sample were injected into the gas chromatograph column. Calibration curves for individual fatty acids and the internal standard were made.

RESULTS AND DISCUSSION

In the procedure described, EFA were specifically hydrolysed by lipoprotein lipase to glycerol and free fatty acids. The glycerol was then measured through its transformation into dihydroxyacetone phosphate, using the coupled enzyme reactions catalysed by glycerol kinase, glycerol-1-phosphate dehydrogenase and diaphorase, in sequence [12]. The amount of glycerol gave the level of triglycerides in the sample. The fatty acids released by the enzymic reaction were trapped in a Carbopack B column, and were selectively and quantitatively recovered in a separate fraction by elution from the column with the organic solvents, to measure both the amount of these fatty acids by GC and the radioactivity content by scintillation counting.

The fatty acids obtained in a sample treated with enzyme gave the TFA content in that sample, whereas the fatty acids of a sample not treated with enzyme gave the FFA content in that sample. The difference in both the fatty acid levels and the radioactivity corresponded to the EFA level.

TABLE I

PERCENTAGE OF RADIOACTIVITY IN EACH FRACTION ELUTED FROM THE CARBOPACK B COLUMN WHEN RADIOLABELED OLEIC ACID AND TRIOLEIN ARE ASSAYED

Fractions A–E are described in Experimental. The results are the mean ± S.E.M. of five determinations.

Compound	Radioactivity (%)				
	A	B	C	D	E
[^{14}C]Oleic acid	0.635 ± 0.180	0.587 ± 0.350	1.41 ± 0.25	95.4 ± 0.6	1.95 ± 0.67
[^{14}C]Oleic acid + plasma	3.66 ± 0.10	0.970 ± 0.140	0.719 ± 0.179	94.5 ± 0.5	0.195 ± 0.040
[^{14}C]Triolein	0.264 ± 0.030	0.322 ± 0.051	0.282 ± 0.075	0.599 ± 0.091	98.0 ± 2.1
[^{14}C]Triolein + plasma	59.9 ± 1.0	11.4 ± 2.1	4.39 ± 0.47	8.18 ± 0.52	16.2 ± 2.2
[^{14}C]Triolein + plasma + enzyme	7.83 ± 1.64	1.38 ± 0.31	0.954 ± 0.757	88.9 ± 2.7	0.966 ± 0.015

In order to validate this method for its application to the determination of EFA in plasma, the following points were considered: (A) fatty acids were quantitatively trapped in the Carbopack B column; (B) discrimination between triglycerides and FFA was reached, and FFA and EFA were separated in different fractions of the Carbopack B column; (C) enzymic lipolysis of triglycerides proved to be specific and quantitative, and interferences from other lipolytic compounds were minimized.

Trapping FFA and EFA in the Carbopack B column

FFA and triglycerides were quantitatively trapped in the Carbopack B column (see Table I): 97% of the [^{14}C]oleic acid and 98.6% of the [^{14}C]triolein were trapped in the column after sample elution (fraction A) and quantitatively recovered in the combined E + D fractions. However, when [^{14}C]triolein or [^{14}C]oleic acid were added to plasma, practically the same amount of the [^{14}C]oleic acid (94.7%) but only 24.4% of the [^{14}C]triolein were trapped in the column. This can be explained because of the existence of FFA–protein and triglyceride–lipoprotein complexes that cannot be trapped in the Carbopack B column [5]. The chylomicrons, very-low-, high- and low-density lipoproteins are the main form of carrying triglycerides in plasma [13–15]. Fatty acids are carried mainly bound to albumin [16,17].

The column conditions used in general were those previously described by Borra *et al.* [5]. However, the composition of the eluting phase was changed in order to recover FFA and triglycerides in different column fractions.

