Formation of oxidation products in edible vegetable oils analyzed as FAME derivatives by HPLC-UV-ELSD

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Abstract

Formation of lipid oxidation products was evaluated in dietary vegetable oils by using a novel analytical approach that consisted of derivatization of TAG into FAME and HPLC analysis with two detectors in series, UV and evaporative light scattering detector (ELSD). Three sunflower oils with different contents of oleic and linoleic acids, i.e. high-linoleic (HLSO), high-oleic (HOSO) and high-stearic high-oleic (HSHOSO), and two oils containing linolenic acid, soybean (SbO) and rapeseed (RO) oils, were heated at 40ºC and analyzed up to the exhaustion of total tocopherols. Results showed that oxidation products of linoleate were predominant in all cases, whereas no significant formation of oleate oxidation products was observed in the five oils in the presence of substantial contents of the tocopherols naturally occurring. Formation of oleate hydroperoxides and monoepoxystearates derived from oleic acid was only detected when tocopherols were exhausted in the monounsaturated oils, *i.e.*, HOSO, HSHOSO and RO. The analysis of the main oxidation products of linoleate by the method applied proved to be a good analytical approach to evaluate the global oxidation extent of oils containing oleic and linoleic acids as the only oxidizable substrates. The method used enabled the quantitative determination of the simple hydroperoxydienes of linoleate and linolenate as a whole. Results suggested occurrence of hydroperoxy compounds other than those determined by the method in the oils containing linolenic acid, showing the low stability of simple hydroperoxydienes and their participation in further reactions.

Key-words: Hydroperoxides; hydroperoxydienes; autoxidation; FAME; ELSD

1. Introduction

Autoxidation of food lipids generates a variety of products that impair the food flavor and may even be involved in chronic and degenerative diseases (Kanazawa, Sawa, Akaike, & Maeda, 2002). Unfortunately, the actual contribution of oxidized lipids coming from diet to diseases is at present unknown because their contents have not yet been defined and therefore it is not known whether they are sufficient to cause detrimental nutritional effects (Dobarganes and Márquez-Ruiz, 2003).

The qualitative analysis of the main oxidation products of the common dietary unsaturated fatty acids has been established using model lipid systems (Frankel, 2005). On the other hand, studies dealing with quantitative analysis in foods are scarce. The principal limitations of the quantitative determination are due to a large number of compounds formed from numerous triacylglycerols (TAG), their low contents as individual species and their relatively low stability.

In a recent report, we have proposed a method of quantitative analysis for the main oxidation products of linoleic acid, *i.e.* hydroperoxy-, keto- and hydroxy- dienes, in oils by using a derivatization step to transform the TAG molecules into fatty acid methyl esters (FAME) and normal-phase HPLC-UV (Morales, Marmesat, Dobarganes, Márquez-Ruiz, & Velasco, 2012a). With the transformation of TAG into FAME the number of analytes is drastically reduced and a considerable concentration effect arises as a result. The effect of the derivatization reaction to the analytes was thoroughly studied. Results showed that losses of hydroperoxydienes lower than 10 wt% occurred at the reaction conditions applied and that formation of keto- and hydroxy- dienes inevitably took place. 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.

While the conjugated diene structure of the oxidation products of linoleic acid can be readily detected by absorption of UV light, those of oleic acid are not detected in the HPLC-UV analysis due to the absence of conjugated double bounds. The incorporation in series of an evaporative light scattering detector (ELSD) to the method was evaluated to examine its possibilities in the direct analysis of model lipids such as oxidized samples of FAME (Morales et al., 2012b). Results showed that the ELSD enables the global determination of the hydroperoxides of oleic and linoleic acid methyl esters. Connected in series with the UV detector, it makes it possible to determine both groups by difference. The ELSD also enabled the determination of the *cis* and *trans* forms of methyl 9,10-epoxystearate, which was indicative of methyl oleate oxidation.

The objective of the present study was to explore the possibilities of the HPLC-UV-ELSD method to evaluate formation of oxidation products in real samples of edible vegetable oils containing different contents of oleic, linoleic and linolenic acids, namely, high-linoleic sunflower oil (HLSO), high-oleic sunflower oil (HOSO), high-stearic high-oleic sunflower oil (HSHOSO), soybean oil (SbO) and rapeseed oil (RO). For the first time in this series of analytical studies oxidation products of linolenic acid are dealt and the ELSD is applied to the analysis of oils, being a much more complex analytical matrix than the purified FAME previously studied (Morales et al., 2012b). A follow-up of oxidation at 40ºC in the five oils was performed to study formation of compounds throughout the induction period, whose end is marked by the total exhaustion of the natural antioxidants, *i.e.* tocopherols. 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 performed as a rapid control measure to discard

samples within advanced oxidation (Márquez-Ruiz, Martín-Polvillo, & Dobarganes, 1996).

2. Materials and Methods

2.1 Chemicals

Methyl linolenate 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), high-oleic sunflower oil (HOSO), high-stearic high-oleic sunflower oil (HSHOSO), soybean oil (SbO) and rapeseed oil (RO) were supplied by the group SOS Cuetara S.A. (Andújar, Jaén, Spain). The five 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, 4.1% C16:0, 4.6% C18:0, 77.6% C18:1, 11.7% C18:2 and 2.0% others for HOSO, 4.4% C16:0, 15.1% C18:0, 71.8% C18:1, 5.0% C18:2 and 3.7% others for HSHOSO, 11.3% C16:0, 0.1% C16:1, 3.5% C18:0, 22.5% C18:1, 54.6% C18:2, 6.1% C18:3 and 1.9% others for SbO and 4.6% C16:0, 0.2% C16:1, 1.5% C18:0, 62.7% C18:1, 19.4% C18:2, 8.6% C18:3 and 3.0% others for RO.

2.3 Methyl linolenate samples

Three samples of 1 g of methyl linolenate containing different concentrations of α-tocopherol were prepared, absence (control), 500 ppm and 5 wt%. The antioxidant was

added directly for the more concentrated sample or in hexane. The hexane was evaporated with nitrogen and stirring under inert headspace with nitrogen was applied in both cases.

