J Am Oil Chem Soc (2010) 87:1271–1279
DOI 10.1007/s11746-010-1624-8

ORIGINAL PAPER

Quantitation of Hydroperoxy-, Keto- and Hydroxy-Dienes During Oxidation of FAMEs from High-Linoleic and High-Oleic Sunflower Oils

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Received: 4 March 2010 / Revised: 16 April 2010 / Accepted: 7 June 2010 / Published online: 23 June 2010

Abstract The objective of this work was to study the quantitative formation of hydroperoxydienes, ketodienes and hydroxydienes during autoxidation at 40 °C of fatty acid methyl esters derived from two sunflower oils with different degree of unsaturation, high-linoleic sunflower oil and high-oleic sunflower oil. The analysis of the oxidation compounds was carried out by NP-HPLC–UV and results were compared to the specific extinction at 232 nm (K_{232}) and the peroxide value (PV). Analysis of FAME polymers by HPSEC was also performed to discard samples of advanced oxidation. Results showed that the contents of hydroperoxydienes with respect to the PV were higher for the high linoleic (HL) sample. At the end of the period of slow polymerization (ΔPol $\leq$ 1 wt%), the content of hydroperoxydienes was found to be 86.0 and 30.7 μg/mg for the HL and high oleic (HO) samples, respectively. Throughout this period, hydroperoxydienes constituted around 90 and 50 wt% of the total hydroperoxides in the HL and HO samples, respectively, suggesting that a significant oxidation of oleic acid also occurred in both samples. The contents of ketodienes and hydroxydienes as a whole constituted 2–3 wt% of the diene compounds analyzed at the end of the period of slow polymerization. Higher contents of ketodienes than of hydroxydienes were found throughout the oxidation time, and the ratio between the contents of ketodienes and hydroxydienes increased with a factor that changed from 1 to 2 throughout the period of slow polymerization.

Keywords Hydroperoxides · Hydroperoxydienes · Ketodienes · Hydroxydienes · Autoxidation · FAMEs

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Introduction

Lipid oxidation is one of the main causes of quality deterioration in foods because it is responsible for the development of products that impair the food flavor. In recent years there has been special concern about the potentially detrimental nutritional effects of oxidized lipids in foods as a consequence of implications of oxidized lipids and other reactive oxygen species in a variety of chronic diseases [1–8]. In order to evaluate their actual contribution to the promotion and/or development of diseases, it is first necessary to know the nature and quantities of lipid oxidation products in foods [4].

The qualitative analysis of the main oxidation products of the major oxidizing fatty acids in foods has been well established through model lipid systems [9]. Nevertheless, the quantitative analysis of lipid oxidation products in real foods finds serious limitations because of the large number of products involved, their low contents as individual species and their relatively high instability.

In previous studies carried out in our lab, the quantitative analysis of lipid oxidation products in thermoxidized and frying oils was tackled through a derivatization step of the triacylglycerol molecules into fatty acid methyl esters (FAMEs) followed by gas–liquid chromatography analysis with flame ionization detection (GC-FID) [10–12]. While the compounds accumulated in thermoxidized oils are

relatively stable secondary oxidation products, the more abundant compounds during autoxidation are hydroperoxides, whose relatively low stability makes their analysis through derivatization steps a challenging task.

Hopia and coworkers [13] have applied normal-phase high-performance liquid chromatography (NP-HPLC) with UV light absorption detection for the direct analysis of the main oxidation products of linoleic acid, i.e. hydroperoxydienes, ketodienes and hydroxydienes, in oxidized samples of methyl linoleate. Due to the diene structure, the hydroperoxydienes and hydroxydienes were detected at 233 nm, while the ketodienes were recorded at 268 nm. Relative contents of the secondary oxidation products, i.e. ketodienes and hydroxydienes, were reported by referring their areas to the area of hydroperoxides and considering their molar extinction coefficients. However, the absolute contents of the oxidation products were not given.

The determination of hydroperoxydienes, ketodienes and hydroxydienes has only been carried out in a pure lipid model system as is methyl linoleate. However, the analysis of these compounds has not yet been performed in more complex lipid systems such as mixtures of FAMEs with different oxidizing fatty acids. FAMEs derived from oils may be used as model systems to study oxidation in oils. They can be analyzed directly by NP-HPLC–UV for the separation and determination of oxidation products containing conjugated double bonds. Nevertheless, the analysis of these analytes in oxidized samples of oils would require the derivatization of triacylglycerols into FAMEs. Therefore, the analysis of oxidized samples of FAMEs derived from oils can be considered as a necessary step previous to the development of the analysis of real samples of oils. As in previous studies, oxidized samples of FAMEs would allow us to investigate how derivatization procedures affect the compounds of interest [14].

