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Influence of Oryzanols Concentrate on the Oxidation of Methyl Ester Linoleic Acid and Study of Their Oxidation Products

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Abstract

Oryzanols are frequently found in rice bran oil but almost completely removed in the neutralization step when the oil is chemically refined. In this way, oryzanols can be recovered from the soapstocks to generate a concentrate. Thereby, they could be used as antioxidants in lipids for specific purposes. In the present work the antioxidant power of oryzanols concentrate (33% purity) was studied together with pure oryzanols and butylhydroxytoulene (BHT). Methyl esters were prepared from regular sunflower oil without antioxidants to which the antioxidants before mentioned were added in an effective concentration of $3x10^{-3}$ M. The samples were oxidized in a heating block with oxygen flow and the hydroperoxides of linoleic acid methyl ester were analyzed. It was observed that all antioxidants were able to protect the sunflower oil methyl esters from oxidation with respect to methyl esters without antioxidants. Oryzanols presented a notoriously lower protection ability compared to BHT. However, the formation of the linoleic acid methyl ester hydroperoxides and their proportion, for the same oxidation stage, did not show differences between the antioxidants used. Therefore, the oxidation kinetics were similar between the different antioxidants studied.

Keywords: oxidation, methyl esters, linoleic acid, hydroperoxides, oryzanols

1. Introduction

It is interesting to study the evolution of the composition of a system subjected to a process of oxidative rancidity and to compare the variations that occur in the presence of an antioxidant whose antioxidant power is to be analyzed. There are precedents related to the monitoring of the concentration of primary oxidation products (hydroperoxides of linoleic acid) as a function of the oxidation time. Xu & Godber (2001) studied the oxidation of high purity linoleic acid at 37 °C under an oxygen atmosphere in the presence of oryzanols, ferulic acid and α-tocopherol. In similar investigations the formation of hydroperoxides from linoleic acid methyl ester was studied (Chan & Gordon, 1976; Brash, 2000; Mäkinen & Hopia, 2000; Morales, Dobarganes, Marquez Ruiz, & Velasco, 2010).

In the case of linoleic acid, the carbons more likely to generate hydroperoxides are those located in positions 9, 11 and 13 (Figure 1). The study of the products formed from linoleic acid showed that hydroperoxides predominate in positions 9 and 13 while the hydroperoxide in position 11 is not formed in significant amount (Bergström, 1945; Bergström, Blomstrand, & Laurell, 1950). This is due to the fact that the formation of the conjugated double bond gives the free radical greater thermodynamic stability and, therefore, the free radicals available for the subsequent reaction with oxygen and with the free hydrogen radical will be located at carbon 9 and in carbon 13 of the linoleic acid molecule (Bascetta, Gunstone, & Walton, 1983; Brash, 2000).

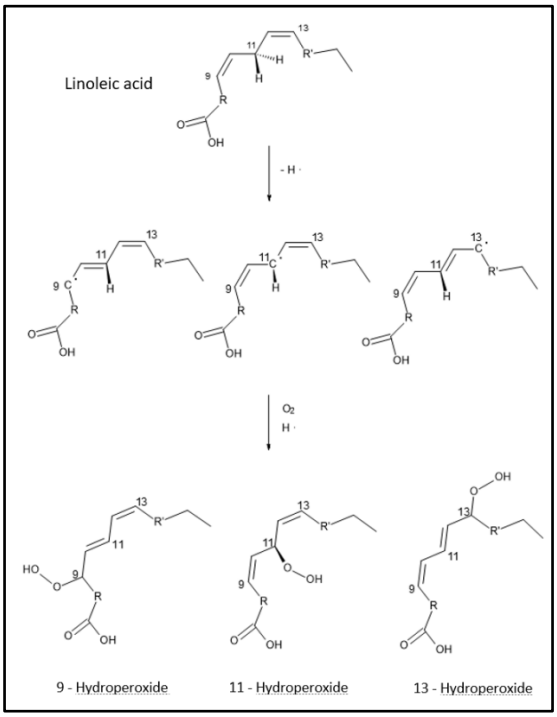

Figure 1. Linoleic acid reaction with molecular oxygen to form 9, 11 and 13-hydroperoxide

The 9 and 13-hydroperoxide of linoleic acid have geometrical isomerism, so that the cis and trans isomers can be formed in each of these positions. This is because when conjugated double bonds are formed, due to their resonance the hydrocarbon chain can be located in a cis or trans position with respect to the other end.

