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Quantitative analysis of hydroperoxy-, keto- and hydroxy-dienes in refined vegetable oils

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ABSTRACT

Quantitative analysis of the main oxidation products of linoleic acid – hydroperoxy-, keto- and hydroxy-dienes – in refined oils is proposed in this study. The analytical approach consists of derivatization of TAGs into FAMEs and direct analysis by HPLC-UV. Two transmethylation methods run at room temperature were evaluated. The reactants were KOH in methanol in method 1 and sodium methoxide (NaOMe) in method 2. Method 1 was ruled out because resulted in losses of hydroperoxydienes as high as 90 wt%. Transmethylation with NaOMe resulted to be appropriate as derivatization procedure, although inevitably also gives rise to losses of hydroperoxydienes, which were lower than 10 wt%, and formation of keto- and hydroxy-dienes as a result. An amount of 0.6–2.1 wt% of hydroperoxydienes was transformed into keto- and hydroxy-dienes, being the formation of the former as much as three times higher. The method showed satisfactory sensitivity (quantification limits of 0.3 μg/mL for hydroperoxy- and keto-dienes and 0.6 μg/mL for hydroxydienes), precision (coefficients of variation ≤6% for hydroperoxydienes and ≤15% for keto- and hydroxy-dienes) and accuracy (recovery values of 85(±4), 99(±2) and 97.0(±0.6) % for hydroperoxy-, keto- and hydroxy-dienes, respectively). The method was applied to samples of high-linoleic (HLSO), high-oleic (HOSO) and high-stearic high-oleic (HSHOSO) sunflower oils oxidized at 40 °C. Results showed that the higher the linoleic-to-oleic ratio, the higher were the levels of hydroperoxy-, keto- and hydroxy-dienes when tocopherols were completely depleted, i.e. at the end of the induction period (IP). Levels of 23.7, 2.7 and 1.1 mg/g oil were found for hydroperoxy-, keto- and hydroxy-dienes, respectively, in the HLSO when tocopherol was practically exhausted. It was estimated that hydroperoxydienes constituted approximately 100, 95 and 60% of total hydroperoxides in the HLSO, HOSO and HSHOSO, respectively, along the IP.

1. Introduction

Implications of oxidized lipids and other reactive oxygen species in a variety of chronic diseases have been demonstrated [1–8]. The relative contribution of dietary oxidized lipids to diseases is however not known, as their nature and, especially, their contents in foods have not yet been defined [4].

While the qualitative analysis of the main oxidation products of the major oxidizing fatty acids in foods has been well established through model lipid systems like fatty acid methyl esters (FAMEs) [9], their quantitative analysis in real foods, in which they occur as acyl chains of triacylglycerols (TAGs), finds serious limitations because of a much larger number of products involved, their low contents as individual species and their relatively low stability. In this respect, a given oxidized product from an unsaturated fatty acyl chain can occur in a variety of triacylglycerol molecules.

In previous studies carried out in our lab, quantitative analysis of lipid oxidation products in thermoxidized and frying oils was tackled through a derivatization step of the TAGs into FAMEs followed by gas–liquid chromatography analysis with flame ionization detection (GC-FID) [10–12]. With the transformation of TAGs into FAMEs the number of analytes is drastically reduced and a considerable concentration effect arises as a result. However, 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.

In a recent report [13], quantitative analysis of the main oxidation products of linoleic acid, i.e. hydroperoxy-, keto- and hydroxy-dienes, was developed to analyze directly oxidized

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samples of model lipid systems like methyl linoleate and FAMEs derived from sunflower oils. The separation and detection of analytes was based upon the NP-HPLC-UV analysis proposed by Hopia et al. to evaluate oxidized samples of methyl linoleate [14]. Quantification was performed by external calibration using synthesized compounds representative of each group [13]. Unlike the direct analysis of oxidized samples of FAMEs, the application of this analytical method to real samples of oils would require a previous derivatization step to transform TAGs into FAMEs.

Due to the absence of conjugated double bounds, oxidation compounds of oleic acid are not detected in the NP-HPLC-UV analysis described above. However, the analysis of total hydroperoxides by the peroxide value can be applied to have by difference an estimate of the contents of oxidation products of oleic acid. The analysis of oxidized samples of FAMEs derived from high-linoleic and high-oleic sunflower oils has shown that in the absence of antioxidants the contribution of hydroperoxydienes to total hydroperoxides was approximately 90 and 50%, respectively, whereas in the presence of α-tocopherol these figures were 90 and 75% [15]. These results suggest that methyl oleate was oxidized at a same extent as methyl linoleate in the high-oleic sample in the absence of antioxidant, whereas in the presence of α-tocopherol methyl oleate was more protected than methyl linoleate, being oxidized in a relative proportion of 1:3.

