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Selection of methylation procedures for quantitation of short-chain glycerol-bound compounds formed during thermoxidation

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Abstract

Five methylation procedures, including base- and acid-catalyzed methods, were tested in thermoxidized methyl linoleate and trilinolein, in order to quantitate major oxidation short-chain glycerol-bound compounds by gas chromatography. Results indicated that transmethylations using KOH in methanol or CH_3ONa–CH_3OH in *tert.*-butylmethyl ether were the most appropriate methods, given the excellent reproducibility and practically complete recovery obtained for the compounds of interest, mainly short-chain fatty acids and aldehydic acids. Also, formation of acids from aldehydes during thermoxidation as well as modifications of aldehydic functions under acidic conditions, such as conversion to acetals, were checked using dodecanal as model aldehyde. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Heating fats and oils at high temperature in the presence of air, a common procedure in culinary practices as frying, results in a complex mixture of new compounds [1–5]. Hydroperoxides, the primary oxidation compounds, readily decompose to a number of secondary oxidation products at high temperature. An important route of hydroperoxide breakdown proceeds by homolytic β-scission of the alkoxy radicals coming from allylic hydroperoxides, to form ultimately volatile and non-volatile oxidation compounds, the latter including short-chain glycerol-bound aldehydes, acids, ketones and alcohols [6–10]. Of particular chemical and nutritional interest are the non-volatile degradation products since they remain in the oil, are absorbed by the food, and subsequently ingested [11,12].

In a previous paper, we emphasized the interest of glycerol-bound aldehydes quantitation in frying fats, being the most abundant oxygenated structures expected to be formed among low-molecular-mass compounds that remain attached to the glyceridic backbone and accumulate during heating [13]. Also, short-chain aldehydes have been reported to be highly reactive physiologically [14] and, although the group of esterified aldehydes has rarely been investigated, some reports on 9-oxononanoic acid, the major aldehydic acid in oxidized lipids, indicated that it could induce hepatic lipid peroxidation [15] and affect hepatic metabolism [16,17].

Information available so far on the analysis of these aldehydic acids is limited to qualitative data. In this context, a combination of thin-layer chromatog-

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raphy (TLC) and high-performance liquid chromatography coupled with mass spectrometry (HPLC–MS) on dinitrophenyl hydrazone derivatives provided qualitative data on the types of aldehydic acids present in intact lipids molecules [18]. However, by applying this methodology, it would be difficult to obtain quantitative data on the occurrence of these compounds in dietary lipids since glycerol-bound aldehydes can be present in hundreds of combinations depending on the other fatty acyl groups esterified, which in turn may or not contain mono- or polyoxygenated functions. Evident advantages over the analysis of intact triacylglycerols could be obtained by converting fats and oils into simpler derivatives, thus concentrating the compounds bearing the oxidized structure. In this line, we reported qualitative analysis of aldehydic acids as fatty acid methyl ester (FAME) derivatives in frying oils by gas–liquid chromatography–mass spectrometry [11] and the need arose for deeper studies on derivatization methods for quantitative purposes.

Essential requirements of the derivatization technique selected should be good repeatability and recovery of the compounds of interest, avoiding artifact formation. Conversion to the simplest volatile derivatives, methyl esters, is by far the most common procedure for gas chromatography analyses but chemical modifications and formation of artifacts in the case of oxidized structures have been reported [19].

The objective of this study was to select methylation procedures suitable for quantitation of major short-chain glycerol-bound oxidation compounds, particularly aldehydic acids, formed during heating. Towards this end, methyl linoleate and trilinolein were subjected to thermoxidation conditions which closely simulate those for frying [20]. Methyl linoleate was used as control since it can be analyzed without further derivatization, also being representative of the main fatty acid undergoing degradation in vegetable oils at high temperature.

2. Experimental

2.1. Samples and reagents

Silica gel 60 for column chromatography (particle size 0.063–0.100 mm) and thin-layer chromatography plates were purchased from Merck (Darmstadt, Germany). Methyl linoleate (ML, $C_{18:2}$ n-6), methyl oleate (MO), trilinolein (LLL), methyl octanoate ($C_{8:0}$), methyl tridecanoate ($C_{13:0}$) and methyl pentadecanoate ($C_{15:0}$) were purchased from Nu-Chek-Prep. (Elysian, MN, USA). Dodecanoic acid, dodecanal, azelaic acid, osmium tetroxide, periodic acid, sodium sulfite and 2-(2-ethoxyethoxy)ethanol were obtained from Sigma (St. Louis, MO, USA). *N*-methyl-*N*-nitroso-*p*-toluene-sulfonamide was obtained from Aldrich (Milwaukee, WI, USA). All other solvents and chemicals (of reagent grade or better quality) were obtained from local suppliers and were used without further purification except for dioxane which was freshly distilled from lithium aluminium hydride to remove peroxides.

2.2. Heating procedure

ML or LLL (2±0.01 g) were weighed directly into standard glass tubes (20 cm×12 mm I.D.) Tubes were introduced into a Rancimat vessel (Metrohm, Herisau, Switzerland) containing 8 g of glycerol to facilitate heat transfer, and inserted in the heating block previously heated at 180±1°C. The reaction vessels were left open to air during heating [20]. After 15 h heating, samples were taken out and kept at −20°C until analysis.

