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Simultaneous determination of molecular species of monoacylglycerols, diacylglycerols and triacylglycerols in human very-low-density lipoproteins by reversed-phase liquid chromatography

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Abstract

The aim of the present study was to investigate the applicability of a previously developed method for the analysis of triacylglycerol molecular species to the simultaneous determination of triacylglycerols, diacylglycerols and monoacylglycerols of human very-low-density lipoproteins (VLDL). Ten elderly women were recruited for the study. Blood was obtained in fasting conditions and VLDL were isolated by ultracentrifugation. Neutral lipids were separated by solid-phase extraction and were subsequently injected on a reversed-phase HPLC system, with an elution system composed of acetone in acetonitrile. The method allowed the separation of four monoacylglycerols, 18 diacylglycerols and 24 triacylglycerols, including the resolution of positional isomers of diacylglycerols. Monoacylglycerols were composed of oleic, linoleic, palmitic and stearic acids. The major diacylglycerols were 1,2-dilinoleoyl-glycerol and 1,3-dilinoleoyl-glycerol (14.24±1.02 and 17.93±1.42%, respectively). The main triacylglycerols quantified were dioleoyl-stearoyl-glycerol (OOS), oleoyl-dipalmitoyl-glycerol (OPP), trilinoleoyl-glycerol (LLL) and linoleoyl-distearoyl-glycerol (LSS), accounting for 11.25±2.15, 10.14±2.05, 9.35±2.30 and 8.56±1.56%, respectively. An inverse relationship between polarity and fatty acid disappearance from triacylglycerols (r^2=0.82, P<0.05) and from diacylglycerols (r^2=0.93, P<0.01) was discovered. In conclusion, the method allowed, for the first time, the easy, rapid and simultaneous determination in a single chromatogram of triacylglycerol, diacylglycerol and monoacylglycerol molecular species of human VLDL by reversed-phase HPLC.

Keywords: Acylglycerols; Very-low-density lipoproteins

1. Introduction

Elevated plasma triacylglycerol concentration is being recognized as a risk factor for coronary heart disease [1,2]. It has been postulated that the atherogenic process might be enhanced by the accumulation of triacylglycerol-rich lipoproteins in plasma, such as VLDL [3,4]. These lipoproteins are formed in the liver and their chief function is the transport of endogenous triacylglycerols through plasma, with the aim of providing peripheral tissues with fatty acids. The release of fatty acids from

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triacylglycerols is dependent upon hydrolysis by LPL, which is attached to the surface of the vascular endothelium [5]. The activity of LPL leads to the transformation of VLDL firstly into intermediate-density-lipoproteins and then, probably with the participation of hepatic lipase, into low-density-lipoproteins (LDL) [6,7].

LPL hydrolyses triacylglycerol molecules releasing diacylglycerols, which undergo a successive hydrolysis to 2-monoacylglycerols. Interestingly, the equilibrium constant controlling the transformation of diacylglycerols into 2-monoacylglycerols is higher than that controlling the transformation of triacylglycerols into diacylglycerols. This mechanism suggests a greater specificity of LPL for diacylglycerols than for triacylglycerols, probably in order to avoid the accumulation of diacylglycerols, maximising the fatty acid release from the VLDL particle [5]. However, VLDL are not completely hydrolysed by LPL in plasma, since they may be taken up by the liver or transformed into LDL, while retaining up to 50% of their initial content in triacylglycerols and a significant amount of diacylglycerols and monoacylglycerols [8].

Traditionally, analyses of VLDL triacylglycerols have been carried out by means of determination of their fatty acid composition. However, as LPL presents a greater specificity for certain fatty acids [5] the determination of the triacylglycerol molecular species composition of VLDL seems of major interest. In 1993, we presented for the first time the molecular species composition of VLDL underivatised triacylglycerol from human origin, analysed by gas–liquid chromatography (GLC) [9]. This method was then employed for the analysis of VLDL triacylglycerols from healthy and hypertensive subjects receiving diets rich in olive oil or high-oleic sunflower oil [10,11]. However, the analysis of triacylglycerols by GLC with flame-ionization detector (FID) presented deficiencies in the detection of most unsaturated triacylglycerols [12]. For that reason, a new method was developed for the determination of triacylglycerol molecular species, using reversed-phase HPLC coupled with an evaporative light-scattering detector (ELSD) [13]. However, that method did not allow the separation of monoacylglycerol and diacylglycerol molecular species. The determination of monoacylglycerols and diacylglycerols has usually been carried out as an intermediate stage in the esteresospecific analysis of triacylglycerols, involving complex derivatization processes with multiple reactions prior to the injection into reversed-phase or chiral-phase HPLC [14]. In addition, thin-layer chromatography coupled to FID has also been successful in the separation of 1,2-diacylglycerols from the 1,3-isomers. However, until now, the resolution of molecular species of triacylglycerols and partial acylglycerols, has not been achieved without derivatisation.