The objective of this work was to propose for the first time a quantitative analysis of hydroperoxy-, keto- and hydroxy-dienes in real samples of refined vegetable oils, taking as an analytical basis the direct method reported to evaluate oxidized samples of model lipid systems like FAMEs [13], outlined above. The main challenge of this analytical approach is to transform the oxidized TAG molecules into FAMEs with minimal formation of analytical artifacts, because the analytes of interest, especially the hydroperoxydienes, are compounds of relatively low stability [9]. Two mild transmethylation methods were studied to obtain the FAME derivatives. The analytical method proposed was validated and applied to study quantitative formation of hydroperoxy-, keto- and hydroxy-dienes in refined sunflower oils with different contents of linoleic and oleic acids, namely, high-linoleic sunflower oil (SO), high-oleic sunflower oil (HOSO) and high-stearic high-oleic sunflower oil (HSHOSO). The oils were oxidized at 40 °C and samples were characterized by applying the specific extinction at 232 nm (K_{232}) and the peroxide value. In addition, analysis of polymers by high-performance size-exclusion chromatography with refractive index detection (HPSEC-RFI) was performed as a rapid control measure in order to discard samples within advanced oxidation [16].

2. Materials and methods

2.1. Chemicals

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) from Carlo Erba Reactifs-SDS (Val de Reuil, France). Both HPLC solvents were used as received.

2.2. Oils

High-linoleic sunflower oil (HLSO), high-oleic sunflower oil (HOSO) and high-stearic high-oleic sunflower oil (HSHOSO) were supplied by Koipe S.A. (Andújar, Jaén, Spain). The fatty acid compositions were 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, 4.1% C16:0, 4.6% C18:0, 77.6% C18:1, 11.7% C18:2 and 2.0% others in HOSO, and 4.4% C16:0, 15.1% C18:0, 71.8% C18:1, 5.0% C18:2 and 3.7% in HSHOSO.

2.3. FAME samples

The FAME samples were obtained from the HLSO according to a previous report [13]. The oil was purified with aluminum oxide [17] and then subjected to transmethylation with 2 M KOH in methanol at room temperature. The FAME sample (20 g) was oxidized in a Petri dish (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 sample was as high as 10 cm^{-1} so that oxidation was developed under non-limiting oxygen conditions. Periodic samplings were carried out and samples showed peroxide values of 21 (sample 1), 45 (sample 2) and 94 mequiv./kg (sample 3), respectively.

2.4. Oil samples

Aliquots of 20 g of the oils were oxidized in Petri dishes (14.5 cm i.d.) at 40 °C in the dark and under non-limiting oxygen conditions. Samplings were carried out periodically and the samples were kept at −35 °C until analyses.

2.5. Transmethylation methods

2.5.1. Method 1. Base-catalyzed transmethylation with KOH

The IUPAC standard method for preparation of the methyl esters of fatty acids at room temperature [18] was applied with slight modifications. A volume of 1 mL of a solution of the lipid sample in hexane at a concentration of 50 mg/mL was added into a 2-mL Eppendorf tube. Then a 50-μL volume of a 2 M KOH solution in methanol was added and vigorous shaking was applied for 20 s. Unlike the IUPAC method, once the solution became clear, the hexane layer was transferred into another tube and neutralized with 25-μL of a 10% HCl solution. After shaking for 10 s and until the solution became clear, the hexane layer was transferred into another tube, 50 mg of anhydrous Na_2SO_4 was added and shaking was applied. A volume of 20 μL of the hexane layer was injected in the HPLC chromatograph.

2.5.2. Method 2. Base-catalyzed transmethylation with NaOMe

Transmethylation with NaOMe at room temperature was applied according to Berdeaux et al. [19]. A 300-mg lipid sample was accurately weighed into a screw-capped centrifuge tube (13 cm × 10 mm I.D.) and a volume of 3 mL of tert-butyl methyl ether (TBME) was added. Then, a 1.5-mL volume of a 0.2 M NaOMe solution in methanol was added, the tube closed, shaken for 1 min, and allowed it to stand for 2 min. The solution was neutralized by adding 0.1 mL of 0.5 M H_2SO_4 in methanol. Finally, 3 mL of distilled water was added, shaken for 10 s and centrifuged at 5000 rpm for 1 min. The organic layer was separated then the solvent was evaporated in a rotary evaporator at 40 °C and taken to dryness with a stream of nitrogen. Solutions of FAME samples in hexane at a concentration of 1–50 mg/mL were analyzed by HPLC.

2.6. Analysis of hydroperoxy-, keto- and hydroxy-dienes by HPLC-UV

Quantitative determination of hydroperoxy-, keto- and hydroxy-dienes was performed by HPLC-UV according to a previous report [13]. FAME samples dissolved in n-hexane in a concentration range of 1–50 mg/mL were analyzed in a Waters 600 HPLC chromatograph. The chromatograph was equipped with a 600 Waters pump, a Rheodyne injector valve (20-μL sample loop), a silica HPLC column (LiChroCART® 250-4, LiChrospher® Si 60, 5 μm particle size) (Merck, Darmstadt, Germany), a 486 Waters tunable absorbance detector (10 mm path length) and a 600 Waters controller. 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 233 nm, whereas ketodienes were recorded at 268 nm. The scale of absorbance units was set at 2000 units/volt. Quantitative data were obtained by applying response factors reported elsewhere [13].

2.7. Analysis of TAG polymers

Analysis of TAG polymers was performed according to IUPAC standard method 2.508 [18]. 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 (30 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.

2.8. Peroxide value

Peroxide value was determined by the iodometric assay according to IUPAC standard method 2.501 [18]. Lipid samples of 500 mg and 1×10^{-2} M $Na_2S_2O_3$ solution were used.

2.9. Ultraviolet light absorption at 232 nm (K_{232})

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

2.10. Analysis of α-tocopherol

Analysis of α-tocopherol was carried out by normal-phase HPLC with fluorescence detection following IUPAC standard method 2.411 [18].