2.3. Synthesis of methyl 9-oxononanoate (9-oxo$C_{9:0}$)

Methyl 9-oxo$C_{9:0}$ was prepared using the tetroxide–periodate method, essentially as described by Kamido et al. [15]. To a solution containing 0.5 mmol of MO in 25 ml of pyridine–dioxane (1:8), 25 ml of a 4 m*M* osmium tetroxide in dioxane solution were added. After stirring for 2 h at room temperature, 220 ml of a suspension of sodium sulfite (40 g–60 ml methanol–240 ml water) were added and mixed. The solution was stirred at room temperature for 1.5 h, filtered and extracted with chloroform–methanol (2:1, v/v). The organic layer was evaporated to dryness under reduced pressure and dissolved in 35 ml of dioxane. A 1.5-g amount of periodic acid in 35 ml water was added and stirred at room temperature for 1 h. The mixture was diluted

with 150 ml chloroform and washed three times with 75 ml of 0.01 *M* NaOH. The chloroformic layer was dried using Na_2SO_4 and evaporated to dryness. The yellow-coloured residue was applied to TLC plates and hexane–diethyl ether (80:20, v/v) was used as developing solvent. Finally, purified methyl 9-oxo$C_{9:0}$ was isolated (51% yield, GC purity >99%).

2.4. Synthesis of methyl 9-oxo$C_{9:0}$ dimethyl acetal

Methyl 9-oxo$C_{9:0}$ (50 mg) were dissolved in 2.5 ml of boron trifluoride reagent–methanol (BF_3–methanol, 14%, w/v) under nitrogen. The mixture was then heated at 95°C for 30 min, cooled, and opened. The dimethyl acetal was extracted by adding 2.5 ml of water and 3×5 ml of hexane. After drying the organic phase over anhydrous sodium sulphate (Na_2SO_4) and evaporation of the solvent on a rotatory film evaporator, the residue was purified on silica TLC plates using hexane–diethyl ether (80:20) as developing solvent, and the dimethyl acetal of 9-oxo$C_{9:0}$ was isolated (60 mg, GC purity >98%).

2.5. Methylation procedures

A standard solution was prepared with a mixture of $C_{13:0}$ and $C_{15:0}$, around 500 μg/ml each in the solvents [diethyl ether, hexane or *tert*.-butyl methyl ether (TBME)] used in the different methylation procedures.

2.5.1. Base-catalyzed methylation using CH_3ONa at room temperature [21]

Samples of 50 mg were accurately weighed into a screwcapped centrifuge tube of 5 ml and dissolved in 0.5 ml of the standard solution prepared in MTBE. A 0.5-ml volume of 0.2 *M* CH_3ONa solution in methanol was added, the vial closed, shaken for 1 min and left for 2 min at room temperature. A 0.1-ml volume of 0.5 *M* H_2SO_4 solution was then added and the mixture was shaken for a few seconds. The mixture was diluted with 1.5 ml of water, shaken for 10 s and centrifuged. A 2-μl volume from the organic layer was injected into the GC system.

2.5.2. Base-catalyzed methylation using KOH at room temperature

The IUPAC standard method [22] was applied with slight modifications. Samples of 100 mg were accurately weighed into a screwcapped vial and dissolved in 0.5 ml of the standard solution prepared in hexane. A 0.1-ml volume of 2 *M* KOH in methanol was added, the vial was closed and shaken vigorously for 15–20 s. Once the solution became clear, 2 μl from the hexane layer were injected in GC. A modification introduced to improve extraction of the compounds of interest consisted in replacing hexane with diethyl ether, and adding 1 ml of brine solution and acetic acid (approximately 0.1 ml) until neutral pH, before shaking the vial. If necessary, the sample was then centrifuged.

2.5.3. Acid-catalyzed methylation using BF_3 [23]

Samples of 20 mg were accurately weighed into a centrifuge tube with a PTFE-lined screw cap and dissolved in 0.1 ml of the standard solution prepared in diethyl ether. After evaporation to dryness under nitrogen, BF_3 reagent [35% BF_3–methanol (14%, w/v), 30% benzene, 35% methanol] was added under nitrogen and the tube was closed with a screw cap. The tube was then heated at 95°C for 30 min and cooled. FAMEs were extracted by adding 2.5 ml of water and 3×5 ml of hexane. Eventually, it was necessary to centrifuge to break the emulsion formed. After drying the organic phase over anhydrous Na_2SO_4, the solvent was removed under a stream of nitrogen and FAMEs were dissolved in diethyl ether (200 mg/ml) and 2 μl were injected into the GC system.

2.5.4. Combination of CH_3ONa and acid-catalyzed methylation with H_2SO_4–methanol [22]

Samples of 50 mg were accurately weighed and dissolved in 0.25 ml of the standard solution prepared in diethyl ether. After evaporation to dryness under nitrogen, sample was refluxed with 1.25 ml of 0.2 *M* CH_3ONa in methanol for 20 min. Then, 1.5 ml of 4% H_2SO_4 in methanol was added and the reaction mixture was refluxed for 20 min. After cooling, the sample was extracted successively with hexane (2×4 ml), hexane–diethyl ether (1:1) (2×4 ml) and diethylether (4 ml). Solvents were removed under a stream of nitrogen and FAMEs were dissolved in diethyl ether (200 mg/ml) and 2 μl were injected into the GC system.

2.5.5. Saponification and methylation using diazomethane

Samples of 20 mg were dissolved in 0.1 ml of the standard solution prepared in diethyl ether. After evaporation to dryness under nitrogen, sample was refluxed with 4 ml of 1 *M* KOH in 95% ethanol at room temperature overnight. The soap solution was acidified with 6 *M* HCl solution in water and free fatty acids extracted with hexane–diethylether (1:1) (3×10 ml). The extract was washed with water, dried over anhydrous Na_2SO_4 and solvents evaporated under reduced pressure. For methylation, a device was used [24] where the first tube was half-filled with diethyl ether (approximately 5 ml) and the second tube contained 3 ml of 2-(2-ethoxyethoxy)-ethanol, 3 ml of diethyl ether and 3 ml of 60% NaOH solution. Approximately 1 g of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide was added to the second tube and both tubes connected. A stream of nitrogen (5–10 ml/min) was applied to the first tube, entered the second tube being saturated with ether and carried the diazomethane generated in the second tube into the vial containing the sample (free fatty acids) diluted in diethyl ether. Methylation was complete upon development of a light yellow colour. In the case of ML, methylation without previous saponification was also simulated to detect any possible artifact formation in this step. FAMEs were dissolved in diethyl ether (200 mg/ml) and 2 μl were injected into the GC system.

