Quantitative determination of major oxidation products in edible oils by direct NP-HPLC-DAD analysis

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Running title: Direct quantification of major oxidation products in oils by HPLC

Abstract

The objective of the present study was to explore the possibilities of the direct analysis of vegetable oils by normal-phase HPLC-DAD to evaluate the amounts of the main oxidation products of triacylglycerols containing linoleate, i.e. hydroperoxy-, keto- and hydroxy-dienes. A follow-up of oxidation at 40 °C of trilinolein, used as a simplified model lipid system, high-linoleic sunflower oil and high-oleic sunflower oil was performed to evaluate samples with different fatty acid compositions and different oxidation levels. The results showed that the HPLC-DAD method proposed allows for determining the concentrations of mono-hydroperoxydienes in edible oils without applying any isolation or derivatization step. The method was found to be direct, sensitive (LOQ 0.06 mmol/kg oil), precise ($CV \leq 5\%$) and also accurate, with 99% of analyte recovery. It also enabled the estimation of the minor amounts of ketodienes, but not those of hydroxydienes, which presented wide chromatographic bands and coeluted with a number of different minor oxidation compounds.

Key-words: Hydroperoxydienes; ketodienes; hydroxydienes; oils; autoxidation; HPLC

1. Introduction

Autoxidation of food lipids causes a variety of products that eventually impair the food flavor and make the food unacceptable for consumption. Oxidized lipids are also involved in pathophysiological processes of chronic and degenerative diseases [1-6]. These can be incorporated through the diet, be formed during the digestion of lipids or be produced in the body, especially in unhealthy individuals. Oxidized food lipids are therefore of concern because they may be detrimental to health. Numerous nutritional studies evidence the toxicological effects of oxidized oils [2, 7-10]. Unfortunately, in most of these studies the characterization of oxidized oils was deficient. This was performed by applying analytical indices such as the peroxide value, anisidine value and others that only provide information about partial aspects of the oxidative degradation. Thereby such indices do not enable to get to know the real oil oxidation state and, more importantly, the chemical nature and amounts of the oxidized lipids that are provided to experimental animals. Despite the known implications of oxidized lipids in a number of degenerative and chronic diseases, the real contribution of those incorporated through the diet is at present unknown. It is not known whether their concentrations are sufficient to cause detrimental nutritional effects [11].

Isolation and structural determination of the main oxidation products of the common dietary unsaturated fatty acids were well established using model lipid systems at the end of the 70's [12]. Complex analytical methods mainly based on the combination of liquid chromatography and mass spectrometry have been reported for the identification of oxidation compounds in a variety of samples, principally those of biological nature [13-15]. Recently, chromatography methods for the analyses of primary [16] and secondary [17] oxidation products of triacylglycerols (TAG) have been reviewed. On the other hand, studies dealing with quantitative analysis of oxidized lipids in foods are rather scarce. The principal limitations of the quantitative determination are due to a large number of compounds formed from

numerous TAG, their low contents as individual species and their relatively low stability. Quantitative analytical studies have been concentrated on specific compounds found in biological samples [13]. Because of their potential toxicity, glycerolipid aldehydes, also known as core aldehydes, and small aldehydes such as acrolein, malodialdehyde and hydroxyalquenals have been the most studied [13, 18]. The analytical approaches based on HPLC-MS used in most of these studies have not been adopted up to now. This is because of the relatively elevated cost of the equipment and the expense involved in its operation and maintenance.

In a previous study we proposed a simple method of quantitative analysis for the main oxidation products of linoleic acid in oils, *i.e.* hydroperoxy-, keto- and hydroxy-dienes. This is based on a derivatization step to convert the TAG molecules into fatty acid methyl esters (FAME) that are analyzed by normal-phase HPLC-UV [19]. The derivatization step caused changes in the composition of the compounds analyzed, as slight losses of hydroperoxydienes and formation of keto- and hydroxy-dienes were observed.

The coupling in series of an evaporative light scattering detector to the HPLC-UV method was useful to also determine oleic acid oxidation products in both model lipids [20] and oils [21]. Results obtained in five edible oils containing different contents of oleate, linoleate and linolenate acyl chains, namely, conventional sunflower, high-oleic sunflower, high-stearic high-oleic sunflower, rapeseed and soybean oils, showed that linoleate oxidation products were predominant in all cases. Substantial formation of oleate oxidation compounds was not observed in the monounsaturated oils until the tocopherols naturally present were completely lost, *i.e.* at advanced global oxidation extent. The analysis of the main oxidation products of linoleate proved to be a good analytical approach to evaluate the global oxidation extent in oils containing oleic and linoleic acids as the only unsaturated fatty acids present at significant

amounts. For monounsaturated oils with low levels of linoleate, it was concluded that the lower the global oxidation extent, the better was the analytical approach [21].