The composition of the eluting phase was changed in order to attain a differential elution of FFA and EFA. Table II shows the [^{14}C]oleic acid and [^{14}C]triolein distribution between fractions D and E, depending on the composition of the

TABLE II

DISTRIBUTION OF TRIOLEIN AND OLEIC ACID BETWEEN D AND E FRACTIONS WHEN ELUTION PHASE COMPOSITION IS CHANGED

Compound	Mobile phase	Distribution (%)	
		D	E
[^{14}C]Oleic acid	Chloroform–methanol (7:3)	99.6	0.4
	Chloroform–methanol (6:3)	99.1	0.9
	Chloroform–methanol (6:4)	98.2	1.8
	Chloroform–methanol (1:1)	97.5	2.5
[^{14}C]Triolein	Chloroform–methanol (7:3)	60.6	39.4
	Chloroform–methanol (6:3)	27.7	72.3
	Chloroform–methanol (6:4)	5.8	94.2
	Chloroform–methanol (1:1)	1.10	98.9

eluting phase. When the chloroform/methanol ratio was decreased, the triolein recovery drastically decreased in fraction D with a minor effect on the oleic acid recovery. Using a ratio of chloroform to methanol of 1:1, only 1.1% of the [^{14}C]triolein and 97.9% of [^{14}C]oleic acid was recovered in the FFA fraction (fraction D). This recovery was considered to be enough. A change in the proportions of chloroform–methanol to 7:3 allows an increase of 5% in the FFA recovery [5], but it is at the expense of increasing the interference due to triglyceride fatty acid overlapping. The 1:1 chloroform–methanol was chosen for radioactivity counting.

Enzymic assay conditions

The enzymic assay conditions were those described in the Triglyceride [INT] method for "In Vitro Diagnostic Use" commercialized by Sigma. It is a modification of the procedure described by Bucolo and David [12]. The lipoprotein lipase used in this method is of microbial origin, and it is used in excess for the complete hydrolysis of EFA. When plasma samples containing [^{14}C]triolein were incubated for 10 min at 37°C with the enzyme preparation, 88.9% of the ^{14}C radioactivity was recovered in fraction D as FFA and only 7% was eluted in fraction A (see Table I). Similar results (97% recovery) using lipoprotein lipase from bovine milk were found by Deckelbaum *et al.* [18].

Specificity

The specificity of the method is directly related to the levels of specificity of the enzyme. Certain drugs and other substances are known to influence triglyceride values measured enzymically [19]. Lipoprotein lipase from mammals, in addition to its primary main function, presents a phospholipase A-1 activity [20] and it could also hydrolyse cholesterol esters [21–23], but in this case only when the enzyme is bound to the cell membrane [24] and not *in vitro*. The molar ratio between the sum of individual EFA *versus* the total plasma triglycerides measured by this procedure is 3.6, which is very close to the ratio expected from the relative composition of esterified glycerol lipids in plasma (triglycerides and phospholipids) [25]. In plasma samples, although diglycerides and monoglycerides are also substrates of the lipase enzyme, these sample constituents can be regarded as negligible in quantity.

Borra *et al.* [5] confirmed the absence of interferences by hydrolysis or transesterification reactions of phospholipids occurring during the analysis. However, they observed some increase in the unsaturated fatty acids when the restored column was left unused and filled with water, and when the purification process was casually interrupted after the acidic washing step, probably owing to the slow hydrolysis of some phospholipids that are strongly retained by the Carbopack B column. We determined the phosphorus content [26] in each fraction eluted from the column. Only 10% of the total phosphorus content was eluted in the FFA fraction (D), so this possible interference of phospholipids in fraction D is negligible in quantity.

To evaluate possible interferences from other compounds present in the plasma samples, radiolabeled saccharose and amino acids, selected in accordance with their chemical structure corresponding to different acid–base and hydrophobic properties, were treated. Table III shows the recovery of these compounds in the different Carbopack B column fractions. The recovery in the FFA fraction (D) was in all cases less than 10% of total eluted compounds. On the other hand, we observed differences in function of the hydrophobicity and ionic charge of the side-chain amino acids. Glutamine and glycine, two neutral side-chain amino acids, were retained by the column in measurable amounts. Polar and ionic side-chain amino acids were practically not retained in the column, and apolar and aromatic amino acids showed intermediate retention.