Mäkinen & Hoppia (2000) and Morales et al. (2010) described the formation and identification of the four isomers obtained from methyl ester linoleic acid: methyl 13-hydroperoxide (Z)-9 (E)-11 octadecadienoate, methyl 13-hydroperoxide (E)-9 (E)-11 octadecadienoate, methyl 9 hydroperoxide (Z)-10 (E)-12 octadecadienoate and 9 hydroperoxide (E)-10 (E)-12 octadecadienoate. These compounds were separated by high performance liquid chromatography on a silica column and detected by HPLC analysis with a diode array detector at a wavelength of 234 nm. These authors also identified (at 234 nm) other oxidation products, such as the methyl ester linoleic acid hydroxydienes. When a greater degree of oxidation is reached it is possible to determine, by this technique, some secondary oxidation compounds derived from methyl ester linoleic acid such as ketodienes which are formed from the aforementioned hydroperoxides, which were identified at a wavelength of 268 nm. Therefore, it is possible to study the formation of hydroxyperoxides from linoleic acid (Gardner & Weileder, 1972; Xu & Godber, 2001; Fukushige, Wang, Simpson, Gardner, & Hildebrand, 2005) as well as from linoleic acid methyl ester (Chan & Gordon, 1976, Wang, Ohshima, Ushio, & Koizumi, 1999; Brash, 2000; Mäkinen & Hopia, 2000; Pajunen, Johansson, Hase, & Hopia, 2008, Morales et al., 2010). Approximately 60 % of regular sunflower oil is linoleic acid. This fatty material is low-cost and easily available.

In this work the evolution of hydroperoxide content in a sample of methyl esters obtained by derivatization of commercial regular sunflower oil (SFOME), which was previously purified to eliminate the presence of antioxidants, was studied. These results were compared with those obtained in identical conditions, but with the addition of oryzanols concentrate and other antioxidants. The latter allowed to get more information regarding the antioxidant activity of these compounds by studying the products formed during the oxidation of a lipid sample and the effect of the presence of oryzanols. This is particularly interesting since through this study it was possible to learn how oryzanols protect this type of lipid matrices at temperatures close to room temperature.

2. Materials and Methods

2.1 Preparation of Fatty Acids Methyl Esters

To obtain the methyl esters, regular refined sunflower oil manufactured by the company COUSA (Uruguay) was used. Before doing so, the oil was purified on an alumina column according to the method described by Yoshida

et al. (1992) and Morales et al. (2010).

Once the oil was purified, 2 grams were weighed in a glass vial with a screw cap and 12.5 mL of 2 N KOH solution in methanol was added. The solution was magnetically stirred for 30 minutes at room temperature. Subsequently, 5 mL of petroleum ether was added and the mix stirred for approximately 1 minute. Phase separation was achieved by centrifugation at 3000 rpm. The organic layer was transferred to a second vial flask and washed with 5 mL of distilled water. This step was repeated three times and the sample then dried with anhydrous sodium sulfate. The solvent was evaporated under a stream of nitrogen until constant weight. Sunflower oil methyl esters (SFOME) were stored under nitrogen atmosphere, at freezer temperature (-20 ℃) and protected from light.

2.2 Addition of Antioxidants to Fatty Acid Methyl Esters

Sigma-Aldrich quality BHT, an oryzanols standard supplied by Dr. Haiko Hense from the Federal University of Santa Catarina (Brazil) and an oryzanols concentrate obtained in our research facility with 33 % purity were used as antioxidants. The oryzanols concentrate purity was determined by HPLC.

An antioxidant solution in isopropanol or chloroform was prepared and no more than 150 μL added directly on SFOME in order to obtain a final concentration of 3 x 10^{-3} M. After adding the antioxidants, samples were placed in an ultrasound bath for 10 min to ensure complete homogenization.

The SFOME were stored under nitrogen atmosphere, at freezer temperature (-20 °C) and protected from light until use.

2.3 Oxidation of Fatty Acids Methyl Esters

The oxidation of the SFOME was carried out in a test tube with a screw cap, provided with a capillary tube (0.25 mm internal diameter x 400 cm long) for oxygen supply. A dry block heater for temperature control was used.

The temperature was set at 50 ℃ and once it was reached, the test tubes containing 1.5 g of SFOME samples were placed. A constant oxygen flow of 6 mL / min was applied so that it bubbled through the samples. At certain time intervals, aliquots of approximately 7 mg were taken. Subsequently, 1 mL of hexane was then added and samples were ready for HPLC analysis.

2.4 Analysis by High Performance Liquid Chromatography

The analysis by high performance liquid chromatography (HPLC) was carried out according to the method reported by Morales et al. (2010). A Shimadzu 20A HPLC, equipped with a Lichrospher-Si column (250 mm x 4.6 mm x 5 μm) and Shimadzu SPD M20A diode array detector was used. Oven temperature was adjusted at 40 ℃. An isocratic program was used with hexane:diethylether (82:18, v:v) and the flow was 2 mL/min.

