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ORIGINAL ARTICLE

Optimization of Oil Oxidation by Response Surface Methodology and the Application of this Model to Evaluate Antioxidants

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Abstract The oxidative stability of oils is a complex process influenced by several factors, making the evaluation of antioxidant effects of new compounds difficult. Thus, the objective of this study was to apply a factorial design to obtain the combination of factors that maximizes the formation of oil oxidation products, and use this model to evaluate the antioxidant activity of different compounds. Temperature, Fe^{2+} and ascorbyl palmitate were evaluated in two full-factorial designs (2^3 and 3^2). The validated optimized oxidation model was obtained by adding 1.47 mmol/L of Fe^{2+} and 1.54 mmol/L of ascorbyl palmitate to flaxseed oil stripped of tocopherol kept at 40 °C for 8 days. Antioxidant activities of six compounds were evaluated using this model. All antioxidant samples were statistically different ($p < 0.001$) at 200 ppm concentration, indicating the efficiency of the optimized model to evaluate the antioxidant action of natural and synthetic compounds.

Keywords Oil · Flaxseed · Minor components · Response surface methodology · Oxidation · Antioxidants

Introduction

Foods and edible oils with high contents of polyunsaturated fatty acids are especially susceptible to oxidative damage [1]. Oxidation of the edible oils is a natural reaction that is favored by many factors including heat, fatty acid composition, oxygen contact, light, absence of minor components such as tocopherols and polyphenols and the presence of free fatty acids, transition metals, pigments, and thermally oxidized compounds [2]. Autoxidation of polyunsaturated fatty acids is a free chain reaction that includes the steps of initiation, propagation and termination (Fig. 1). In the initiation, a free radical or initiator ($In^{\cdot}$) abstracts the hydrogen atom from the fatty acids or acylglycerol (LH) producing a lipid alkyl radical ($L^{\cdot}$), that will react very quickly with oxygen forming lipid peroxy radicals ($LOO^{\cdot}$). In the autoxidation process, the lipid alkyl radical abstracts hydrogen from other lipid molecules and reacts with oxygen to form hydroperoxides (LOOH) and another lipid alkyl radical ($L^{\cdot}$). In the presence of the metals or under high temperature the hydroperoxides are readily decomposed to alkoxyl radicals ($LO^{\cdot}$) and then form aldehydes, ketones, acids, esters and others secondary compounds [2].

Besides the alterations caused in the quality properties of flavor, color, texture and nutritional value, lipid oxidation leads to the formation of several secondary products that have mutagenic capacity and high toxicological potential [3, 4]. For this reason, many strategies have been developed to avoid the oxidation of oils and food emulsions. One of these strategies involves the addition of antioxidants (AH), which are compounds that extend the induction period of oxidation or slow the oxidation rate [4]. Antioxidants (AH) can donate hydrogen atoms to free radicals ($In^{\cdot}$) or react with lipid peroxyl radicals ($LOO^{\cdot}$) faster than lipids (LH). Antioxidants can also convert metal ions into insoluble complexes [2], through scavenging activity (Fig. 1). Generally, antioxidants have reduction potentials ($\approx$500 mV) lower than those of free radicals ($\approx$600–1,600 mV), and their own radicals are stabilized by resonance [2].

