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Effect of laccase from *Trametes versicolor* on the oxidative stability of edible vegetable oils

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ABSTRACT

The increasing demand of natural food from consumers has limited the use of traditional methods to control the oxidation of lipids, such as synthetic antioxidants and hydrogenation. Besides, it has been reported that the use of enzymes is efficient to eliminate dissolved oxygen in foods such as vegetable oils. Laccase is a polyphenol oxidase and the reduction of oxygen to water is accompanied by the oxidation, typically, of a phenolic substrate. Laccase have become important, industrially relevant enzymes that can be used for a number of diverse applications such waste detoxification, textile dye transformation, food technologic uses, biosensor and analytical applications, bioethanol production, among others. The target of this study was to evaluate the effect of laccase enzyme from Trametes versicolor, on the oxidative stability of sesame, chia, peanut and sunflower oils, measured through the peroxide value (PV) and conjugated dienes (K232) and trienes (K270). The samples of oil with laccase showed higher PV, K232 and K270 than their corresponding controls, under the conditions evaluated (room temperature and 60 °C). The results suggest that fungal laccase has an unexpected pro-oxidant effect on vegetable oils, possibly promoted by products derived from the oxidation of phenols by enzymatic action.

1. Introduction

A lot of food is prepared with vegetable oils that contain a large amount of unsaturated fatty acids that react with dissolved oxygen generating strange odors and flavors, decreasing shelf life and the nutritional value [1]. These degraded products have harmful effects on human health [2,3].

The process of oxidation of lipids in food occurs through a series of chain reactions of free radicals. The classic route of autoxidation includes initiation (production of lipid free radicals), propagation and termination (production of non-radical products) reactions. This process starts with small amounts of oxygen, which is difficult to avoid; however, it can be controlled or delayed by using properly the different conservation techniques, combined with the use of antioxidants [4]. Among the most used synthetic antioxidants in the food industry are the BHA (Butylated hydroxyanisole), BHT (Butylated hydroxytoluene) and TBHQ (Tertiary-butyl hydroquinone). The use of these compounds is being increasingly questioned because negative effects on health are attributed to them [5]. For this reason, in recent years, there has been a growing interest in the study and application of natural antioxidants or the incorporation of non-toxic natural components for the processing of food [6].

Laccase (EC 1.10.3.2, p-diphenol oxidase) catalyze the oxidation of phenolic substrates and the reduction of molecular oxygen to water [7–10]. According to phylogenetic analyzes most fungal laccase enzymes are found in white rot fungi [11]. It is one of the main enzymes involved in delignification; furthermore, on occasions, the presence of laccase has been described as the only ligninolytic activity in fungi that degrade lignin or other aromatic compounds with great environmental impact [12].

Many reviews discuss the potential applications of laccase in different forms in the food industry such as wine stabilization, beverage processing, sugar beet pectin gelation, baking and as a biosensor [13–18].

Laccase scavenges O_2 which would otherwise react with fatty acids, amino acids, proteins and alcohols to form off-flavour precursors. This application can be found in the commercial preparation Flavourstar (Novozymes), which was developed for brewing and based on the laccase from *Myceliophthora thermophila* [19]. More-

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over, it has been shown that oxygen-reducing enzymes, such as glucose oxidase and laccase, have the potential to catalyze the elimination of oxygen in active packages of food packages [20–22].

Petersen and Mathiasen [23] demonstrated that laccase can particularly improve the sensory attributes of foods that are high in fat and contain endogenous phenolic compounds, by reducing the available oxygen. Cold pressed seed oils contain phenols at a concentration range from 18 to 99 ppm caffeic acid equivalents (CAEs) [24]. The depletion of intrinsic phenols in vegetables oils, catalyzed by exogenous enzymes in contact with the oil, was also reported [25,26].

The objective of this work was to obtain a laccase extract from one strain of white rot fungi belonging to the *Trametes* genus and to analyze the effect of this on the oxidative stability of several vegetable oils.

2. Materials and methods

2.1. Oil samples

The vegetable oils were obtained by cold pressing from sesame, chia, peanut and sunflower seeds. These were obtained by a manual hydraulic press of 20 Tn.

Cold-pressed oils may retain higher levels of natural antioxidants that may be removed during the refining steps of a conventional oil processing procedure. In addition, cold pressing involves no organic solvent, which results in a product that is chemically contaminant free [27].