The objective of this work was to study the quantitative formation of hydroperoxydienes, ketodienes and hydroxydienes during autoxidation of FAMEs derived from sunflower oils with different degree of unsaturation, high-linoleic sunflower oil (HLSO) and high-oleic sunflower oil (HOSO). The analysis of the oxidation compounds was carried out by NP-HPLC with a UV-light detector following the studies of Hopia and coworkers in methyl linoleate [13]. Unlike the studies of Hopia et al., in this study we tackle the quantitative analysis of the compounds of interest. To this end, compounds representative of each group of products were synthesized and the corresponding HPLC response factors were determined. In order to relate the contents of the oxidation products under study to the oxidation state of the samples, oxidation was characterized by the specific extinction at 232 nm (K_{232}) and the peroxide value. In addition, the analysis of polymers by high-performance size-exclusion chromatography with refraction-index detection (HPSEC-RFI) was performed as a rapid control measure in order to discard samples within advanced oxidation [15].

Materials and Methods

Chemicals

Linoleic acid (≥99%) and soybean lipoxygenase (lipoxidase preparation Type I-B) were purchased from Sigma-Aldrich Química (Madrid, Spain). Methyl linoleate (≥99%) was acquired from Nu-Chek-Prep (Elysian, MN, USA). Diethyl ether stabilized with 1% v/v ethanol (Super purity solvent, HPLC grade) was purchased from Romil LTD (Cambridge, UK) and *n*-heptane (99% purity, HPLC grade) was purchased from Carlo Erba Reactifs-SDS (Val de Reuil, France). Both HPLC solvents were used as received.

Samples

High linoleic sunflower oil (HLSO) and high oleic sunflower oil (HOSO) were supplied by Koipe S.A. (Andújar, Jaén, Spain). The fatty acid composition was determined to be 6.7% C16:0, 0.2% C16:1, 3.6% C18:0, 33.0% C18:1, 55.2% C18:2 and 1.3% others in HLSO and 4.1% C16:0, 4.6% C18:0, 77.6% C18:1, 11.7% C18:2 and 2.0% others in HOSO.

Sample Treatments

The oils were purified with aluminum oxide according to Yoshida et al. [16]. Then the purified oils were subjected to a transmethylation procedure to obtain FAMEs. This procedure consisted of the reaction of the oils with 2 M KOH in methanol at room temperature. First, an aliquot of 30 g of purified oil was dissolved in 100 mL *n*-hexane. A volume of 10 mL of the KOH solution was added and stirring was applied for 30 min at room temperature and in the dark. The medium was neutralized with 30 mL of 0.5 M H_2SO_4 in MeOH. The organic phase was separated, filtered through filter paper containing anhydrous Na_2SO_4, and the solvent was evaporated in a rotary evaporator at 40 °C. Finally, the lipid sample was bubbled with N_2 to remove the residual solvent. The transmethylation reaction was found to be complete by thin layer chromatography (TLC). Silica plates and hexane:diethyl ether:acetic acid (80:20:1, v/v) as the mobile phase were used. The spots on the plate were revealed by iodine vapor.

Oxidation Conditions

Aliquots of 20 g of FAME samples were oxidized in Petri dishes (14.5 cm i.d.) at 40 °C in the dark by using an oven with continuous air circulation. The surface-to-volume ratio of the FAME samples was as high as 10 cm^{-1}, so oxidation was developed under non-limited oxygen conditions.

Synthesis of Oxidation Compounds

Methyl 13-Hydroperoxy- (Z)-9, (E)-11-Octadecadienoate

Methyl 13-hydroperoxy- (*Z*)-9, (*E*)-11-octadecadienoate was obtained through the enzymatic oxidation of linoleic acid with soybean lipoxygenase following the procedure described by Hidalgo et al. [17]. First, linoleic acid was oxidized by lipoxygenase in aqueous solution under basic conditions (pH 9), oxygen saturation and at a low temperature (0 °C). The reaction was stopped by adding 2 M HCl up to pH 3 and the hydroperoxide formed was extracted with diethyl ether. Then, the corresponding methyl ester was obtained by the reaction of the free fatty acid with diazomethane according to the procedure described by Cohen [18]. Finally, methyl 13-hydroperoxy- (*Z*)-9, (*E*)-11-octadecadienoate was purified by fractionation in a glass chromatographic column filled with silica gel. A blend of hexane and diethyl ether (7:3, v/v) was used as the elution solvent. The hydroperoxydiene fraction obtained was shown to be pure by TLC. Confirmation of identity and purity was performed by ^{1}H and ^{13}C NMR.

Methyl 13-Hydroxy- (Z)-9, (E)-11-Octadecadienoate

Methyl 13-hydroxy- (*Z*)-9, (*E*)-11-octadecadienoate was obtained from the reduction of 13-hydroperoxy- (*Z*)-9, (*E*)-11-octadecadienoic acid with $NaBH_4$ following the procedure described by Hidalgo et al. [17]. Briefly, before stopping the lipoxygenase reaction, $NaBH_4$ was added to the reaction medium and stirring was applied for 1 h under a nitrogen atmosphere. Then, 2 M HCl was added up to an acidic pH and methyl 13-hydroxy- (*Z*)-9, (*E*)-11-octadecadienoate was obtained applying the same procedure as the one described above for the hydroperoxide. Likewise, the hydroxydiene fraction was shown to be pure by TLC. Confirmation of identity and purity was performed by ^{1}H and ^{13}C NMR.