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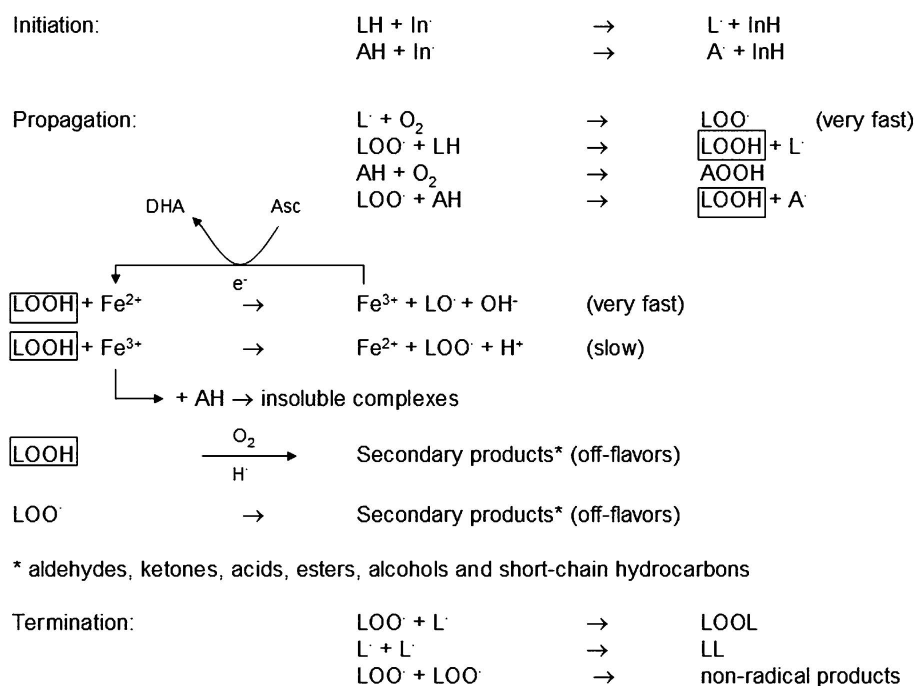

Fig. 1 Scheme of lipid oxidation considering the prooxidant effect of Fe^{2+} and ascorbyl palmitate (Fe^{2+}, ferrous ion; Fe^{3+}, ferric ion; LH, lipid alkyl; L·, lipid alkyl radical; LO·, alkoxyl radical; LOO·, peroxyl radical; LOOH, hydroperoxide; AH, antioxidant; A·, antioxidant radical, Asc; ascorbic acid; DHA, dehydroascorbic acid; In·, initiator; InH, stable initiator). (Adapted from 5, 3, 33)

Synthetic food additives such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butyl hydroquinone (TBHQ) are widespread chain-breaking antioxidants used in food systems [1, 5]. These compounds may be toxic [6, 7] and must be declared on the oil or food labels, causing rejection by consumers who associate a “clean label” with “safety and health” [4]. Several researchers have reported the antioxidant activities of a number of natural compounds, suggesting their use to replace synthetic antioxidants in foods [1, 8].

A large number of studies apply in vitro methodologies to evaluate the potential antioxidant action of compounds, using a wide variety of free radical generating systems, different methods of inducing oxidation and different end-points or chemical markers [9]. The most widely applied methods have been DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, ORAC (oxygen radical absorption capacity), $ABTS^{+}$ (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)) cation radical and FRAP (ferric reducing antioxidant power) [10]. Although these in vitro methodologies are very simple and allow the assessment of several samples at the same time, correlations with results observed in bulk oils or emulsions are not always high or significant. Confusing results have been reported depending on the protocols, methods and conditions used to test the antioxidant activity [9]. Therefore, despite numerous studies on this topic, there is no general and official method for assessing antioxidant capacity [11]. This discrepancy can be attributed to many factors, including fatty acid composition, surface area, storage conditions, and the presence of minor components. One factor less discussed in the literature is the short range of variation of the chemical markers or end-points used to measure the oxidation. In other words, the magnitude between minimum and maximum values of the chosen markers might be too narrow to reveal statistically significant differences in the antioxidant activities of different compounds. For this reason, a stripped oil rich in polyunsaturated fatty acids as the oxidizable substrate could be useful, if the oxidation conditions are optimized, as a model for oxidation. Among the factors able to increase the oil oxidation are temperature, iron and ascorbic acid content. Temperature is probably the most influential factor when assessing the extent of oxidation. Transition metals fully participate in the radical autoxidation mechanism via ·OH radicals. Ascorbic acid is a well-known prooxidant rather than an antioxidant in the presence of transition metal ions, such as iron [10].