For that reason, the objective of the present study was to develop a new method for the separation, identification and quantification of underivatized monoacylglycerol, diacylglycerol and triacylglycerol molecular species of human VLDL in a single chromatogram, in order to facilitate the study of the metabolism of this proatherogenic lipoprotein.

2. Experimental

2.1. Samples

Ten women were enrolled from Residencia Heliópolis (Junta de Andalucía, Seville, Spain), a residential home for the elderly, where they had been living for at least 5 years. Consequently, the diet was controlled and all their food habits were completely known. The subjects gave written informed consent to a protocol approved by the Institutional Committee on Investigation in Humans (Hospitales Universitarios Virgen del Rocío, Seville, Spain). The participants were free-living and we gave them no payment. None of them were diabetic or suffered from glucose intolerance or hypothyroidism. Cigarette smokers were excluded from the study and no case of alcohol abuse was detected among participants.

The regular dietary intake of the participants was recorded during four consecutive weeks before the study, using a 24-h recall and food frequency questionnaires. The energy consumption was approximately 1800 kcal/day and nutrient consumption was calculated as 30% of the energy as fat (7% SFA, 20% MUFA and 3% PUFA), 55% as carbohydrates and 15% as proteins. Fat was provided mainly by sunflower oil and corn margarine for bread slices at breakfast. The intake of cholesterol was approxi-

mately 307 mg/day. Food calculations were carried out from Ref. [15].

2.2. Plasma lipid and lipoprotein analyses

Venous blood was obtained in fasting status after an overnight period and collected in Vacutainer® tubes (1 g/l EDTA-K_3). Plasma was obtained by centrifugation at 1500 rpm during 30 min at 4 °C and immediately frozen below −80 °C until analysis. Plasma concentrations of total, LDL- and HDL-cholesterol, as well as total triacylglycerol concentrations, were measured by conventional enzymatic methods and using the Friedewald formula [16,17].

2.3. Isolation of very-low-density lipoproteins (VLDL)

VLDL were isolated from 4 ml of plasma layered with 6 ml of a NaCl solution (density 1.006 g/ml) by a single ultracentrifugation spin (40 000 rpm, 18 h, 15 °C). Ultracentrifugation was carried out using a SW 41 Ti rotor in a BECKMAN L8-70M preparative ultracentrifuge (Beckman Instruments, Palo Alto, CA, USA).

2.4. Isolation of neutral lipids

Total lipids were extracted following a modification of the method of Rose and Oaklander [18], using 2,6-di-*tert.*-butyl-*p*-cresol (BHT) as antioxidant. Neutral lipids (a mixture of triacylglycerols, diacylglycerols and monoacylglycerols) were isolated by solid-phase extraction (SPE) diol columns (Supelclean™ LC-Diol, Supelco, Bellefonte, USA). A diol bonded-phase SPE column was placed in a vacuum elution apparatus and was equilibrated by washing with 4 ml hexane under vacuum. The sample (200 μl lipids) was layered on the column and the solvent drawn through. Two volumes of hexane/methylene chloride (9:1, v/v) were applied to the column and the neutral lipid fraction was collected. The SPE column was then successively washed with 4 ml of ethanol and 3 ml of acetone and dried by passing nitrogen through. The eluate was evaporated to dryness under a stream of nitrogen and redissolved in hexane. An aliquot was taken for the analysis of fatty acids by GLC. A second aliquot was stored at −20 °C for further determination of the triacylglycerol, diacylglycerol and monoacylglycerol molecular species composition by HPLC.

2.5. Fatty acid analysis of neutral lipids

Neutral lipids were transmethylated and the resulting fatty acid methyl esters (FAME) analysed by GLC as described by Ruiz-Gutiérrez et al. [9], using a model 5890 series II gas chromatograph (Hewlett-Packard Co, Avondale, USA) equipped with a flame ionisation detector (FID) and a capillary silica column Supelcowax 10 (Sulpelco) of 60 m length and 0.25 mm internal diameter. The splitting ratio was 1:25. Individual FAME were identified by means of comparison of their retention times with those of standards. FAME for which no standard was available were identified by gas chromatography–mass spectrometry on a Konik KNK-2000 chromatograph (Konik Co, Barcelona, Spain) interfaced directly to an AEJ MS30/790 VG mass spectrometer (VG Analytical, Manchester, UK) using electron impact ionization mode. The ion source temperature was maintained at 200 °C, the multiplier voltage was 4.0 kV, the emission current was 100 μA and the electron energy was 70 eV. The data were processed with a VG 11/250 data system. FAME were quantified by means of their relative peak areas.