Methyl 13-keto- (Z)-9, (E)-11-Octadecadienoate

Methyl 13-keto- (*Z*)-9, (*E*)-11-octadecadienoate was obtained from oxidation of 13-hydroxy- (*Z*)-9, (*E*)-11-octadecadienoic acid with chromium (VI) oxide according to the procedure described by Hidalgo et al. [17]. Thus, oxidation was performed in dry pyridine under stirring for 24 h at room temperature. The reaction was stopped with water and the ketodiene was extracted with diethyl ether. The corresponding methyl ester was obtained and purified following the same procedure as described above. The ketodiene fraction was shown to be pure by TLC. Confirmation of identity and purity was also performed by ^{1}H and ^{13}C NMR.

Mixture of Hydroperoxydienes from Methyl Linoleate

Methyl linoleate was oxidized in the dark at room temperature. An aliquot of 5 g of methyl linoleate was weighed in a 250-mL beaker and oxidized for 3 d. Formation of hydroperoxides was checked by TLC using a blend of hexane, diethyl ether and acetic acid (80:20:1, v/v) as mobile phase. The spots were revealed by iodine vapor and the formation of hydroperoxydienes was previously confirmed by UV light. Hydroperoxides were isolated by fractionation in a glass chromatographic column filled with silica gel following the same procedure as described above. Unreacted methyl linoleate was oxidized again under the same oxidation conditions. Hydroperoxides were isolated and added to the first batch. This procedure was repeated three times in total. The fraction obtained was found to be pure by TLC and confirmation of identity and purity was also performed by ^{1}H and ^{13}C NMR. The hydroperoxides were kept under nitrogen at −32 °C until analysis.

Analysis of FAME Polymers

Analysis of polymers was performed according to IUPAC standard method 2.508 [19]. An HPSEC chromatograph equipped with a Rheodyne 7725i injector with a 10-μL sample loop, a Knauer 120 HPLC pump (Knauer, Berlin, Germany) and a Merck L-7490 refractive index detector (Merck, Darmstadt, Germany) was used. The separation was performed on two 100- and 500-Å Ultrastyragel columns (25 cm × 0.77 cm i.d.) packed with porous, highly cross-linked styrene-divinylbenzene copolymers (10 μm) (Agilent Technologies, Palo Alto, CA) connected in series by using tetrahydrofuran as the mobile phase at a flow rate of 1 mL/min.

Peroxide Value

The peroxide value was determined by the iodometric assay according to IUPAC standard method 2.501 [19]. Samples of 500 mg FAMEs and 1×10^{-2} M $Na_2S_2O_3$ solution were used.

Ultraviolet Light Absorption at 232 nm (K_{232})

Specific extinction at 232 nm was determined in cyclohexane as a measure of the total conjugated dienes according to AOCS standard method Ch 5-91 [20].

Analysis of Oxidation Compounds by HPLC

A Waters 600 HPLC chromatograph (Waters Corp., Milford, MA, USA) equipped with a 600 Waters pump, a Rheodyne injector valve (20-μL sample loop), a silica HPLC column (Si 60, 5 μm particle size) (Merck, Darmstadt, Germany), a 486 Waters tunable absorbance detector (10 mm path length) and a 600 Waters controller was used. The mobile phase was n-heptane:diethyl ether (82:18, v/v) with a flow rate of 1 mL/min. Ethanol present in diethyl ether as a stabilizer was not removed. Hydroperoxydienes and hydroxydienes were monitored at 234 nm, while ketodienes were recorded at 268 nm. The FAME samples were dissolved in *n*-hexane in the range 1–50 mg/mL prior to analysis. The scale of absorbance units was set at 2,000 units/volt.

^{1}H and ^{13}C NMR

^{1}H- and ^{13}C-NMR spectra were recorded at 300 and 75.4 MHz, respectively, in a Bruker AC-300P (Karlsruhe, Germany). Tetramethylsilane was used as an internal standard. Two-dimensional NMR was used to assign the ^{13}C-NMR spectra.

Statistical Analysis

Analytical determinations were carried out in triplicate and results were expressed as mean values. Linear regression analysis was performed in Microsoft Excel 2000 (Microsoft Corporation, Redmond, WA, USA). Comparisons between the slopes obtained in the linear regression analysis were made by the Student's *t* test in Microsoft Excel 2000 (Microsoft Corporation, Redmond, WA, USA). Significance was defined at $p < 0.05$.