According to Frankel and Finley [9], more valid and rigorous guidelines and assay protocols are required to shed light on discrepancies found in antioxidant literature. Thus, the objective of this study was to apply response surface methodology (RSM) to optimize oil oxidation, and then to use this optimized model to evaluate the antioxidant activities of different compounds.

Experimental Procedures

Materials

Iso-octane, 2-propanol, methanol, hexane and 1-butanol were obtained from Merck & Co. (Whitehouse Station, NJ,

Table 1 Primary and secondary products of the oil oxidation observed in the first $2^{(3-0)}$ full factorial design

Assay	Coded Factors			Original Range			Oil Oxidation Products			
	Temp (x_1)	$Fe^{2+}(x_2)$[a]	Asc (x_3)[b]	Temp (x_1) °C	$Fe^{2+}(x_2)$[a] mmol kg^{-1} oil	Asc (x_3)[b] mmol kg^{-1} oil	$LOOH^{c}_{max}$ meq kg^{-1} oil	Time[d] days	$TBARS_{max}$ mmol kg^{-1} oil	AUC^{e}_{LOOH} meq days kg^{-1} oil
1	−1.0	−1.0	−1.0	40	0.0	0.0	655.35	08	15.7	2365.60
2	1.0	−1.0	−1.0	60	0.0	0.0	569.34	06	14.0	1209.57
3	−1.0	+1.0	−1.0	40	1.0	0.0	700.35	07	19.9	1677.52
4	+1.0	+1.0	−1.0	60	1.0	0.0	617.36	07	15.3	1287.17
5	−1.0	−1.0	+1.0	40	0.0	1.5	626.97	10	18.7	3426.68
6	+1.0	−1.0	+1.0	60	0.0	1.5	458.36	09	15.0	1863.68
7	−1.0	+1.0	+1.0	40	1.0	1.5	740.57	09	27.0	2924.06
8	+1.0	+1.0	+1.0	60	1.0	1.5	492.17	04	11.7	1217.43
9[f]	+1.0	+1.0	+1.0	60	1.0	1.5	476.92	04	12.4	1211.06
Pooled SD							98.49	–	16.6	800.60
P (Hartley)[g]							0.731	0.999	0.440	0.174
P (ANOVA)[h]							<0.001	<0.001	<0.001	<0.001

[a] Iron (II) sulfate heptahydrate (Fe^{2+})

[b] Ascorbyl palmitate

[c] Maximum hydroperoxides concentration

[d] Days to achieve the maximum hydroperoxide concentration

[e] Area under the hydroperoxides formation curve (relative only to the ascendant part)

[f] Replicate of the assay number 8

[g] Probability value obtained from Tests of Homogeneity of Variances (Hartley)

[h] Probability value obtained from one-way ANOVA

[i] Values expressed as means ± SD ($n = 2$)

USA). The soybean oil was purchased at a local market. Flaxseed oil (stored in the dark at 4 °C), ammonium thiocyanate, barium chloride, iron(II) sulfate heptahydrate, 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), silicic acid, activated charcoal, 1,1,3,3-tetraethoxypropane (TEP), cumene hydroperoxide, ascorbyl palmitate, α-tocopherol, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), tocopherol, caffeic acid, gallic acid, catechin and *tert*-butyl hydroquinone (TBHQ) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The organic solvents and water were HPLC grade. All other reagents used in the experiment were analytical grade.

Crude and refined oils contain endogenous antioxidants that may compete with the tested antioxidants, which could markedly bias the results [10]. Thus, we opted to use an oil that was stripped of its minor compounds. Flaxseed (or linseed) was used in this model because of its high content of α-linolenic acid and high susceptibility to oxidation [12]. The oil was stripped of tocopherol according to the method proposed by Khan and Shahidi [13] and modified by Waraho et al. [14].