2.6. HPLC analysis of triacylglycerol, diacylglycerol and monoacylglycerol molecular species

Neutral lipids were passed through a filter with a pore size of 0.2 μm (Millipore, Bedford, USA). The chromatographic system consisted of a model 2690 Alliance liquid chromatograph (Waters, Milford, USA), provided with a Novapack (250×4.6 mm) of 4-μm particle size (Waters). The liquid chromatograph was coupled to a light-scattering detector model DDL31 (Eurosep Ins., Cergy-Pontoise, France). The system was controlled by computer through the Millenium System (Waters). The mobile phase consisted of an initial elution gradient of 20% of acetone in acetonitrile raising the percentage of acetone to 45% in 12 min and then to 80% after 60 min. This percentage was held up to the end of the analysis. The flow-rate was 1 ml/min. Standard

solutions were of Sigma Grade (99% pure) of tritridecanoyl-glycerol (DDD), 1,3-dioleoyl-2-palmitoyl-glycerol (OPO), trimyristoyl-glycerol (MMM), 1,3-dioleoyl-2-stearoyl-glycerol (OSO), 1,3-dioleoyl-2-linoleoyl-glycerol (OLO), tripentadecanoyl-glycerol (TTT), tripalmitoyl-glycerol (PPP), trioleoyl-glycerol (OOO), trilinoleoyl-glycerol (LLL), 1,2-dipalmitoyl-glycerol (PP), 1,2-dioleoyl-glycerol (OO), 1,2-dilinoleoyl-glycerol (LL), 1-monopalmitoyl-glycerol (P) and 1-monooleoyl-glycerol (O). In order to calibrate the detector and to establish the capacity factor (k') of the system, tripled runs of six concentrations of these standards, between 0.25 and 2.5 mg/ml (0.25, 0.5, 1, 1.5, 2 and 2.5 mg/ml), were injected in hexane. Various regression models were tested and finally non-linear fourth regression curves (r^2=0.999) were chosen. Equations resulting from these curves were employed for quantification. When the corresponding standard for a chromatograph peak identified was not available, the curve of the standard with a closer retention time was applied. Response factors for each standard relative to closer standards in retention time were calculated, being always between 0.98 and 1.02.

2.7. Calculation of triacylglycerol, diacylglycerol and monoacylglycerol molecular species composition

The triacylglycerol, diacylglycerol and monoacylglycerol molecular species compositions were calculated as follows. For identification purposes, fatty acids resulting from GLC analysis of FAME were combined in threes to calculate all possible triacylglycerols, or in couples to calculate all possible diacylglycerols. Only those fatty acids present with content higher than 1% were considered for the analysis. The equivalent carbon number (ECN) of each triacylglycerol, diacylglycerol and monoacylglycerol was calculated according to the following equation:

$$\mathrm{ECN} = \mathrm{CN} - 2 \times \mathrm{DB} - 0.2 \times \mathrm{NUFA} \quad (1)$$

where CN is the total carbon number of the three fatty acids, DB the total number of double bonds and NUFA the number of unsaturated fatty acids of the acylglycerol molecules. A simple linear regression analysis was applied to relate ECN with log k' of the standards analyzed under the same conditions. The result was Eq. (2) for diacylglycerols and monoacylglycerols and Eq. (3) for triacylglycerols.

$$\mathrm{ECN} = 13.802 + 22.956 \times \log k' \ (r^2 = 0.999,$$
standard error of estimate: 0.42, standard error of slope: 0.82, standard error of intercept: 0.47) (2)

$$\mathrm{ECN} = 5.130 + 32.147 \times \log k' \ (r^2 = 0.995,$$
standard error of estimate: 0.37, standard error of slope: 0.90, standard error of intercept: 1.09) (3)

The values of ECN obtained from these equations were employed to assign triacylglycerols, diacylglycerols and monoacylglycerols to chromatographic peaks. In order to complete the prediction process, 1,3- and 1,2-diacylglycerol isomers were injected and the random composition was used to establish the probability of the presence of the triacylglycerols in each HPLC peak when more than one molecular species was predicted.

2.8. Statistical analysis

Results are expressed as means and SD (n=10). Pearson's correlation coefficients were calculated for linear regression to explore the relationship between variables. To assess for statistical significance, Student's t-test was applied at 95% of the confidence level. Statistical treatments were carried out with Statistica for Windows 6.0 (Statsoft, Tulsa, OK, USA).

3. Results

The study was carried out on normal elderly subjects without known metabolic disorders and who showed no hyperglycaemia at the time when the sample was taken. Table 1 shows the general data and the blood parameters of the subjects under study.

The result of the GLC analysis of fatty acids of neutral lipids from VLDL is shown in Table 2. Palmitic (16:0) and oleic (18:1, n-9) acids were the most abundant, each accounting for nearly 30%.