2.2. Laccase extract

2.2.1. Organism

The fungal strain and culture media were selected taking into account previous data from the working group, and other authors, on the laccase production of some native rot fungi [28]. The fungal strain was *Trametes versicolor* BACF (Buenos Aires Facultad de Ciencias) 2234 belonging to the cepary of the Facultad de Ciencias Exactas y Naturales de la Universidad de Buenos Aires and contributed by the Instituto Misionero de Biodiversidad (IMiBio), Puerto Iguazú, Misiones-Argentina. The inoculum was maintained at 4 °C on 2% (*m/V*) potato agar solid medium, until required.

2.2.2. Media and culture conditions

Laccase was produced in a liquid medium containing ($g\,L^{-1}$): glucose, 40; yeast extract, 5; meat peptone, 5; $MgSO_4{\cdot}7H_2O$, 1 [29]. It was added with ($mg\,L^{-1}$) $CuSO_4{\cdot}5H_2O$, 0.4; to induce even more laccase activity [30–32]. The pH of the solution was adjusted to 6.3 ± 0.4. Although the optimal pH for oxidation of phenolic substrates is around 4 but *T. versicolor* laccase is most stable at pH 6–7 [33,34]. The medium was sterilized by autoclaving at 120 °C for 20 min.

The inoculation was done in 300 mL of medium contained in 1000 mL Erlenmeyer flasks. Three agar blocks, of 0.5 cm^3 each, were removed from edge of a colony grown on solid medium. The cultures were incubated at 28 °C under static and dark conditions for 21 days.

2.2.3. Extract preparation

The culture liquid was separated from the mycelium by gentle filtration through Whatman filter paper and concentrated by dialysis against sucrose through a semi-permeable regenerated cellulose membrane of 25 mm × 16 mm of Sigma-Aldrich [35]. The extract was filtered by syringe filter of 0.45 μm × 25 mm of polyvinyl difluoride (PVDF) from Biopore. This concentrated extract was taken as the source of laccase.

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2.2.4. Determination of laccase activity

Laccase extract activity assayed using 5 mmol/L 2,6-Dimetoxyphenol (DMP) (77.1 mg/100 mL acetate buffer pH 3.6) (E469 = 27,500 mol/L·cm) [36]. The enzymatic reaction was carried out at room temperature (25 °C) and one unit of enzyme activity (U) was defined as the amount of enzyme oxidizing one μmoL of substrate per min. The laccase activity was triplicate by the enzymatic concentration process carried out reaching 32 U/mL.

2.3. Oxidative stability analysis

2.3.1. Peroxide value (PV)

Peroxides are formed in the primary phase of oxidation. It is the measure used most frequently during the production of vegetable oils [37]. This was determined using the analytical methods described by El-Shattory et al. [38]. In this case, the sample (0.025 g) was mixed in a glass tube with 9.8 mL chloroform-methanol (7:3) on a vortex for 10 s. Then 50 μL of NH_4SCN 30% *m/V* solution and $FeCl_2$ 0.2% solution were added, and the sample was mixed on a vortex for 10 s. The reaction mixture was incubated at room temperature for 5 min. Absorbance of the red complex was read in a Shimadzu UV 1800 spectrophotometer at 500 nm against a blank containing the whole reagent, except the sample. The procedure occurred in low light and completed in 10 min. The quantification was referred to a standard calibration curve made with $FeCl_3$. The PV was expressed as milliequivalents of peroxides per kilogram of oil (mEq Px/kg).

$$PV = \frac{(A_s - A_b) \times m}{55.84 \times W} \times 2 \quad (1)$$

Where: A_s: absorbance of the sample; A_b: absorbance of the target; m: slope of the calibration curve; 55.84: iron atomic weight; W: grams of oil in the sample. The factor **2** is necessary to express the peroxide value as milliequivalents of peroxides instead of milliequivalents of oxygen.

2.3.2. Conjugated dienes (K232) and conjugated trienes (K270)

After peroxides formation, in the primary phase of oxidation, the rearrangement of the double and triple bonds of the fatty acids originate the conjugated dienes and trienes [39]. In this experience, an aliquot (20 μL) of the reaction mixture was placed in a volumetric flask and weighed accurately. It was prepared with 10 mL of hexane and stirred for 5 min, and then the absorption spectrum was recorded [40]. The results was

$$K_\lambda = \frac{A_\lambda}{b.c.} \quad (2)$$

Where: K_λ: specific extinction coefficient at the wavelength λ; A_λ: absorbance read in the spectrophotometer at the wavelength λ; b: thickness in cm of the cuvette c: oil concentration of the solution in g/100 mL.