2.4 Oxidation conditions

Aliquots of 20 g of the oils were placed in Petri dishes (14.5 cm i.d.) open to the air and oxidized at 40 ºC in the dark in an oven with air circulation. A high surface-to-oil-volume ratio (7 cm^{-1}) guaranteed high oxygen availability and therefore non-limiting oxygen conditions. Three independent samples were used in each oil. An aliquot of each sample was taken in each sampling and the three aliquots were mixed and kept at -35 ºC until analyses. The samples were oxidized until the total depletion of tocopherols.

Aliquots of 1 g of the methyl linolenate samples were oxidized in beakers at ambient temperature and in the dark using a surface-to-oil-volume ratio of 5 cm^{-1}. The control sample, i.e. without α-tocopherol, was oxidized until the notorious occurrence of complex chromatographic bands in the zone of hydroxydienes. The samples containing α-tocopherol were oxidized until the simple hydroperoxydienes reached levels similar to those of the control.

2.5 Derivatization of oils

Oxidized oil samples were transformed into FAME by transmethylation with NaOMe at room temperature, according to a previous report (Morales et al., 2012a). A 300-mg sample was accurately weighed into a screw-capped centrifuge tube (13 cm x 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 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 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, and then the solvent was evaporated in a rotary evaporator at 40 ºC and taken to dryness with a stream of nitrogen. Solutions of FAME samples dissolved in hexane at a concentration of 1-50 mg/mL were analyzed by HPLC.

2.6 HPLC-UV-ELSD analyses

The derivatized oil samples or oxidized methyl linolenate were analyzed in a Waters 600 HPLC chromatograph. The chromatograph was equipped with a Waters 600 pump, a Rheodyne injector valve (20-μL sample loop), a silica HPLC column (LiChrospher® Si 60, 250 mm x 4 mm i.d., 5 μm particle size) (Merck, Darmstadt, Germany), an HP 1050 Series variable wavelength UV detector (8 mm path length) (Agilent Technologies Inc., Palo Alto, CA), a Waters 2424 Evaporative Light Scattering detector (ELSD), an Agilent 35900E A/D Converter (Agilent Technologies Inc.) and a Waters 600 controller. The separation of analytes was performed in isocratic elution using 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 recorded by the UV detector at 234 nm, while ketodienes were at 268 nm. The ELSD conditions applied were 35ºC for nebulizer temperature, 40ºC for tube temperature and 25 psi for gas flow. The gas used for nebulization was nitrogen (purity 99.99%). The gain was set at 50. Quantitative data by the UV detector were obtained by applying response factors reported elsewhere (Morales, Dobarganes, Márquez-Ruiz, & Velasco, 2010), while external calibration was applied for quantitative determination of compounds by the ELSD (Morales et al., 2012b).

145 *2.7 Analysis of polymers*

146 Analysis of TAG polymers in oils was performed by HPSEC with refractive index detector
147 according to IUPAC standard method 2.508 (IUPAC, 1992).

148 *2.8 Peroxide value*

149 Peroxide value was determined by the iodometric assay according to IUPAC standard
150 method 2.501 (IUPAC, 1992). Oil samples of 500 mg and 1 x 10^{-2} M $Na_2S_2O_3$ solution
151 were used.

152 *2.9 Ultraviolet light absorption at 232 nm (K_{232})*

153 Specific extinction at 232 nm was determined in cyclohexane as a measure of total
154 conjugated dienes according to AOCS standard method Ch 5-91 (AOCS, 1998).

155 *2.10 Analysis of tocopherols*

156 Analysis of tocopherols was carried out by normal-phase HPLC with fluorescence
157 detection following IUPAC standard method 2.411 (IUPAC, 1992).

158 *2.11 Statistical analysis*

159 The oil samples were analyzed in triplicate to determine lipid oxidation products by
160 HPLC and results were expressed as mean values followed by the standard deviation.
161 For the other analytical determinations the oil samples were analyzed in duplicate and
162 results were expressed as mean values. Comparisons of means were performed by the
163 Student's *t* test using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA). Significance
164 was defined at $p < 0.05$.

165

3. Results and Discussion

Oils containing oleic and linoleic acids as oxidizable substrates

Three sunflower oils, HLSO, HOSO and HSHOSO, were chosen in this study because they contained oleic and linoleic acids as the only oxidizable fatty acids at significant concentrations and because of their different contents in these two substrates. The oils were oxidized at 40ºC and analyzed along their induction periods (IPs), whose end is determined by the exhaustion of antioxidants, *i.e.* tocopherol, and a sharp increase in the content of polymerization compounds (Márquez-Ruiz and Dobarganes, 2005). The samples analyzed at the end of the assay presented amounts of α-tocopherol equal or close to zero and concentrations of polymers that increased approximately in 1% with respect to the initial samples.

The three oils presented very low peroxide values (PVs), which were typical of fresh refined oils (< 10 meq/kg) (**Tables 1-3**). As observed in a previous report (Morales et al., 2012a), minor amounts of hydroperoxy-, keto- and hydroxy- dienes were recorded by UV in the fresh oils, showing slight incipient oxidation. As expected, the ELSD did not enable to detect the low amounts of compounds found by UV in the initial samples, due to a much lower sensitivity (Morales et al., 2012b).

The quantitative analysis of hydroperoxides by light scattering detection was possible in the oxidized oils. As it will be dealt later, significant differences between the concentrations of hydroperoxides found by UV and light scattering detection were not found in the oils along the IPs (**Tables 1-3**). Nevertheless, the concentrations of hydroperoxides determined by the ELSD in the two monounsaturated oils, i.e. HOSO and HSHOSO, were significantly larger than those by UV in samples of advanced oxidation, suggesting the formation of oleic hydroperoxides (Morales et al, 2012b). The

ELSD also enabled the evaluation of ketodienes from concentrations larger than 0.4 mg/g oil and the results obtained (not shown) were similar to those determined by UV. Regarding hydroxydienes, the amounts found by UV were below the limit of quantification in the ELSD (0.9 mg/g oil) (Morales et al., 2012b). Furthermore, the chromatograms obtained by the ELSD in all samples of the three oils showed peaks that interfered with the hydroxydienes. These peaks can be attributed to compounds that form the unsaponifiable fraction of the oils and make it not possible to determine hydroxydienes by light scattering detection.