Chromatograms were recorded at a wavelength of 234 nm and 268 nm to determine the SFOME primary and secondary oxidation compounds. Hydroperoxides of methyl ester linoleic acid were identified according Morales et al. (2010).

3. Results and Discussion

3.1 Hydroperoxide Formation Rate

Lipid oxidation process starts with relatively slow kinetics. The advance of the oxidation reaction accelerates the kinetics until at some point the rate of formation of the oxidation compounds strongly increases. As previously mentioned, Mäkinen & Hopia (2000) and Morales et al. (2010) identified by HPLC the four major hydroperoxides of methyl ester linoleic acid (Table 1).

Therefore, the variation of the area corresponding to these peaks as a function of time allowed to compare the kinetics of the oxidation process in different conditions (without the addition of antioxidants or with the presence of antioxidants of different nature).

Table 1. Identification of the four hydroperoxides formed in the oxidation of methyl linoleate

Hydroperoxide	Abbreviation
Methyl 13-hydroperoxy-(Z)-9,(E)-11-octadecadienoate	13H (Z)-9 (E)-11
Methyl 13-hydroperoxy-(E)-9,(E)-11-octadecadienoate	13H (E)-9 (E)-11
Methyl 9-hydroperoxy-(E)-10,(Z)-12-octadecadienoate	9H (E)-10 (Z)-12
Methyl 9-hydroperoxy-(E)-10,(E)-12-octadecadienoate	9H (E)-10 (E)-12

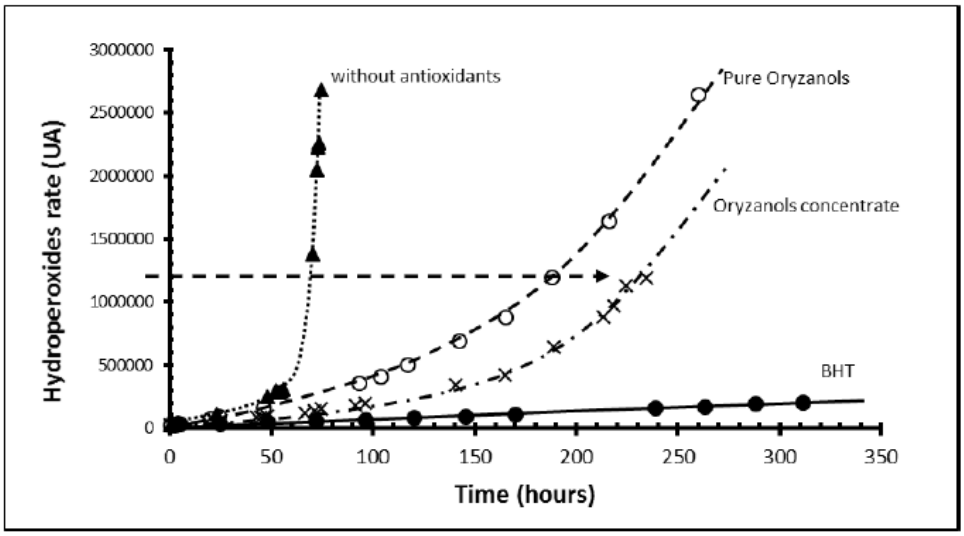

Figure 2. Total hydroperoxides rate in SFOME without antioxidants and with addition of different antioxidants during oxidation in oxygen flow at 50 ℃: a) BHT, b) pure oryzanols and c) oryzanols concentrate, all in concentration 3x10-3 M (UA: arbitrary units)

Figure 2 shows the evolution of total hidroperoxides of methyl ester linoleic acid corresponding to the oxidation of SFOME at 50 ℃ without antioxidants and with the addition of different antioxidants (BHT, pure oryzanols or oryzanols concentrate).

The ordinates axis is showed in "arbitrary units" (UA), since the values were determined as the total area (areas of the four mentioned hydroperoxides) "normalized" with respect to methyl esters concentration (g/mL) in the sample analyzed. This normalization allowed to compare the results obtained from different analysis.

Analogous to the analysis of accelerated rancidity tests, from these graphs it was possible to determine an induction time, corresponding to the intersection point of the slopes between the initial stage and the final stage. The induction time value for SFOME without antioxidants was 64 minutes.