Table 1
General data, blood parameters and plasma lipid levels of the subjects enrolled in the study

Parameter	Mean	SD	Range
Age (years)	84.0	7.4	65.0–98.0
BMI (kg/m^2)	27.4	3.4	19.8–32.8
WHR	0.91	0.06	0.85–1.01
Glucose (mmol/l)	5.24	0.74	4.28–7.13
Total cholesterol (mmol/l)	4.82	0.99	4.17–5.59
LDL-cholesterol (mmol/l)	2.92	0.92	2.00–3.62
HDL-cholesterol (mmol/l)	1.49	0.46	0.82–1.83
Total triacylglycerols (mmol/l)	0.90	0.18	0.53–1.73
VLDL triacylglycerols[a] (mmol/l)	0.27	0.03	0.22–0.31
VLDL diacylglycerols[a] (mmol/l)	0.19	0.01	0.17–0.21
VLDL monoacylglycerols[a] (mmol/l)	0.17	0.01	0.15–0.19

$n=10$; BMI, body mass index; WHR, waist to hip ratio; SD, standard deviation.

[a] Calculated from HPLC analysis of triacylglycerols, diacylglycerols and monoacylglycerols.

Linoleic acid (18:2, *n*-6) represented 20% of total fatty acids, with stearic acid (18:0) representing approximately 8%. These fatty acids, along with arachidonic (20:4, *n*-6), linolenic (18:3, *n*-3) and palmitoleic (16:1, *n*-9) acids were considered for the identification of molecular species of triacylglycerols, diacylglycerols and monoacylglycerols. Since reversed-phase HPLC does not distinguish among different positions of double bonds, oleic acid (O) stands for 18:1, *n*-9 and 18:1, *n*-7, palmitoleic acid (Po) for 16:1, *n*-9 and 16:1, *n*-7 and linolenic acid (Ln) for 18:3, *n*-6 and 18:3, *n*-3.

Table 2
Fatty acid composition (%) of neutral lipids from human VLDL

Peak	Fatty acid	Mean	SD
1	14:0	0.07	0.04
2	14:1, *n*-5	0.19	0.02
3	16:0	30.01	2.35
4	16:1, *n*-9	0.77	0.09
5	16:1, *n*-7	1.94	0.29
6	18:0	8.71	2.18
7	18:1, *n*-9	29.44	4.31
8	18:1, *n*-7	2.03	0.29
9	18:2, *n*-6	20.04	1.13
10	18:3, *n*-6	0.90	0.84
11	18:3, *n*-3	0.61	0.31
12	20:0	0.15	0.04
13	20:1, *n*-9	0.39	0.10
14	20:1, *n*-7	0.50	0.06
15	20:2, *n*-6	0.84	0.32
16	20:3, *n*-6	0.32	0.14
17	20:4, *n*-6	2.17	0.09
18	22:1, *n*-9	0.10	0.00
19	20:5, *n*-6	0.17	0.03
20	20:5, *n*-3	0.37	0.09
21	22:4, *n*-6	0.13	0.01
22	22:5, *n*-3	0.12	0.04

$n=10$; SD, standard deviation.

Fig. 1 illustrates the molecular species composition of triacylglycerols, diacylglycerols and monoacylglycerols of human VLDL as determined by reversed-phase HPLC after isolation of neutral lipids by SPE on diol columns. Four monoacylglycerols, 18 diacylglycerols and 24 triacylglycerols were resolved in 55 min. Eight minutes were enough to elute all chromatographic peaks corresponding to monoacylglycerols, and all diacylglycerols were separated in approximately 25 min. The rest of the chromatogram corresponded to triacylglycerol molecular species.

Table 3 describes the result of the identification process of diacylglycerol and monoacylglycerol molecular species. The four chromatographic peaks corresponding to monoacylglycerols were identified as L, O, P and S. The proportion of L, O and P was close to 30% and only the content of S was considerably lower ($6.75\pm1.10\%$). The diacylglycerol molecular species composition of human VLDL is also depicted in Table 3. In addition to the fatty acids found in the monoacylglycerol fraction, three diacylglycerols containing arachidonic or linolenic acids were found. Although, generally, one diacylglycerol was assigned to a single chromatographic peak, diacylglycerols containing arachidonic acid could not be separated from those containing linolenic acid (LnL/AL, LnO/AO and LnP/AP). However, the method allowed the separation of other diacylglycerols presenting the same ECN and even positional isomers of diacylglycerols, such as LL (1,2-LL from 1,3-LL), LO (1,2-LO from 1,3-LO), LP (1,2-LP from 1,3-LP), OP (1,2-OP from 1,3-OP) or PP (1,2-PP from 1,3-PP). The major diacylglycerols quantified were 1,2-LL and 1,3-LL (14.24 ± 1.02 and $17.93\pm1.42\%$, respectively) followed by 1,2-OP ($10.97\pm0.92\%$) and LnP/AP ($10.17\pm2.20\%$). Consequently, linoleic acid-containing diacylglycerols (LX), along with those containing palmitic acid (PX), were the most abundant.