In contrast to what has been found in purified lipids in the absence of antioxidants, *i.e.*, FAME obtained from high oleic sunflower oil (Morales et al., 2012b), formation of epoxides was not observed by the ELSD in the two monounsaturated oils, i.e. HOSO and HSHOSO, during the IP. Occurrence of monoepoxystearates was however detected in samples of both HOSO and HSHOSO that had already lost the tocopherol completely (**Figure 1).** As a result, formation of epoxystearates at the conditions assayed appeared to be inhibited in the presence of the tocopherol naturally occurring in the oils. This is consistent with the results obtained by Rovellini and Cortesi (2004), who did not observe the occurrence of epoxy compounds in a number of extra virgin olive oils during storage.

In agreement with a previous study (Morales et al., 2012a), the concentration of hydroperoxydienes showed excellent linear correlation with the PV during the IPs of the oils (data not shown). Once tocopherol was completely depleted, the PV increased more rapidly than the content of hydroperoxydienes (**Figure 2**), indicating the formation of compounds with hydroperoxy functions other than those analyzed by HPLC, mainly polymerization products and compounds with more than one oxygen-containing functional group. The lower slopes for the two monounsaturated oils, i.e. HOSO and

HSHOSO, during the IP suggest the presence of oleic hydroperoxides (Morales et al., 2012a). The differences between the three oils were larger as the two monounsaturated oils got closer to the end of the IPs, *i.e.*, when the content of tocopherol was low. On the contrary, no substantial differences were found in samples with relatively low oxidation (PV < 50 meq/kg) (**Figure 2**). As a conclusion, the results seem to indicate that the oxidation of oleic acid in the monounsaturated oils was low in samples with low oxidation levels, when tocopherol was present in substantial contents, and significant from low concentrations of tocopherol. These results are coherent with those reported elsewhere (Morales et al., 2012c), which showed that for a given global oxidation extent the relative oxidation of oleic acid in FAME obtained from high oleic sunflower oil was much less in the presence of α-tocopherol. It was suggested that the hydrogen donor capacity of methyl oleate was reduced in the presence of α-tocopherol because the antioxidant competes much more efficiently for peroxyl radicals (Kamal-Eldin et al., 2003).

Even though the relationship between the concentration of hydroperoxydienes and the PV suggested the formation of oleic acid hydroperoxides at significant amounts in the two monounsaturated oils at the end of the IP, no significant differences were found between the contents of hydroperoxides determined by UV and light scattering detection. The ELSD only showed significantly higher values in samples that had lost tocopherols completely, *i.e.*, in advanced oxidation extents (**Tables 2-3**). The absence of compounds that can be used as markers for the oxidation of oleic acid, like monoepoxystearates, support the low oxidation of such a substrate during the IPs of HOSO and HSHOSO. Therefore, it can be said that, at the analytical conditions applied, the ELSD does not enable the determination of the low concentrations of oleic acid hydroperoxides that are formed in monounsaturated oils during the IPs. Apart from the

low sensitivity of the ELSD, this can be attributed to the low global oxidation levels of monounsaturated oils reached at the end of the IP compared to polyunsaturated oils (Martín-Polvillo, Márquez-Ruiz, & Dobarganes, 2004; Morales et al., 2010).

Oils containing oleic, linoleic and linolenic acids as oxidizable substrates

The presence of α-linolenic acid at significant contents in vegetable oils such as those dealt in the present study, SbO and RO, and its larger susceptibility to oxidation than linoleic acid make it of interest to study the possibilities of the HPLC method to evaluate their oxidation products. Likewise, the main oxidation products of linolenic acid, i.e. hydroperoxides, have two conjugated double bonds and so the same chromophore which is detected by UV in the method. Nevertheless, due to the high reactivity of the hydroperoxides or peroxide precursors, bifunctional compounds, *i.e.* with more than one oxygen-containing functional group, are formed in linolenic acid, increasing considerably the degree of analytical complexity (Neff, Frankel, & Weisleder, 1981; Frankel, 2005). A few of these compounds like monocyclic endoperoxides keep the conjugated diene structure, whereas others like bicyclic endoperoxides lose it.

To explore the possibilities of the HPLC method in the evaluation of oxidation products of linolenic acid in oils it was first necessary to study model lipids. In this regard, samples of methyl linolenate were oxidized and analyzed directly by the method. The analysis showed two major chromatographic peaks in the zone of hydroperoxides (**Figure 3A**). These two peaks may be assigned to the so called external hydroperoxides, 9- and 16-hydroperoxide, since the internal 12- and 13-hydroperoxides form at lower amounts due to their participation in intramolecular 1,3-cyclization reactions to give monocyclic endoperoxides (Frankel, 2005). These hydroperoxides have two conjugated double bonds with *cis,trans* or *trans,cis* configuration and a third

intact double bound. Unlike linoleic acid, the *cis,trans*-hydroperoxides of linolenate are not readily isomerized to the *trans,trans* form, apparently because cyclization is much more favored (Frankel, 2005). The chromatogram also showed wide bands at longer retention times, in the zone of hydroxydienes, indicating the presence of numerous compounds of high polarity, probably due to more than one oxygen-containing function such as those of monocyclic endoperoxides or hydroperoxy epidioxides. Regarding detection of compounds at 268 nm, very minor peaks in the zone of ketodienes were only detected in a wide range of oxidation levels (not shown), indicating that secondary structures similar to those of linoleic acid were not significant. This fact can be attributed to the much lower stability of methyl linolenate hydroperoxides, which readily participate in reactions of intramolecular cyclization, originating endoperoxides, and polymerization (Frankel, 2005). Nevertheless, formation of ketones other than ketodienes should not be ruled out, being possible the formation of carbonyl groups in molecules with more than one functional group. The ELSD only recorded the signals of hydroperoxides (not shown), which were similar to those displayed by UV. Due to a lower sensitivity, the bands corresponding to those at retention times longer than hydroperoxydienes were not observed by light scattering detection.