2.3.3. Statistical analysis

The set of analytic determinations was carried out in triplicate. All data are presented as means ± SD and were derived with the Infostat statistical software [41]. Difference between means was performed by ANOVA. The level of statistical significance was accepted at $P \leq 0.05$.

Table 1
Peroxide Value (mEq Px/kg) in vegetable oils treated whit laccase.[1] Different letters indicate significant difference ($P \leq 0.05$)[2].

	Sample	Days			
		0	3	11	18
Chia	LAC w/air	2.1 ± 0.4 [a]	3.3 ± 0.7 [a]	5.4 ± 0.6 [a]	16.7 ± 0.1[a]
	LAC w/o air	1.9 ± 0.1 [a]	2.9 ± 0.2 [a]	5.3 ± 0.8 [a]	15.8 ± 0.5[a]
	Fresh w/air	1.9 ± 0.2 [a]	2.3 ± 0.3 [a]	4.8 ± 0.1 [a]	13.9 ± 0.9 [b]
	Fresh w/o air	1.9 ± 0.2 [a]	2.2 ± 0.3 [a]	4.2 ± 0.4 [a]	12.1 ± 0.9 [b]
Sunflower	LAC w/air	1.5 ± 0.4 [a]	1.7 ± 0.1 [a]	3.2 ± 0.1 [a]	8.1 ± 0.4 [a]
	LAC w/o air	1.5 ± 0.3 [a]	1.6 ± 0.5 [a]	3.0 ± 0.3 [a]	7.8 ± 0.3 [a]
	Fresh w/air	1.2 ± 0.6 [a]	1.4 ± 0.1 [a]	2.7 ± 0.1 [a]	6.8 ± 0.3 [b]
	Fresh w/o air	1.2 ± 0.4 [a]	1.4 ± 0.1 [a]	2.7 ± 0.1 [a]	7.0 ± 0.3 [b]
Peanut	LAC w/air	3,5 ± 0.3 [a]	4.6 ± 0.1 [a]	5.3 ± 0.5 [a]	11.1 ± 0.5 [a]
	LAC w/o air	3.5 ± 0.1 [a]	4.4 ± 0.1 [a]	5.2 ± 0.5 [a]	10.9 ± 0.4 [a]
	Fresh w/air	3.3 ± 0.5 [a]	4.2 ± 0.1 [a]	5.1 ± 0.5 [a]	9.7 ± 0.3 [b]
	Fresh w/o air	3.4 ± 0.4 [a]	4,0 ± 0.1 [a]	5.0 ± 0.5 [a]	9.2 ± 0.3 [b]
Sesame	LAC w/air	4.0 ± 0.4 [a]	6.6 ± 0.1 [a]	8.5 ± 0.4 [a]	11.8 ± 0.6 [a]
	LAC w/o air	3.7 ± 0.1 [a]	6.6 ± 0.2 [a]	8.1 ± 0.9 [a]	10.4 ± 0..2 [a]
	Fresh w/air	3.5 ± 0.1 [a]	6.4 ± 0.3 [a]	8.1 ± 0.1 [a]	10.0 ± 0.2 [b]
	Fresh w/o air	3.5 ± 0.1 [a]	5.9 ± 0.3 [a]	7.8 ± 0.1 [a]	9.5 ± 0.2 [b]

Legend: LAC w/air, oil with laccase and whit atmospheric air; LAC w/o air, oil with laccase and without atmospheric air; Fresh w/air, oil fresh with atmospheric air; Fresh w/o air, oil fresh without atmospheric air.
*1 Each sample "LAC" contained 3.2 U/mL laccase from *Trametes versicolor*.
*2 ANOVA was between LAC and Fresh (w/air and w/o air) in each oil and each time.

Table 2
Conjugated dienes (K232) in vegetable oils treated with laccase[1] Different letters indicate significant difference ($P \leq 0.05$)[2].