2.6. Thin-layer chromatography

Analytical TLC was performed on 0.25 mm pre-coated silica gel 60 plates, eluted with hexane–diethyl ether–acetic acid (80:20:1, v/v/v) and visualized by exposure to iodine vapours.

2.7. Gas–liquid chromatography

FAMEs were analyzed by GC using a HP 6890 chromatograph on a HP Innowax capillary column (cross-linked polyethylene glycol, 30 m×0.25 mm I.D., film thickness 0.25 μm) (Hewlett-Packard, USA), under the following temperature programme: 90°C (2 min), 4°C/min to 240°C (25 min). Samples were introduced to the column via a split injector (split ratio 1:40) at 250°C and the flow-rate of hydrogen, used as carrier gas, was 1 ml/min. Temperature of both split injector and flame ionization detector was 250°C.

2.8. Gas–liquid chromatography–mass spectrometry

GC–MS analyses were performed with a Finnigan MAT95 double focusing mass spectrometer (Finnigan MAT, Bremen, Germany) operating in the electron-ionization mode. Electron energy was 70 eV, multiplier voltage 1500 V, source temperature 200°C and transfer line 250°C. Spectral data was acquired over a mass range of 28–600 at a scan rate of 1 s/scan. Chromatographic conditions were the same as those used for the GC analyses.

3. Results and discussion

Fig. 1 presents a representative gas chromatogram showing the separation achieved of the main structures found in ML thermoxidized for 15 h. Apart from $C_{13:0}$ and $C_{15:0}$, added as internal standards, all peaks eluting at retention times shorter than that for the intact ML (32 min) corresponded to oxidation compounds of lower molecular mass. Among the most relevant compounds identified were short-chain FAMEs, methyl $C_{7:0}$ and $C_{8:0}$; and aldehydic FAMEs, methyl 8-oxooctanoate (8-oxo$C_{8:0}$) and 9-oxo$C_{9:0}$. As to those compounds of molecular mass higher than that of the starting ML, a complex mixture of degradation products eluted at retention times beyond 32 min, where the main groups identified corresponded to epoxyacids, ketoacids and hydroxyacids. The predominant epoxyacid structures found at high temperature in model systems of triacylglycerols and fatty acid methyl esters have been recently the subject of detailed identification and quantitation [25].

Table 1 lists the low-molecular-mass compounds attached to triacylglycerol backbone coming from linoleate hydroperoxide breakdown through homolytic β-scission of the alkoxy radicals formed. Volatile compounds produced concurrently have been omitted. From the most abundant 9-hydroperoxy-10,12-dodecadienoate (9-HOPD) and 13-hydroperoxy-9,11-dodecadienoate (13-HPOD), the ex-

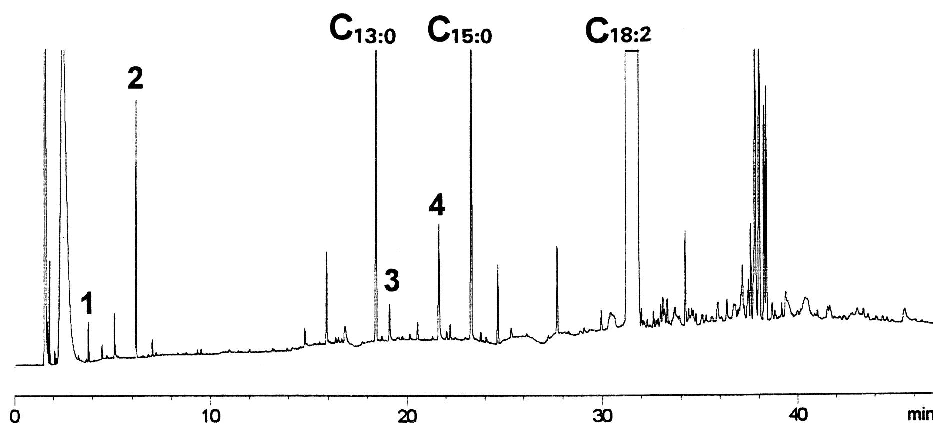

Fig. 1. Representative gas chromatogram of thermoxidized methyl linoleate (control). Peak assignments: $C_{13:0}$, methyl tridecanoate, internal standard (t_R = 18.4 min); $C_{15:0}$, methyl pentadecanoate, internal standard (t_R = 23.3 min); $C_{18:2}$, methyl linoleate (t_R = 32.1 min); 1, methyl heptadecanoate (t_R = 4.4 min); 2, methyl octadecanoate (t_R = 6.1 min); 3, methyl 8-oxooctanoate (t_R = 19.1 min); 4, methyl 9-oxononanoate (t_R = 21.6 min).

pected compounds are 8-hydroxyoctanoate, $C_{8:0}$, 12-oxododec-9-enoate and dodeca-9,11-enoate through route A, and 9-oxo$C_{9:0}$ and 13-oxotrideca-9,11-dienoate through route B. Thus, special attention was placed to the identification of these compounds. Nevertheless, under the conditions used, only $C_{8:0}$ and 9-oxo$C_{9:0}$ coming from 9-HOPD were found in significant amounts. The presence of 8-oxo$C_{8:0}$ and traces of $C_{7:0}$ along with the absence of the expected diunsaturated compounds from 13-HPOD may be explained by further oxidation of such diunsaturated structures and decomposition of the alkoxy radicals formed through the same pathway above commented. Fig. 2 shows this tentative mechanism in the case of 13-oxotrideca-9,11-dienoate, which may oxidize to an allylic hydroperoxide that would further yield, among other compounds, $C_{7:0}$ and 8-oxo$C_{8:0}$, through routes A and B, respectively.

