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Determination of rat liver triglycerides by gas–liquid chromatography and reversed-phase high-performance liquid chromatography

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Abstract

Rats fed with a fat-free or an olive oil-rich diet were employed to compare the response of two chromatographic techniques in the determination of rat liver triglyceride (TG) molecular species composition. Gas–liquid chromatography (GLC) on polarizable liquid phase and reversed-phase high-performance liquid chromatography (RP-HPLC) have been commonly employed for TG analysis, obtaining a similar number of chromatographic peaks when used for animal tissue TG determination. In the present study similar results were achieved with regard to most relevant chromatographic peaks, however, important differences were found in the content of minor TGs. Indeed, RP-HPLC permitted separation of long chain polyunsaturated fatty acids, which were not detected by GLC, while the latter technique reported a higher number of myristoyl-containing TG species. RP-HPLC analysis reported a greater number of TGs, with more similarity to a random composition, made up from the liver fatty acid composition. Therefore, it was concluded that utilization of both techniques would be helpful for liver TG analysis as the use of only one of them does not provide a complete profile of liver TGs. Nevertheless RP-HPLC seems to be more useful for this purpose since revealed a more extensive profile. © 1998 Elsevier Science B.V.

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1. Introduction

The progress in chromatographic techniques has enormously facilitated the analysis of triglycerides (TGs) [1]. Reversed-phase high-performance liquid chromatography (RP-HPLC) is becoming commonly employed for this purpose. The separation mechanism includes chain length and degree of unsaturation of their constituent fatty acids [1]. RP-HPLC offers important advantages as a qualitative and also as a quantitative tool in TG analysis. The wide range of column sizes and materials along with the variety of eluting systems allow the separation of TG species. In addition, sample does not need to be derivatized and the detectors employed permit recovery of components [2]. One of the most challenging research areas in RP-HPLC of natural samples of TGs is the identification of the molecular species. An important number of researchers use the theoretic prediction of TGs from the RP-HPLC peaks as a

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useful tool in the TG identification process providing a number of possible TGs for each chromatographic peak. The more or less accuracy in the prediction will depend on the complexity of the fat and the mathematic equations employed [3–6].

However, a considerable number of authors still use gas–liquid chromatography (GLC) for TG molecular species analysis [7–9]. Capillary column GLC has been used to separate TGs according to their degree of unsaturation and to the carbon number [10,11]. The separation is made by the total number of carbons, and within each TG class with the same number of carbons the retention is higher in growing order of unsaturation degree. Since the 1970s, capillary GLC using apolar columns has become a routine mechanism for characterising the TG profile of fats [12]. Today, capillary columns filled with high-temperature polarisable phenylmetylsilicone have been shown to be as effective as RP-HPLC for the quantification of saturated and monounsaturated TGs, except for long chain fatty acid TGs [13,14].

GLC and RP-HPLC are being both recently employed for animal tissues TG composition evaluation. Adipose tissue [15], very low density lipoproteins (VLDLs) [7,16] and liver [17,18] TGs have been analyzed by GLC. Although some significative results have been obtained in liver TG investigation by RP-HPLC with light-scattering detection [19,20], they were not much more relevant than those obtained by GLC.

It is well established that diets containing very low levels of fat promote alterations on the liver lipid metabolism [21]. One of the most significative effects of administration of these diets is the increase of hepatic TG levels, correlated with a subsequent increase in VLDL TGs. Although these effects have been reported in animals fed a fat-free diet [22–25], there is actually no information on the TG molecular species composition of the liver after feeding animals with such a diet. On the other hand, high-fat diets also cause modifications on the lipid metabolism. Indeed, high-monounsaturated fatty acid diets have been shown to establish a less atherogenic serum lipid profile [26,27].

This study was carried out to determine changes in the liver TG composition of rats fed a fat-free diet compared with rats fed a MUFA-rich diet (olive oil). In addition, we evaluated the different responses obtained when GLC or RP-HPLC were applied for rat liver TGs determination in order to establish the suitability of both techniques in the TG analysis of a complex tissue, as the liver is.