With the aim of avoiding any derivatization step that may result in analytical artifacts and so modify the composition of analytes, the objective of the present study was to explore the possibilities of the direct analysis by normal-phase HPLC-DAD of the amounts of the main oxidation products of linoleate in intact samples of edible vegetable oils, i.e. as TAG molecules instead of FAME derivatives. In this regard, the direct analysis of main oxidation products by a simple method as is HPLC-DAD, using a common chromatograph easily available in research laboratories, would be of great convenience in oxidation studies and would also contribute to improving the characterization of oxidized oils in nutritional studies. A comparison with the analysis of the FAME derivatives mentioned above, which was validated elsewhere [19], was made to assess its utility. Prior to the analysis of oils, which are constituted by a number of different TAG, trilinolein was first assayed as a simplified lipid system. High-linoleic sunflower oil (HLSO) and high-oleic sunflower oil (HOSO) were the oils studied. A follow-up of oxidation at 40 °C of the model lipid and the two oils was performed to evaluate samples with different oxidation levels. The samples were characterized by applying the specific extinction at 232 nm (K_{232}) and the peroxide value. Analysis of polymers by high-performance size-exclusion chromatography (HPSEC) with refractive-index detection was also applied as a rapid control measure to discard samples in advanced oxidation states [22].

2. Materials and Methods

2.1 Chemicals

Trilinolein (> 99%) was purchased from Nu-Check-Prep (Elysian, MN, USA) and used as received. Diethyl ether stabilized with 1% v/v ethanol (Super purity solvent, HPLC grade)

was acquired 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. Any other chemical used was of analytical grade at least.

2.2 Oil samples

High-linoleic sunflower oil (HLSO) and high-oleic sunflower oil (HOSO) were supplied by a local refinery (Andújar, Jaén, Spain). The two oils were edible refined oils. 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 for HLSO, and 4.1% C16:0, 4.6% C18:0, 77.6% C18:1, 11.7% C18:2 and 2.0% others for HOSO.

2.3 Oxidation conditions

A 10-g sample of trilinolein was placed in a 250-mL beaker and oxidized at 40 °C in the dark in an oven with air circulation. The surface-to-lipid-volume ratio was 2 cm⁻¹. Aliquots were withdrawn periodically and kept at -25°C until analyses.

Samples of 20 g of the oils were placed in Petri dishes (14.5 cm i.d.) that were left open to the air and oxidized at 40 °C in the dark in the oven with air circulation. The surface-to-oil-volume ratio was 7 cm⁻¹. An aliquot of each sample was taken in each sampling and kept at -25 °C until analyses. The samples were oxidized until the tocopherols naturally present were almost or completely depleted.

2.4 Reduction of samples with NaBH₄

Oxidized samples were selected and reduced with NaBH₄ to identify hydroxy- compounds. The procedure followed was the one reported by Hämäläinen et al. [23] with a few modifications. A volume of 6 mL of a saturated NaBH₄ solution in isopropanol (10 mg/mL) was added to a 200-mg aliquot of an oxidized oil sample. The isopropanol had been previously dried with anhydrous Na₂SO₄. The reaction mixture was stirred at room

temperature under nitrogen for one hour. The reaction was stopped with 6 mL water and the excess of the reagent was neutralized with aqueous HCl (5 %, w/w). Saturated aqueous solution of NaCl (5 mL) was added and the lipids transformed were extracted using one extraction with diethyl ether (10 mL). The organic phase was washed with 10 mL water, dried over anhydrous Na₂SO₄ and filtered with paper filter. The solvent was first evaporated in a rotary evaporator at 40 °C and then taken to dryness with a stream of nitrogen.

2.5 Direct HPLC analysis

2.5.1 Chromatographic conditions

The trilinolein and the oil samples were dissolved in n-heptane at a concentration of 1-50 mg/mL and directly analyzed in an Agilent 1260 Infinity HPLC chromatograph (Agilent Technologies, USA). The chromatograph was equipped with a 1260 quaternary pump VL (G1311C), a 1260 standard autosampler (G1329B), a 1260 thermostatted column compartment (TCC) (G1316A) and a 1260 diode array detector (DAD) VL (G1315D) with a standard measure cell (13 µL volume, 10 mm cell path length). A silica HPLC column (LiChrospher[®] Si 60, 250 mm x 4 mm i.d., 5 µm particle size) (Merck, Darmstadt, Germany) was used. The volume of sample analyzed was 20 µL. The temperature of the TCC was set at 25 °C. The separation of analytes were performed using isocratic elution with 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. Hydroperoxy- and hydroxy- dienes were evaluated at 234 nm, while ketodienes were at 268 nm.