2.11. Statistical analysis

Analytical determinations were carried out in triplicate and results were expressed as mean values followed by the standard deviation. Error bars in figures represent the standard deviation of the mean. Comparisons of means between control and treated samples in Section 3.1 were performed by the Student's *t* test using the analysis of paired samples of SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA). Linear regression analyses were performed in Microsoft Excel 2000 (Microsoft Corporation, Redmond, WA, USA). Significance was defined at $P < 0.05$.

3. Results and discussion

3.1. Selection of the derivatization method

The analysis of hydroperoxy-, keto- and hydroxy-dienes in oils proposed in this study is based upon transformation of the TAG molecules into FAMEs. Due to their relatively low stability, especially that of hydroperoxydienes, the effect of the transmethylation procedure on the contents of these compounds was studied. The analytical strategy followed consisted of applying the reaction conditions of the transmethylation method to the analytes themselves. Towards this end, FAME samples derived from a sunflower oil were oxidized at different extents and directly analyzed by HPLC before and after applying the conditions of the derivatization reaction.

Two different transmethylation methods were evaluated for being simple, fast and, especially, because in both mild reaction conditions are applied, as they are run at room temperature. The two procedures had shown satisfactory results in the quantitative analysis of relatively stable compounds like core aldehydes and other short-chain glycerol-bound compounds in lipid samples heated at high temperature [11,19]. In one of the methods, KOH in methanol was used as reactant (method 1), whereas in the other the reactant was sodium methoxide (MeONa) (method 2).

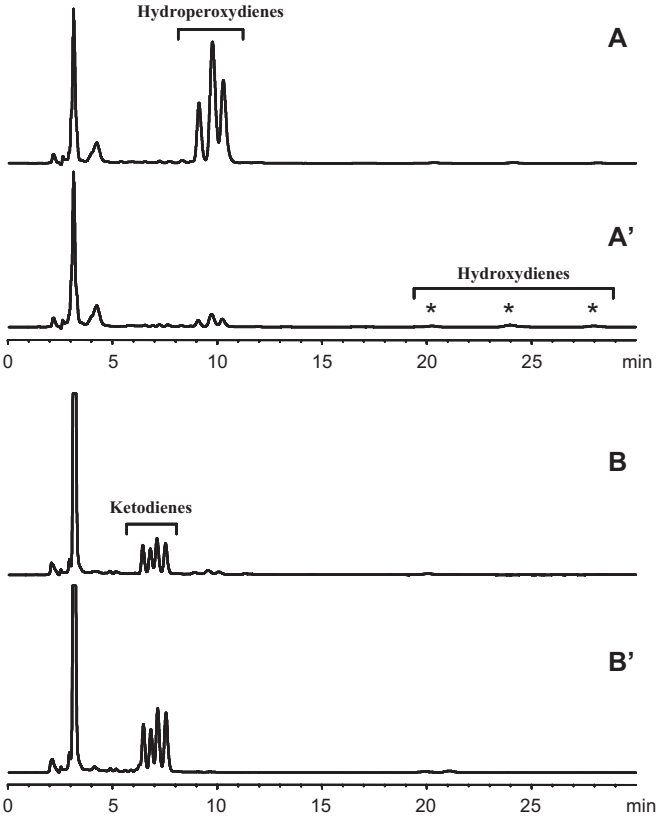

Fig. 1. Influence of transmethylation method 1 (KOH/MeOH) on the chromatographic profile of a FAME sample derived from a high-linoleic sunflower oil and oxidized at 40 °C. Control (A and B) and treated (A′ and B′) samples analyzed at 234 and 268 nm, respectively.

Figs. 1 and 2 illustrate the HPLC chromatograms obtained for an oxidized sample of FAMEs before (control) and after (treated) applying methods 1 and 2, respectively. Regardless of the transmethylation method used, the chromatogram profiles of the control and treated samples were not different in terms of peaks detected or the distribution of the isomeric forms of the compounds. Method 1 showed a great reduction of the signal of hydroperoxydienes and a slight increase in the signals of hydroxy- and keto-dienes. Substantial losses of the signal of hydroperoxydienes were not observed in method 2 and a slight increase in the signals of hydroxy- and keto-dienes was also found.

Quantitative data showing the influence of the transmethylation methods are listed in Tables 1 and 2. The losses of hydroperoxydienes in method 1 were as high as 90%, whereas only losses that ranged from 4 to 9% were obtained in method 2. These differences may be attributed to the higher concentration of the base in method 1, which was ten times higher than in method 2. Unlike method 2, in which the reaction medium is comprised of an only phase, in method 1 the lipid substrate and the reactant are dissolved in immiscible solvents (hexane and methanol, respectively), being necessary a high base concentration for an effective transmethylation reaction.

As for keto- and hydroxy-dienes, significantly higher contents were found in the treated samples in both methods, which can be due to formation by transformations of hydroperoxydienes

Table 1
Influence of transmethylation method 1 (KOH/MeOH) on the contents of hydroperoxy-, keto- and hydroxy-dienes in oxidized FAME samples.