The SFOME with added BHT presented a slight increase in the hydroperoxide rate as a function of time, without it being possible to determine an induction period, as occurred with the sample without antioxidants. This indicates that the degree of advance of the oxidation was not sufficient to reach the acceleration phase in the formation of hydroperoxides. For the SFOME without antioxidants (Figure 2) a hydroperoxides rate of $2x10^6$ U.A. immediately after the induction period was obtained, while in the case of the SFOME additivated with BHT at a concentration $3x10^{-3}$ M (Figure 2) they never reached this rate.

The curves corresponding to the SFOME with pure oryzanols or with oryzanols concentrate (Figure 2), showed a higher increase in the hydroperoxides rate than the sample with BHT. However, even after 350 h of oxidation the sample with BHT showed no significant increase in the hydroperoxides rate. Therefore, the determination of an induction period was not possible.

Finally, to reach a hydroperoxides rate of $1.2x10^6$ U.A. took 60, 188 and 235 h for SFOME without antioxidants, pure oryzanols and oryzanols concentrate, respectively.

These results indicated that all the antioxidants protected the SFOME under the conditions of the analysis effectively and that the BHT was the one with the highest antioxidant power.

It was also observed that oryzanols concentrate protected SFOME more efficiently than pure oryzanols. This was surely because the oryzanols concentrate had small amounts of tocopherols and tocotrienols. The concentrate was analyzed and the presence of a small amount of tocopherols was confirmed.

3.2 Hydroperoxide Composition

The peaks corresponding to 13H (E)-9 (E)-11 and 9H (E)-10 (Z)-12 overlapped, making it difficult to quantify separately. This coincides with the reports made by Morales et al., 2010. For this reason, the total area corresponding to the sum of both peaks was considered.

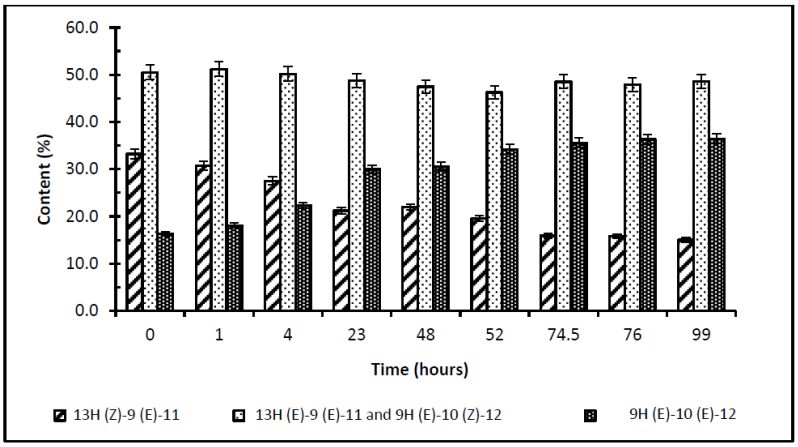

Figure 3. Content of each hydroperoxide during the oxidation of the SFOME without antioxidants

Figure 3 shows the percentage of each hydroperoxide (calculated as the percentage of the corresponding peak area with respect to the sum of areas of the four peaks) during the oxidation of SFOME without antioxidants as a function of the oxidation time at 50 ℃.

The percentage of 13H (E)-9 (E)-11 and 9H (E)-10 (Z)-12 was maintained between 46 and 51 % throughout the period of oxidation studied. On the other hand, the percentage of 9H (E)-10 (E)-12 presented a continuous increase from 16 to 37 %, due to a gradual and continuous decrease of 13H (Z)-9 (E)-11 from 33 to 15 %. However, after 74.5 h the percentages did not change significantly.