In order to confirm that the bands observed at long retention times (**Figure 3A**) can be assigned to hydroperoxy epidioxides, the formation of cyclization compounds was inhibited by addition of a powerful hydrogen donor like α-tocopherol at elevated concentration (5 % p/p) (Frankel, 2005). At these conditions, the internal peroxides are readily transformed into their corresponding hydroperoxides and these in turn are stabilized. The results obtained confirmed the starting hypothesis (**Figure 3B**). Only peaks in the zone of hydroperoxides were recorded, although these unexpectedly eluted in two peaks instead of four, probably due to coelution of compounds. Unlike the

290 control sample, *i.e.*, in the absence of antioxidants, both peaks presented similar areas,
291 indicating that the four hydroperoxides eluted two by two, given that under these
292 conditions they form at similar amounts (Frankel, 2005).

293 The concentration of α-tocopherol tested was much larger than that commonly
294 found in edible vegetable oils (<0.1 % p/p). For a more realistic situation, samples of
295 methyl linolenate containing 500 ppm α-tocopherol were also studied. The
296 chromatograms obtained were quite similar to those for the control (**Figure 3C**). The
297 intramolecular cyclization was not completely inhibited at these conditions. Likewise,
298 secondary structures similar to those of methyl linoleate, i.e. ketodienes, were not
299 detected at 268 nm in a significant way (not shown).

300 The analysis of blends of oxidized samples of methyl linolenate (**Figure 3A**) and
301 linoleate (**Figure 3D**) showed overlapping between both groups of hydroperoxides
302 (**Figure 3E**), indicating that it is not possible to differentiate them. However, the
303 slightly higher polarity of linolenate hydroperoxides gave rise to a peak that eluted
304 immediately after the linoleate hydroperoxides. This could be used as a marker for
305 linolenic acid oxidation. In addition, there was expected overlapping between the bands
306 attributed to monocyclic endoperoxides and the hydroxydienes of methyl linoleate.

307 The results obtained show that the HPLC method enables the evaluation of simple
308 hydroperoxides of linoleic and linolenic acids as a whole. Given that both groups of
309 compounds have practically the same chromophore, an only response factor for
310 hydroperoxydienes, *i.e.*, that reported elsewhere (Morales et al., 2010), was assumed for
311 quantitative purposes in oils.

312 Results for the oxidation of soybean oil (SbO) and rapeseed oil (RO) are presented in
313 **Tables 4-5**. Both oils exhibited quality parameters that were typical of fresh refined

314 oils. Like the sunflower oils, the RO and SbO showed by light scattering detection
315 chromatographic peaks that overlapped with hydroxydienes. As opposed to the rest of
316 the oils tested in the present study, the chromatograms of SbO by ELSD also showed
317 peaks that overlapped with the ketodienes (**Figure 4**). Even though the SbO presented
318 quality parameters appropriate for well refined vegetable oils, with PV equal to zero and
319 a value for acidity of 0.04% on oleic acid, the HPLC analysis of the initial sample
320 remarkably exhibited the presence of hydroxydienes at relatively high concentrations
321 (**Figure 4**). Because the content of hydroperoxydienes was low, as it was also indicated
322 by the PV, the presence of these compounds may be attributed to modifications of
323 hydroperoxides in the crude oil during refining. Nevertheless, it is remarkable the fact
324 that apart from hydroxydienes other secondary oxidation compounds like ketodienes
325 were not observed. In this regard, previous studies have shown that hydroperoxydienes
326 are partially transformed into keto- and hydroxy- dienes at basic conditions (Morales et
327 al., 2012a).

328 The IPs determined by the total loss of tocopherols were 53 and 40 days for SbO and
329 RO, respectively (**Tables 4-5**). The contents of hydroperoxydienes accumulated during
330 the IP and the PV were larger for SbO, supporting the fact that polyunsaturated oils
331 accumulate more contents of oxidation products during the IP than monounsaturated
332 oils (Martín-Polvillo et al., 2004).

333 Apart from the hydroperoxides of linoleic acid, the chromatograms also showed
334 formation of linolenic hydroperoxides (**Figure 5**). Compared to RO, the presence of
335 linolenate hydroperoxides was not so evident in SbO, probably due to its larger content
336 of linoleic acid that resulted in higher relative proportions of linoleate hydroperoxides.
337 Similarly, the bands at longer retention times that were observed in oxidized methyl
338 linolenate (**Figure 3C**) were hardly noticeable in both oils because of the relatively high

339 contents of linoleate hydroxydienes. In the zone of hydroxydienes, the chromatograms
340 of the ELSD (not shown) were not different from those of the initial samples, showing
341 peaks that may well be attributed to compounds of the unsaponifiable fraction.

342 Like in the sunflower oils, significant differences between the contents of
343 hydroperoxides determined by UV and light scattering detection were not found in the
344 two oils during the IP. Likewise, the ELSD only showed higher hydroperoxide
345 concentrations after the total depletion of tocopherols in the monounsaturated oil, *i.e.*,
346 RO. In agreement with the results found in the sunflower oils, these results suggest that
347 the oxidation of oleic acid was low in both poly- and mono- unsaturated oils during the
348 IP, *i.e.* in the presence of tocopherols.

349 Consistently with the sunflower oils, the relationship between the content of
350 hydroperoxydienes and the PV was practically linear in the SbO and RO during the IP
351 and after the exhaustion of tocopherols the PV increased more rapidly (not shown).
352 With the progressive loss of tocopherols the concentration of hydroperoxydienes was
353 significantly lower in the RO for a given PV, suggesting the formation of oleic acid
354 hydroperoxides (Morales et al., 2012a). However, hydroperoxides other than
355 hydroperoxydienes were not observed by the ELSD in the RO until tocopherol was
356 completely depleted.

357 As compared to results reported elsewhere for the relationship between
358 hydroperoxydienes and the PV in samples of pure linoleate hydroperoxides (Morales et
359 al., 2010), both SbO and RO showed lower hydroperoxydiene concentrations for a
360 given PV. This fact was indicative of the occurrence of compounds with hydroperoxy
361 functions other than those detected by either UV or the ELSD in the method, probably
362 due for the latter to its low sensitivity. Both monocyclic and bicyclic endoperoxides
363 contribute to increasing the PV. The low stability of simple linolenate hydroperoxides

364 provided by the double bounds can also result in formation of hydroperoxides with
365 other additional oxygen functions and polymerization products bearing the hydroperoxy
366 group (Frankel, 2005).