Experimental Design and Statistical Analysis

Two different experimental designs were applied in this study. The objective of the first design was to screen the variables that had a significant influence on all of the evaluated responses. Usually, designs containing two levels (2^k) are applied to screen variables. Once the variables were selected, a second design was carried out to identify the curvature of the responses so as to obtain a better adjustment of the models to the experimental data and for further optimization.

First Design

The objective of the first design was only to identify which of the selected factors were relevant to the oxidative reaction and to determine the behavior of these variables. For this reason, a full-factorial design ($2^{(3-0)}$) was applied to evaluate the effect of three factors on stripped flaxseed oil oxidation at two variation levels: −1 and +1. The factors were temperature (x_1), Fe^{2+} (ferrous ion) content (x_2) and ascorbyl palmitate content (x_3); and the design is shown in Table 1. The responses were firstly submitted to a

homogeneity test (Hartley) and to an one-way ANOVA, adopting 0.05 as the p value. These proceedings are necessary because there is no reason to use a regression model when the results do not present variation. In our study, all the measurements were replicated, and the results were separated into two blocks. In addition, an extra assay was performed to add one more degree of freedom, which was useful for checking the model's quality. This extra additional assay is usually carried out in the central point, but in our study we opted to duplicate the last point. This decision was because in the beginning of the experimental design some qualitative variables (with no central point) had been included, however, they were removed after preliminary tests. Based on the results from the nine assays with replication, a total of seventeen degrees of freedom were used to fit the model:

$$\hat{y}i = \beta_0 + \beta_1 \times x_1 + \beta_2 \times x_2 + \beta_3 \times x_3 + \beta_{12} \times x_1x_2 + \beta_{13} \times x_1x_3 + \beta_{23} \times x_2x_3 + \beta_{123} \times x_1x_2x_3 \quad (1)$$

where $\hat{y}i$ = estimated response, b_i = coefficients estimated by the least square method and x_i = dependent variables. Non-significant coefficients were excluded and the models were readjusted. The coefficient of determination (Adjusted R^2) and ANOVA were applied to check the models′ quality. Based on the first design analysis, the temperature was kept at 40 °C. Both Fe^{2+} and ascorbyl palmitate were the variables selected for the second experimental design, in order to determine what relative content of these two compounds would yield the highest oil oxidation.

Second Design

A new factorial design ($3^{(2-0)}$) was applied to the variables selected from the first design. Fe^{2+} and ascorbyl palmitate were chosen as factors based on the significance of their β's coefficients observed to all responses, showing contrary signals to $LOOH_{max}$ (maximum concentration of hydroperoxides), $TBARS_{max}$ (maximum concentration of thiobarbituric acid reactive substances) and AUC_{LOOH} (area under the curve), while the effect of temperature (β_1) presented negative signals for all responses. Fe^{2+} and ascorbyl palmitate had a range within three levels (Table 2), designated as −1, 0 and +1. This new design allows the model curvature to be detected and requires nine assays. There were other design options to investigate the model curvature, such as the central composite or Box Behnken designs, but we applied a 3^2 full factorial design because it seemed rapid enough to achieve the objectives of this study. Similar to the first design, an additional point was included, and all of the assays were performed twice. Thus, nineteen degrees of freedom were used to adjust the following second-order model:

$$\hat{y}i = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j}\sum + \beta_{ij} x_i x_j \quad (2)$$

where $\hat{y}i$ = estimated response, b_i = coefficients estimated by the least square method, x_i = dependent variables. The same proceedings applied in the first design were also used to check the fitness of the models obtained from this second design. In addition, the models obtained for the four responses were optimized based on methodology proposed by Derringer and Suich [15]. Optimization was achieved by looking for the maximum values for AUC_{LOOH}, $LOOH_{max}$ and $TBARS_{max}$ as well as the minimum values for time to achieve the $LOOH_{max}$ (TIME). Validation was carried out based on three randomized points ($x_1 = 0.73$ mmol/L and $x_2 = 0.71$ mmol/L; $x_1 = 0.20$ mmol/L and $x_2 = 2.60$ mmol/L; and $x_1 = 1.81$ mmol/L and $x_2 = 1.32$ mmol/L) by applying the same experimental procedures used to build the models. The observed and estimated values were compared by a Chi square test. Statistical analysis and graphical representations were calculated using STATISTICA v.9 software (Statsoft Inc., Tulsa, OK).