2. Experimental

2.1. Animals and diets

Wistar rats weighing 30–35 g purchased from Letica (Barcelona, Spain) were used. They were randomly distributed into two groups of twelve rats and were individually housed in a room at 21±2°C with a light/dark cycle of 12 h. Animals were fed with a fat-free diet (FF) or FF supplemented with 10% virgin olive oil (OO). Feeding was ad libitum. Fat-free diet contained 20.8% milk casein, 19.6% corn starch, 37% glucose, 5.3% cellulose, 6.3% mineral mix and 1% vitamin mix (Panlab, Barcelona, Spain). The fatty acid composition of the employed oil is shown in Table 1. After twelve weeks of feeding rats fed with FF diet weighed 301.3±9.0 g while the weight of rats fed OO was 313.2±11.4 g. The ratio liver weight/total weight was similar for all rats (0.014 for rats fed FF diet vs. 0.020 for rats fed OO). This means that liver weight was in all cases proportional to rat weight. Rats were sacrificed by decapitation and livers obtained and preserved at −80°C until used.

Table 1
Fatty acid composition of virgin olive oil (VOO)

Fatty acid	VOO (%)
16:0	11.9
16:1 $n-7$	0.9
18:0	2.8
18:1 $n-9$	79.3
18:2 $n-6$	3.6
18:3 $n-3$	0.6
20:0	0.3
20:1 $n-9$	0.2
24:0	0.4

Percentage of total fatty acid methyl esters.

2.2. Sample preparation

Total lipids were extracted following the method described by Folch et al. [28]. Different lipid fractions were separated by thin-layer chromatography (TLC) on silica gel 60 plates (Kieselgel 60 F_{254}, Merck) using an elution system of hexane–diethyl ether–acetic acid (80:20:1, v/v/v), according to the method of Ruiz-Gutiérrez et al. [7].

2.3. RP-HPLC analysis

TGs fraction, vacuum-evaporated to dryness, at a temperature below 30°C, was redissolved in *n*-hexane and passed through a filter with a pore size of 0.2 μm (Millipore). Injections of 10-μl volume containing 60 μg of TGs were used for HPLC analysis. The chromatographic system consisted of two pumps, Model 422M and 422S (Kontron), a Model 7161 injector (Rheodyne), two 20-cm long stainless steel columns connected in series with an internal diameter of 4.6 mm containing a bonded-phase of Spherisorb ODS-2 (Phase Separations) with a particle size of 3 μm, and a light scattering detection system Model Sedex 45 (Sedere) and an automatic air compressor Model Compact 106 (Cedime). The system was controlled by a computer through MT-450 Data System (Kontron), and connected to a matrix printer model LQ 570 (Epson). The columns were submerged in a thermostatted water bath at 30°C. The mobile phase consisted of an initial elution gradient of 20 to 80% (v/v) of acetone–acetonitrile during 100 min. After this time, the percentage of acetone was raised to reach the 100% at minute 105, and held to the end of the analysis. The flow-rate was 1.0 ml/min.

Quintupled runs of 10 μl of *n*-hexane solution containing 0.5 mg/ml of pure rac-TGs (Sigma Grade, 99% pure) tritridecanoin, 1,2-dipalmitoyl–oleoyl–glycerol, trimiristin, 1,2-distearoyl–3-miristoyl–glycerol, triheptadecanoin, 1,2-distearoyl–3-palmitoyl–glycerol, 1-palmitoyl–2-oleoyl–3-stearoyl–glycerol, 1,2-dioleoyl–3-palmitoyl–glycerol, 1,2-dipalmitoyl–3-miristoyl–glycerol, 1,2-dimiristoyl–3-palmitoyl–glycerol, tripentadecanoin, 1,3-distearoyl–2-oleoyl–glycerol, 1,2-dimiristoyl–3-lauroyl–glycerol, 1-miristoyl–2-oleoyl–3-palmitoyl–glycerol, tripalmitin, triolein and 1,3-dipalmitoyl–2-linoleoyl–glycerol were analysed in order to calculate de equations used in the prediction of the TG molecular species of rat liver.