These potential interferences are eliminated provided that the results are obtained by comparing two samples run in parallel, one incubated with Triglyceride [INT] reagent and another without. Only the substrates specifically attacked by lipoprotein lipase enzyme could be detected, and of these only those that were recovered in fraction D from the Carbopack B column.

TABLE III

RECOVERY OF AMINO ACIDS AND SACCHAROSE ELUTED FROM CARBOPACK B COLUMN

Fractions A–E are described in Experimental. The results are mean ± S.E.M. of three determinations.

Compound	Recovery (%)				
	A	B	C	D	E
[^{14}C]Phenylalanine	54.7 ± 0.6	19.8 ± 1.0	20.5 ± 1.4	4.72 ± 0.17	0.359 ± 0.139
[^{14}C]Lysine	83.3 ± 0.6	14.9 ± 0.4	0.739 ± 0.024	0.801 ± 0.168	0.278 ± 0.074
[^{14}C]Alanine	78.8 ± 1.2	18.1 ± 1.4	1.18 ± 0.16	1.67 ± 0.02	0.233 ± 0.008
[^{14}C]Glycine	54.4 ± 0.6	21.9 ± 0.1	14.4 ± 0.7	9.10 ± 0.09	0.256 ± 0.095
[^{14}C]Leucine	68.1 ± 2.8	17.8 ± 1.8	7.14 ± 0.73	6.76 ± 0.27	0.259 ± 0.008
[^{14}C]Glutamine	50.7 ± 3.4	29.1 ± 4.5	9.80 ± 1.08	9.75 ± 0.32	0.694 ± 0.294
[^{14}C]Glutamate	3.12 ± 0.05	87.6 ± 0.3	5.68 ± 0.17	3.38 ± 0.06	0.205 ± 0.032
[^{14}C]Saccharose	79.8 ± 0.7	15.2 ± 0.5	3.35 ± 0.09	1.43 ± 0.34	0.231 ± 0.074

Fatty acid gas chromatography determination

Fig. 1 shows a typical chromatogram of a plasma sample fatty acid content following lipase treatment. All methyl fatty acid esters can be separated with the chromatographic conditions used in this procedure. The internal standard can be efficiently determined without interference from other fatty acid esters. However, it is necessary to apply the response factor (f) of each individual fatty acid methyl ester *versus* the internal standard response.