Sample		Hydroperoxydienes (mg/g)	Ketodienes (mg/g)	Hydroxydienes (mg/g)
1	Control	2.20 ± 0.03	0.129 ± 0.003	<0.05
	Treated	0.31 ± 0.04	0.295 ± 0.012	0.140 ± 0.018
2	Control	4.39 ± 0.18	0.209 ± 0.004	0.079 ± 0.002
	Treated	0.49 ± 0.08	0.378 ± 0.007	0.184 ± 0.006
3	Control	10.22 ± 0.10	0.222 ± 0.002	0.216 ± 0.009
	Treated	0.97 ± 0.26	0.871 ± 0.035	0.420 ± 0.016

The statistical analysis showed significant differences between the control and treated samples: $P = 0.002$ for hydroperoxydienes; $P = 0.004$ for ketodienes; $P = 0.001$ for hydroxydienes.

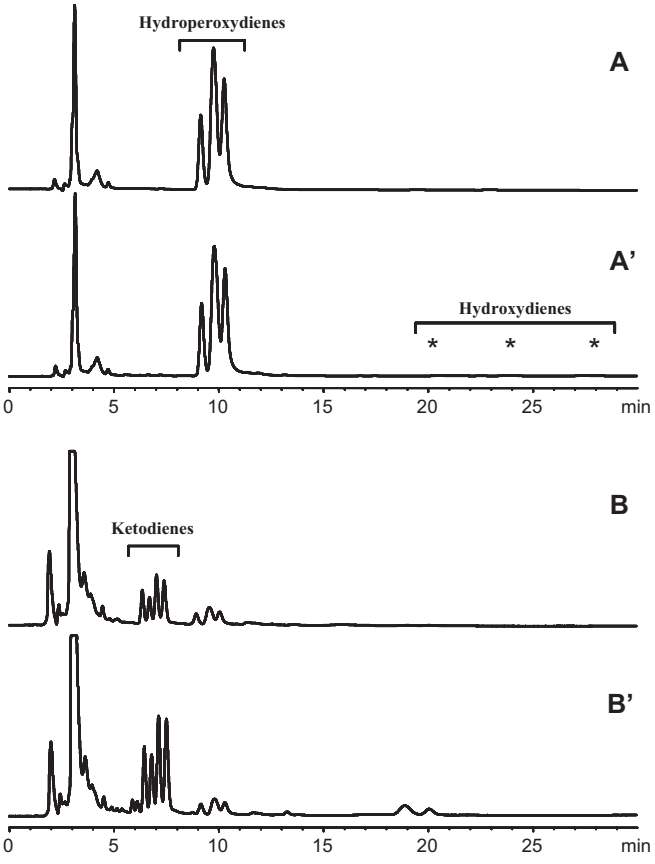

Fig. 2. Influence of transmethylation method 2 (MeONa/MeOH) on the chromatographic profile of a FAME sample derived from a high-linoleic sunflower oil and oxidized at 40 °C. Control (A and B) and treated (A′ and B′) samples analyzed at 234 and 268 nm, respectively.

during the treatments. The contents of keto- and hydroxy-dienes were higher in method 1, which may account for the larger losses of hydroperoxydienes observed. In this respect, the content of ketodienes increased in a factor that ranged between 2 and 4 for method 1 and between 1.1 and 1.8 for method 2; whereas the

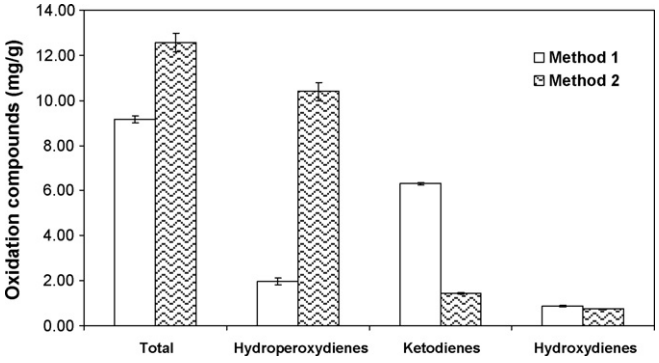

Fig. 3. Application of transmethylation methods 1 (KOH/MeOH) and 2 (MeONa/MeOH) to a sample of high-linoleic sunflower oil oxidized at 40 °C.

content of hydroxydienes increased in a factor of approximately 2 and 1.3 in methods 1 and 2, respectively. Formation of ketodienes was therefore more favored than that of hydroxydienes, being approximately as much as three times higher. Comparing the contents of keto- and hydroxy-dienes formed during the treatment with the initial content of hydroperoxydienes, it can be drawn that transformation of hydroperoxydienes into keto- and hydroxy-dienes, as a whole, was of approximately 6–12% in method 1 and 0.6–2.1% in method 2. It was evident that the higher the content of hydroperoxydienes, the higher the contents of keto- and hydroxy-dienes formed during the treatment. Therefore, the transmethylation reaction exerts a more marked influence on these compounds in samples with higher oxidation extents.

Fig. 3 shows the results obtained by applying both transmethylation methods to an oxidized oil sample. As observed above in the FAME samples, the content of hydroperoxydienes found in method 1 was far lower than that in method 2. The content of hydroxydienes was similar with both methods, but that of ketodienes was approximately four times higher in method 1. As expected, the total content of the analyzed compounds was quite lower in method 1, indicating that a substantial part of hydroperoxydienes was transformed into compounds without conjugated double bounds.