All of the solvents used, both in dissolving sample TGs and in the mobile phase were HPLC grade (Normasolv, Scharlau). The dead volume (v_0) was calculated as the difference in column weight when saturated in methanol and when saturated in water.

2.4. GLC analysis

The analysis of TGs by GLC was performed by a Model 5890 series II gas chromatograph (Hewlett-Packard) equipped with a flame ionization detection (FID) system and a 400 65 HT aluminium-clad capillary 65% phenylsilica column (Quadrex, New Haven, CT, USA) of 25 m×0.25 mm I.D. Modifications of the method proposed by Ruiz-Gutierrez et al. [8] were made in order to improve resolution of the analysis.

Lipids were transmethylated and the resulting fatty acid methyl esters (FAME) analyzed by GLC as described by Ruiz-Gutierrez et al. [7] using a Model 5890 series II gas chromatograph (Hewlett-Packard) equipped with a flame ionization detection (FID) system and a capillary silica column Omegawax 320 (Sulpelco) of 50 m×0.32 mm I.D.

2.5. Calculation of TG composition

TG composition of rat liver by HPLC was predicted by means of relationships between the capacity factors (k') and molecular variables of the pure TGs. The equivalent carbon number (ECN) was calculated according to the following equation:

$$\mathrm{ECN} = \mathrm{CN} - a_1' \cdot \mathrm{DB} - a_2' \cdot \mathrm{NUFA} \quad (1)$$

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 TG molecule. The values of the constants a_1' and a_2' were calculated by multiple linear regression analysis of the experimental values of the dependent variable, log k', and the independent variables CN, ND and NUFA for the pure TGs ($\log k' = q' + b' \cdot \mathrm{CN} + c' \cdot \mathrm{ND} + d' \cdot \mathrm{NUFA}$), where a_1' is the quotient between

the coefficients c' and b', and a_2' between the coefficients d' and b'.

Firstly, a simple linear regression analysis was applied to relate ECN with log k' of the pure TGs. According to the methodology proposed by Takahashi et al. [4], a multiple linear regression was secondly applied to relate chain length (CL) and number of double bounds (DB) of each of the three fatty acids of the TG molecule to log k' of the pure TGs. The stereoespecific positions in the glycerol molecule were equivalent because HPLC analysis cannot separate positional and conformational isomers [29]. Finally, the random composition was used to establish the probability for the presence of the TGs in each HPLC peak when more than one molecular species was predicted.

2.6. Statistical analysis

Data were evaluated with Student's two-tailed paired *t*-test. The significance of the differences between the groups were assessed by analysis of variance (ANOVA).

3. Results and discussion

GLC permitted separation of 22 chromatographic peaks from the rat liver TGs fraction. Our method is a modification of one previously proposed by Ruiz-Gutierrez et al. [8] for the analysis of TGs of rat caecal mucosa, where 21 chromatographic peaks were already separated. The same method was successfully applied for TGs determination of VLDLs from human serum [7]. This result improves those reported by other authors who reported no more than 17 chromatographic peaks when GLC was employed for the separation [17,18] (Table 2). Despite the high resolution achieved by GLC, RP-HPLC could resolve 30 chromatographic peaks. Our RP-HPLC method improved those reported for determination of rat liver TG molecular species [19,20,30] (Table 3), of rat adipose tissue [16] and VLDLs [30]. Nevertheless, Yang et al. [30] were able to describe up to four triglycerides containing docosahexaenoic acid in rat liver.

Fifteen fatty acids were identified by GLC analysis of rat liver TGs fraction. Only the fatty acids with percentages equal or higher than 0.5 were used for the TGs prediction. These fatty acids were oleic (29.5%), linoleic (28.7%), palmitic (23.5%), palmitoleic (6.0%), arachidonic (4.0%), stearic (2.0%), docosahexaenoic (1.5%), linolenic (1.1%) and myristic (0.5%) acids. Making all the possible combinations between these fatty acids, the total number of possible TGs in rat liver was 220, considering stereospecific positions equivalent. The range of ECN, calculated by the equation [2] and its standard error, provided the selection of the possible molecular species for each RP-HPLC peak. The equations proposed by Takahashi et al. in 1985 [31] for TG prediction were applied.