The different individual fatty acids showed

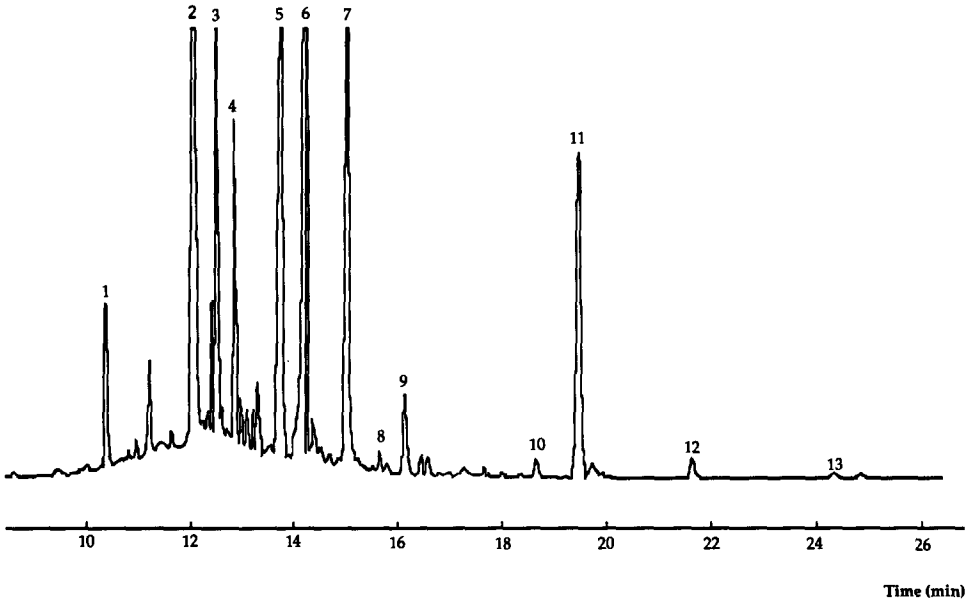

Fig. 1. Typical chromatogram of plasma fatty acid content following lipase treatment. The plasma sample was from the hepatic vein of 320-day-old Wistar rats. Peaks: 1 = myristic acid ($C_{14:0}$); 2 = palmitic acid ($C_{16:0}$); 3 = palmitoleic acid ($C_{16:1}$); 4 = heptadecanoic acid ($C_{17:0}$); 5 = stearic acid ($C_{18:0}$); 6 = oleic acid ($C_{18:0}$); 7 = linoleic acid ($C_{18:2}$); 8 = arachidic acid ($C_{20:0}$); 9 = linolenic acid ($C_{18:3}$); 10 = eicosatrienoic acid ($C_{20:3}$); 11 = eicosatetraenoic acid ($C_{20:4}$); 12 = erucic acid ($C_{22:1}$); 13 = lignoceric acid ($C_{24:0}$).

TABLE IV

PLASMA FFA AND EFA COMPOSITION FROM HEPATIC VEIN OF WISTAR RATS, AND STANDARD CHOW TOTAL FATTY ACID COMPOSITION

Acid	FFA (μM)	EFA (μM)	Standard chow (μmol/g)
$C_{14:0}$	19.1 ± 1.6	89.1 ± 9.4	72.9 ± 4.6
$C_{16:0}$	235 ± 14	1871 ± 238	466 ± 29
$C_{16:1}$	49.5 ± 7.0	277 ± 57	27.4 ± 1.7
$C_{18:0}$	94.0 ± 7.4	875 ± 57	158 ± 10
$C_{18:1}$	167 ± 17	1401 ± 195	525 ± 33
$C_{18:2}$	129 ± 10	1036 ± 158	624 ± 39
$C_{18:3}$	15.2 ± 1.1	76.8 ± 9.9	100 ± 6
$C_{20:0}$	4.69 ± 0.61	7.29 ± 2.19	133 ± 8
$C_{20:3}$	2.92 ± 0.41	22.4 ± 3.0	2.81 ± 0.18
$C_{20:4}$	30.3 ± 2.1	1216 ± 190	10.3 ± 0.7
$C_{22:1}$	74.8 ± 11.8	41.9 ± 5.0	5.92 ± 0.37
$C_{24:0}$	79.3 ± 9.4	16.4 ± 3.8	7.95 ± 0.50
Total fatty acids	763 ± 43	6930 ± 664	2134 ± 135

values of f that linearly decreased when the fatty acid chain length increased.

Table IV shows the FFA and EFA compositions of rat plasma from hepatic vein samples. The Wistar rats were on standard chow-fed conditions (food composition is also shown in the table). The standard deviation from the mean represents less than 10% for most individual fatty acids, and *ca.* 5% of the total combined values of fatty acids. This accuracy is similar to that obtained with other methods, such as the method we applied to determine the standard chow fatty acid composition [27].

The method proposed is rapid, reliable and useful for the analysis of a large number of samples. It can also allow both the determination of the pattern of the individual fatty acid compositions of the triglycerides by GC separation after methylation of the fatty acids purified in the Carbopack B column and, also, the radioactivity content in this fraction.

ACKNOWLEDGEMENTS

This work was supported by Grant No. PM91-0070 from DGICYT (Dirección General de Investigación Científica y Técnica) and Grant No. PTR91-0091 from CICYT (Comisión Interministerial de Ciencia y Tecnología) of the Government of Spain.

