**OXIDATION OF A FUNCTIONAL, CLA-RICH OIL: DETERMINATION OF VOLATILE AND NON-VOLATILE COMPOUNDS**

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**Abstract**

The objective of this work was to monitor and compare formation of non-volatile and volatile oxidation compounds in a conjugated linoleic acid (CLA)-rich oil, Tonalin® oil (TO), and in a linoleic acid (LA)-rich oil, safflower oil (SO) at 40ºC in the dark. In TO, formation of hydroperoxides was negligible and the first and major compounds formed were polymerization products. Thus, when tocopherols were exhausted, SO showed 152 meq O2/kg oil and 3% polymers, values consistent with the expected progress of oxidation in unsaturated oils under these conditions, while TO showed only 19 meq O2/kg oil of peroxide value and as much as 15% polymers. In relation to volatile profile, that found in SO was close to that expected from the cleavage of the alkoxyl radicals formed from the LA-derived hydroperoxides, being hexanal the main compound. However, the volatile profile of TO was characterized by the occurrence of heptanal and *t*-2-nonenal, otherwise absent in SO. An alternative route of formation for these distinct volatile oxidation compounds in TO could be scission of dioxetanes coming from 1,2 cycloadditions of CLA with oxygen. Overall results obtained in this study, both on non-volatile and volatile compounds, support that oxidation kinetics of CLA-rich oils differ substantially from that expected according to the hydroperoxide theory. Oxidation of CLA seems to proceed preferentially by the addition of the peroxyl radical to a double bond during propagation reactions, thus supporting formation of oligomeric peroxides from the early events of lipid degradation.

**Introduction**

Oils rich in conjugated linoleic acid (CLA) such as Tonalin® oil (TO) are functional ingredients nowadays added to a variety of foods due to their health-promoting effects (Ozer and Kilmazi 2010, Koba and Yanagita 2014, Yang et al 2015). CLA is a mixture of positional and geometric isomers of octadecadienoic acid with conjugated double bounds and oils rich in CLA are normally obtained through alkaline isomerization of safflower oil (SO) - an oil rich in linoleic acid (LA) (Saebo 2003). Commercial CLA-rich oils consist almost entirely of the two biologically active CLA isomers (cis-9, trans-11 and cis-10, trans-12 linoleic acids) in approximately equal amounts (about 45% each). In particular, dairy products with added CLA have nowadays tremendous potential within the emerging nutraceutical and functional food markets (Ozer and Kilmazi 2010).

Despite the growing consumption of CLA-rich oils, safety concerns regarding the use of CLA persist and specifically in relation to the formation of oxidized compounds that could lead to adverse physiological effects in cardiovascular and cancerous processes, which are precisely the same targets for potential health benefits of CLA (Dobarganes and Márquez-Ruiz 2003; García-Martínez and Márquez-Ruiz 2009). Little is known about oxidation kinetics of CLA and the main variables affecting oxidative stability of CLA-fortified or CLA-added foods. In fact, it is only generally agreed that oxidation pathways of CLA are unclear (Eulitz 1999, Yurawecz 1999, Brimberg 2003, Yurawecz 2003, 2007, Luna 2007, Pajunen and Kamal-Eldin 2008, García-Martinez et al 2009, Márquez-Ruiz et al 2014).

Measurement of oxidation in CLA-rich oils is carried out by a variety of methods that do not often indicate the real level of oxidation and provide contradictory results. Fatty acid composition is routinely used by manufacturers just to ensure that the labeled level of CLA in functional products is within the range claimed and the only oxidation parameter used as quality specification is the peroxide value. However, hydroperoxides seem to be only minor compounds in the oxidation of CLA, in contrast with what occurs with LA, while formation of polymers occurs from the very beginning of the oxidation process (Brimberg 2003, Suzuki et al, 2004, Luna 2007, Márquez-Ruiz et al 2014). Regarding measurement of CLA loss based on fatty acid analysis, it lacks of sufficient sensitivity to detect oxidation levels at initial stages (Luna 2007, Brimberg 2003). Still, fatty acid composition and peroxide value determination remain as the most widely used methods to evaluate oxidation of CLA substrates in research studies (Lee et al., 2003, Minemoto et al 2003, Tsuzuki et al 2004; Yetella et al 2011, Lele et al 2014). As to volatile oxidation compounds, we published the first study in this context and found that CLA-rich oils showed a distinct profile and that heptanal could be used as maker of their oxidation progress (García Martinez et al 2009). Other authors have recently confirmed these results for CLA in different chemical forms (Cossignani et al 2014, Martínez-Monteagudo et al 2015)