2.5.2 Response factors

Following a previous study [24], quantitative data were obtained by applying the response factors determined for methyl-linoleate oxidation products, i.e. methyl 13-hydroperoxy-(Z)-9,(E)-11-octadecadienoate, methyl 13-hydroxy-(Z)-9,(E)-11-octadecadienoate and methyl 13-

keto-(Z)-9,(E)-11-octadecadienoate. The standards were prepared in the lab according to the previous study [24]. Expressed in $\text{nmol}^{-1} \text{ mL}$, the response factors obtained were $38.3(\pm 0.3)$ and $40.6(\pm 0.2)$ for hydroperoxy- and hydroxy-dienes, respectively, at 234 nm and $39.2(\pm 0.1)$ for keto-dienes at 268 nm. The response factor for hydroperoxydienes at 268 nm was found to be $0.172(\pm 0.007) \text{ nmol}^{-1} \text{ mL}$.

2.5.3 Linearity

Linearity of chromatographic response for hydroperoxy- and hydroxy-dienes was evaluated by starting from the most oxidized sample of trilinolein, i.e. a sample oxidized at 40 °C for 30 h. Different solutions of this sample were prepared by dilution with n-heptane and analyzed by HPLC-DAD. Similarly, linearity for ketodienes was evaluated from the most oxidized sample of HLSO, i.e. a sample that was oxidized at 40 °C for 35 d. Also, different solutions of this sample prepared by dilution with n-heptane were analyzed.

2.5.4 Sensitivity

The limits of detection (LOD) and quantification (LOQ) were estimated for each group of compounds following the signal-to-noise ratio criterion. LOD and LOQ were estimated for a signal-to-noise ratio of 3 and 10, respectively. They were calculated from the noise magnitude that was taken as an estimate of the blank standard deviation [25]. An oil sample concentration of 50 mg/mL was considered for the determinations of LOD and LOQ.

2.5.5 Precision

Repeatability, expressed as coefficient of variation (CV), was determined from six replicates in a series of oil samples oxidized at different oxidation extents.

2.5.6 Accuracy

Accuracy was estimated for hydroperoxydienes only. A trilinolein sample oxidized at the conditions described above at a low oxidation extent was used to isolate hydroperoxydienes.

The sample presented a level of ketodienes close to their LOQ and hydroxydienes were not detected. Trilinolein hydroperoxides were isolated by silica-packed column chromatography by applying the same procedure as that used for the determination of polar compounds in used frying oils, following IUPAC standard method 2.507 [26]. The isolation of the polar fraction was performed using a simultaneous fractionation of four samples of 2.5 g oxidized trilinolein each in four columns. The polar fractions were joined to have approximately 40 mg isolated sample. An HPLC direct analysis of the polar fraction showed hydroperoxydienes and ketodienes only. The levels of ketodienes present in the isolated sample were 1.5 mol%. Therefore, the purity of hydroperoxydienes was estimated to be 98.5 mol%. The isolated hydroperoxydienes were added to a fresh sample of HLSO at different concentrations, which ranged from 5 to 40 mmol/kg. The samples were analyzed by HPLC and the recovery value was calculated as the mean value of all the determinations.

2.6 HPLC analysis of derivatized samples

For comparative purposes, the oxidized fatty chains in the trilinolein and oil samples were also analyzed. A derivatization reaction to transform the TAG into FAME was applied according to a previous study [19]. A 200-mg sample was accurately weighed into a screw-capped centrifuge tube (13 cm × 10 mm I.D.) and a volume of 3 mL of methyl *tert*-butyl ether was added. Then, a 1.5-mL volume of 0.2 M NaOMe 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 H₂SO₄ 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 and the solvent was first evaporated in a rotary evaporator at 40 °C and then taken to dryness with a stream of nitrogen. Solutions of the FAME samples in n-heptane at a concentration of 1–50 mg/mL were analyzed by HPLC. The chromatograph and the chromatographic conditions were the same as

those described above for the direct analysis of the lipid or oil samples. Also, quantitative data were obtained by applying the response factors outlined above.

2.7 Analysis of polymers

Analysis of TAG polymers was performed by HPSEC with refractive index detection according to IUPAC standard method 2.508 [26].

2.8 Peroxide value

Peroxide value was determined by the iodometric assay according to IUPAC standard method 2.501 [26]. Oil samples of 500 mg and 1×10^{-2} M $\text{Na}_2\text{S}_2\text{O}_3$ 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 [27].

2.10 Analysis of tocopherols

Analysis of tocopherols was carried out by normal-phase HPLC with fluorescence detection following IUPAC standard method 2.411 [26].

2.11 Statistical analysis

The trilinolein and oil samples were analyzed by HPLC in triplicate to determine lipid oxidation products and results were expressed as mean values followed by the standard deviation. As to the other analytical determinations applied for chemical characterization, the samples were analyzed in duplicate and results were expressed as mean values. Comparisons of means were performed by the Student's *t* test. Comparisons between the TAG and FAME analyses were performed by applying a mixed model analysis of repeated measurements, i.e. those for the analytical method and the three analytical replications, using the oxidation time and the type of analytical method as fixed factors. This analysis was performed separately for

each type of lipid sample, namely, trilinolein, HLSO and HOSO. For practical purposes, the HOSO sample showing the most advanced oxidation extent (82 days) was not considered in the statistical analysis. Linear regression analysis was applied between the concentrations of hydroperoxydienes determined by the TAG analysis and those by the FAME analysis to establish relationships between both parameters. Similarly, the total amounts of the compounds determined by the two analytical methods received the same statistical treatment. The 24.0 SPSS Statistics program was used (SPSS Inc., Chicago, IL, USA). Significance was defined at $p < 0.05$.