The results have definitely shown that the more adequate transmethylation procedure for the analysis in oils is method 2

Table 2
Influence of transmethylation method 2 (NaOMe/MeOH) on the contents of hydroperoxy-, keto- and hydroxy-dienes in oxidized FAME samples.

Sample		Hydroperoxydienes (mg/g)	Ketodienes (mg/g)	Hydroxydienes (mg/g)
1	Control	2.45 ± 0.04	0.117 ± 0.006	<0.05
	Treated	2.35 ± 0.02	0.132 ± 0.017	<0.05
2	Control	4.16 ± 0.03	0.173 ± 0.003	0.052 ± 0.001
	Treated	3.81 ± 0.22	0.226 ± 0.003	0.069 ± 0.008
3	Control	10.14 ± 0.22	0.199 ± 0.004	0.166 ± 0.011
	Treated	9.43 ± 0.08	0.358 ± 0.031	0.220 ± 0.003

The statistical analysis showed significant differences between the control and treated samples: $P = 0.004$ for hydroperoxydienes; $P = 0.009$ for ketodienes; $P = 0.010$ for hydroxydienes.

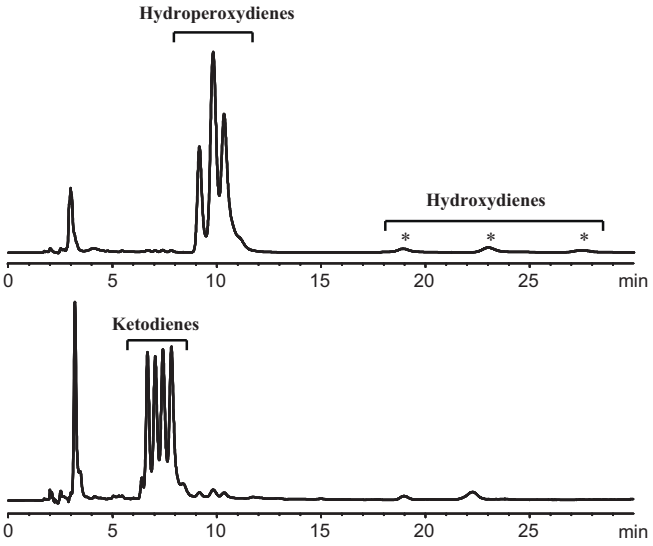

Fig. 4. HPLC chromatogram obtained in the analysis of a sample of high-linoleic sunflower oil oxidized at 40 °C after applying derivatization method 2 (MeONa/MeOH).

(NaOMe/MeOH). Application of this method inevitably results in a slight transformation of the compounds of interest. Losses of hydroperoxydienes lower than 10 wt% are expected and formation of keto- and hydroxy-dienes occurs as a result. In this study an amount of 0.6–2.1 wt% of hydroperoxydienes was transformed into keto- and hydroxy-dienes, being the formation of the former as much as three times higher.

3.2. Chromatographic conditions

Fig. 4 illustrates the HPLC chromatogram of an oxidized oil sample after derivatization by transmethylation method 2. Due to the specificity provided by the conjugated diene structure, the profile of chromatographic peaks was similar to that obtained in model lipid systems like oxidized samples of methyl linoleate and FAMEs obtained from oils. The isocratic regime conditions applied by using a blend of n-heptane:diethyl ether 82:18 (v/v) enabled a complete separation of the three groups of compounds, i.e. keto-, hydroperoxy- and hydroxy-dienes. Therefore the absence of peaks overlapping with those of the analytes made unnecessary to modify the chromatographic conditions.

3.3. Validation of the analytical method

The validation of the method for the analysis of oils was mainly based on that carried out in the analysis of model lipid systems like FAMEs proposed in a recent report [13]. Quantitation is performed by applying the HPLC response factors determined for synthesized FAME compounds that represent each group of interest. These compounds are exactly the same analytes as those to be measured in the analysis of oils.

3.3.1. Linearity

Calibration curves of the synthesized compounds were made in a concentration range from 10 to 300 μg/mL for hydroperoxy- and hydroxy-dienes and from 10 to 100 μg/mL for ketodienes. Linearity was held in the whole range of concentrations studied, showing linear regression coefficients that were higher than 0.999 [13].

3.3.2. Sensitivity

The detection limits, defined as a signal-to-noise ratio of 3, for each group of compounds as a whole were 0.1 μg/mL for hydroperoxy- and keto-dienes and 0.2 μg/mL for hydroxydienes. The quantification limits, defined as a signal-to-noise ratio of 10, were 0.3 μg/mL for hydroperoxy- and keto-dienes and 0.6 μg/mL for hydroxydienes.

3.3.3. Precision

Repeatability, expressed as coefficient of variation (CV), was determined from three replicates in a series of oil samples oxidized at different oxidation extents. The CVs determined were ≤6% for hydroperoxydienes and ≤15% for keto- and hydroxy-dienes.

3.3.4. Accuracy

Accuracy was estimated by assays of recovery of the synthesized compounds in a fresh sample of HSHOSO, added at a concentration of 3 mg/g oil. The recovery values found were 85(±4), 99(±2) and 97.0(±0.6) % for hydroperoxy-, keto- and hydroxy-dienes, respectively.