Results and Discussion

Determination of HPLC Response Factors

Methyl 13-hydroperoxy-(*Z*)-9,(*E*)-11-octadecadienoate (13-*cis*,*trans*-MeLOOH), methyl 13-keto-(*Z*)-9,(*E*)-11-octadecadienoate (13-*cis*,*trans*-MeLO) and methyl 13-hydroxy-(*Z*)-9,(*E*)-11-octadecadienoate (13-*cis*,*trans*-MeLOH) were synthesized and used as compounds representative of the hydroperoxides, ketodienes and hydroxydienes of methyl linoleate, respectively, for the determination of HPLC response factors. The analysis of these compounds by HPLC showed the presence of minor contents of position and geometrical isomers due to the formation of the 9-hydroperoxide and the double-bound isomerization to the *trans,trans* forms, respectively (Fig. 1). The presence of isomers at variable quantities would not affect significantly the value of a global response factor for each group of compounds because the absorption spectra of the different isomers are quite similar. As an example, the spectra of the *cis, trans* and the *trans, trans* isomers of the hydroperoxydienes have been reported to be broad bands with very close absorption maxima, namely, at 235 and 230 nm, respectively [13]. Therefore, the total area of each group of compounds was used for the determination of the response factors.

Table 1 lists the purity of each compound and the content of the different isomers, the response factors and the limit of detection (LOD) and quantification (LOQ) defined at a signal-to-noise ratio of 3 and 10, respectively. For comparative purposes, the extinction coefficients were also determined in cyclohexane and included in Table 1.

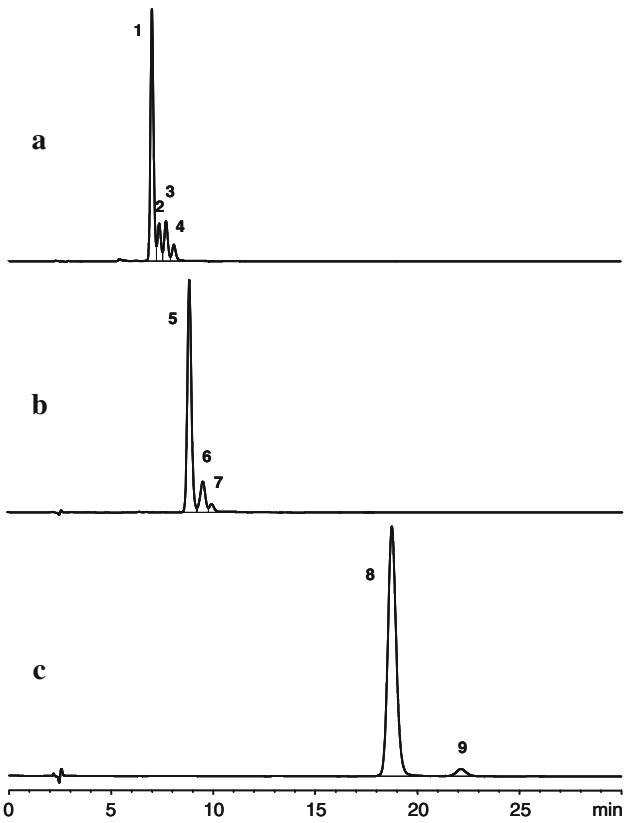

Fig. 1 HPLC chromatograms of the synthesized compounds. **a** Methyl 13-keto-(*Z*)-9,(*E*)-11-octadecadienoate recorded at 268 nm. **b** Methyl 13-hydroperoxy-(*Z*)-9,(*E*)-11-octadecadienoate recorded at 234 nm. **c** Methyl 13-hydroxy-(*Z*)-9,(*E*)-11-octadecadienoate recorded at 234 nm. For chromatographic peak numbers see Fig. 2

Table 1 HPLC response factors, limits of detection and quantification, and molar extinction coefficients of the synthesized compounds

	Ketodienes				Hydroperoxydienes			Hydroxydienes		
	1	2	3	4	5	6	7	8	9	10
% $Area_{HPLC}$	71.0	11.2	12.5	5.3	82.8	13.9	3.4	96.7	2.1	ND
Conc. range (μg mL^{-1})	10–100				10–300			10–300		
F_R (μg^{-1} mL)	223.6 ± 1.3				219.2 ± 1.0			226.9 ± 2.5		
r^2	0.9997				0.9998			0.9992		
LOD (μg mL^{-1})	0.1				0.1			0.2		
LOQ (μg mL^{-1})	0.3				0.3			0.6		
ε (mol^{-1} L cm^{-1})	25,700				25,300			26,200		

F_R response factor determined at 268 nm for ketodienes and at 234 nm for hydroperoxydienes and hydroxydienes, r^2 linear regression coefficient, *LOD* limit of detection, *LOQ* limit of quantification, ε molar extinction coefficient in cyclohexane at 268 nm for ketodienes and at 233 nm for hydroperoxydienes and hydroxydienes, *ND* not detected. For chromatographic peak numbers see Fig. 2