3. Results and Discussion

3.1 Chromatographic conditions

The chromatographic conditions applied in the direct analysis of oils were the same as those employed in the analysis of FAME derivatives, previously reported [19]. It was not necessary to change the conditions because the results obtained were satisfactory. Thus, on a silica HPLC column, the isocratic regime conditions employed using a blend of n-heptane:diethyl ether 82:18 (v/v) enabled the elution of the analytes in a reasonable time. Oxidized samples of trilinolein showed chromatograms that were not complex because of the specificity of the detection of conjugated diene structures (**Fig. 1**). Hydroperoxydienes were grouped in a similar way to the analysis of the FAME derivatives [19], but at shorter retention times due to a lower polarity. Eight different hydroperoxide isomers would be expected in trilinolein, the four stereoisomers of linoleate, i.e. *cis,trans*- and *trans,trans*-9- and 13-isomers, located on the 1(3)- or 2-positions of the TAG molecule [28]. The chromatographic conditions applied in the present study did not allow for the separation between the eight isomers, although the number of unresolved peaks observed on the band was eight. As oxidation progressed, a band formed by a number of different chromatographic peaks of low intensity was also observed at retention times much longer than those of hydroperoxydienes (**Fig. 1A**). Because of its

similarity to the FAME analysis (**Fig. 1B**), such a chromatographic band was first assigned to hydroxydienes, although more complex oxidation compounds could not be ruled out, as it will be dealt later. Ketodienes were also grouped at the chromatographic conditions applied. Unlike the FAME analysis, they were not completely separated from hydroperoxydienes. However, the overlapping between both bands was small and so the measure of the ketodiene area was not significantly affected (**Fig. 1a-b**).

Despite the number of different TAG present in oils, the chromatograms of the oxidized oils were not very different from those of oxidized trilinolein, as shown in **Fig. 2A** for HLSO. Hydroperoxydienes were grouped in a band of unresolved chromatographic peaks. Similarly, a band of peaks of low intensity was also observed in the hydroxy- region of the chromatograms as oxidation progressed. Unlike the trilinolein, the ketodienes presented a wider band with clear overlapping with that of hydroperoxydienes as a consequence of a greater number of keto- and hydroperoxy-diene TAG in oils (**Fig. 2B**). Also, it was found that the greater the oxidation extent, the more was affected the band of keto- by that of hydroperoxy-dienes. In contrast, the band of hydroperoxydienes was unaffected by that of ketodienes due to the comparatively low levels of the latter and their low light absorption at 234 nm.

With the aim of identifying compounds in the hydroxy- region different oil samples were reduced with NaBH₄. All the hydroperoxydienes were practically transformed into their parent hydroxydienes and were displaced to the hydroxy- region, as shown in **Fig. 2C** for HLSO. The relative intensities of the unresolved peaks suggested that the first part of the band corresponded to the transformed mono-hydroperoxides. The second part could be due to TAG with more than one hydroxy function, probably formed from bis- and tris-hydroperoxydienes [28-31]. It can also be observed that the peaks at longer retention times disappeared with the NaBH₄ reduction, suggesting the presence of compounds of greater structural complexity.

3.2 Validation of the analytical method

The validation of the direct analysis of oils was mainly based on that carried out for the analysis of the FAME derivatives proposed in a previous publication [19]. Quantitation was performed by applying the HPLC response factors determined for synthesized FAME compounds that represent each group of the compounds of interest. These were assumed to have the same response factors as when forming part of TAG molecules. In fact, intact and derivatized samples of oxidized trilinolein presented chromatographic responses for hydroperoxydienes whose areas under the curve showed similar values, which indicated that the chromophore remained unchanged when the TAG were transformed into the FAME derivatives. Similarly, the same was assumed for hydroxy- and keto-dienes. Therefore, quantitative data were obtained assuming the same response factors as those used in the FAME analysis, but obviously considering a molar basis.

3.2.1. Linearity

Hydroperoxydienes exhibited an HPLC-DAD response that was linear in the range of concentrations tested, i.e. from 3 to 4000 μM . Similar results were obtained for methyl 13-hydroperoxy-(Z)-9,(E)-11-octadecadienoate synthesized in the lab and used in the validation of the analysis of the FAME derivatives [24]. The band assigned to hydroxydienes and that of ketodienes also presented linearity in all the range of concentrations assayed, i.e. from 50 to 500 μM and from 5 to 50 μM , respectively. Linear regression coefficients were higher or equal to 0.999 in the three cases (not presented).