3.4. Application of the analytical method in sunflower oils with different fatty acid composition

The method proposed was applied to oxidized samples of three sunflower oils with different contents in linoleic acid. The samples were oxidized at 40 °C and analyses were performed throughout the whole induction period of the oils, whose end was determined by the exhaustion of natural antioxidants, i.e. tocopherols, and a significant increase in the polymerization compounds [21]. For comparative purposes, the peroxide value (PV) and the extinction coefficient at 232 nm were also determined. The samples chosen at the end of the assay showed contents of α-tocopherol close to zero and contents of polymers of approximately 1 wt%. Samples in advanced oxidation states were therefore ruled out. Results obtained are listed in Tables 3–5.

Contents of hydroperoxydienes of 0.1–0.2 mg/g were detected in the fresh samples, whose PVs were typical for fresh refined oils (<10 mequiv./kg). Ketodienes were also detected in the fresh samples at levels that ranged from 0.04 to 0.13 mg/g, as well as hydroxydienes in the HLSO and HOSO at 0.36 and 0.13 mg/g, respectively. The HSHOSO also showed the presence of hydroxydienes, although at levels lower than the limit of quantification, i.e. <0.05 mg/g at the analytical conditions applied.

Table 3
Autoxidation of high-linoleic sunflower oil (HLSO) at 40 °C in the dark.

Time (days)	Pol (wt%)	α-Toc (mg/kg)	PV (mequiv./kg)	K_{232}	Hydroperoxy-dienes (mg/g)	Keto-dienes (mg/g)	Hydroxy-dienes (mg/g)
0	0.5	632	0.7 ± 0.1	3.56 ± 0.65	0.09 ± 0.01	0.12 ± 0.01	0.36 ± 0.01
9	0.6	604	22.8 ± 0.8	5.55 ± 0.05	3.19 ± 0.08	0.41 ± 0.02	0.43 ± 0.01
14	0.7	567	43.3 ± 0.1	8.51 ± 0.03	6.15 ± 0.05	0.82 ± 0.13	0.49 ± 0.01
21	0.7	619	79 ± 2.5	12.98 ± 0.38	10.82 ± 0.22	1.66 ± 0.27	0.68 ± 0.03
33	0.8	233	142 ± 2.2	22.56 ± 0.68	18.60 ± 0.72	2.10 ± 0.06	0.95 ± 0.02
41	1.0	23	195 ± 1.2	26.96 ± 1.13	23.73 ± 0.53	2.67 ± 0.43	1.12 ± 0.16

Pol, polymers; α-Toc, α-tocoferol; PV, peroxide value; K_{232}, specific extinction coefficient at 232 nm.

Table 4
Autoxidation of high-oleic sunflower oil (HOSO) at 40 °C in the dark.

Time (days)	Pol (wt%)	α-Toc (mg/kg)	PV (mequiv./kg)	K_{232}	Hydroperoxy-dienes (mg/g)	Keto-dienes (mg/g)	Hydroxy-dienes (mg/g)
0	0.7	704	2.8 ± 0.3	2.26 ± 0.03	0.20 ± 0.01	0.13 ± 0.01	0.13 ± 0.00
9	0.6	672	10.3 ± 0.2	2.62 ± 0.79	1.16 ± 0.01	0.16 ± 0.01	0.15 ± 0.01
21	0.4	606	21.0 ± 0.8	4.53 ± 0.04	2.61 ± 0.04	0.31 ± 0.01	0.21 ± 0.03
41	0.5	459	41.9 ± 0.1	7.19 ± 0.23	5.01 ± 0.03	0.59 ± 0.03	0.28 ± 0.01
61	0.6	238	66.1 ± 0.4	9.97 ± 0.13	7.32 ± 0.20	0.79 ± 0.10	0.45 ± 0.02
79	0.7	70	81.2 ± 0.1	11.87 ± 0.75	9.41 ± 0.04	1.08 ± 0.15	0.57 ± 0.08
86	0.8	3	102.6 ± 0.5	14.60 ± 0.70	11.70 ± 0.47	1.34 ± 0.07	0.72 ± 0.03

Pol, polymers; α-Toc, α-tocoferol; PV, peroxide value; K_{232}, specific extinction coefficient at 232 nm.

Hydroperoxydienes increased steadily with oxidation time and reached different contents in the three oils at the end of the induction period. The amounts found when tocopherols were practically exhausted were 23.7, 11.7 and 6.5 mg/g in HLSO, HOSO and HSHOSO, respectively (Tables 3–5). Therefore, the higher the content of linoleic acid, the higher was the content of hydroperoxydienes at the end of the induction period. These results are in agreement with previous studies carried out in our lab, which showed that the levels of oxidation compounds accumulated during the induction period in different sunflower oils at 25 °C increased with the degree of unsaturation of the oil [22].

Likewise, keto- and hydroxy-dienes increased gradually and higher contents were also found with the degree of unsaturation of the oil, i.e. in the order HSHOSO < HOSO < HLSO. Therefore, the highest amounts were detected in the HLSO, which presented contents of keto- and hydroxy-dienes of 2.7 and 1.1 mg/g, respectively, at the end of the assay.