367 **4. Conclusions**

368 Oxidation products of linoleate were predominant in all cases, whereas no significant
369 formation of oleate oxidation products was observed in the five oils in the presence of
370 substantial contents of the tocopherols naturally occurring, *i.e.*, at low global oxidation
371 extents. The analysis of the main oxidation products of linoleate by the method applied
372 proved to be a good analytical approach to evaluate the global oxidation extent in oils
373 containing oleic and linoleic acids as the only oxidizable substrates present at
374 significant contents. For monounsaturated oils with low contents of linoleic acid, the
375 lower the oxidation extent, the better will be the analytical approach.

376 The analysis of oils containing linolenic acid suggested the formation of hydroperoxy
377 compounds other than those determined by the method, showing the low stability of
378 simple linolenate hydroperoxides and their participation in further reactions.

379 The HPLC method enables a more specific quality determination of fresh refined oils
380 than the control parameters normally applied in the industry, such as the PV. Detection
381 of secondary oxidation products, which can act as oxidation catalysts, may account for
382 losses of stability unexpectedly found in fresh refined oils.

383

Acknoledgements

This work was funded by “Junta de Andalucía” through project P09-AGR-4622 and by “Ministerio de Ciencia e Innovación” through project AGL 2010-18307.

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435 **Figure captions**

436 **Fig. 1** HPLC-ELSD chromatograms of HOSO oxidized at 40ºC for 82 days (**A**) and
437 HSHOSO for 143 days (**B**).

438 **Fig. 2** Formation of hydroperoxydienes (filled) and loss of tocopherol (hollow) relative
439 to the PV during oxidation at 40ºC in HLSO (circles), HOSO (triangles) and HSHOSO
440 (squares).

441 **Fig. 3** HPLC-UV_{234} chromatograms of samples of methyl linolenate oxidized at
442 ambient temperature in the absence of antioxidants (**A**), containing 5 wt% (**B**) and 500
443 ppm of α-tocopherol (**C**). HPLC-UV_{234} chromatograms of a sample containing methyl
444 linoleate hydroperoxides and methyl 13-hydroxy-(*Z*)-9,(*E*)-11-octadecadienate (**D**) and
445 a sample containing a mixture of samples C and D (**E**).

446 **Fig. 4** HPLC-UV-ELSD chromatograms of the initial sample of SbO.

447 **Fig. 5** HPLC-UV_{234} chromatograms of SbO oxidized at 40ºC for 53 days (**A**) and RO
448 for 40 days (**B**).

Table 1 Autoxidation of high-linoleic sunflower oil (HLSO) at 40 ºC in the dark.

Time (days)	Pol[a] (%)	α-Toc (mg/kg)	PV[b] (meq/kg)	K_{232}[c]	UV			ELSD
					Hydroperoxy-dienes (mg/g)	Keto-dienes (mg/g)	Hydroxy-dienes (mg/g)	Hydroperoxides (mg/g)
0	0.6	614	1.7	2.8	0.08 ± 0.01	0.11 ± 0.01	0.37 ± 0.01	ND[d]
7	0.6	611	30.7	6.3	3.19 ± 0.02 a	0.31 ± 0.01	0.39 ± 0.02	3.20 ± 0.48 a
17	0.7	432	91.7	13.5	10.86 ± 0.14 a	0.81 ± 0.06	0.48 ± 0.05	11.07 ± 0.28 a
21	0.8	349	125	17.3	13.77 ± 0.79 a	1.02 ± 0.04	0.58 ± 0.00	14.10 ± 1.00 a
31	0.9	93	188	24.0	22.45 ± 0.80 a	1.47 ± 0.04	0.66 ± 0.00	23.92 ± 1.20 a
35	1.0	25	216	41.1	24.12 ± 1.27 a	1.57 ± 0.04	0.74 ± 0.01	25.25 ± 1.59 a

[a]polymers, [b]peroxide value, [c]specific extinction at 232 nm, [d]not detected. Different letters for hydroperoxy-dienes and hydroperoxides for a given sample show significant differences ($p < 0.05$).

Table 2 Autoxidation of high-oleic sunflower oil (HOSO) at 40 ºC in the dark.

Time (days)	Pol (%)	α-Toc (mg/kg)	PV (meq/kg)	K_{232}	UV			ELSD
					Hydroperoxy-dienes (mg/g)	Keto-dienes (mg/g)	Hydroxy-dienes (mg/g)	Hydroperoxides (mg/g)
0	0.5	630	2.4	2.3	0.16 ± 0.01	0.13 ± 0.00	0.14 ± 0.02	ND
10	0.5	566	17.0	3.7	1.33 ± 0.01 a	0.15 ± 0.01	0.12 ± 0.01	1.35 ± 0.12 a
21	0.6	483	30.9	5.5	3.06 ± 0.26 a	0.25 ± 0.01	0.16 ± 0.02	2.95 ± 0.46 a
36	0.6	356	57.8	8.0	5.31 ± 0.06 a	0.43 ± 0.03	0.23 ± 0.01	5.71 ± 0.35 a
55	0.7	158	83.9	10.4	7.75 ± 0.31 a	0.57 ± 0.02	0.36 ± 0.02	7.55 ± 0.64 a
72	0.8	10	110	12.6	9.42 ± 0.34 a	0.77 ± 0.01	0.46 ± 0.02	9.45 ± 0.62 a
82	1.4	0	157	21.7	12.21 ± 0.11 a	0.98 ± 0.06	0.67 ± 0.05	13.27 ± 0.77 b

For abbreviations see Table 1. Different letters for hydroperoxy-dienes and hydroperoxides for a given sample show significant differences ($p < 0.05$).

Table 3 Autoxidation of high-stearic high-oleic sunflower oil (HSHOSO) at 40 ºC in the dark.