Vegetable Oil	Sample	Time in Days			
		0	3	11	18
Chia	LAC	1.3 ± 0.4[a]	1.3 ± 0.5 [a]	1.4 ± 0.6 [a]	4.7 ± 0.1 [a]
	Fresh	1.2 ± 0.2 [a]	1.3 ± 0.3 [a]	1.3 ± 0.1 [a]	3.5 ± 0.9 [b]
Sunflower	LAC	0.8 ± 0.4 [a]	1.1 ± 0.1 [a]	2.2 ± 0.1 [a]	4.3 ± 0.5 [a]
	Fresh	0.8 ± 0.6 [a]	0.9 ± 0.2 [a]	1.9 ± 0.2 [a]	3.2 ± 0.3 [b]
Peanut	LAC	1,5 ± 0.3 [a]	1.6 ± 0.1 [a]	2.0 ± 0.5 [a]	2.7 ± 0.6 [a]
	Fresh	1.4 ± 0.4 [a]	1.5 ± 0.1 [a]	1.8 ± 0.5 [a]	2.0 ± 0.3 [b]
Sesame	LAC	1.8 ± 0.2 [a]	2.1 ± 0.1 [a]	3.0 ± 0.4 [a]	3.8 ± 0.6 [a]
	Fresh	1.7 ± 0.1 [a]	1.9 ± 0.3 [a]	2.8 ± 0.1 [a]	3.1 ± 0.2 [b]

Legend: LAC, oil with laccase; Fresh, oil fresh.
*1 Each sample "LAC" contained 3.2 U/mL laccase from *Trametes versicolor*.
*2 ANOVA was between LAC and Fresh in each oil and each time.

2.4. Experimental design

The samples treated with laccase (LAC) contained 3 mL of vegetable oil, 100 μL of enzymatic extract of *Trametes versicolor BACF 2234* (32 U/mL) and 100 μL of citrate-phosphate buffer pH 5. This corresponds to the optimum pH for the action of the fungal laccase, according to the bibliography [9]. The control samples (Fresh) were prepared the same manner except the enzymatic extract was inhibited with 0.1 mmol/L sodium azide [42].

According to the reaction conditions, two treatments were tested:

- **Treatment 1:** the samples were incubated at room temperature of 28 °C for 18 days; in static and dark conditions, covered.
- **Treatment 2:** the samples were kept under accelerated conditions of 60 °C in the oven, for 72 h. Hypothetically, one day at 60–65 °C should correspond to one month of storage at room temperature of a vegetable oil [43].

3. Results and discussion

3.1. Peroxide value (PV) in Treatment 1

The peroxide values observed in vegetable oils, were collected in Table 1.

As can be observed, the increase in the PVs was gradual until day 11 and it was significant on day 18, for the treated and untreated samples.

As for the effect of the enzyme, the PV was surprisingly increased. However, the differences between LAC and Fresh were statistically significant ($P \leq 0.05$) from day 18 of reaction.

In detail, in LAC, the amount of peroxides was between 8 and 17 mEq Px/kg on day 18, while in Fresh, the values were between 7 and 14 mEq Px/kg approx.

Chia oil showed a higher PV, these were 13.9 and 16.7 mEq Px/kg in Fresh and LAC, respectively. This may be due to its high content of linolenic acid ($C_{18:3}$) (60% of total fatty acids) that confers susceptibility to oxidation [44].

The effect of the absence of oxygen, by vacuum application, was also determined in this treatment. The LAC and Fresh showed no significant differences on the PV with respect to them in the presence of oxygen (Table 1).

Assuming that laccase treatment is capable of playing the role of oxygen scavenger, it was expected to be more efficient than phenolic compounds in protecting oils from oxidation. On the contrary, a pro-oxidant effect was observed, which could be promoted by a route other than oxygen from the air and related to the phenols oxidized by the enzyme, under the conditions tested.

Other authors also found contrary results to those expected. They studied the oxidation of linoleic acid in presence of laccase and