3.2.2. Sensitivity

The HPLC-DAD analysis presented elevated sensitivity for the three groups of compounds, especially for hydroperoxy- and keto-dienes. The determination of hydroxydienes was obviously less sensitive because of the greater width of the chromatographic band. The

estimated LOD values were 0.03 mmol/kg oil for hydroperoxy- and keto-dienes and 0.22 mmol/kg oil for hydroxydienes, whereas the LOQ values were 0.06 mmol/kg oil for hydroperoxy- and keto-dienes and 0.75 mmol/kg oil for hydroxydienes.

3.2.3. Precision

The CVs were found to be $\leq 5\%$ for hydroperoxydienes and $\leq 7\%$ for both keto- and hydroxydienes, whereas such figures were reported to be respectively $\leq 6\%$ and $\leq 15\%$ in the FAME analysis [19].

3.2.4. Accuracy

Accuracy was estimated by assays of recovery of isolated hydroperoxydienes added to a fresh sample of HLSO. The recovery value found was $99(\pm 1)\%$.

3.3 Quantitative determination of oxidation compounds in trilinolein samples

Formation of hydroperoxy-, keto- and hydroxy-dienes was studied in trilinolein and the direct analysis was compared to the analysis of FAME derivatives. The results obtained are listed in **Table 1**.

The results of hydroperoxydienes were quite similar to those of the FAME analysis, although, as expected [19], slightly higher concentrations were obtained as oxidation progressed. A linear regression analysis between both measures indicated that the FAME concentrations were on average approximately 94% of the TAG results ($[\text{OOH}]_{\text{FAME}} = 0.938(\pm 0.009) [\text{OOH}]_{\text{TAG}}$, $r^2 = 0.9996$). As outlined above, such analytical differences were close to the precisions of both methods and so to the experimental analytical error. In addition, such differences suggested losses of approximately 6 molar% for hydroperoxydienes in the derivatization reaction, which supports the results found in a previous study in which the transmethylation reaction was applied to oxidized FAME samples to assess chemical transformations [19].

The concentrations of ketodienes were significantly lower in the TAG analysis, which was expected because of the formation of ketodienes in the derivatization reaction in the FAME analysis [19]. However, the concentrations found in the latter were significantly greater than those expected from the results obtained in a previous study, in which it was observed that not more than 2.1 wt% of hydroperoxydienes were transformed into keto- and hydroxy-dienes as a whole when the transmethylation conditions were applied to oxidized FAME samples [19]. In contrast, the results in the present study suggested that 10-11 molar% of simple hydroperoxydienes were transformed into simple ketodienes. This is not consistent with the fact that the results for simple hydroperoxydienes suggested losses of as low as 6 molar% on average as a consequence of the derivatization reaction. Consequently, the direct analysis of the oil samples pointed out that the determination of ketodienes in the FAME analysis requires further research, which is out of the scope of the present study.

The concentrations of the group of compounds determined as hydroxydienes in the TAG analysis were substantially greater compared to the FAME analysis. This was clearly indicative of the coelution of compounds other than simple or mono-hydroxydienes. TAG holding more than one oxidized acyl chain could have been involved [28-31]. According to studies by Neff et al. [28], formation of bis- and tris-hydroperoxides, i.e. TAG with two and three acyl chains holding the hydroperoxy function, were found in trilinolein oxidized at 40 °C from peroxide values (PVs) as low as 18 meq/kg. Although quantitative data were not unfortunately provided, the relative area percentage in reverse-phase HPLC chromatograms indicated that the relative proportions of mono-, bis- and tris-hydroperoxydienes were approximately 69, 21 and 10%, respectively, at PV 125 meq/kg. Therefore formation of compounds with more than one hydroperoxy group seems not to be negligible during autoxidation of trilinolein. Such compounds are obviously of higher polarity than their parent mono-hydroperoxides and could elute in the hydroxy- region of the chromatogram. In

addition, their derivatization to FAME would enable the grouping of compounds in the FAME analysis and so it could be explained the fact that the HPLC chromatograms of the FAME derivatives were not complex in terms of appearance of unidentified chromatographic peaks (**Fig. 1B**). Also, bis- and tris-hydroperoxides could also contribute to the relatively high concentrations of ketodienes found in the FAME analysis.

It is of interest to emphasize that bis- and tris-hydroperoxydienes would have respectively response factors twice and three times greater than that of the mono-hydroperoxides, expressed on a molar basis. Therefore, the method proposed in the present study allows for determining the molar concentrations of oxidized acyl chains on TAGs, which correspond to the same molar concentrations of TAG for mono- hydroperoxy, keto and hydroxy compounds.