Time (days)	Pol (%)	α-Toc (mg/kg)	PV (meq/kg)	K_{232}	UV			ELSD
					Hydroperoxy-dienes (mg/g)	Keto-dienes (mg/g)	Hydroxy-dienes (mg/g)	Hydroperoxides (mg/g)
0	0.4	561	1.6	2.5	0.13 ± 0.01	0.04 ± 0.00	< 0.05	ND
31	0.4	496	18.1	3.7	1.50 ± 0.07 a	0.23 ± 0.01	0.12 ± 0.01	2.00 ± 0.07 b
55	0.7	395	33.1	4.6	2.47 ± 0.10 a	0.44 ± 0.03	0.20 ± 0.01	2.46 ± 0.02 a
80	0.8	237	52.1	5.9	3.97 ± 0.13 a	0.60 ± 0.01	0.28 ± 0.01	4.25 ± 0.34 a
101	0.9	129	63.6	6.7	4.81 ± 0.09 a	0.76 ± 0.08	0.37 ± 0.01	5.17 ± 0.28 a
143	1.5	0	130	14.9	6.09 ± 0.40 a	0.99 ± 0.02	0.59 ± 0.03	8.29 ± 0.30 b

For abbreviations see Table 1. Different letters for hydroperoxy-dienes and hydroperoxides for a given sample show significant differences ($p < 0.05$).

Table 4 Autoxidation of soybean oil (SbO) at 40 ºC in the dark.

Time (days)	Pol (%)	Toc (mg/kg)	PV (meq/kg)	K_{232}	UV			ELSD
					Hydroperoxy-dienes (mg/g)	Keto-dienes (mg/g)	Hydroxy-dienes (mg/g)	Hydroperoxides (mg/g)
0	0.5	915	0	4.5	0.07 ± 0.03	0.07 ± 0.01	1.02 ± 0.02	ND
11	0.7	853	23.7	5.7	1.85 ± 0.09 a	0.17 ± 0.02	1.10 ± 0.04	2.16 ± 0.20 b
21	0.6	667	92.4	11.2	6.99 ± 0.11 a	0.56 ± 0.04	1.19 ± 0.02	8.35 ± 0.27 b
33	0.7	417	160	18.5	13.19 ± 0.26 a	1.04 ± 0.07	1.42 ± 0.06	13.65 ± 0.35 a
40	1.0	260	219	20.1	15.94 ± 0.73 a	1.41 ± 0.07	1.62 ± 0.11	16.65 ± 0.66 a
46	1.3	144	275	27.3	20.15 ± 0.78 a	1.86 ± 0.12	1.88 ± 0.02	21.83 ± 1.69 a
49	1.5	94	335	27.9	24.77 ± 0.23 a	2.21 ± 0.03	2.24 ± 0.05	26.61 ± 0.58 b
53	2.4	0	492	36.2	30.62 ± 1.24 a	2.97 ± 0.03	3.53 ± 0.23	32.50 ± 1.39 a

For abbreviations see Table 1. The initial sample presented 151, 19, 560 and 185 mg/kg of α-, β-, γ- and δ-tocopherol, respectively. Different letters for hydroperoxy-dienes and hydroperoxides for a given sample show significant differences ($p < 0.05$).

Table 5 Autoxidation of rapeseed oil (RO) at 40 ºC in the dark.

Time (days)	Pol (%)	Toc (mg/kg)	PV (meq/kg)	K_{232}	UV			ELS
					Hydroperoxy-dienes (mg/g)	Keto-dienes (mg/g)	Hydroxy-dienes (mg/g)	Hydroperoxides (mg/g)
0	0.3	522	1.4	4.29	0.10 ± 0.03	< 0.02	ND	ND
11	0.5	450	35	6.41	2.46 ± 0.13 a	< 0.02	ND	2.24 ± 0.18 a
18	0.6	312	81	11.41	5.07 ± 0.06 a	0.37 ± 0.05	0.17 ± 0.01	4.77 ± 0.24 a
25	0.6	180	114	12.77	6.41 ± 0.32 a	0.44 ± 0.08	0.24 ± 0.03	6.04 ± 0.45 a
40	1.2	0	203	16.28	10.25 ± 0.03 a	0.78 ± 0.02	0.73 ± 0.03	15.85 ± 0.40 b
46	4.3	0	611	32.56	24.24 ± 0.34 a	2.10 ± 0.02	3.71 ± 0.01	35.19 ± 0.10 b

For abbreviations see Table 1. The initial sample presented 252 and 270 mg/kg of α- and γ-tocopherol, respectively. Different letters for hydroperoxy-dienes and hydroperoxides for a given sample show significant differences ($p < 0.05$).