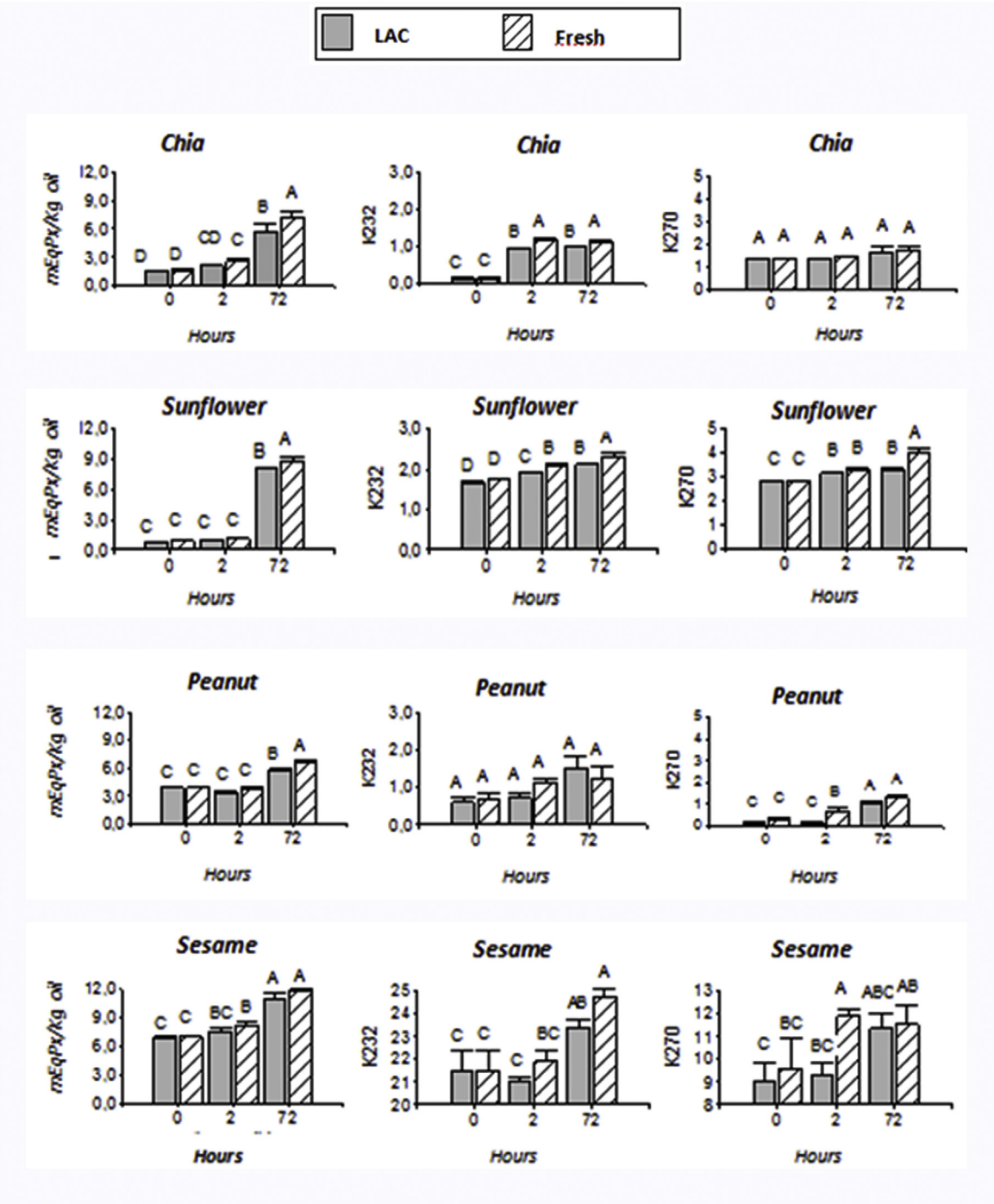

Fig. 1. Evolution of Peroxide Values (mEqPx/kg Oil), conjugated dienes (K232) and conjugated trienes (K270) in vegetable oils (chia, sunflower, peanut, sesame) treated whit laccase (LAC) and its corresponding controls (Fresh), stored during 72 h at 60 °C. Columns with different letter are statistically different ($P \leq 0.05$) according to Fisher's test. The bars represent the error. The results are based on triplicate measurements.

p-hydroxycinnamic, sinapic, ferulic and p-coumaric acids, which are substrates of the enzyme and also powerful antioxidants, however the results showed that p-coumaric acid has a high capacity to peroxidate unsaturated lipids once it is oxidized by laccase [45].

3.2. Conjugated Dienes (K232) and Conjugated Trienes (K270) in Treatment 1

The evolution of oxidation at 28 °C through the specific extinction coefficients at 232 nm and 270 nm are shown in the Tables 2 and 3, respectively.

K232 showed higher values in the samples with the enzyme. The differences were significant on day 18, as was the case with the peroxides. Chia oil, followed by sunflower oil, showed the greatest alteration about this coefficient. In detail, these were 4.7 and 4.3 mEq Px/kg, respectively. This could be explained by the high content of polyunsaturated fatty acids in these oils, which are 90% for sunflower oil and 80% for chia oil, approx [47,44].

Other authors measured the relationship between the composition of the fatty acids, PV and K232 during the oxidation of various sunflower oils and found a lower slope for the oil with a lower degree of unsaturation [48].

Table 3
Conjugated trienes (K270) in vegetable oils treated with laccase[1] Different letters indicate significant difference ($P \leq 0.05$)[2].