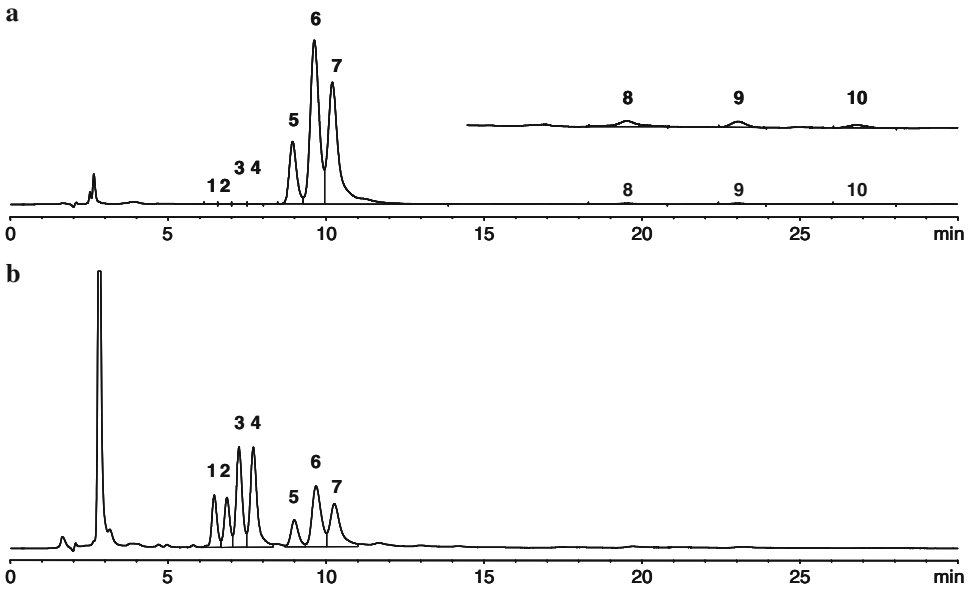

Fig. 2 HPLC chromatograms of a FAME sample derived from HLSO and oxidized at 40 °C for 68 h. **a** Chromatogram recorded at 234 nm. **b** Chromatogram recorded at 268 nm. Peak assignation: ***1*** methyl 13-keto-(*Z*)-9,(*E*)-11-octadecadienoate, ***2*** methyl 9-keto-(*E*)-10,(*Z*)-12-octadecadienoate, ***3*** methyl 13-keto-(*E*)-9,(*E*)-11-octadecadienoate, ***4*** methyl 9-keto-(*E*)-10,(*E*)-12-octadecadienoate, ***5*** methyl 13-hydroperoxy-(*Z*)-9,(*E*)-11-octadecadienoate, ***6*** methyl 13-hydroperoxy- (*E*)-9,(*E*)-11-octadecadienoate and methyl 9-hydroperoxy-(*E*)-10,(*Z*)-12-octadecadienoate, ***7*** methyl 9-hydroperoxy-(*E*)-10,(*E*)-12-octadecadienoate, ***8*** methyl 13-hydroxy-(*Z*)-9,(*E*)-11-octadecadienoate, ***9*** methyl 13-hydroxy-(*E*)-9,(*E*)-11-octadecadienoate and methyl 9-hydroxy-(*E*)-10,(*Z*)-12-octadecadienoate, ***10*** methyl 9-hydroxy-(*E*)-10,(*E*)-12-octadecadienoate

Linearity was found in the whole range of concentrations assayed. The response factors for hydroperoxydienes and hydroxydienes were very similar to each other, as well as between these determined at 234 nm and the one for ketodienes determined at 268 nm.

The values found for LOD and LOQ were as low as 0.1–0.2 and 0.3–0.6 μg/mL, respectively, or 0.3–0.6 and 1.0–2.0 expressed in μM. The molar extinction coefficients were coherent with those reported in the literature [21, 22], as well as their relative values with the HPLC response factors obtained in this study.

HPLC Chromatograms of an Oxidized FAME Sample

Figure 2 illustrates HPLC chromatograms recorded at 234 and 268 nm of an oxidized FAME sample. Because of the specificity of the light absorption of conjugated diene structures, the chromatograms of mixtures of oxidized FAMEs were not complex. At the chromatographic conditions applied, ketodienes and hydroxydienes were completely resolved or separated between each other. Ketodienes eluted as four chromatographic peaks, while hydroperoxydienes were only resolved as three peaks.

Likewise, hydroxydienes eluted as three peaks, the second of which was split depending on the concentration. Although the determination of the different isomers was out of the scope of this study, we attempted to assign the different chromatographic peaks to oxidation compounds. Thus, assignation was performed according to the work of Hopia et al. [13] and Mäkinen and Hopia [23]. Because of its changing profile along oxidation, the second peak of hydroxydienes can be assigned to the coelution of the 9-*cis*,*trans* and 13-*trans*,*trans isomers*, and the same was adopted for the hydroperoxydienes. Regarding ketodienes, Mäkinen and Hopia [23] obtained two pairs of peaks that were assigned in order of elution to the *cis,trans* and *trans,trans* isomers, so a specific assignation to the two regioisomers was not given. In this study, the analysis of the 13-*cis,trans*-MeLO that was synthesized showed that the major peak corresponded to the peak of the shortest retention time (Fig. 1), so the first and the second peaks were assigned to the 13-*cis,trans* and the 9-*cis,trans*-MeLO, respectively, and the same was tentatively adopted for the *trans,trans*-MeLO isomers.