The objective of the present work was, for the first time to our knowledge, to monitor jointly formation of non-volatile and volatile oxidation compounds in TO, a CLA-rich oil, as compared with its parent, LA-rich oil, SO. Oxidation conditions simulating ambient, shelf-life storage were selected (40ºC in the dark). Analytical evaluations included identification and quantification of volatiles by solid phase microextraction (SPE) and GC-FID/GC-MS and, in the case of non-volatile compounds, measurement of peroxide value (PV) and quantification of polymers by high-performance size-exclusion chromatography. Additionally, tocopherols were determined throughout the oxidation process.

**Materials and Methods**

**Materials**

Tonalin® TG 80 oil (TO) was acquired from Cognis Nutrition & Health (Cincinnati, OH, USA), and refined safflower oil (SO) was purchased from Interfat S.A. (Barcelona, Spain). Solid phase microextraction (SPME) fibers coated with 75µm CarboxenTM/Polydimethylsiloxane (CAR/PDMS) were obtained from Supelco (Bellefonte, PA, USA). Tridecanoin was purchased from Sigma Chemical Co. (St. Louis, MO) and bromobenzene from Fluka Chemika (Buchs, Switzerland). Other chemicals and reagents used were of analytical grade and obtained from local suppliers.

**Oxidation assays**

Triplicate samples of TO and SO (40 g each) were placed in 50 ml dark vials and stored in a temperature-controlled chamber at 40ºC ± 3 ºC in the dark. Aliquots (1g) were withdrawn for analyses along the oxidation experiment.

**Methods**

*Analysis of fatty acid composition*

Fatty acid composition was determined by GC-FID analysis. The oils were converted into fatty acid methyl esters using 2M KOH in methanol (IUPAC, 1992). FAME were analysed on an HP-6890 chromatograph (Hewlett Packard, Avondale, PA, USA) equipped with a split/splitless injector and a FID detector. Fatty acids were separated using an HP Innowax capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness (Hewlett Packard, Avondale, PA, USA). The column was held at 180°C for 2 min after injection, temperature-programmed at 3°C/min to 230°C and held there for 20 min. A split ratio of 1:40 was applied and hydrogen was used as carrier gas (1 mL/min). The injector temperature was set at 250ºC and detector temperature was set at 270ºC.

*Determination of the peroxide value*

Peroxide value was determined by the iodometric assay following IUPAC standard method 2.501 [IUPAC].

*Determination of tocopherols*

Tocopherols were determined by normal-phase HPLC with fluorescence detection according to IUPAC Standard Method 2.411 [IUPAC].

*Quantitation of triacylglycerol polymers*

Aliquots of 50 mg oil were dissolved in 1 mL tetrahydrofuran for direct analysis by HPSEC. A 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 500Å Ultrastyragel columns (25 cm x 0.77 cm i.d.) packed with porous, highly cross linked styrene-divinylbenzene copolymers (particle size: 5 μm) (Hewlett-Packard, Avondale, PA, USA) connected in series, with tetrahydrofuran (1 mL/min) as the mobile phase according to IUPAC Standard Method 2.508 [IUPAC]. The groups of compounds quantified were dimers and higher oligomers of triacylglycerols [Márquez-Ruiz and Dobarganes 2007]. The sum of dimers and higher oligomers will be referred to as polymers.