The amounts obtained for keto- and hydroxy-dienes were relatively high as compared to those found in oxidized samples of FAMEs obtained from HLSO and HOSO in a previous report [13]. With the exception of the fresh oils, the secondary oxidation products constituted 14–20 wt% of the analyzed compounds in oils, whereas 2–3 wt% was found in FAME samples. Taking into account the influence of the derivatization method on the compounds studied, the expected contents of keto- and hydroxy-dienes as a whole would not be higher than 6 wt% of the analyzed compounds, since as outlined above, not more than 2 wt% of hydroperoxydienes were transformed into these secondary products during the treatment. As a consequence, the results suggest that most keto- and hydroxy-dienes were formed during oxidation of oils rather than in the derivatization step. In addition, the relationship between keto- and hydroxy-dienes found in the oils was similar to that determined in oxidized FAME samples, increasing the ratio between keto- and hydroxy-dienes from 1 to 2.5 throughout the induction periods of the oils.

One of the main differences between oils and samples of FAMEs derived from oils is the presence of the fraction of minor components in the former, whose composition can be quite variable even for different batches of a same kind of oil. In this regard, the formation of secondary oxidation products not only depends on the contents of their precursor hydroperoxides, but also on the presence of metals that catalyze hydroperoxide decomposition [9]. The higher contents of keto- and hydroxy-dienes found in the oils might be related to a greater decomposition of hydroperoxydienes catalyzed by metals, since these are mostly removed in the purification step of oils applied in the preparation of the FAMEs [23]. Nevertheless, the results of the present study showed that similar amounts of the secondary oxidation products relative to that of their precursor hydroperoxydienes were found for the three oils.

Another minor component which might account in part for the differences between the oils and FAME samples is the presence of α-tocopherol in the oils. Kamal-Eldin et al. [24] found that addition of α-tocopherol to methyl linoleate resulted in an increase of the formation of keto- and hydroxy-dienes during the induction period that was dependent on the antioxidant concentration. In order to explain this increase the authors suggested that owing to its high hydrogen-donating capability α-tocopherol may participate in side reactions with hydroperoxides to yield α-tocopheroxyl, alkoxyl radical and water; i.e. exerting a decomposing effect on hydroperoxides. Such an effect has been suggested to account in part for the loss of antioxidant efficiency of α-tocopherol at high concentrations [24,25].

The content of hydroperoxydienes showed a satisfactory linear correlation with the PV in the three oils (Fig. 5A). For a given PV, higher contents of hydroperoxydienes were found in the oil with the highest content of linoleic acid. For instance, when the PV was 50 mequiv./kg the estimated contents of hydroperoxydienes were 6.1, 5.7 and 3.8 mg/g in HLSO, HOSO and HSHOSO, respectively. These results indicate the occurrence of hydroperoxides other than hydroperoxydienes. According to a previous report, the differences between hydroperoxydienes and total hydroperoxides may be attributed to the oxidation of oleic acid, whose monoenic hydroperoxides are not detected in the HPLC-UV analysis [13]. Comparing the slopes of the straight lines in Fig. 5A with that found for pure hydroperoxydienes, the contents of hydroperoxides from oleic acid can be estimated [13]. The slope for HLSO is quite similar to that found for pure hydroperoxydienes (0.119 ± 0.004), suggesting that oxidation of oleic acid was quite negligible. Likewise, the

Table 5
Autoxidation of high-stearic high-oleic sunflower oil (HSHOSO) at 40 °C in the dark.

Time (days)	Pol (wt%)	α-Toc (mg/kg)	PV (mequiv./kg)	K_{232}	Hydroperoxy-dienes (mg/g)	Keto-dienes (mg/g)	Hydroxy-dienes (mg/g)
0	0.4	836	0.0 ± 0.0	2.21 ± 0.06	0.11 ± 0.00	0.04 ± 0.00	<0.05
9	0.5	826	–	–	–	–	–
21	0.4	822	6.3 ± 0.1	2.56 ± 0.01	0.65 ± 0.02	0.11 ± 0.01	<0.05
41	0.6	775	11.7 ± 0.1	3.00 ± 0.04	1.43 ± 0.08	0.19 ± 0.03	0.14 ± 0.01
61	0.7	677	–	–	–	–	–
79	0.5	551	38.5 ± 0.7	4.94 ± 0.11	3.10 ± 0.09	0.27 ± 0.06	0.15 ± 0.01
100	0.7	243	68.9 ± 0.7	6.93 ± 0.05	4.82 ± 0.10	0.37 ± 0.02	0.23 ± 0.01
120	0.8	199	–	–	–	–	–
140	0.9	156	–	–	–	–	–
156	1.1	4	77 ± 1.4	7.55 ± 1.21	6.54 ± 0.12	0.79 ± 0.02	0.47 ± 0.01

Pol, polymers; α-Toc, α-tocoferol; PV, peroxide value; K_{232}, specific extinction coefficient at 232 nm.