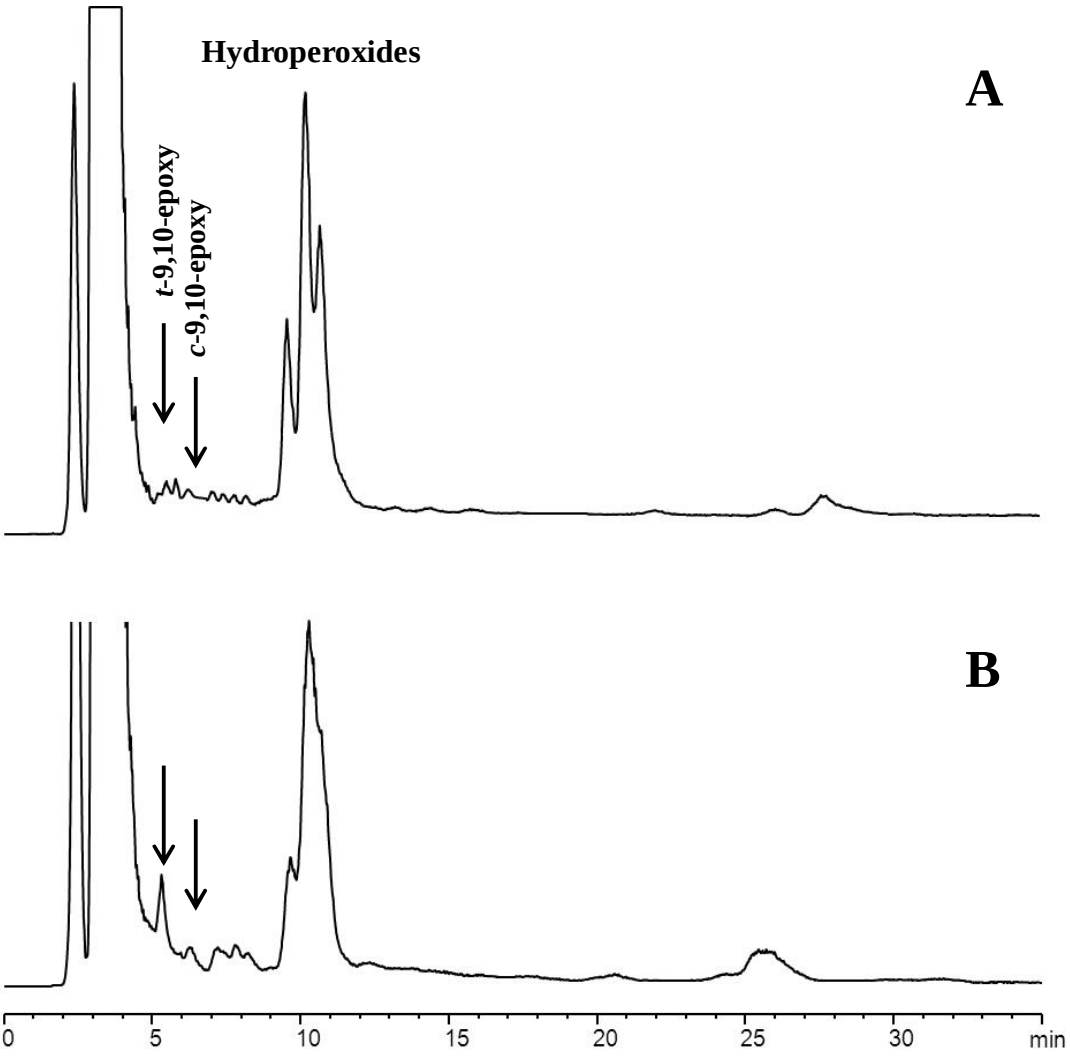

Figure 1

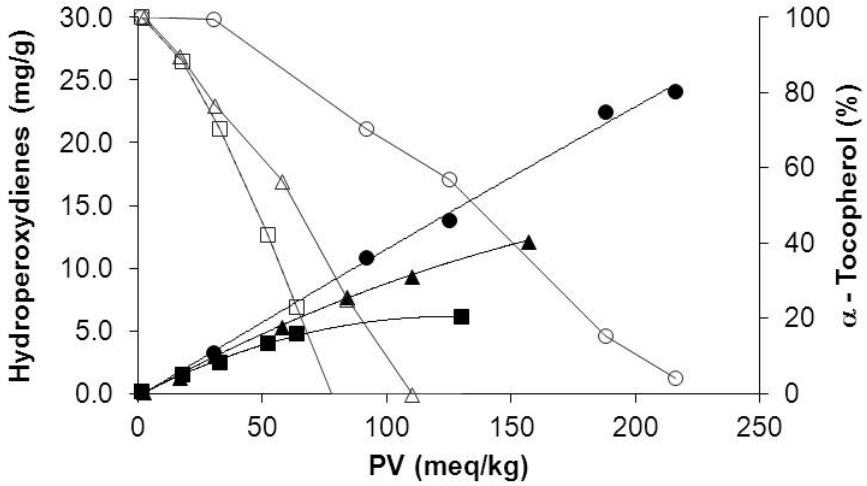
Hydroperoxydienes (mg/g)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0
50
100
150
200
250
PV (meq/kg)
100
80
60
40
20
0
α - Tocopherol (%)

Figure 2

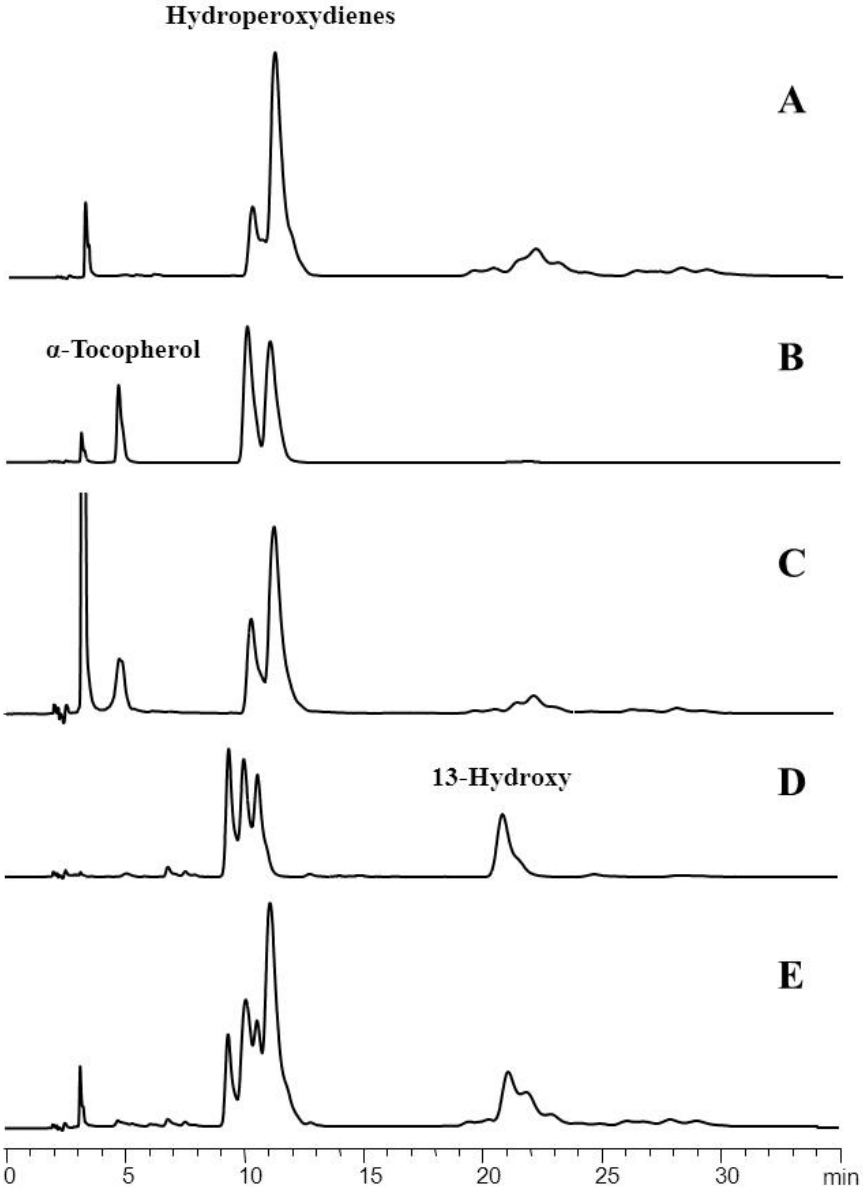
Hydroperoxydienes
A
α-Tocopherol
B
C
13-Hydroxy
D
E
0
5
10
15
20
25
30
min