$$\mathrm{ECN} = -18.6190 + 46.7656 \cdot \log k' \quad \mathrm{S.E.} = 0.6593 \tag{2}$$

Table 2
Comparison of triglyceride identification in rat liver by GLC

No.	Our results	Chen et al. [17]	Chen and Cunnane [18]
1	MPP	–	–
2	MMO	–	–
3	MML	–	–
4	PPP	PPP	–
5	MPO	MPO/PPPo	–
6	–	PoPoPo	–
7	MPL	–	–
8	MPoL	–	–
9	MSS	–	–
10	–	PPS	PPS
11	PPO	PPO	PPO
12	PPL/PPoO	PPL	PPL
13	MOL/PPoL	PPoL	PPoL
14	MLL	–	–
15	PSO	PSO	PSO
16	POO	POO	POO
17	POL	POL	POL
18	PLL	PLL	PLL
19	SSO	–	–
20	SOO	SOO	SOO
21	OOO	OOO	OOO
22	SOL	–	–
23	OOL	OOL	OOL
24	–	–	SLL
25	OLL	OLL	OLL
26	–	LLL	LLL

Fatty acids: M = myristic acid, 14:0; P = palmitic acid, 16:0; S = stearic acid, 18:0; Po = palmitoleic acid, 16:1; O = oleic acid, 18:1; L = linoleic acid, 18:2; Ln = linolenic acid, 18:3; A = arachidonic acid, 20:4; D = docosahexaenoic acid, 22:6. Triglycerides: PPP = sn-glycerol–tripalmitate; MPL = sn–glycerol–myristate–palmitate–linoleate; POL = sn-glycerol–palmitate–oleate–linoleate.

Table 3
Comparsion of triglyceride identification in rat liver by RP-HPLC

No.	Our results	Huang et al. [19]	Yang et al. [30]
1	–	–	OLD
2	OOD/OOA	–	OOD
3	LLA/PoLA/LLLn	LLA	–
4	PLD	–	PLD
5	PPoD	–	–
6	MMA/MMLn	–	–
7	OLA	OLA	OLA
8	LLL	LLL	–
9	PoLL	PoLL	–
10	POD	–	–
11	PLA	PLA	–
12	PLLn/PPoA	PLLn	–
13	PPD/MML	–	PPD
14	–	MLL	–
15	MMM	–	–
16	OLL	OLL	–
17	–	POA	POA
18	–	POLn	–
19	PLL/PPoL	PLL/PPoL	PLL
20	PPA/MPL	–	–
21	PPLn/MPL	–	–
22	MMP	–	–
23	OOL	OOL	OOL
24	POL	POL	POL/SLL
25	PPL/MPO	PPL	PPL
26	MPP	–	–
27	OOO	OOO	OOO
28	POO	POO	POO/SOL
29	PPO	–	PPO
30	PPP	–	–
31	SOO	–	SOO
32	SSL	–	–
33	PSO	–	POS
34	MSS	–	–

Abreviations as in Table 2.

On the other hand, the experimental values of log k' for the TG standards, and the logic sequence of elution in growing order of ECN, were also taken into account for the final estimation of TGs from the individual chromatographic peaks [32]. Finally, in the cases in which the prediction systematics assigned more than one TG to only one HPLC peak, the random composition was used to establish the highest probability for the existence of TGs in the peaks. Although it has been reported that significant differences do occur between experimental quantitative composition and theoretical random composition for the natural TGs [33,34], the random composition may, from a qualitative standpoint, furnish extremely useful information to complete the TG molecular prediction.