*Analysis of volatiles*

Solid phase microextraction (SPME) of volatiles was performed using a 75µm carboxen/polydimethylsiloxane (CAR/PDMS) fiber mounted in a SPME manual holder assembly (Supelco, Poole, U.K.). The fiber was conditioned for 30 min in the injection port of the GC at 250 ºC as recommended by the manufacturer. A 500 mg-aliquot of sample was weighed in a 20 ml dark vial. Ten µl of internal standard solution (0.75 mg/ml of bromobenzene in methanol) and a stirring bar were added. The vial was capped with a PTFE septum (Qmx Laboratories, Thaxted, UK) and placed in a water bath (40 ºC) on a magnetic stirrer, and the sample was equilibrated for 5 min at the required temperature before SPME sampling. The septum was manually pierced with the SPME needle and the fibre was exposed to the sample headspace for 30 min. Volatiles were analysed with a Hewlett-Packard (Palo Alto, CA, USA) 5890 series II gas chromatograph equipped with FID detector, split/splitless injector and a CP–Sil 8 CB low bleed/MS fused-silica capillary column (5% phenyl/95% PDMS; 60 m×0.25 mm I.D., 0.25 μm film thickness; Varian Chrompack). A section of column nearest to the injection port was cooled in a beaker of powdered solid carbon dioxide to cryofocus the volatiles. Volatile compounds were desorbed from the SPME fiber onto the front of column for 3 min. The injection port was in splitless mode, and split flow was programmed to turn on after 0.5 min. The temperature of the injector was 250 °C. After fiber desorption, the solid carbon dioxide was removed and the GC program started. The oven was maintained at 40°C for a further 2 min and then the temperature was raised to 120 ºC at 4°C/min and then at 20 ºC/min to 250 ºC, and held at 250 ºC for 10 min. The FID temperature was 280 ºC. Helium at 16 psig was used as the carrier gas. *n*-Alkanes (C5–C25) were run under the same conditions to obtain linear retention index (LRI) values for the components (Hashizume, Gordon & Mottram, 2007). The identification of compounds was performed on a Hewlett-Packard (Palo Alto, CA, USA) 5972 mass spectrometer, coupled to a 5890 Series II gas chromatograph and a G1034C Chemstation. The mass spectrometer was operated in electron impact mode with an electron energy of 70 eV and an emission current of 50 μA. The ion source was maintained at 170°C. The mass spectrometer scanned from *m/z* 29 to *m/z* 400 at 1.9 scans/s. Compounds were identified by first comparing their mass spectra with those contained in the National Institute of Standards and Technology (NIST) followed by comparing LRI values with *n*-alkanes (C5–C25) (Hashizume, Gordon & Mottram, 2007).

Statistical analysis

Data of fatty acid composition, tocopherols, peroxide value and polymers of the starting oils were obtained by using three determinations. Data reported for the oxidation experiments were obtained from triplicate samples and expressed as mean values and SDs. Excel 2000 (Microsoft Corporation, Redmond, WA, USA) was used for data analyses.

Results and Discussion

**Characterization of oils**

Table 1 shows fatty acid and tocopherol compositions, and oxidative parameters of SO and TO. The content of linoleic acid (C18:2 9*c*, 12*c*) in SO was similar to that of total CLA (C18:2 9*c*, 11*t* and C18:2 10*t*, 12*c*) in TO. However, TO contained approximately twice as much of total tocopherols as SO, and  and  homologues were the most abundant tocopherols. The PV and initial polymer content were low in both oils and typical for fresh refined oils.

**Evolution of PV, tocopherols and polymers during oxidation**

Figure 1 shows changes of PV and tocopherols during oxidation of SO and TO at 40ºC. As expected, SO showed a progressive rise in the PV from the beginning and increased markedly once tocopherols were only present in very low amounts. At the end of the induction period, 35 days, when tocopherols were exhausted, PV was 152 meq O2/kg. In sharp contrast, PV did not increase significantly during the induction period in the case of TO oxidation and, at 40 days, when tocopherols were exhausted, PV was only 19 meq O2/kg.

Figure 2 shows changes in polymers together with loss of tocopherols during oxidation of SO and TO at 40ºC. In SO, amounts of polymers remained practically constant and under 3% till the end of the induction period and in fact a significant rise in polymers marked the onset of advanced oxidation. This oxidation pattern has been repeatedly observed in our previous studies on oils and model triacylglycerols [Márquez-Ruiz et al., 1996, 2003; Martín-Polvillo et al., 2004]. Nevertheless, TO showed a very different oxidation pattern since polymers started to increase from the beginning, reaching amounts as high as 15% at the point of tocopherols exhaustion.