Figure 3

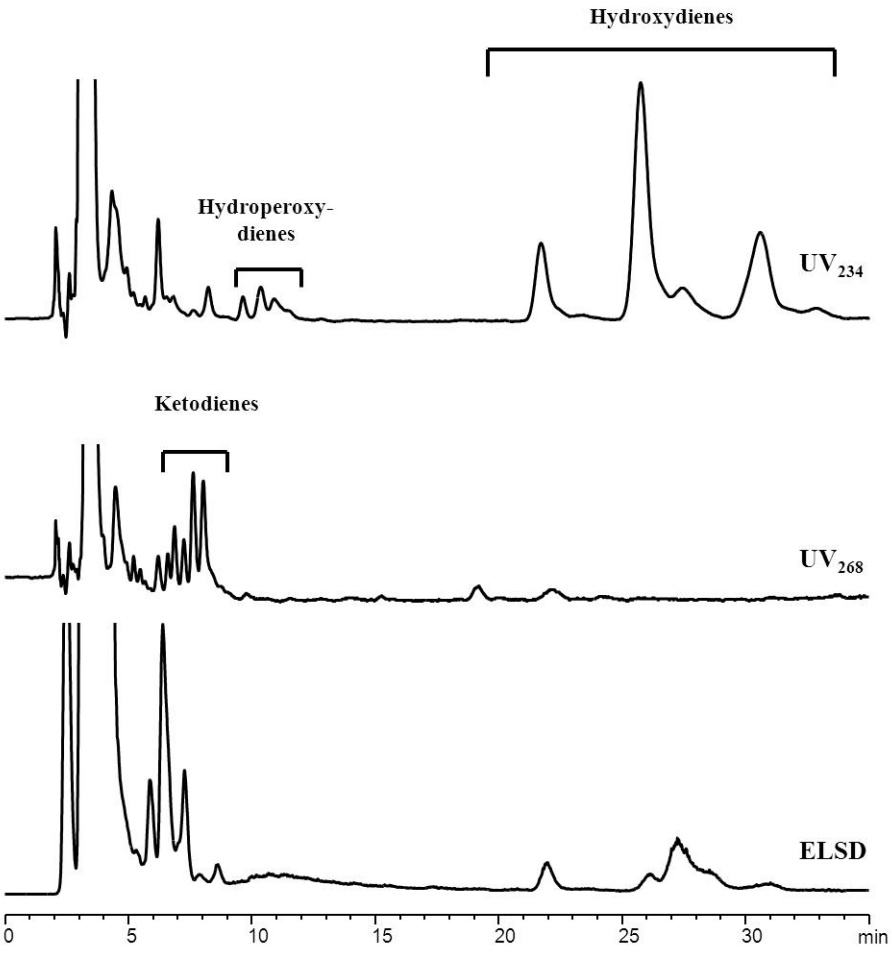
Hydroxydienes
Hydroperoxy-
dienes
UV234
Ketodienes
UV268
ELSD
0
5
10
15
20
25
30
min

Figure 4

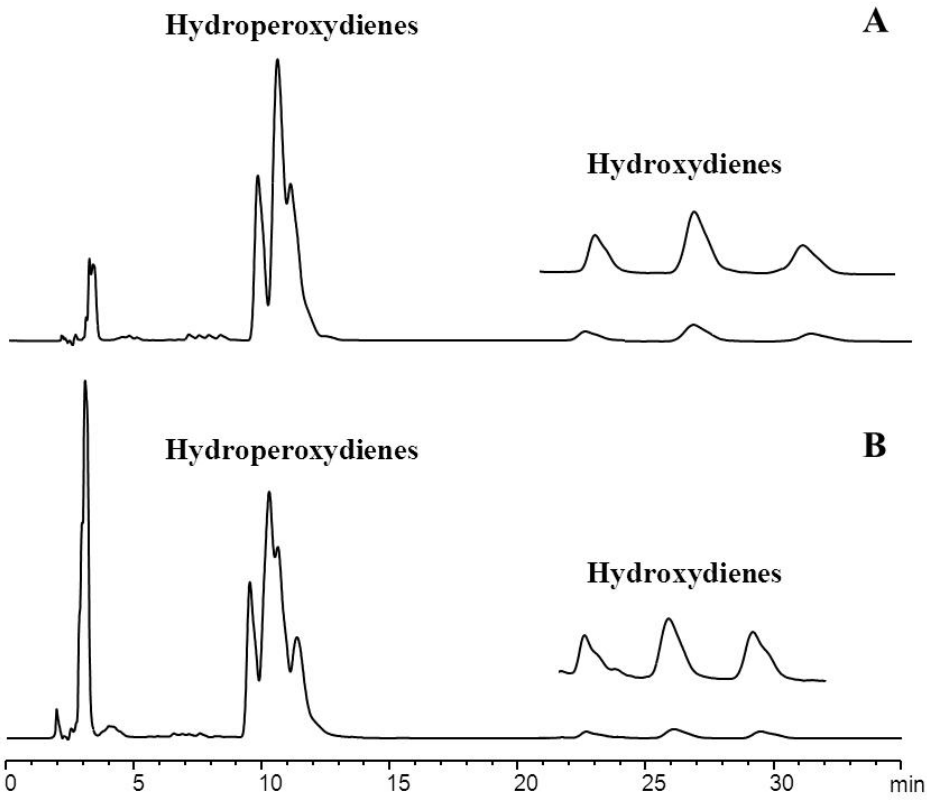
Hydroperoxydienes
A
Hydroxydienes
Hydroperoxydienes
B
Hydroxydienes
0
5
10
15
20
25
30
min

Figure 5