The total amounts of oxidation compounds determined by the direct analysis of oxidized trilinolein were similar to those of the FAME analysis, although slightly higher as shown by the statistical analysis (**Table 1**). A linear regression analysis between both measures exhibited that the total concentrations of the oxidation compounds evaluated in the FAME analysis were on average approximately 95% of the TAG results ($[Total]_{FAME} = 0.949(\pm 0.007) [Total]_{TAG}$, $r^2 = 0.9998$). In addition, the results found in the TAG analysis showed that the mono-hydroperoxides evaluated constituted more than 90 molar% of the compounds quantified when polymerization was not yet significant.

3.4 Quantitative determination of oxidation compounds in oil samples

Quantitative results found in oxidized HLSO and HOSO samples are listed in **Tables 2-3**. For comparative purposes, the chromatographic band formerly assigned to hydroxydienes was divided into two parts, the one formed by mono- and other hydroxydienes and the band due to compounds of greater structural complexity (**Fig. 2A**). Because of their polarity, TAG with

more than one hydroperoxy function should not be discarded in the hydroxy region, as the results obtained in the trilinolein samples suggested. Also, the concentrations of ketodienes were determined by difference from the total area of the band formed by keto- and hydroperoxy-dienes at 268 nm (**Fig. 2B**). The area of hydroperoxydienes at 268 nm was estimated from their concentration values determined at 234 nm and their response factor at that wavelength, which was approximately two hundred times lower than that at 234 nm.

As observed in the trilinolein samples, the amounts of hydroperoxydienes determined in the two oils by the TAG analysis were similar to those found in the FAME analysis. Although slightly higher concentrations were found for the former in a few samples, a global statistical analysis of the data obtained for each type of oil separately showed no significant differences between the two methods. This was supported by the fact that the differences found between the two methods in the trilinolein samples were close to the experimental analytical error. In addition, the results of the FAME analysis were similar to those found in previous studies, in which they were expressed on a weight basis [19,21].

In the vicinity of the end of the induction period, defined as the time at which the oil becomes unprotected by the total loss of the naturally occurring antioxidants (tocopherol) [32], the amounts of hydroperoxydienes were found to be 78 and 32 mmol/kg in the HLSO and HOSO, respectively. It has been found in previous studies that hydroperoxydienes constitute by far the most abundant compounds formed in common vegetable oils containing linoleate and oleate as the only oxidizable substrates present at significant amounts [19,21]. Oxidation of oleate seems to be efficiently inhibited in the presence of substantial amounts of the tocopherols naturally present in oils, i.e. at low global oxidation extents [33]. Even in monounsaturated oils in which the levels of linoleate are relatively low, such as the HOSO tested in the present study, the oxidation of oleate was not relatively significant until the PV was higher than 50 meq/kg [19,21]. Therefore, the quantification of the main oxidation

products of linoleate seems to be a good analytical approach to evaluate the global oxidation extent in this kind of oils. For monounsaturated oils containing low levels of linoleate, the lower the oxidation extent, the better will be this analytical approach. Against analytical indices, such as the PV and K_{232} , the measure of hydroperoxydienes is not subjected to possible analytical interferences that may occur especially when food lipid extracts are analyzed. Such interferences are normally due to food components other than hydroperoxides that are extracted along with the lipids and have a response in the test [34].

The estimated amounts of ketodienes in the direct analysis were significantly lower compared to the FAME analysis. In addition, the levels of ketodienes in the FAME analysis were similar to those found in previous studies, but higher than expected [19], as also observed in the model lipid system. While the amounts of ketodienes found by the TAG analysis at the end of the induction periods were respectively 1.0 and 0.82 mmol/kg in HLSO and HOSO, these figures were found to be 5.1 and 2.58 mmol/kg, respectively, in the FAME analysis. The results obtained in both oils suggested that approximately 5 molar% of monohydroperoxydienes on average were transformed into ketodienes in the derivatization step of the FAME analysis. Therefore, the amounts of ketodienes found by the TAG analysis were more reliable and realistic than those determined in the FAME analysis.

The compounds analyzed as hydroxy- in the TAG analysis were detected at concentrations that were below the LOQ in the fresh samples and the samples with low oxidation (Tables 2-3). Substantial formation was not observed until the PV was higher than 90 meq/kg oil in the HLSO and 80 meq/kg oil in the HOSO. The concentrations of hydroxy- compounds were similar between the two methods, although obviously significantly greater levels were obtained with the FAME analysis as oxidation progressed as a result of hydroperoxydiene transformations. Also, the concentrations of hydroxydienes found in the FAME analysis were

similar to those found in previous studies, in which they were expressed on a weight basis [19,21].

Concerning the group determined as other compounds, it was observed an increase that was in parallel to the formation of hydroxy- compounds. When the HOSO became unprotected by the total loss of tocopherols, their concentrations substantially increased along with the polymerization compounds. However, whether these correspond to, for example TAG dimers, is a subject that would require further research. If so and the TAG were linked between each other by a peroxide bridge, this would explain the fact that they disappeared with the NaBH₄ reduction. Consequently, they would contribute to the band of hydroxy- compounds in reduced samples.