The results obtained in the present work agree with those we previously found in oxidation studies on conjugated and non-conjugated methyl linoleate [Luna 2007] and those reported by Suzuki et al on conjugated linolenic acid-rich oil (bitter gourd oil) and non-conjugated linolenic acid-rich oil (soybean oil) [Suzuki 2004]. It is well-known that the mechanism of autoxidation of methylene-interrupted fatty acid double bonds involves a catalytic process which proceeds *via* a free radical mechanism [Frankel 2005]. The initiation step consists of alkyl radical formation by the abstraction of a hydrogen radical from the carbon adjacent to the double bond. In the propagation step, oxygen is added to form alkylperoxyl radicals and the oxygen consumed is primarily converted to hydroperoxides. However, the low occurrence of hydroperoxides and high polymerization observed in the present work support that different mechanisms could be involved. In this regard, pioneering studies on conjugated polyunsaturated fatty acids already established that oxygen-containing polymers were main oxidation products [Miller 1928], and suggested that carbon-to-oxygen polymerization occurred rather than carbon-to-carbon polymerization [Allen 1949] and that the peroxide decomposition is not a major factor in the mechanism of oxidation of conjugated substances [Jackson and Kummerow 1949].

Figure 3 shows the HPSEC chromatograms obtained at 35 days for SO and TO. At that point, close to the end of the induction period for both oils, amounts of polymers were markedly different, 3.1 *vs.* 15%, respectively. Moreover, it is especially noticeable that, while polymers were essentially dimers in the case of SO, polymers were constituted almost exclusively of trimers and higher oligomers in the case of TO. This finding was observed from the beginning of the oxidation progress. These results are consistent with those we previously obtained in conjugated methyl linoleate, which showed for the first time the large degree of polymerization (higher than three) occurring from the beginning of the oxidation process under mild storage conditions [Luna 2007]. In this regard, it is important to comment that Muizebelt and Nielen, in studies inducing polymerization with Co/Ca/Zr drier and using various mass spectrometry techniques, reported formation of up to hexamers in a conjugated fatty acid ester and SIMS traces indicated that up to 15 oxygens were incorporated in the hexamer group [Muizebelt and Nielen, 1996]. Apart from the contribution of oxygenated functional groups in fatty acyls, the large number of oxygens observed with SIMS in oligomers suggests abundance of peroxyl crosslinks.

Figure 4 shows a schematic representation which help illustrate the different results found for SO and TO. These differences could be explained in terms of bond dissociation energy (BDE) as suggested by Oyman and coworkers [Oyman 2005]. For LA oxidation, the most favourable reaction of the peroxyl radical formed in the propagation step is the H-abstraction from the double allylic group of LA (BDE: 272 KJ/mol) to yield hydroperoxides as primary oxidation products. However, in CLA the preferred reaction pathway for the peroxyl radical formed is addition to a conjugated double bond (BDE: 284 KJ/mol) other than abstracting a monoallylic H atom (BDE: 322 KJ/mol). Also, in CLA, addition of the peroxyl radical to the conjugated diene system leads to formation of resonance-stabilized allylic radical intermediates. These differences in oxidation mechanisms would account for the low hydroperoxide amounts (PV) formed throughout oxidation of TO in favour of formation of peroxyl radical dimers leading ultimately to oligomeric peroxides. Such polymers cannot be therefore considered termination products characteristic of advanced oxidation stages but primary oxidation compounds, formed during the propagation stage.