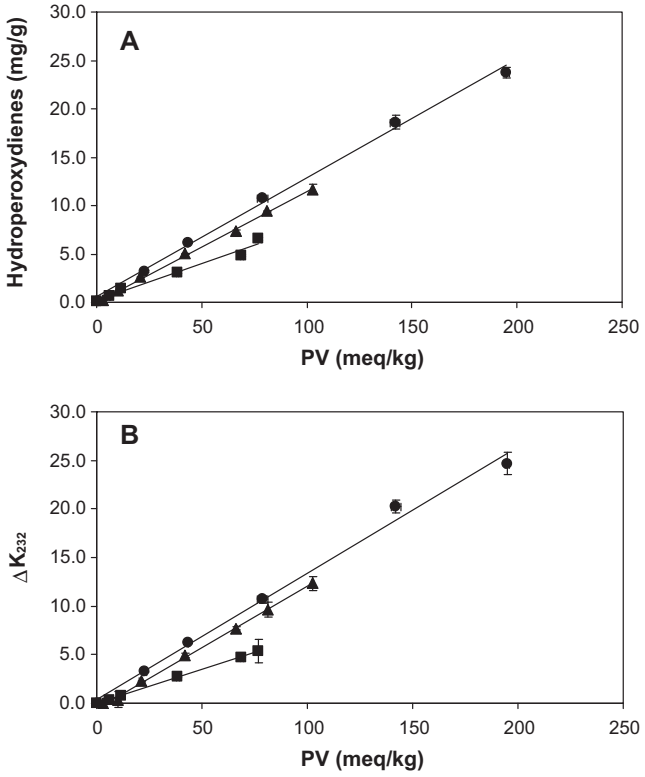

Fig. 5. Linear correlation analyses between the content of hydroperoxydienes and the peroxide value (PV) (A) and between the increase of the specific extinction coefficient at 232 nm (ΔK_{232}) and the PV (B) in HLSO (●), HOSO (▲) and HSHOSO (■) during oxidation at 40 °C. (HLSO: [Hydroperoxydienes](mg/g) = 0.122(±0.004)PV(mequiv./kg), r = 0.998; HOSO: [Hydroperoxydienes] (mg/g) = 0.114(±0.002)PV(mequiv./kg), r = 0.999; HSHOSO: [Hydroperoxydienes] (mg/g) = 0.075(±0.006)PV(mequiv./kg), r = 0.988; HLSO: ΔK_{232} = 0.130(± 0.006)PV(mequiv./kg), r = 0.996; HOSO: ΔK_{232} = 0.125(±0.002)PV(mequiv./kg), r = 0.999; HSHOSO: ΔK_{232} = 0.0695(±0.0008)PV(mequiv./kg), r = 0.999).

estimated value found in HOSO was as low as 5%, whereas that in HSHOSO was of approximately 40% of total hydroperoxides. Therefore, the higher the oleic-to-linoleic ratio the larger the oxidation of oleic acid was for a given PV.

Oxidation of oleic acid was less significant in the HLSO and HOSO than in samples of FAMEs derived from HLSO and HOSO reported in a previous report [13]. This fact can be attributed in part to the presence of α-tocopherol in the oils, not present in the FAME samples, which has shown a higher protection on oleic acid than linoleic acid in FAME samples derived from oils. In fact, addition of α-tocopherol to FAME samples derived from HOSO showed an antioxidant efficacy that was approximately three times higher on methyl oleate [15].

The relationship between the increment of the specific extinction coefficient at 232 nm (ΔK_{232}) and the PV was quite similar to that between the content of hydroperoxydienes and the PV (Fig. 5B). Unlike the determination of hydroperoxydienes, the measure of K_{232} does not require any treatment, so the ΔK_{232} values supported the quantitative results obtained for hydroperoxydienes.

4. Conclusions

The results of this study have shown that transmethylation with NaOMe at room temperature is an appropriate approach to obtaining FAME in the analysis of the main oxidation products of linoleic acid – hydroperoxy-, keto- and hydroxy-dienes – in refined oils. Application of this procedure inevitably results in a slight transformation of the compounds of interest. Losses of hydroperoxydienes lower than 10 wt% are expected and formation of keto- and hydroxy-dienes occurs as a result. An amount of 0.6–2.1 wt% of hydroperoxydienes was transformed into keto- and hydroxy-dienes, being the formation of the former as much as three times higher. It was evident that the transmethylation reaction exerts a more marked influence on these compounds in samples with higher oxidation extents.

Validation of the quantitative analysis of these compounds in oils by HPLC-UV has shown satisfactory sensitivity (quantification limits of 0.3 μg/mL for hydroperoxy- and keto-dienes and 0.6 μg/mL for hydroxydienes), precision (coefficients of variation ≤6% for hydroperoxydienes and ≤15% for keto- and hydroxy-dienes) and accuracy (recovery values of 85(±4), 99(±2) and 97.0(±0.6) % for hydroperoxy-, keto- and hydroxy-dienes, respectively).

Application of the analytical method to oxidized samples of sunflower oils with different contents of the oxidizing substrates linoleic and oleic acids showed that the higher the linoleic-to-oleic ratio, the higher were the levels of hydroperoxy-, keto- and hydroxy-dienes when tocopherols were practically exhausted, i.e. at the end of the induction period. In this regard, the highest contents were found for the HLSO, which showed levels of 23.7, 2.7 and 1.1 mg/g oil for hydroperoxy-, keto- and hydroxy-dienes when tocopherol was practically exhausted.

Likewise, for a given oxidation extent, as determined by the PV, higher levels of hydroperoxydienes were found when the linoleic-to-oleic ratio increased. As an example, when the PV was 50 mequiv./kg the estimated contents of hydroperoxydienes were 6.1, 5.7 and 3.8 mg/g in HLSO, HOSO and HSHOSO, respectively. It was estimated that hydroperoxydienes constituted approximately 100, 95 and 60% of total hydroperoxides in the HLSO, HOSO and HSHOSO, respectively.

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