Quantitative analyses were focused on the three major compounds formed, i.e., the predominant short-chain FAMEs, and the two short-chain aldehydic FAMEs. Correction factors for methyl $C_{8:0}$ and synthesized methyl 9-oxo$C_{9:0}$ were calculated against the internal standards selected, $C_{13:0}$ and $C_{15:0}$, and the values found for methyl $C_{8:0}$ (0.98) and methyl 9-oxo$C_{9:0}$ (1.80) were applied for quanti-

Table 1
Short-chain glycerol-bound compounds formed by decomposition of hydroperoxides from linoleate, through homolytic β-scission of the alkoxy radical on either side of the carbon bearing the oxygen (A or B)

Hydroperoxides	Route A[a,b]		Route B
	+OH˙	+ H˙	
8-HOPE	7-Hydroxyheptanoate	Heptanoate	8-Oxooctanoate
9-HOPE[c]	8-Hydroxyoctanoate	Octanoate	9-Oxononanoate
10-HOPE	9-Oxononanoate	Non-8-enoate	10-Oxodec-8-enoate
11-HOPE	10-Oxodecanoate	Dec-9-enoate	11-Oxoundec-9-enoate
12-HOPE	11-Hydroxyundec-9-enoate	Undec-9-enoate	12-Oxododec-9-enoate
13-HOPE[c]	12-Oxododec-9-enoate	Dodeca-9,11-enoate	13-Oxotrideca-9,11-dienoate
14-HOPE	13-Oxotridec-9-enoate	Trideca-9,12-dienoate	14-Tetradeca-9,12-dienoate

[a] Scission on the bond closer to the carboxylate function.
[b] Radicals produced via route A further react with OH˙ or H˙.
[c] Outer and predominant 9- and 13-hydroperoxides of linoleate.

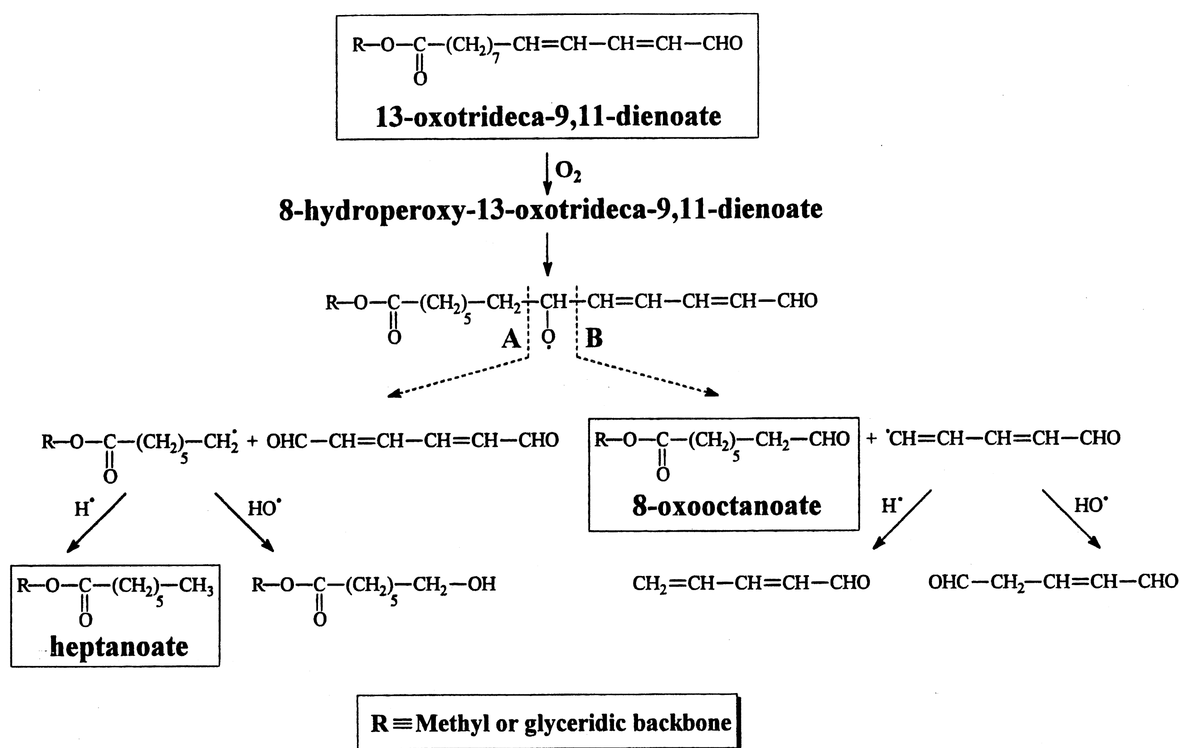

Fig. 2. Tentative mechanism for indirect formation of 8-oxooctanoate and heptanoate, by oxidation and decomposition of 13-oxotrideca-9,11-dienoate.

tation. Similar weight response was assumed for methyl 8-oxo$C_{8:0}$ than that for methyl 9-oxo$C_{9:0}$.