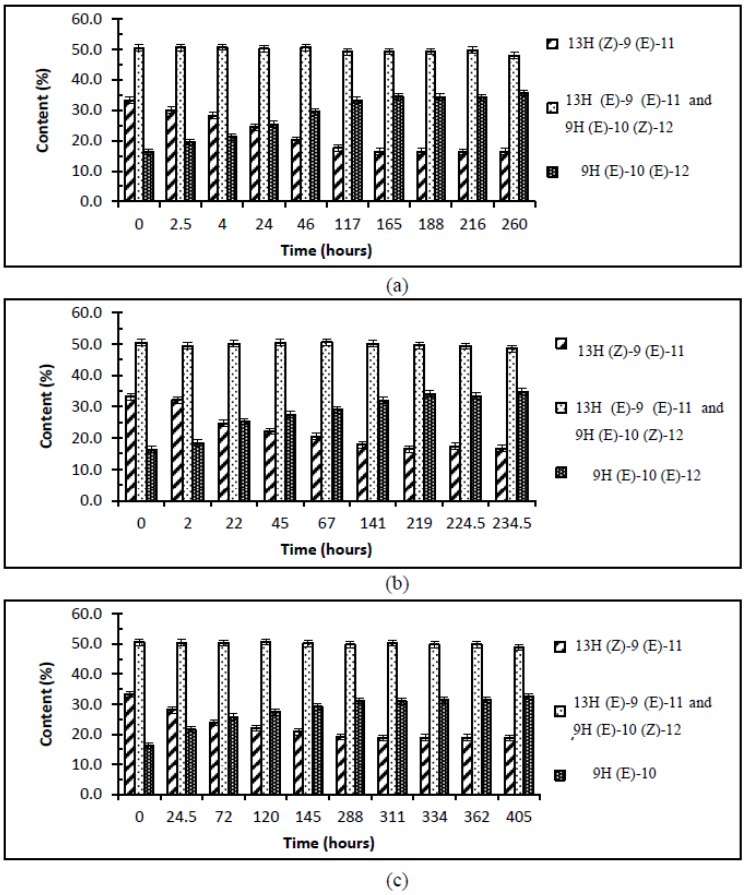

Figure 4. Content of each hydroperoxide during the oxidation of SFOME with addition of: a) pure oryzanols, b) oryzanols concentrate and c) BHT

Figure 4 shows the percentages for oxidation compounds of SFOME with the addition of different antioxidants. The behavior was similar to that of the SFOME without antioxidants: 9H (E)-10 (E)-12 increased until a maximum value and 13H (Z)-9 (E)-11 decreased until a minimum value, from which they remained constant. These values were achieved at 117, 141 and 288 hours for pure oryzanols, oryzanols concentrate and BHT, respectively.

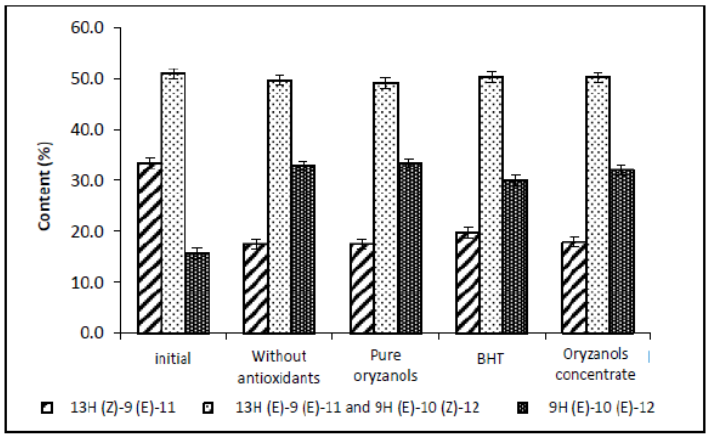

Figure 5. Content of the initial hydroperoxides and each hydroperoxide formed from SFOME for the same oxidation degree

Figure 5 compares the percentage contents of the different hydroperoxides in un-oxidized SFOME without antioxidants (initial) and oxidized SFOME (with and without antioxidants) for an oxidation time at which a similar total concentration of hydroperoxides was achieved (expressed as total area of hydroperoxides vs. concentration). In the initial sample, the percentage content of 13H (E)-9 (E)-11 and 9H (E)-10 (Z)-12 did not change after a certain time of oxidation, both in SFOME without antioxidants and with the addition of antioxidants. However, 13H (Z)-9 (E)-11 decreased by half with respect to initial SFOME, whereas 9H (E)-10 (E)-12 increased twice as much. This suggested that the oxidation mechanism favored the 9H 10-trans 12-trans formation with respect to 13H 9-cis 11-trans. On the other hand, it was observed that SFOME without antioxidants presented a similar variation in hydroperoxides percentage compared to SFOME with the addition of antioxidants.

4. Conclusions

Through this oxidation study at 50 ℃ it was possible to observe a sustained increase in the oxidation compounds that in some cases allowed to calculate an "apparent" induction period. This method useful for comparing the efficiency of antioxidants in the oxidation of those oils that had a certain content of linoleic acid, although it must be taken into account that it took a long time to achieve important advances in oxidation when some antioxidants were added to the methyl esters. It should also be noted that the study involved methyl esters rather than the oil itself.

The oryzanols concentrate presented a better protective effect on methyl esters than pure oryzanols. This showed that the other compounds present in the oryzanols concentrate did not affect the antioxidant power of the oryzanols in it. The increase in antioxidant power was probably due to the presence of tocopherols. However, oryzanols presented a much lower antioxidant power compared to BHT.

In addition, the hydroperoxides proportion obtained from methyl ester linoleic acid was similar in all cases and for this reason it was possible to conclude that the oxidation mechanism did not vary with respect to the different antioxidants studied.

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