Formation of Hydroperoxydienes, Ketodienes and Hydroxydienes: Influence of the Degree of Unsaturation

Results for FAMEs derived from HLSO and HOSO during autoxidation at 40 °C are listed in Tables 2 and 3, respectively. The analysis of polymers by HPSEC, which was used to discard samples with advanced oxidation, showed two well-defined oxidation periods. A first stage was characterized by very low contents of polymers, reaching values up to around 1.0% with respect to the initial content. Then, polymerization was accelerated in the second period. The end of the first period was detected at 76 h in the high linoleic (HL) sample (Table 2) and at 144 h in the high oleic sample (HO) (Table 3).

Hydroperoxydienes were already detected in the starting samples at very low concentrations (0.10 μg/mg HL sample and 0.02 μg/mg HO sample), even when the PV was zero. Ketodienes and hydroxydienes were also detected in the starting samples, however, their contents were below the LOQ, i.e. lower than 0.02 and 0.05 μg/mg,

Table 2 Autoxidation of purified FAMEs derived from high linoleic sunflower oil at 40 °C in the dark

Time (h)	Pol (%)	PV (meq/kg)	K_{232}	Hydroperoxydienes (μg/mg)	Ketodienes (μg/mg)	Hydroxydienes (μg/mg)
0	1.0	0.0 ± 0.0	2.23 ± 0.09	0.10 ± 0.00	<0.02	<0.05
20	1.0	11.3 ± 1.2	2.73 ± 0.09	1.04 ± 0.01	<0.02	<0.05
44	1.3	128 ± 4.7	11.60 ± 0.11	12.74 ± 0.20	0.082 ± 0.005	0.060 ± 0.005
51	1.3	184 ± 1.4	17.96 ± 0.89	19.01 ± 0.90	0.112 ± 0.007	0.066 ± 0.005
68	2.0	485 ± 1.4	44.84 ± 3.90	55.65 ± 0.31	0.662 ± 0.026	0.212 ± 0.011
76	2.0	809 ± 7.8	73.77 ± 3.20	86.04 ± 0.95	1.107 ± 0.004	0.550 ± 0.030
91	6.5	1453 ± 17.4	107.69 ± 8.58	131.33 ± 1.73	3.094 ± 0.099	1.338 ± 0.080
100	8.6	–	–	–	–	–
115	19.3	–	–	–	–	–

Pol FAME polymers, *PV* peroxide value, K_{232} specific extinction coefficient at 232 nm

Table 3 Autoxidation of FAMEs derived from purified high oleic sunflower oil at 40 °C in the dark

Time (h)	Pol (%)	PV (meq/kg)	K_{232}	Hydroperoxydienes (μg/mg)	Ketodienes (μg/mg)	Hydroxydienes (μg/mg)
0	0.9	0.0 ± 0.0	2.19 ± 0.03	0.02 ± 0.00	<0.02	<0.05
25	1.1	20.0 ± 0.7	2.83 ± 0.08	0.90 ± 0.01	<0.02	<0.05
48	1.1	79.0 ± 4.1	4.45 ± 0.07	4.15 ± 0.16	0.031 ± 0.003	0.056 ± 0.004
72	1.2	163 ± 1.4	8.32 ± 0.00	8.66 ± 0.06	0.084 ± 0.006	0.096 ± 0.005
96	1.3	275 ± 0.0	13.37 ± 0.00	15.16 ± 0.49	0.231 ± 0.030	0.162 ± 0.003
120	1.5	384 ± 7.8	18.27 ± 0.38	21.36 ± 0.06	0.397 ± 0.001	0.198 ± 0.002
144	1.8	558 ± 2.1	26.12 ± 0.03	30.71 ± 0.07	0.664 ± 0.010	0.291 ± 0.005
168	2.3	818 ± 0.7	39.82 ± 0.99	36.45 ± 0.33	1.239 ± 0.027	0.459 ± 0.004
192	4.0	1053 ± 16.3	45.13 ± 0.85	42.29 ± 0.08	1.726 ± 0.030	0.590 ± 0.035
216	5.3	–	–	–	–	–
240	6.7	–	–	–	–	–

Pol FAME polymers, *PV* peroxide value, K_{232} specific extinction coefficient at 232 nm

respectively, at the analytical conditions applied. The coefficients of variation found for the three groups of compounds were satisfactory, being ≤5% for hydroperoxydienes and ≤7% for ketodienes and hydroxydienes, respectively.

Hydroperoxydienes increased exponentially over oxidation time, reaching different values for the HL and HO samples at the end of the period of slow polymerization. Thus, while 86.0 μg/mg was found for the HL sample, the content of hydroperoxydienes was as low as 30.7 μg/mg for the HO sample. Likewise, ketodienes and hydroxydienes increased exponentially throughout the oxidation time, but, as expected, in a much lower range of concentrations. In fact, the content of ketodienes and hydroxydienes as a whole constituted 2–3 wt% of the analyzed compounds at the end of the period of slow polymerization. Higher contents of ketodienes than of hydroxydienes were found throughout oxidation time, and the ratio between the contents of ketodienes and hydroxydienes increased with a factor that changed from 1 to 2 throughout the period of slow polymerization. A faster formation of ketodienes, as compared to hydroxydienes, has also been found by Mäkinen et al. [24] during oxidation of methyl linoleate at 40 °C. Although the exact mechanism responsible for the formation of hydroxy- and ketodiene-products from lipid hydroperoxides has not yet been established, a few mechanisms have been proposed [25, 26].