The analytical method proposed in the present study can be applied using common high-performance liquid chromatographs. It would be necessary for it to determine the response factors from the standards synthesized in the lab as FAME, as described elsewhere [19,24]. Alternatively, an estimate of the response factors could be obtained from methyl linoleate oxidized at PVs not higher than 100 meq/kg. Determining the PV in such samples, the concentrations of hydroperoxydienes can be estimated from the curve that experimentally relates both parameters ($[\text{LOOH}] \text{ (mmol/kg)} = 0.365(\pm 0.012) \text{ PV (meq/kg)}$, $r = 0.998$), obtained from pure methyl linoleate hydroperoxides in a previous report [24].

4. Conclusions

The HPLC-DAD method proposed enables to determine the concentrations of mono-hydroperoxydienes in edible oils without applying any isolation or derivatization step. It is therefore direct, rapid and sensitive, showing values of 0.03 and 0.06 mmol/kg oil for LOD and LOQ, respectively. The results have shown that it is precise ($\text{CV} \leq 5\%$) and also more accurate when compared to the FAME analysis, which involves chemical transformations.

The method also enables the determination of the minor levels of ketodienes. As intact TAG molecules are measured, the results are more reliable than those provided by the FAME analysis, in which ketodienes are formed in part in the derivatization step. In contrast, the analysis of TAG hydroxydienes is not as simple as that of the FAME analysis. A wide chromatographic band is formed by TAG hydroxydienes and a number of minor compounds with more structural complexity, probably including TAG with more than one oxidized chain and different functional groups. Nevertheless, such a band is indicative of the progress of oxidation and so it could be useful for qualitative purposes.

Lastly, using common HPLC devices, the method proposed can be applied to determine the amounts of the more abundant oxidized lipids in a variety of oils and food lipids, to estimate the oxidation extent, and in kinetic and other oxidation studies.

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532 **Figure captions**

533 **Fig. 1.** HPLC chromatograms of trilinolein oxidized at 40 °C for 30 h. Intact sample recorded
534 at 234 nm (**A**) and 268 nm (**a**). Sample derivatized to FAME recorded at 234 nm (**B**) and 268
535 nm (**b**).

536 **Fig. 2.** HPLC chromatograms of high-linoleic sunflower oil oxidized at 40 °C for 21 days.
537 Intact sample recorded at 234 nm (**A**) and 268 nm (**B**). Sample reduced with NaBH₄, recorded
538 at 234 nm (**B**).

Table 1 Analyses of trilinolein oxidized at 40 °C in the dark.

Time (h)	Pol (%)	K ₂₃₂	TAG analysis				FAME analysis			
			Hydroperoxy- (mmol/kg)	Keto- (mmol/kg)	Hydroxy-* (mmol/kg)	Total (mmol/kg)	Hydroperoxy- (mmol/kg)	Keto- (mmol/kg)	Hydroxy- (mmol/kg)	Total (mmol/kg)
0	0.1	3.39	0.97 ± 0.02a	0.065 ± 0.004b	ND	1.04 ± 0.05a	0.90 ± 0.05a	0.15 ± 0.02a	< 0.16	1.05 ± 0.07a
5	0.1	3.40	4.13 ± 0.13a	0.068 ± 0.004b	< 0.75	4.20 ± 0.14a	4.01 ± 0.13a	0.47 ± 0.02a	< 0.16	4.48 ± 0.16a
10	0.1	3.96	8.08 ± 0.03a	0.064 ± 0.004b	0.89 ± 0.06	9.04 ± 0.09a	7.53 ± 0.26b	0.94 ± 0.03a	< 0.16	8.47 ± 0.30b
15	0.1	5.78	15.28 ± 0.20a	0.063 ± 0.004b	1.47 ± 0.01a	16.81 ± 0.21a	13.82 ± 0.43b	1.71 ± 0.12a	0.16 ± 0.03b	15.69 ± 0.57b
20	0.1	9.52	28.13 ± 0.79a	0.074 ± 0.002b	3.12 ± 0.13a	31.33 ± 0.93a	25.56 ± 0.76b	3.29 ± 0.13a	0.34 ± 0.03b	29.18 ± 0.92b
25	0.2	15.87	49.53 ± 0.11a	0.085 ± 0.002b	5.70 ± 0.10a	55.31 ± 0.02a	47.39 ± 0.71b	5.28 ± 0.43a	0.74 ± 0.00b	53.41 ± 1.14b
30	0.5	25.49	79.61 ± 1.61a	0.119 ± 0.004b	11.41 ± 0.30a	91.14 ± 1.92a	74.20 ± 1.93b	9.24 ± 0.31a	1.48 ± 0.06b	86.39 ± 2.82b

Pol, polymers; K₂₃₂, specific extinction at 232 nm. *Group of compounds formed by simple hydroxydienes and other TAG with similar or higher polarity. ND, not detected. Different letters in the concentrations of each group of compounds for a given sample denote significant differences between the TAG and FAME analyses (p < 0.05).