The triacylglycerol molecular species composition of VLDL is illustrated in Table 4. All chromato-

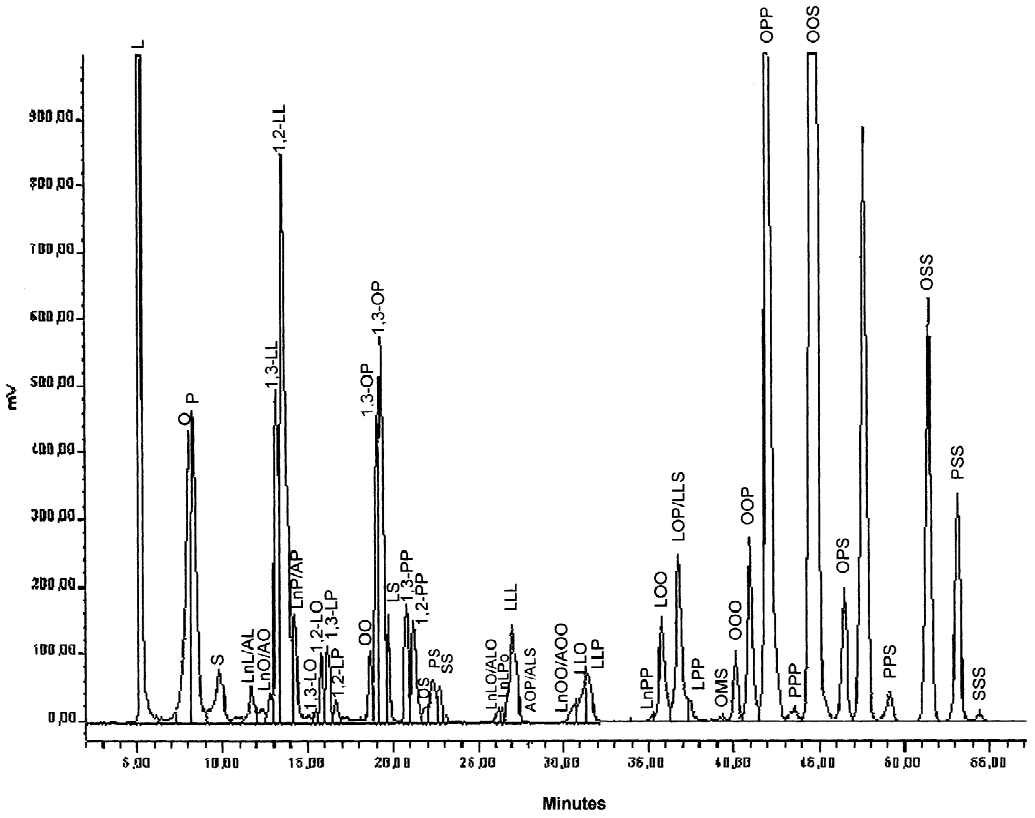

Fig. 1. Reversed-phase HPLC chromatogram of monoacylglycerol, diacylglycerol and triacylglycerol molecular species of human VLDL. Details of analysis conditions are described in Section 2. For nomenclature, see Tables 4 and 5.

graphic peaks could be identified and only to five of them two triacylglycerols had to be assigned (LnLO/ALO, LnOO/AOO, AOP/ALS, LOP/LLS and OOP/LOS). Although, as a general rule, triacylglycerols containing arachidonic or linolenic acids could not be separated, APP was resolved from LnPP. The main triacylglycerols quantified in human VLDL were again those containing oleic, linoleic, palmitic and stearic acids, namely, OOS, OPP, LLL and LSS, accounting for 11.25±2.15, 10.14±2.05, 9.35±2.30 and 8.56±1.56%, respectively. Therefore, nearly equivalent amounts of triacylglycerols containing these fatty acids were found.

The fatty acid composition of each fraction of triacylglycerols, diacylglycerols and monoacylglycerols (Table 5) was calculated on the basis of the percentage share of each molecular species (Tables 3 and 4). According to this calculation, palmitic, oleic, linoleic and stearic acids contributed to a similar extent to the fatty acid composition of triacylglycerols (close to 25%), although the content of oleic acid was the highest (28.50%) and that of stearic acid the lowest (20.21%). The amount of linolenic/arachidonic acid was only 3.72%. However, these proportions were not maintained in diacylglycerols and monoacylglycerols. In the former fraction, stearic acid represented only 8.44% and the content of linolenic/arachidonic acid increased to 7.91%. In the monoacylglycerol fraction the amount of palmitic and oleic acids was maintained, but that of stearic acid was reduced to 6.75%, whereas the proportion of linoleic acid was elevated to 36.60%. In order to establish a possible relationship between the presence of fatty acids in each lipid class and their polarity, the fatty acids concentration in diacylglycerols relative to triacylglycerols (TAG/DAG) and in monoacylglycerols relative to diacylglycerols (DAG/MAG) were plotted against the ECN of each fatty

Table 3
Monoacylglycerol and diacylglycerol molecular species composition (%) of human VLDL