The content of hydroperoxydienes showed, as expected, a significant linear correlation with the extinction coefficient at 232 nm (K_{232}) during the period of slow polymerization for the two FAME samples. For comparative purposes, the increment of the extinction coefficient with respect to the starting sample (ΔK_{232}) was examined (Fig. 3a). Results showed no significant differences in the linear correlation analysis between the HL and HO samples, indicating that the compounds analyzed, i.e. hydroperoxydienes, were to a great extent the most abundant compounds absorbing light at 232 nm.

The content of hydroperoxydienes also showed a significant linear correlation with the PV during the period of slow polymerization, but clear differences were found between the HL and HO samples (Fig. 3b). As an example, when the PV was 100 meq/kg the content of hydroperoxydienes was determined to be 11.5 and 5.6 μg/mg for the HL and HO samples, respectively. These differences indicate that a significant formation of hydroperoxides other than hydroperoxydienes occurred in the HO sample. As the only oxidizing substrates were oleic and linoleic acids and the formation of polymerization compounds was very low, the differences between the HL and HO samples can be mainly attributed to the oxidation of oleic acid, whose hydroperoxides do not possess conjugated double bonds and are not detected in the HPLC–UV analysis.

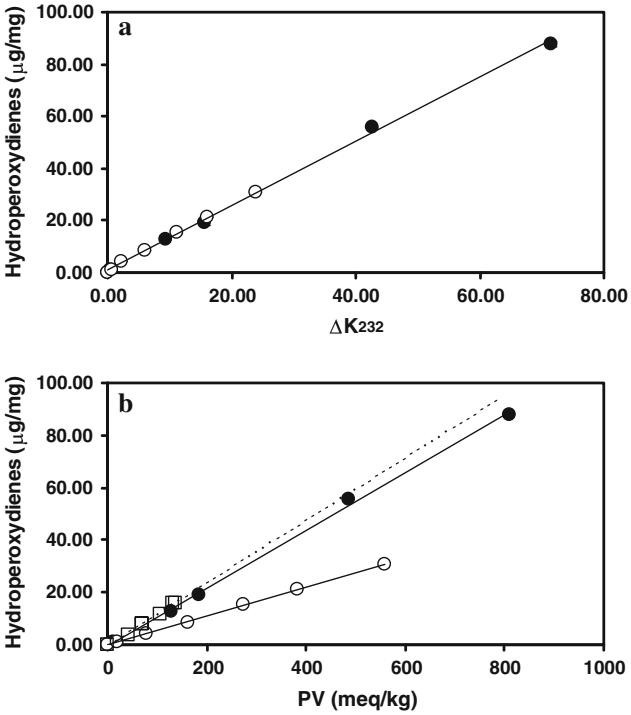

Fig. 3 **a** Linear correlation between the content of hydroperoxydienes and ΔK_{232} in FAMEs derived from HLSO (*solid*) and HOSO (*hollow*) during autoxidation at 40 °C (HL: [LOOH] (μg/mg) = 1.24(±0.02) ΔK_{232}, $r = 0.999$; HO: [LOOH] (μg/mg) = 1.28(±0.02) ΔK_{232}, $r = 0.999$). **b** Linear correlation between the content of hydroperoxydienes and the PV in samples of FAMEs derived from HLSO (*solid circles*) and HOSO (*hollow circles*) during autoxidation at 40 °C, and in solutions of pure hydroperoxydienes (*squares*) in hexane (HL: [LOOH] (μg/mg) = 0.110(±0.002) PV (meq/kg), $r = 0.999$; HO: [LOOH] (μg/mg) = 0.0555(±0.0003) PV (meq/kg), $r = 0.999$; Pure hydroperoxydienes: [LOOH] (μg/mg) = 0.119(±0.004) PV (meq/kg), $r = 0.998$)

Although oxidation of oleic acid is known to be much slower than that of linoleic acid when both substrates are tested independently [27], the results showed that oxidation of oleic acid was favored in mixtures of oleic and linoleic acids and that the participation of oleic acid in the oxidation chain was a function of its concentration.

In order to determine the relationship between pure hydroperoxydienes and PV, methyl linoleate was oxidized and hydroperoxides were isolated and purified. Then solutions of known concentrations of the pure hydroperoxydienes in hexane were titrated with sodium thiosulfate applying the same conditions as those of the PV test. For comparative purposes, the results of the titration were expressed supposing the same amount of lipid sample that was used in the PV test for the HO and HL samples, i.e. 0.5 g. Likewise, the hydroperoxydiene concentrations (μg/mL) were also referred to as a hypothetical amount of sample of 0.5 g. As both parameters were modified by a same factor, the relationship between each other remained unaffected. Results showed an excellent linear correlation