REFERENCES

1 F. Serra, A. Palou and A. Pons, *Anal. Chem.*, 59 (1987) 1841.
2 P. Roca, M. Gianotti and A. Palou, *Anal. Biochem.*, 148 (1985) 190.
3 M. Gianotti, P. Roca and A. Palou, *J. Biochem. Biophys. Methods*, 10 (1984) 181.
4 S. Quevedo, A. Palou and P. Roca, *Anal. Chem.*, 65 (1993) 992.
5 C. Borra, G. Cosmi, A. Di Corcia and R. Samperi, *J. Chromatogr.*, 311 (1984) 9.
6 G. Cosmi, A. Di Corcia, L. Ripani, R. Samperi and G. Vinci, *Clin. Chim. Acta*, 126 (1982) 101.
7 A. Di Corcia, R. Samperi, G. Vinci and G. D'Ascenzo, *Clin. Chem.*, 28 (1982) 1457.
8 G. Cosmi, A. Di Corcia, R. Samperi and G. Vinci, *Clin. Chem.*, 29 (1983) 319.
9 F. Andreolini, A. Di Corcia, A. Lagana, R. Samperi and G. Raponi, *Clin. Chem.*, 29 (1983) 2076.
10 F. Andreolini, C. Borra, A. Di Corcia, A. Lagana, R. Samperi and G. Raponi, *Clin. Chem.*, 30 (1984) 742.
11 A. F. Mckay, *J. Am. Chem. Soc.*, 1948 (1974) 70.
12 G. Bucolo and H. David, *Clin. Chem.*, 19 (1973) 476.
13 K. W. Miller and D. M. Small, *Plasma Lipoproteins*, Elsevier, Amsterdam, 1st ed., 1987, Ch. 1.

14 R. W. Mahley, T. L. Innerarity, S. C. Rall and K. H. Weisgraber, *J. Lipid Res.*, 25 (1984) 1277.
15 S. J. Robins, J. M. Fasulo, V. F. Robins and G. M. Patton, *Am. J. Physiol.*, 257 (1989) E860.
16 B. J. Potter, D. Sorrentino and P. D. Berk, *Ann. Rev. Nutr.*, 9 (1989) 253.
17 M. Jeevanandam, Y. Hsu, L. Ramias and R. Schiller, *Clin. Chem.*, 35 (1989) 2228.
18 R. J. Deckelbaum, S. Eisenberg, M. Fainaru, Y. Barenholz and R. Olivecrona, *J. Biol. Chem.*, 254 (1979) 6079.
19 D. S. Young, L. C. Pestaner and V. Gibberman, *Clin. Chem.*, 21 (1975) 368D.
20 M. Shinomiya, L. R. McLean and R. L. Jackson, *J. Biol. Chem.*, 258 (1983) 14178.
21 G. Friedman, T. Chajek-Shaul, O. Stein, T. Olivecrona and Y. Stein, *Biochim. Biophys. Acta*, 666 (1981) 156.
22 C. J. Fielding and P. E. Fielding, *Biochemical Interactions at the Endothelium*, Elsevier, Amsterdam, 1983, p. 275.
23 T. Chajek-Shaul, G. Friedman, O. Stein, T. Olivecrona and Y. Stein, *Biochim. Biophys. Acta*, 712 (1982) 200.
24 T. Chajek-Shaul, G. Friedman, G. Halperin, O. Stein and Y. Stein, *Biochim. Biophys. Acta*, 666 (1981) 147.
25 K. W. Miller and D. M. Small, *J. Biol. Chem.*, 258 (1983) 13772.
26 G. R. Bartlett, *J. Biol. Chem.*, 234 (1959) 466.
27 E. Peuchant, R. Wolff, C. Salles and R. Jensen, *Anal. Biochem.*, 181 (1989) 341.