Peak	Molecular species	ECN	Mean	SD	RSD (%)
Monoacylglycerol species					
M1	L	13.8	36.60	2.63	7.18
M2	O	15.8	27.78	1.96	7.07
M3	P	16.0	28.71	1.70	5.92
M4	S	18.0	6.75	1.10	16.35
Diacylglycerol species					
D1	LnL/AL	25.6	3.19	0.75	23.96
D2	LnO/AO	27.6	3.41	0.30	9.11
D3	1,3-LL	27.6	14.24	1.02	7.31
D4	1,2-LL	27.6	17.93	1.42	8.13
D5	LnP/AP	27.8	10.17	2.20	22.14
D6	1,3-LO	29.6	1.17	0.15	13.42
D7	1,2-LO	29.6	6.28	0.62	10.09
D8	1,3-LP	29.8	8.34	0.46	5.67
D9	1,2-LP	29.8	2.20	0.14	6.39
D10	OO	31.6	2.35	0.28	12.27
D11	1,3-OP	31.8	6.43	0.74	11.81
D12	1,2-OP	31.8	10.97	0.92	8.58
D13	LS	31.8	3.42	0.30	8.93
D14	1,3-PP	32.0	3.52	0.83	24.02
D15	1,2-PP	32.0	2.03	0.49	24.63
D16	OS	33.8	0.67	0.15	22.39
D17	PS	34.0	2.11	0.24	11.51
D18	SS	36.0	1.56	0.12	7.80
	LnX/AX		16.77	3.25	19.38
	LX		56.77	4.86	8.56
	OX		31.28	3.16	10.10
	PX		45.77	6.02	13.15
	SX		7.76	0.81	10.44

$n=10$; SD, standard deviation; RSD, relative standard deviation; ECN, equivalent carbon number. Fatty acids: A: arachidonic acid (20:4); Ln: linolenic acid (18:3); L: linoleic acid (18:2); O: oleic acid (18:1); P: palmitic acid (16:0); S: stearic acid (18:0). Monoacylglycerols: L: monolinoleoyl-glycerol; O: monooleoyl.glycerol; P: monopalmitoyl-glycerol; S: monostearoyl-glycerol. Diacylglycerols: LL: dilinoleoyl-glycerol; OP: oleoyl-palmitoyl-glycerol; PX: palmitoyl-acyl-glycerol.

acid (Fig. 2). A negative relationship between these parameters was observed, presenting correlation coefficients (r^2) of 0.82 for TAG/DAG ($P<0.05$, standard error of estimate: 30.00, slope: -23.87, standard error of slope: 6.36, intercept: 376.32, standard error of intercept: 96.77) and of 0.93 for DAG/MAG ($P<0.01$, standard error of estimate: 6.76, slope: -13.88, standard error of slope: 1.43, intercept: 224.71, standard error of intercept: 21.82).

4. Discussion

Although VLDL are triacylglycerol-rich lipoproteins with relevance in the development of atherosclerosis, the study of their triacylglycerol composition has usually been accomplished by analysing their fatty acid or total triacylglycerol content. Only exceptionally has their triacylglycerol molecular species composition been determined [9–11,19,20]. In the present study we report the achievement of the simultaneous determination of molecular species of triacylglycerols, but also of diacylglycerols and monoacylglycerols, which are products of the catabolism of VLDL triacylglycerols by LPL. Four chromatographic peaks corresponding to monoacylglycerols, 18 corresponding to diacylglycerols and 24 corresponding to triacylglycerols could be separated

Table 4
Triacylglycerol (TAG) molecular species composition (%) of human VLDL

Peak	TAG	ECN	Mean	SD	RSD (%)
T1	LnLPo	39.4	3.01	0.67	22.21
T2	LnLO/ALO	41.4	3.08	0.51	16.68
T3	LLL	41.4	9.35	2.30	24.60
T4	LnOO/AOO	43.4	1.04	0.37	35.02
T5	LLO	43.4	3.03	0.49	16.25
T6	LLP	43.6	6.21	1.23	19.75
T7	AOP/ALS	43.6	0.45	0.13	29.05
T8	APP	43.8	0.20	0.04	20.56
T9	LnPP	43.8	1.00	0.19	18.72
T10	LOO	45.4	4.45	0.78	17.40
T11	LOP/LLS	45.6	4.35	1.02	23.49
T12	LPP	45.8	2.53	0.20	8.05
T13	OMS	46.8	0.44	0.13	30.66
T14	OOO	47.4	3.09	0.35	11.45
T15	OOP/LOS	47.6	5.58	0.69	12.33
T16	OPP	47.8	10.14	2.05	20.26
T17	PPP	48.0	3.30	0.64	19.34
T18	OOS	49.6	11.25	2.15	19.13
T19	OPS	49.8	5.41	1.03	19.08
T20	LSS	49.8	8.56	1.56	18.28
T21	PPS	50.0	3.07	0.61	19.96
T22	OSS	51.8	4.49	0.71	15.93
T23	PSS	52.0	3.88	0.25	6.34
T24	SSS	54.0	2.14	0.51	23.61
	LnXX/AXX		8.78	0.91	10.36
	LXX		50.6	8.58	16.96
	OXX		56.73	9.57	16.87
	PXX		46.12	7.08	15.35
	SXX		49.62	7.79	15.07

$n=10$; SD, standard deviation; RSD, relative standard deviation; ECN, equivalent carbon number. Fatty acids: A: arachidonic acid (20:4); Ln: Linolenic acid (18:3); L: linoleic acid (18:2); O: oleic acid (18:1); P: palmitic acid (16:0); S: stearic acid (18:0). Triacylglycerols: LLL: trilinoleoyl-glycerol; OOP: dioleoyl-palmitoyl-glycerol; AOP: arachidonoyl-oleoyl-palmitoyl-glycerol; PXX: palmitoyl-diacyl-glycerol.