between the content of pure hydroperoxydienes and the PV (Fig. 3b). The slope of the curve was quite close to that of the HL sample, but statistically different. From the slopes of the curves we can estimate the content of hydroperoxydienes relative to that of the compounds giving response in the PV test, i.e. total hydroperoxides. Thus, the hydroperoxydienes analyzed in this study constituted 92% (±3) of total hydroperoxides in the HL sample and 47% (±3) in the HO sample. Therefore, this approach suggests that formation of hydroperoxides from oleic acid was around 10 and 50% in the HL and HO samples, respectively. These figures should be considered as estimations, since accurate results would also require the direct determination of the hydroperoxides from oleic acid. The results obtained in this study are in agreement with those of a previous report, in which oxidation of purified HLSO, HOSO and methyl linoleate was studied at 40 °C in the dark [28]. Results for the relationship between K_{232} and the PV for the three lipid substrates were similar to those of the present study (data not shown). In addition, the loss of fatty acids in HLSO and HOSO exhibited that while the oxidation of oleic acid was very low in HLSO, the loss of oleic acid was similar to that of linoleic acid in HOSO.

The contents of both ketodienes and hydroxydienes with respect to hydroperoxydienes were significantly different for the HO and HL samples. Surprisingly, for a given content of their precursor hydroperoxydienes, the contents of ketodienes and hydroxydienes were higher for the sample with a lower content of linoleic acid, i.e. the HO sample (Fig. 4). Therefore, factors controlling the formation of these secondary oxidation products other than the content of hydroperoxydienes were involved. Among them, a lower tendency to polymerization of monounsaturated oils as compared to polyunsaturated oils has been clearly observed at high temperature [29, 30]. Under these circumstances, the monomers-to-polymers ratio would be favored for the less unsaturated substrate in the complex set of oxidation reactions.

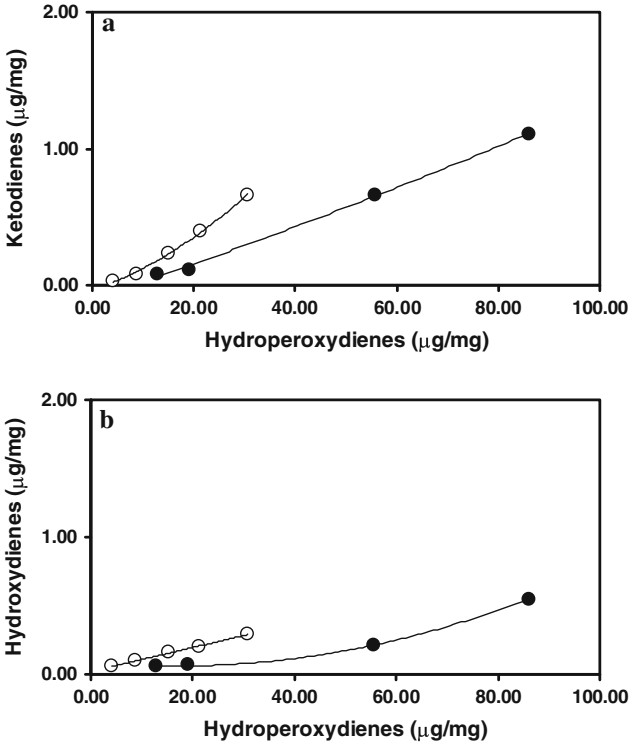

Fig. 4 Contents of ketodienes (**a**) and hydroxydienes (**b**) as a function of the content of hydroperoxydienes in FAMEs derived from HLSO (*solid*) and HOSO (*hollow*) during autoxidation at 40 °C

Conclusions

The quantitative analysis of hydroperoxydienes during autoxidation of FAMEs derived from HLSO and HOSO has showed that with respect to the oxidation extent, as detected by the PV, the formation of hydroperoxydienes depends on the relative composition of the oxidizing fatty acids, i.e. oleic and linoleic acids. Thus, for a given PV, higher contents of hydroperoxydienes were found in the sample with a higher content of linoleic acid. At the end of the period of slow polymerization ($\Delta Pol \leq 1$ wt%), the content of hydroperoxydienes was found to be 86.0 and 30.7 μg/mg for the HL and HO samples, respectively. Throughout this period hydroperoxydienes constituted around 90 and 50 wt% of total hydroperoxides in the HL and HO samples, respectively, suggesting that a significant oxidation of oleic acid also occurred in both lipid substrates.

Formation of the secondary oxidation products, i.e. ketodienes and hydroxydienes, was already detected in the non-heated samples, when the content of hydroperoxydienes was as low as 0.02 μg/mg and the PV was zero. The content of ketodienes and hydroxydienes as a whole constituted 2–3 wt% of the diene compounds analyzed at the end of the period of slow polymerization. Higher contents of ketodienes than of hydroxydienes were found throughout the oxidation time, and the ratio between the contents of ketodienes and hydroxydienes increased by a factor that changed from 1 to 2 throughout the period of slow polymerization.

Acknowledgments This work was funded by "Ministerio de Ciencia e Innovación" through project AGL 2007-62922/ALI and by "Junta de Andalucía" through project AGR-4622. The authors thank Prof. Javier Hidalgo and Prof. Rosario Zamora for assistance in the synthesis and identification of compounds.

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