Taking into account the results of Table 4 it could be asserted that the main TGs in the rat liver were palmitoyl–oleoyl–linoleoyl–glycerol (POL), dioleoyl–linoleoyl–glycerol (OOL), palmitoyl–dioleoyl–glycerol (POO), triolein (OOO), palmitoyl–dilinoleoyl–glycerol (PLL), dipalmitoyl–oleoyl– glycerol (PPO) and oleoyl–dilinoleoyl–glycerol (OLL). Three main TGs containing arachidonic acid (20:4, $n-6$) were predicted: dioleoyl–arachidonoyl–glycerol (OOA), dipalmitoyl–arachidonoyl–glycerol (PPA) and oleoyl–linoleoyl–arachidonoyl–

Table 4
Mean triglyceride composition of the liver of rats fed a fat-free diet (FF) or an olive oil diet (OO) determined by RP-HPLC

Peak	Triglyceride	FF (%)	OO (%)
1	OOD/OAA	n.q.	n.q.
2	LLA/PoLA/LLLn	n.q.	n.q.
3	PLD	0.22±0.00	0.06±0.00
4	PPoD	0.11±0.04	n.q.
5	MMA/MMLn	0.03±0.00	n.q.
6	OLA	0.08±0.00	n.q.
7	LLL	0.13±0.00	n.q.
8	PoLL	0.33±0.36	0.11±0.09
9	POD	0.14±0.12	0.05±0.00
10	PLA	0.17±0.09	n.q.
11	PLLn/PPoA	n.q.	n.q.
12	PPD/MML	0.54±0.13	0.03±0.01*
13	MMM	0.91±0.16	0.21±0.12*
14	OLL	3.38±1.02	0.78±0.28*
15	PLL/PPoL	12.55±4.23	1.23±0.59*
16	PPA/MPL	n.q.	0.44±0.08
17	PPLn/MPL	1.00±0.14	0.54±0.19
18	MMP	n.q.	0.08±0.00
19	OOL	5.87±0.47	12.23±1.75**
20	POL	38.65±5.32	20.00±3.53**
21	PPL/MPO	1.97±0.42	0.25±0.10**
22	MPP	1.09±0.00	0.11±0.07
23	OOO	1.81±0.85	18.17±7.41*
24	POO	22.89±6.19	44.61±4.14**
25	PPO	3.36±0.83	0.99±0.67*
26	PPP	0.29±0.07	0.08±0.06
27	SOO	0.03±0.00	0.24±0.06
28	SSL	0.48±0.05	0.27±0.28
29	PSO	0.56±0.56	0.12±0.05
30	MSS	0.08±0.00	0.03±0.00

Results are given as mean±S.D. for three separate determinations. Abreviations as in Table 2.
n.q.=Not quantified. *: $p<0.05$; **: $p<0.01$.

glycerol (OLA); and other three main TGs containing docosahexaenoic acid (22:6, $n-3$): dipalmitoyl–docosahexaenoyl–glycerol (PPD), palmitoyl–oleoyl–docosahexaenoyl–glycerol (POD) and palmitoyl–linoleoyl–docosahexaenoyl–glycerol (PLD). In both cases arachidonic and docosahexaenoic acids were combined with oleic, linoleic or palmitic acids to form the TG molecules.

Table 5 shows the TGs of the rat liver after GLC analysis. The main molecular species were palmitoyl–oleoyl–linoleoyl–glycerol (POL), dioleoyl–linoleoyl–glycerol (OOL), palmitoyl–dioleoyl–glycerol (POO) and triolein (OOO). This finding is in agreement with that found by RP-HPLC analysis (Table 4). However, no TGs containing arachidonic or docosahexaenoic acid could be detected. This is in concordance with previously cited works performed by GLC [8,17,18] or RP-HPLC [19,20,30], which indicate that GLC causes a lack in the detection of long-chain polyunsaturated fatty acids. One probable reason for this effect is that these fatty acids are very unstable at the high temperatures employed for the analysis by GLC and may be destroyed [13,35]. The only tissue in which long chain polyunsaturated fatty acid-containing TGs have not been found after RP-HPLC was adipose tissue [16]. As these fatty acids are preferentially incorporated into phospholipids, only when the intake of linoleic acid, and its subsequent transformation into arachidonic acid, is very high does the latter accumulate in adipose tissue [15]. Taking all these results as a whole, we may consider that RP-HPLC results are more reliable than GLC for the determination of polyunsaturated TGs.