Vegetable Oil	Sample	Time in Days			
		0	3	11	18
Chia	LAC	0.4 ± 0.05[a]	0.7 ± 0.07 [a]	1.0 ± 0.3 [a]	1.7 ± 0.2 [a]
	Fresh	0.4 ± 0.04[a]	0.6 ± 0.06 [a]	0.9 ± 0.1 [a]	1.1 ± 0.4 [b]
Sunflower	LAC	0.2 ± 0.05 [a]	0.3 ± 0.05 [a]	0.4 ± 0.1 [a]	1.5 ± 0.3 [a]
	Fresh	0.2 ± 0.04 [a]	0.2 ± 0.04 [a]	0.3 ± 0.1 [a]	0.9 ± 0.2 [b]
Peanut	LAC	0.1 ± 0.06 [a]	0.1 ± 0.05 [a]	0.2 ± 0.1 [a]	0.9 ± 0.6 [a]
	Fresh	0.1 ± 0.05[a]	0.1 ± 0.03 [a]	0.2 ± 0.1 [a]	0.4 ± 0.2 [b]
Sesame	LAC	0.1 ± 0.02 [a]	0.3 ± 0.06 [a]	0.6 ± 0.6 [a]	1.9 ± 0.4 [a]
	Fresh	0.1 ± 0.03 [a]	0.2 ± 0.05 [a]	0.4 ± 0.5 [a]	1.3 ± 0.2 [b]

Legend: LAC, oil with laccase; Fresh, oil fresh.
[*1] Each sample "LAC" contained 3.2 U/mL laccase from *Trametes versicolor*.
[*2] ANOVA was between LAC and Fresh in each oil and each time.

K270 also showed greater increase in the samples with the enzyme, with significant differences towards day 18 (Table 3). Sesame oil obtained the highest values of conjugated trienes in LAC (1.9 ± 0.4).

3.3. Peroxide value (PV) in Treatment 2

Results relative to PV in the accelerated oxidation conditions carried out at 60 °C and 72 h were collected in Fig. 1.

The samples with the enzyme showed higher PVs, with significant differences ($P \leq 0.05$) at 72 h for chia, sunflower and peanut. The PVs were higher in this treatment, compared to the treatment 1. At 72 h, these were between 7–12 mEq Px/kg versus 1–6 mEq Px/kg for the LAC, in treatment 1 and 2 respectively.

De Leonardis *et al.* [46] reported in 2013, about the effect of laccase on the oxidative stability of olive oil and the role of endogenous phenols in the oxidation process of these oils. They found that olive oil with laccase had a lower resistance to oxidation at higher temperatures, as a consequence of partial depletion of endogenous polyphenols; however, oxidative stability was significantly lower than in chemically desfenolized oil analogues. These results suggested that the presence of newly formed compounds in oils treated with laccase could promote a pro-oxidant effect.

3.4. Conjugated Dienes (K232) and Conjugated Trienes (K270) in Treatment 2

Experimental data of the oven test in 60 °C have been analyzed according to the Eq. (2), and are illustrated in Fig. 1.

The increase in conjugated dienes was higher in the treated samples. The differences was statistically significant ($P \leq 0.05$) in chia and sunflower oils after 2 h of reaction. However, sesame oil showed the highest coefficients at 232 nm (>21 nm), suggesting that it has low resistance to storage at high temperatures.

The formation of conjugated trienes (K270) showed the same trend as for K232 (Fig. 1). This confirmed the negative effect of laccase on the oxidative stability of vegetable oils.

Petersen *et al.* [49] reported that laccase enzyme is adequate to prevent the oxidation of foods with a high content of vegetable oils because to its effective ability to take dissolved oxygen from the headspace, but those authors did not measure the direct effect on the oxidative stability of the oils.

4. Conclusions

Oxidative stability of vegetables oils depends on several factors such as; the unsaturation degree of fatty acids, the amount and type of antioxidants present, the enzymes action such us polyphenoloxidases, the availability of oxygen, light and exposure to elevated temperatures.

Our results indicate that laccase enzyme impairs the oxidative stability of edible vegetable oils, due to the increase of peroxide values and conjugated dienes and trienes, after the application of an enzymatic extract of *Trametes versicolor* containing laccase.

It may be argued that several partially oxidized by-products yielded by the enzymatic activity on the phenolic substrates could be responsible for the observed oxidative behavior.

Nevertheless, the application of polyphenol oxidases directly in vegetable oils can be exploited both for analytical purposes and to better understand the antioxidant role played by natural phenolic compounds.

Declaration of Competing Interest

The authors have declared no conflict of interest.

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