Table 2 Analyses of high linoleic sunflower oil oxidized at 40 °C in the dark.

Time (days)	Toc (ppm)	Pol (%)	PV (meq/kg)	K ₂₃₂	TAG analysis				FAME analysis		
					Hydroperoxy- (mmol/kg)	Keto- (mmol/kg)	Hydroxy- [*] (mmol/kg)	Others (mmol/kg)	Hydroperoxy- (mmol/kg)	Keto- (mmol/kg)	Hydroxy- (mmol/kg)
0	614	0.6	1.7	2.8	< 0.06	0.381 ± 0.007a	< 0.75	ND	0.24 ± 0.02	0.34 ± 0.02b	1.15 ± 0.07
7	611	0.6	30.7	6.3	11.55 ± 0.27a	0.408 ± 0.001b	< 0.75	ND	9.89 ± 0.06b	1.05 ± 0.03a	1.48 ± 0.08
17	432	0.7	91.7	13.5	39.63 ± 0.33a	0.536 ± 0.003b	0.77 ± 0.04b	< 0.75	33.85 ± 0.48b	2.75 ± 0.15a	1.60 ± 0.12a
21	349	0.8	125	17.3	44.88 ± 1.57a	0.582 ± 0.001b	0.96 ± 0.06b	1.00 ± 0.01	47.28 ± 1.62a	3.41 ± 0.16a	1.81 ± 0.05a
31	93	0.9	188	24.0	68.62 ± 2.09a	0.928 ± 0.035b	1.40 ± 0.03b	2.17 ± 0.09	65.00 ± 0.51b	4.92 ± 0.12a	2.12 ± 0.04a
35	25	1.0	216	41.1	77.58 ± 1.14a	1.002 ± 0.105b	2.24 ± 0.07a	2.59 ± 0.13	77.85 ± 1.93a	5.07 ± 0.45a	2.28 ± 0.07a

Toc, tocopherol; Pol, polymers; PV, peroxide value; K₂₃₂, specific extinction at 232 nm. ^{*}Group of compounds located in the hydroxydiene region of the chromatograms, probably formed by TAG with one or more acyl chains holding a hydroxy- function and by TAG with more than one acyl chain holding the hydroperoxy- function. ND, not detected. Different letters in the concentrations of each group of compounds for a given sample denote significant differences between the TAG and FAME analyses (p < 0.05).

Table 3 Analyses of high oleic sunflower oil oxidized at 40 °C in the dark.

Time (days)	Toc (ppm)	Pol (%)	PV (meq/kg)	K ₂₃₂	TAG analysis				FAME analysis		
					Hydroperoxy- (mmol/kg)	Keto- (mmol/kg)	Hydroxy- [*] (mmol/kg)	Others (mmol/kg)	Hydroperoxy- (mmol/kg)	Keto- (mmol/kg)	Hydroxy- (mmol/kg)
0	630	0.5	2.4	2.3	2.53 ± 0.05a	0.328 ± 0.002b	< 0.75	ND	2.55 ± 0.01a	0.41 ± 0.00a	0.24 ± 0.03
10	566	0.5	17.0	3.7	6.85 ± 0.25a	0.376 ± 0.004b	< 0.75	ND	6.12 ± 0.05b	0.50 ± 0.02a	0.36 ± 0.02
21	483	0.6	30.9	5.5	11.43 ± 0.13a	0.430 ± 0.001b	< 0.75	< 0.75	11.42 ± 0.94a	0.83 ± 0.04a	0.49 ± 0.00
36	356	0.6	57.8	8.0	17.65 ± 0.41a	0.546 ± 0.015b	< 0.75	0.75 ± 0.03	17.84 ± 0.14a	1.43 ± 0.09a	0.74 ± 0.03
55	158	0.7	83.9	10.4	25.97 ± 0.24a	0.728 ± 0.035b	1.12 ± 0.14a	1.87 ± 0.01	25.79 ± 0.99a	1.88 ± 0.05a	1.10 ± 0.02a
75	0	0.8	115	14.2	31.88 ± 1.59a	0.825 ± 0.042b	1.19 ± 0.06b	3.12 ± 0.25	31.77 ± 1.15a	2.58 ± 0.02a	1.59 ± 0.01a
82	0	1.4	157	21.7	34.62 ± 0.28b	0.881 ± 0.008b	1.26 ± 0.03b	6.13 ± 0.39	39.80 ± 0.53a	3.24 ± 0.06a	2.24 ± 0.02a

For abbreviations see Table 2. Different letters in the concentrations of each group of compounds for a given sample denote significant differences between the TAG and FAME analyses ($p < 0.05$).

Figure 1

Figure 2