The analysis of ML directly after thermoxidation, referred as to ML control, provided an excellent control for the detection of artifact formation from the compounds of interest due to the use of derivatization techniques. Table 2 lists, in the first row, the quantitative results obtained for ML control (expressed as mg/g of initial sample), showing an excellent repeatability. Surprisingly, the contents of methyl 9-oxo$C_{9:0}$ were low as compared to those of methyl $C_{8:0}$ considering the route of formation. In effect, after the homolytic β-scission of the alkoxy radical from allylic hydroperoxides (Table 1), in this

Table 2
Major short-chain oxidation compounds (mg/g) quantitated in thermoxidized methyl linoleate after different methylation procedures[b]

Procedures	$C_{8:0}$	8-oxo$C_{8:0}$	$C_{8:0}$ acetal	$C_{8:0}$ diester	9-oxo$C_{9:0}$	$C_{9:0}$ acetal	$C_{9:0}$ diester
Control[a]	3.33±0.01	0.95±0.01	ND	ND	3.11±0.03	ND	ND
CH_3ONa	3.32±0.02	0.93±0.06	ND	0.20±0.03	3.09±0.08	ND	0.67±0.03
KOH	3.22±0.07	0.85±0.08	ND	0.33±0.03	2.81±0.22	ND	1.28±0.17
BF_3	Tr.	0.14±0.02	0.51±0.02	0.79±0.06	0.44±0.05	2.38±0.06	3.39±0.17
CH_3ONa–H_2SO_4	Tr.	0.43±0.12	0.30±0.23	1.25±0.16	1.06±0.30	0.53±0.24	4.37±0.51
Saponification–diazomethane	1.00±0.36	Tr.	ND	1.20±0.23	0.53±0.27	ND	4.58±1.03
Diazomethane	1.14±0.54	0.74±0.06	ND	0.54±0.04	2.48±0.24	ND	1.76±0.13

[a] Samples of methyl linoleate analyzed after thermoxidation (without applying methylation procedures).
[b] Values are means±standard deviation ($n=4$). Tr: traces (<0.1 mg/g). ND: not detected. Abbreviations: $C_{8:0}$, methyl octanoate; 8-oxo$C_{8:0}$, methyl 8-oxooctanoate; $C_{8:0}$ acetal, methyl 8-oxooctanoate dimethyl acetal; $C_{8:0}$ diester, suberic acid dimethyl ester; $C_{9:0}$, methyl nonanoate; 9-oxo$C_{9:0}$, methyl 9-oxononanoate; $C_{9:0}$ acetal, methyl 9-oxononanoate dimethylacetal; $C_{9:0}$ diester, azelaic acid dimethyl ester.

case the 9-HOPE, the aldehydic acids are directly formed via route B, while the radical attached to the glyceridic backbone formed via route A needs to abstract a hydrogen radical, in competition with other radicals, to form saturated short-chain fatty acyls. The results obtained could be explained in part by further oxidation of aldehydes to acids, but special comments on this subject have been included ahead in this paper.

The other results included in Table 2 correspond to the amounts of compounds obtained after applying the different methylation procedures.

The first method tested was a rapid base-catalyzed transesterification procedure using CH_3ONa in methanol at room temperature [21]. Among the inert solvents tested were hexane, which gave poor recovery (only 40%) of aldehydic FAMEs, or diethyl ether, which was too soluble in water. Finally, the low solubility in water and medium polarity of TBME allowed to recover the compounds of interest at levels practically identical to those found in the control. This is illustrated in the gas chromatogram obtained (Fig. 3a), showing practically the same profile than that of control thermoxidized ML (Fig. 1). Only two new small peaks (X and Y) were detected, which were identified as dimethyl esters of dicarboxylic acids, namely, suberic acid dimethyl ester and azelaic acid dimethyl ester, previously