Table 5
Mean triglyceride composition of the liver of rats fed a fat-free diet (FF) or an olive oil diet (OO) determined by GLC

Peak	Triglyceride	FF (%)	OO (%)
1	MPP	0.15±0.12	0.01±0.00
2	MMO	0.20±0.10	0.07±0.03
3	MML	0.18±0.07	0.12±0.04
4	PPP	0.45±0.24	0.26±0.09
5	MPO	1.49±0.36	0.57±0.08
6	MPL	1.83±0.15	0.44±0.10**
7	MPoL	1.18±0.23	0.28±0.08**
8	MSS	0.34±0.02	0.09±0.02
9	PPO	4.71±0.67	2.77±0.50
10	PPL+PPoO	9.30±0.36	3.95±0.29**
11	MOL+PPoL	6.52±0.91	1.91±0.35**
12	MLL	3.44±0.29	0.59±0.31**
13	PSO	1.27±0.08	0.52±0.18**
14	POO	13.92±1.23	28.37±1.81**
15	POL	21.91±0.25	21.06±1.82
16	PLL	18.36±0.85	7.63±2.97**
17	SSO	0.54±0.34	n.q.
18	SOO	0.53±0.05	1.19±0.32*
19	OOO	1.29±0.09	9.78±2.30**
20	SOL	2.23±0.31	7.19±0.98**
21	OOL	5.93±0.19	10.97±0.80**
22	OLL	4.24±0.41	2.24±1.24

Results are given as mean±S.D. for three separate determinations. Abreviations as in Table 2.
n.q.=Not quantified. *: $p<0.05$; **: $p<0.01$.

The present study reveals that an important number of saturated TGs containing myristic acid is present in rat liver (Tables 4 and 5). Indeed, myristic acid was always combined with palmitic or stearic acid or forming dimyristoyl species. A greater number of saturated TGs containing mirystic acid (14:0) were found after the analysis by GLC (Table 4) compared with RP-HPLC analysis. Nevertheless, by RP-HPLC seven TGs containing this saturated fatty acid could be found. This is relevant since mirystic acid-rich TG had only been detected previously in rat liver by RP-HPLC by Yang et al. [36], who found increased biosynthesis of myristic acid containing TGs by the liver of rats on high glucose or fructose diets.

The variations produced in rat liver TG composition by the OO diet compared with FF diet were virtually the same, independently of the chromatographic technique employed for the analysis. Oleic acid-containing TGs (POO, OOO and OOL) were lower in rat liver after the fat-free diet when compared with rats consuming OO diet, due to the content of this fatty acid in OO diet. On the contrary, those TGs composed mainly of palmitic acid or linoleic acid (PLL, POL, PPL and PPO) were significantly higher. It has been reported that the absence of fat in the diet promote the synthesis of TGs in the liver [22–25], however the way these TGs are distributed remains unknown. The enhancement of plasma and hepatic TG content after a low fat diet has been attributed to an increase in their hepatic secretion, without changes in hepatic lipase and lipoprotein lipase [37]. As the diet contains no fat, fatty acids for TG synthesis should come from adipose tissue or de novo synthesis. Linoleic acid (18:2) cannot be synthesized by mammals, so it must

come from adipose tissue. Chen and Cunanne [18], observed lower activity of $\Delta-6$ and $\Delta-5$ desaturases in fasting rats. For this reason, and considering an increase in linoleic acid content, they affirm that arachidonic acid must come also from adipose tissue. In the present study, arachidonic acid-containing TGs could not be efficiently compared. HPLC permitted identification but not quantification of such TGs, while GLC just did not show them.

In conclusion, RP-HPLC and GLC have been demonstrated as being suitable for TG analysis of rat liver. Both techniques allowed comparison of main TGs, in spite of reporting slightly different profiles. Differences between the techniques were found in the composition of minor TG. GLC registered more TGs containing myristic acid while RP-HPLC particularly reported TGs containing long-chain polyunsaturated fatty acids. For this reason, complementation of both techniques should be advisable. Nevertheless RP-HPLC showed more usefulness for rat liver TG molecular species determination since it revealed a more extensive profile.

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