**Evolution of volatile compounds during oxidation**

Figure 5 shows time course of formation of the main volatile compounds in SO and TO. Major volatiles found in TO were not those expected from theoretically stable hydroperoxides formed in CLA, as we already reported in samples oxidized at 60°C (García-Martínez et al., 2009). In SO, the volatile profile was close to that expected from the cleavage of the alkoxyl radicals formed from the hydroperoxides of autoxidized LA, being hexanal the main volatile oxidation product. Pentanal, *t*-2-heptanal and *t*-2-octenal were other major volatiles formed during oxidation of SO, also produced by decomposition of the most abundant LA hydroperoxides (Frankel 2005). However, the volatile profile of oxidized TO was characterized by the joint occurrence of two major volatile oxidation products, i.e., hexanal and heptanal, and the latter compound was absent in oxidized SO. Another important difference was the presence of *t*-2-nonenal in TO, while it was totally absent in SO. According to the hydroperoxide theory, formation of hexanal and pentanal is predictable from the expected major 13-hydroperoxides formed in both 9*c*, 11*t*-CLA and 10*t*, 12*c*-CLA, both isomers present in equal amounts in TO. In contrast, the presence of heptanal and *t*-2-nonenal, as well as that of other minor peaks, is not easily accounted for. Heptanal could come from **-scission of the alkoxyl radical formed from 12-hydroperoxy-8-*t*,10-*t*-octadecadienoate, in turn reported to be one of the hydroperoxides formed in oxidised 9-*c*, 11-*t* CLA (Hämäläinen et al 2002). However, *t*-2-nonenal would be a product derived from the 10-hydroperoxy-8-*t*,11-*t*-octadecadienoate or 10-hydroperoxy-8-*t*,11-*c*-octadecadienoate in oxidised 9-*c*, 11-*t* CLA, and it has been reported such hydroperoxides are unlikely to be formed because of the instability of the resonance structures in which double bonds are not conjugated in the pentadienyl radicals (Hämäläinen et al 2002). Likewise, *t*-2-octenal would come from the supposedly unexpected 11-hydroperoxy-9,12-octadecadienic fatty acyl (Tallman et al 2001; Tallman et al 2004) from both 9-*c*, 11-*t* CLA and 10-*t*, 12-*c* CLA, which would also contribute to formation of further amounts of heptanal. An alternative route of formation for the oxidation volatile compounds found in TO could be based on the mechanisms proposed by Yurawecz and coworkers. They suggested that CLA may undergo 1,2 cycloadditions with oxygen resulting in dioxetanes that would lead to volatile formation and proposed that heptanal would be a scission product of a 11,12-dioxetane whereas 2-nonenal would result from the scissions of 9,10-dioxetane (Yurawecz et al., 2003).

It is also of great relevance that much higher amounts of oxidation volatiles were formed in SO than in TO. This could be explained as the result of the strong build-up of hydroperoxides in SO leading to higher amount of volatiles via -scission in contrast to the predominant route of formation of oligomeric peroxides in the case of SO (Figure 4). In view of these results, it is not strange that sensory analyses of CLA-enriched products had not reported increased oxidized flavour (García-Martínez and Márquez-Ruiz 2009; Jones et al 2005).

**Conclusions**

The results obtained in this study show that different oxidation mechanisms are involved in LA-rich and CLA-rich oils. Polymers formation and not hydroperoxide formation occurred in TO from the beginning of the oxidation process thus invalidating peroxide value as oxidation measurement for CLA-rich oils. This is of the utmost importance since, at present, PV is the only determination included in quality specifications of commercialized CLA-rich oils. Results on volatile formation showed a markedly different profile for TO and SO, being heptanal distinctive of the oxidation progress of TO. Since volatile patterns of TO and SO greatly differed qualitatively and quantitatively, it is necessary to reconsider the sensory analyses currently used as a tool to detect oxidation of functional food products with CLA-rich oils.

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**Compliance with ethical standards**

**Conflict of interest** None.

**Compliance with Ethics Requirements**

This article does not contain any studies with human or animal subjects.

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**Table 1.**- Composition and oxidative parameters of Safflower and Tonalin® oils.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | Safflower oil |  | Tonalin oil |
| Fatty acid composition (%): | |  |  |  |
| 16:0 |  | 7.2 ± 0.2 |  | 2.4 ± 0.1 |
| 18:0 |  | 2.6 ± 0.1 |  | 2.6 ± 0.1 |
| 18:1 |  | 13.7 ± 0.5 |  | 14.2 ± 0.6 |
| 18:2 *9c, 12c* |  | 74.7 ± 0.7 |  | 0.5 ± 0.1 |
| 18:2 *9c, 11t* (CLA) | |  |  | 38.2 ± 0.7 |
| 18:2 *10t, 12c* (CLA) | |  |  | 38.6 ± 0.7 |
| Others |  | 1.8 ± 0.1 |  | 3.5 ± 0.2 |
| Tocopherols (mg/kg): | |  |  |  |
|  |  | 266 ± 13 |  | 28 ± 2 |
|  |  |  |  | 324 ± 17 |
|  |  |  |  | 215 ± 13 |
| Peroxide value (meq O2/kg) | | 2.9 ± 0.2 |  | 2.5 ± 0.3 |
| Polymers (%) | | 1.0 ± 0.3 |  | 1.1 ± 0.4 |

Data are expressed as Means ± Standard Deviations (n = 3).

*c*: *cis*; *t*: *trans*; CLA: conjugated linoleic acid.