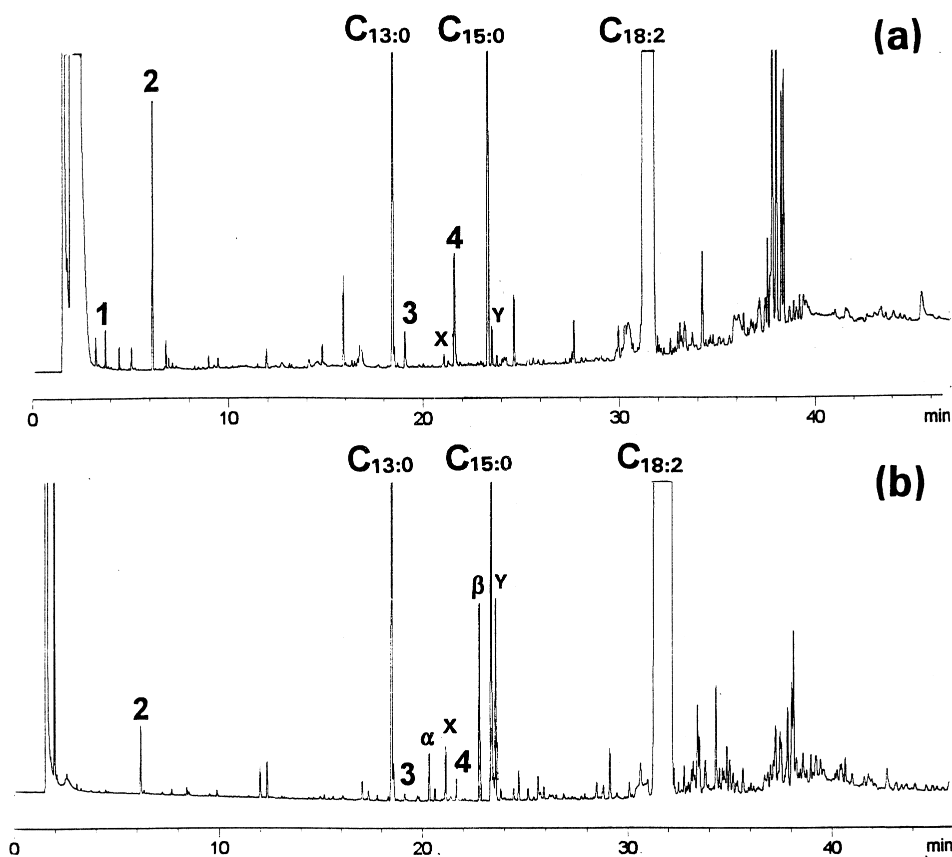

Fig. 3. Representative gas chromatograms of thermoxidized methyl linoleate after base-catalyzed methylation using CH_3ONa at room temperature (a), or acid-catalyzed methylation using BF_3 (b). Peak assignments: $C_{13:0}$, methyl tridecanoate, internal standard (t_R = 18.4 min); $C_{15:0}$, methyl pentadecanoate, internal standard (t_R = 23.3 min); $C_{18:2}$, methyl linoleate (t_R = 32.1 min); 1, methyl heptadecanoate (t_R = 4.4 min); 2, methyl octadecanoate (t_R = 6.1 min); 3, methyl 8-oxooctanoate (t_R = 19.1 min); 4, methyl 9-oxononanoate (t_R = 21.6 min); α, methyl 8-oxooctanoate dimethyl acetal (t_R = 20.4 min); β, methyl 9-oxononanoate dimethyl acetal; X, suberic acid dimethyl ester (t_R = 21.2 min); Y, azelaic acid dimethyl ester.

described as oxidation products [26]. Correction factor for azelaic acid dimethyl ester was calculated against the internal standards selected, $C_{13:0}$ and $C_{15:0}$, and the value found (1.53) was applied for quantitation. Similar weight response was assumed for suberic acid dimethyl ester.

Since this base-catalyzed procedure does not effect methylation of free fatty acids, the question arose as to whether these two saturated diesters were methyl ester derivatives of oxidation compounds resulting from thermoxidation or artifacts formed during transesterification. On the other hand, this transmethylation method did not practically change the profile of compounds eluting at retention times longer than that of intact ML.

The second method assayed was also a rapid base-catalyzed transesterification procedure using 2 *M* methanolic KOH at room temperature, principally designed to prepare FAMEs for analysis by GC. However, using hexane for extraction, following the IUPAC method, losses of aldehydes were observed (data not shown), probably because they partially remained in the methanolic layer. A more polar solvent, diethyl ether, was selected but, since it was very soluble in methanol, water was added and separation facilitated by centrifugation. A practically identical GC chromatogram than that for the first method was obtained here (not shown). As can be observed in Table 2, repeatability and recovery were excellent for $C_{8:0}$, 8-oxo$C_{8:0}$ and 9-oxo$C_{9:0}$ in both base-catalyzed methods.

The third method used was an acid-catalysed methylation procedure with methanol containing BF_3, while the fourth one was a method intended to be used for transesterification of triacylglycerols in a first step, followed by methylation of free fatty acids using CH_3ONa and H_2SO_4–methanol, respectively.

Fig. 3b shows the important changes occurred by using either of the two acid-catalyzed methods, including evident losses of low-molecular-mass compounds, enlargement of peaks X and Y, appearance of two new peaks (α and β) and a totally different picture of compounds eluting at long retention times. The loss of methyl $C_{8:0}$ (Table 2) was probably due to the aqueous extraction step, because short chain FAMEs are more soluble in water than longer chain FAMEs, and/or to heating for evaporation of the extraction solvents [19]. Further, peaks α (21.1 min) and β (23.6 min), identified as methyl 8-oxo$C_{8:0}$ dimethyl acetal and methyl 9-oxo$C_{9:0}$ dimethyl acetal, respectively, were likely artifacts coming from their respective aldehydic FAME under acidic conditions in the presence of a large excess of methanol. It was then necessary to determine the acetals mass response, and hence pure methyl 9-oxo$C_{9:0}$ dimethyl acetal was prepared. Correction factor for methyl 9-oxo$C_{9:0}$ dimethyl acetal against the internal standards ($C_{13:0}$ and $C_{15:0}$) was 1.02, which was also applied to methyl 8-oxo$C_{8:0}$ dimethyl acetal. Results showed that a large part of aldehydic functions were converted to acetals but, in the case of CH_3ONa–H_2SO_4, even considering together aldehydic FAME and their derived acetals, only half of the amount expected was recovered. On the other hand, very high values were found for diacid dimethyl esters using either of the acid-catalyzed methylation procedures.

The gas chromatogram in Fig. 3b also reflects important changes in the structures eluting after intact ML. In fact, epoxy fatty acids, very sensitive to acidic conditions, may have reacted with opening of the oxirane ring to give a mixture of methoxy-hydroxy products. Moreover, acidic catalyzed methylation can lead to isomerizations to conjugated dienes [27–29]. Lowering of temperature during methylation reduced the extent of this conversion [29,30]. Therefore, to avoid isomerization and incomplete methylation of conjugated linoleic acid, base-catalyst reagent such as CH_3ONa at room temperature is recommended [31,32]. On the other hand, methanol containing BF_3 or other strong acids have been reported to cause addition of methanol to conjugated diene systems [33].

Regarding combination of saponification–methylation with diazomethane, recoveries of methyl $C_{8:0}$ and both aldehydic FAMEs were very poor. However, when diazomethane was used alone, the recovery of aldehydic FAMEs was good while significantly lower amounts of methyl $C_{8:0}$ were found as compared to control. Therefore, while the loss of $C_{8:0}$ could be attributed to evaporation of solvents in both cases, modifications of the aldehydic functions seemed to be due to the saponification step. As to diacid dimethyl esters, levels found for the diazomethane method alone were intermediate as compared to those for base and acid-catalyzed procedures.