Table 5
Calculated fatty acid composition (%) of molecular species of triacylglycerols (TAG), diacylglycerols (DAG) and monoacylglycerols (MAG) of human VLDL

Fatty acid	TAG	DAG	MAG
16:0	24.32	30.01	28.71
18:0	20.21	8.44	6.75
18:1	28.50	27.47	27.78
18:2	24.25	26.16	36.60
18:3/20:4	3.72	7.91	n.d.

$n=10$; n.d., not detected.

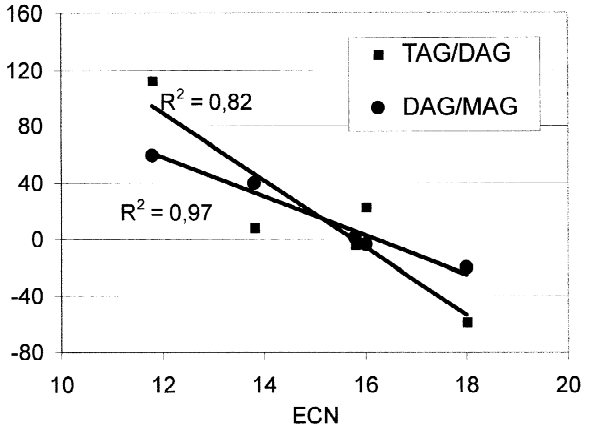

Fig. 2. Fatty acid concentrations (%) in diacylglycerols relative to triacylglycerols (TAG/DAG) ($P<0.05$) and in monoacylglycerols relative to diacylglycerols (DAG/MAG) ($P<0.01$) plotted against their equivalent carbon number (ECN). ECN was calculated as described in Section 2; R^2, correlation coefficient.

by the HPLC method proposed here. As expected, the most polar molecules were eluted with lower retention times. Hence, monoacylglycerols eluted first, then diacylglycerols and finally triacylglycerols. These molecules were identified following a methodology previously employed with success in the identification of triacylglycerols from fish oil [13], rat liver [21] and adipose tissue [22] and from human chylomicrons [23,24]. The result of this methodology resulted in the identification of monoacylglycerols constituted by the four main fatty acids found in neutral lipids (Table 2).

Although this method allowed the identification of all chromatographic peaks, there were difficulties in separating diacylglycerols and triacylglycerols containing arachidonic or linolenic acids, with the exception of APP, which was separated from LnPP. In a reversed-phase column, analytes are separated on the basis of their partition number (PN), that is, depending on the chain length and number of double bounds of the constituting fatty acids [25,26]. However, several authors have provided experimental data in which triacylglycerols with the same PN were separated by reversed-phase HPLC according to the number of unsaturated fatty acids (NUFA) [21,25,27,28], the triacylglycerols with higher NUFA showing lower retention times. Thus, these authors defined the term equivalent carbon number (ECN) as

a function of PN and NUFA (ECN = PN − 0.2 × NUFA). Therefore, it is often difficult to separate molecules presenting the same ECN, such as those carrying arachidonic or linolenic acids. Something similar occurred with positional isomers of diacylglycerols. Although generally it is not possible, in the present work, some *sn*-1,2 isomers could be separated from the *sn*-1,3. Positional isomers of VLDL-diacylglycerols have been successfully resolved by chiral-phase HPLC by Yang et al. [19,20]. In combination with reversed-phase HPLC, these authors achieved the determination of molecular species of enantiomeric diacylglycerols. However, that method implies the derivatization of diacylglycerols to 3,5-dinitrophenyluretanes. Lin et al. [29] were the first to separate positional isomers of underivatised diacylglycerols by HPLC using a single reversed-phase column. They reported the retention times of 45 synthetic diacylglycerols and triacylglycerols eluted with a mixture of methanol and isopropanol. Among diacylglycerols, they separated positional isomers of dioleoylglycerol, dipalmitoylglycerol and distearoylglycerol, observing that *sn*-1,3 isomers eluted earlier than the *sn*-1,2 ones.