Table 3 shows parallel results obtained when LLL

Table 3
Major short-chain oxidation compounds (mg/g) quantitated in thermoxidized trilinolein after different methylation procedures[a]

Procedures	$C_{8:0}$	8-oxo$C_{8:0}$	$C_{8:0}$ acetal	$C_{8:0}$ diester	9-oxo$C_{9:0}$	$C_{9:0}$ acetal	$C_{9:0}$ diester
CH_3ONa ($n=6$)	3.42±0.04[b]	0.65±0.04	ND	0.11±0.02	2.64±0.12	ND	0.41±0.04
KOH ($n=10$)	3.19±0.25	0.52±0.04	ND	0.13±0.01	2.10±0.22	ND	0.46±0.02
BF_3 ($n=6$)	1.13±0.53	0.12±0.02	0.33±0.02	0.23±0.02	0.43±0.08	1.91±0.07	1.33±0.07
$CH_3ONa–H_2SO_4$ ($n=3$)	0.42±0.28	0.32±0.04	0.16±0.05	0.39±0.03	0.89±0.11	0.45±0.13	2.00±0.15
Saponification–diazomethane ($n=4$)	1.42±0.32	Tr.	ND	0.48±0.10	0.64±0.11	ND	2.39±0.40

[a] For abbreviations, see Table 2.
[b] Values are means±standard deviation; Tr, traces (<0.1 mg/g); ND, not detected.

was used under the same heating conditions. As expected, results were of the same order of magnitude than those found for ML (Table 2). Regardless of the procedure used, transmethylation of LLL model was effective and complete (no partial glycerides were detected by TLC). In general, lower values were obtained as compared to thermoxidized ML but results confirmed all the observations made. That is, the two base-catalyzed transesterification procedures at room temperature were the best methods to quantitate aldehydic FAMEs, and gave excellent results for methyl $C_{8:0}$ as compared to the others while acid-based methylation gave rise to acetal formation.

Additional tests were carried out using dodecanal as model aldehyde in order to investigate changes occurring in the aldehydic function during the different methylation treatments and the saponification step previous to methylation with diazomethane (Table 4), particularly to gain some insight into the important occurrence of acetals and diacids. GC–MS analysis showed that the commercially available dodecanal used contained 2.3% of 2-decyltetradec-2-enal, resulting from dehydration of the aldol condensation product formed between two dodecanal molecules (3-hydroxy-2-decyltetradecanal), and 1.9% of dodecanoic acid. During treatment with CH_3ONa and KOH, dodecanal was almost completely recovered, although KOH gave rise to a small amount of 2-decyltetradec-2-enal. In contrast, using BF_3, virtually all dodecanal was found as dodecanal dimethyl acetal and around 50% in the case of $CH_3ONa–H_2SO_4$. These results were consistent to those found in ML and LLL samples (Tables 2 and 3). The amounts of dodecanal converted to the aldol condensation product were very high for $CH_3ONa–H_2SO_4$ and especially for alkali saponification. These results suggest that methylation procedures using such alkali conditions could favor aldol condensation and hence the significantly lowest amounts of aldehydic FAMEs found for ML and LLL samples treated with $CH_3ONa–H_2SO_4$ and saponification–diazomethane (Tables 2 and 3). Dimeric condensation products thus formed could have involved reactions between two molecules of the same aldehydic acid, or two different aldehydic acids, or between one aldehydic acid and other carbonyl compound.

On the other hand, none of these procedures produced a significant amount of dodecanoic acid from dodecanal, suggesting that diacid dimethyl esters are not likely to be artifacts produced during the transmethylation step but methyl ester derivatives of compounds formed during thermoxidation. This

Table 4
Major compounds quantitated (wt%) in dodecanal sample treated with different methylation procedures

	Dodecanal	Dodecanal dimethyl acetal	Dodecanoic acid	Dodecanoic acid methyl ester	2-Decyl-tetradec-2-enal
Original sample	95.1±0.59		1.86±0.41		2.3±0.08
CH_3ONa	93.5±1.21		2.01±0.21		4.5±0.05
KOH	88.7±3.32			Tr.[a]	9.4±2.40
BF_3	0.5±0.01	94.3±0.51		2.3±0.18	2.8±0.06
$CH_3ONa–H_2SO_4$	0.9±0.15	49.8±2.73		4.5±0.21	38.7±2.55
Saponification	25.0±1.21		3.9±0.56		70.2±2.50

[a] Tr., traces (<0.05 mg/g).

hypothesis was supported by the results obtained for dodecanal heated under the same conditions used to prepare thermoxidized ML or LLL, showing that after 15 h heating, 80% dodecanal had been oxidized to dodecanoic acid. These results could also explain in part the unexpected low contents of methyl 9-oxo$C_{9:0}$ as compared to those of methyl $C_{8:0}$ in thermoxidised ML or LLL models (Table 2 and 3) already commented. Such modifications of aldehydic acids due to further oxidation during heating have been reported [26].

Special comments deserve the results obtained for diacid dimethyl esters, found in considerably high amounts in ML and LLL samples. In the original thermoxidized ML and LLL molecules, one carboxylic acid group would remain esterified to the methyl group or glyceridic backbone of ML and LLL, respectively, while the second carboxylic acid group formed by oxidation of the aldehyde group would be free. By means of BF_3, CH_3ONa–H_2SO_4 and saponification–diazomethane procedures, dimethyl esters would be obtained, while base-catalysed methods could only transmethylate the esterified carboxylic acid group. In fact, as expected, the highest values were found for BF_3, CH_3ONa–H_2SO_4 and saponification–diazomethane (Tables 2 and 3). Nevertheless, the presence of dimethyl esters after base-catalyzed transmethylations is a clear indication on the participation of free carboxylic groups in ester formation during thermoxidation. Such reactions have already been reported to occur under heating conditions, similar to those used here, being formation of estolides through esterification between a hydroxy group and a carboxylic group one of the major reactions described in the presence of fatty acids [34–36]. On the other hand, using diazomethane alone would lead to a situation just opposite to that using base-catalyzed procedures, i.e., only free carboxylic groups would be esterified. Thereby, differences between dimethyl esters obtained after saponification–diazomethane and those obtained after just diazomethane would provide an estimate of the free carboxylic groups esterified during thermoxidation.

In conclusion, from quantitation of dimethyl esters in ML models, diazomethane alone would allow measurement of the originally diacid monoesterified compounds, base-catalyzed procedures would provide values of the originally diacid diesterified compounds and acid-catalyzed methods of the total compounds.

Overall, the results obtained in this study showed that the two base-catalyzed transmethylation methods were excellent procedures for quantitation of short-chain fatty acids and aldehydic acids, originally glycerol-bound. In view of this, these methods are now being applied to study the evolution of such compounds during thermoxidation of model methyl esters, triacylglycerols and oils, and also to determine their levels in real used frying fats.

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References

[1] S.S. Chang, R.J. Peterson, C.T. Ho, J. Am. Oil Chem. Soc. 55 (1978) 718.
[2] M.C. Dobarganes, G. Márquez-Ruiz, in: E.G. Perkins, M.D. Erickson (Eds.), Deep Frying: Chemistry, Nutrition and Practical Applications, AOCS Press, Champaign, 1996, pp. 89–111.
[3] A. Gasparoli, Grasas Aceites 49 (1998) 303.
[4] W.W. Nawar, Grasas Aceites 49 (1998) 271.
[5] E.N. Frankel (Ed.), Lipid Oxidation, Oily Press, Dundee, 1998.
[6] E.N. Frankel, in: D.B. Min, T.H. Smouse (Eds.), Flavor Chemistry of Fats and Oils, AOCS Press, Champaign, 1985, pp. 1–37.
[7] M.C. Dobarganes, J.J. Rios, M.C. Pérez-Camino, Grasas Aceites 37 (1986) 61.
[8] P. Capella, Rev. Fr. Corps Gras 36 (1989) 313.
[9] G. Márquez-Ruiz, M.C. Dobarganes, J. Sci. Food Agric. 70 (1992) 120.
[10] A. Kamal-Eldin, L.-A. Appelqvist, Grasas Aceites 47 (1996) 342.
[11] G. Márquez-Ruiz, M.C. Pérez-Camino, M.C. Dobarganes, J. Am Oil Chem. Soc. 69 (1992) 930.
[12] G. Márquez-Ruiz, M.C. Dobarganes, in: E.G. Perkins, M.D. Erickson (Eds.), Deep Frying: Chemistry, Nutrition and Practical Applications, AOCS Press, Champaign, 1996, pp. 160–182.

[13] A. Kamal-Eldin, G. Márquez-Ruiz, M.C. Dobarganes, L.-A. Appelqvist, J. Chromatogr. A 776 (1997) 245.
[14] H. Esterbauer, R.J. Schaur, H. Zollner, Free Rad. Biol. Med. 11 (1991) 841.
[15] S. Minamoto, K. Kanazawa, H. Ashida, G. Danno, M. Natake, Agric. Biol. Chem. 49 (1985) 2747.
[16] S. Minamoto, K. Kanazawa, H. Ashida, M. Natake, Biochim. Biophys. Acta 958 (1988) 199.
[17] K. Kanazawa, H. Ashida, Arch. Biochem. Biophys. 288 (1991) 71.
[18] H. Kamido, A. Kuksis, L. Murai, J.J. Myher, H. Pang, Lipids 27 (1992) 645.
[19] W.W. Christie, in: W.W. Christie (Ed.), Advances in Lipid: Methodology — 2, Oily Press, Dundee, 1993, pp. 8–111.
[20] D. Barrera-Arellano, G. Márquez-Ruiz, M.C. Dobarganes, Grasas Aceites 48 (1997) 231.
[21] G. Cecchi, S. Biasini, J. Castano, Rev. Fr. Corps Gras 32 (1985) 163.
[22] IUPAC (Ed.), Standard Methods for the Analysis of Oils, Fats and derivatives, 1st Supplement to the 7th edition, International Union of Pure and Applied Chemistry, Pergamon Press, Oxford, 1992.
[23] W.R. Morrison, L.M. Smith, J. Lipid Res. 5 (1964) 600–608.
[24] J.D. Cohen, J. Chromatogr. 303 (1984) 193.
[25] O. Berdeaux, G. Márquez-Ruiz, M.C. Dobarganes, Grasas Aceites 50 (1999) 53.
[26] S. Passi, M. Picardo, C. De Luca, M. Nazzaro-Porro, L. Rossi, G. Rotilio, Biochim. Biophys. Acta 1168 (1993) 190.
[27] C.R. Kepler, S.B. Tove, J. Biol. Chem. 242 (1967) 5686.
[28] N.C. Shantha, E.A. Decker, B.J. Hennig, AOAC Int. 76 (1993) 644.
[29] S.A. Werner, L.O. Luedecke, T.D. Shultz, J. Agrc. Food Chem. 40 (1992) 18.
[30] N. Sehat, M.P. Yurawecz, J.A.G. Roach, M.M. Mossoba, J.K.G. Kramer, Y. Ku, Lipids 33 (1998) 217.
[31] N. Sehat, J.K.G. Kramer, M.M. Mossoba, M.P. Yurawecz, J.A.G. Roach, K. Uulitz, K.M. Morehouse, Y. Ku, Lipids 33 (1998) 963.
[32] S. Banni, J.-C. Martin, in: W.W. Christie, J.L. Sébédio (Eds.), *Trans* Fatty Acids in Human Nutrition, Oily Press, Dundee, 1998, pp. 261–302.
[33] S. Koritala, W.K. Rohwedder, Lipids 7 (1972) 274.
[34] J. Graille, M. Naudet, Ann. Fals. Exp. Chim. 69 (1976) 179.
[35] P. Ottaviani, J. Graille, P. Perfetti, M. Naudet, Chem. Phys. Lipids 24 (1979) 57.
[36] J.-L. Perrin, P. Perfetti, M. Naudet, Rev. Fr. Corps Gras. 32 (1985) 205.