The most abundant triacylglycerols in VLDL confirmed the habitual consumption of sunflower oil (rich in linoleic acid) by the participants (Table 4). In view of these major triacylglycerols, and assuming a random action of LPL, it might be speculated that major diacylglycerols would be composed of the same fatty acids. The possible diacylglycerols that might be formed from the main triacylglycerols yielded the following concentration order: OO ≈ OP > LL ≈ OS > PP ≈ SS > OL. In contrast, the real concentration order recorded from the HPLC analysis was: LL ≫ OP > LnP/AP ≈ PL > LO > OO, revealing an unexpected accumulation of linoleic and linolenic/arachidonic acids and a practical absence of stearic acid in diacylglcyerols. The same calculation was carried out to predict the concentration of monoacylglycerols. The calculated values yielded the following concentration order: L > P > O > Ln/A > S. In this case, the experimental concentration of monoacylglycerols was very close to that predicted with the exception of the lack of detection of monoacylglycerols containing linoleic/arachidonic acid.

Data presented in the present study confirm the postulation of Wang et al. [5] suggesting a greater specificity of LPL for diacylglycerols than for triacylglycerols. In our study, we have observed that there is a selective accumulation of the most polar fatty acids in diacylglycerols and that such accumulation is conserved in monoacylglycerols. Concomitantly, we observed a lower concentration of diacylglycerols and monoacylglycerols containing stearic acid, but also of those containing oleic acid (Tables 3 and 4). This fact could be indicative of a preference of LPL for stearic and, in a lower grade, for oleic acid.

The calculation of the reconstituted fatty acid composition from each acylglycerol fraction (Tables 3 and 4) was comparable to the direct measurement of neutral lipid fatty acids (Table 2) and confirmed the higher disappearance of stearic acid and the accumulation of linoleic and linolenic acids in diacylglycerols and monoacylglycerols (Table 5). In addition, a significantly ($P < 0.05$ TAG/DAG and $P < 0.01$ DAG/MAG) higher disappearance of fatty acids with a lower polarity (higher ECN) was observed (Fig. 2). Thus, fatty acid disappearance from triacylglycerols and diacylglycerols was inversely proportional to the polarity.

The putative atherogenicity of VLDL is attributable to the possibility that the VLDL remnants, formed by the catalytic action of LPL, enter the subendothelial space of the vascular vessel [30]. This hydrolytic activity of LPL depends to a great extent on the composition and structure of triacylglycerols [31]. Wang et al. [5] found that LPL hydrolysed fatty acids preferentially in the following order: 18:1 > 18:3 > 18:2 > 14:0 > 16:0 > 18:0. These experiments were carried out by in vitro incubation of cow-milk LPL with pure triacylglycerols. However, more recently, Sato et al. [32] observed that chylomicrons and VLDL enriched with palmitic acid were more rapidly hydrolysed than those enriched in oleic acid and these ones, more than those enriched in linoleic acid. These authors related the elevated rate of hydrolysis to a reduction in the fluidity of palmitic acid-rich lipoproteins, which would increase the affinity between lipoproteins and LPL. Similarly, we previously observed a more rapid disappearance of triacylglycerol molecular species containing palmitic and oleic acid than those containing linoleic acid during the postprandial metabolism of chylomicrons formed after the ingestion of virgin olive oil and

high-oleic sunflower oil [23]. The more effective disappearance of triacylglycerol-rich lipoproteins rich in saturated or apolar species, might facilitate the removal of these species from these particles towards adipose tissue, and also the incorporation of more polar fatty acids, including essential linoleic and linolenic acids, to other non-storage tissues. It is unlikely that the more rapid removal of saturated fatty acids is due to a more rapid uptake of VLDL remnants. The liver is the organ involved to a greater extent in the uptake of lipoprotein remnants, via apo-E recognition by VLDL and LDL receptors and the LDL receptor-related protein (LRP) [33]. However, the fatty acid composition of lipoprotein remnants seems to have little or no influence on these receptors [34].

In conclusion, the method described here has proved a useful and rapid tool for the simultaneous determination of molecular species of underivatised monoacylglycerols, diacylglycerols and triacylglycerols in a single HPLC chromatogram. The method can be applied to the study of the metabolism of either VLDL or postprandial triacylglycerol-rich lipoproteins, but also to the activity and specificity of LPL.

5. Nomenclature

ECN	equivalent carbon number
CN	carbon number
DB	number of double bonds
NUFA	number of unsaturated fatty acids

Fatty acids

A	arachidonic acid (20:4)
Ln	linolenic acid (18:3)
L	linoleic acid (18:2)
O	oleic acid (18:1)
P	palmitic acid (16:0)
S	stearic acid (18:0)

Monoacylglycerols

L	monolinoleoyl-glycerol
O	monooleoyl-glycerol
P	monopalmitoyl-glycerol
S	monostearoyl-glycerol

Diacylglycerols

LL	dilinoleoyl-glycerol
OP	oleoyl-palmitoyl-glycerol

Triacylglycerols

LLL	trilinoleoyl-glycerol
OOP	dioleoyl-palmitoyl-glycerol
AOP	arachidonoyl-oleoyl-palmitoyl-glycerol

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