Preparation of Minor Polar Component-Stripped Oils

Flaxseed oil was stripped of tocopherol according to the methodology proposed by Khan and Shahidi [13] and modified by Waraho et al. [14]. Briefly, samples were prepared by dissolving 30 g of oil in 100 mL of hexane. Then, the mixture was passed through a chromatographic column (29.5-mm ID, 350 mm in length; Wilmad Labglass No.:LG-4567T-130 w/Fritted Disc and PTFE Stopcock). The process was repeated adding 100 mL of hexane through the chromatographic column, and after adding another 70 mL of hexane. The bottom layer of the column was packed with 22.5 g of silicic acid that had been washed three times with distilled water, filtered and activated at 110 °C for 24 h according to Waraho et al. [14]. A middle layer of 5.625 g of activated charcoal and a top layer of 22.5 g of washed, filtered and activated silicic acid were used in the column. The solvent from the eluate was removed using a rotary evaporator (Rotavac valve tec, Heildolph, Germany) at 30 °C. Traces of hexane were removed by flushing the column with nitrogen. The stripped and non-stripped oils were stored at −80 °C until use. The column containing the oil was protected from light using aluminum foil paper. The stripped oil was received in an Erlenmeyer flask also covered by foil paper and immersed in an ice bath.

Table 2 Primary and secondary products of the oil oxidation observed in the second $3^{(2-0)}$ full factorial design

Assay	Coded factors		Original range		Oil oxidation products (responses)			
	$Fe^{2+}(x_1)$[a]	Asc (x_2)[b]	$Fe^{2+}(x_1)$[a] mmol kg^{-1} oil	Asc (x_2)[b] mmol kg^{-1} oil	$LOOH_{max}$[c] meq kg^{-1} oil	Time[d] days	$TBARS_{max}$ mmol kg^{-1} oil	AUC_{LOOH}[e] meq days kg^{-1} oil
1	−1.0	−1.0	0.0	0.0	655.35	8	15.67	2365.88
2	−1.0	0.0	0.0	1.5	626.97	10	18.69	3424.89
3	−1.0	+1.0	0.0	3.0	675.51	8	25.45	1620.63
4	0.0	−1.0	1.0	0.0	700.35	7	18.63	1680.11
5	0.0	0.0	1.0	1.5	740.57	9	26.99	2925.74
6	0.0	+1.0	1.0	3.0	536.62	10	38.25	2663.57
7	+1.0	−1.0	2.0	0.0	760.70	9	35.44	2858.70
8	+1.0	0.0	2.0	1.5	737.01	7	30.82	1688.05
9	+1.0	+1.0	2.0	3.0	728.66	8	39.33	2475.52
10[f]	+1.0	+1.0	2.0	3.0	721.41	8	39.01	2357.77
Pooled SD					66.18	–	8.86	584.83
P (Hartley)[g]					0.531	0.998	0.073	0.189
P (ANOVA)[h]					<0.001	<0.001	<0.001	<0.001

[a] Iron (II) sulfate heptahydrate (Fe^{2+})

[b] Ascorbyl palmitate

[c] Maximum hydroperoxides concentration

[d] Days to achieve the maximum hydroperoxide concentration

[e] Area under the hydroperoxides formation curve (relative only to the ascendant part)

[f] Replicate of the assay number 9

[g] Probability value obtained from Tests of Homogeneity of Variances (Hartley)

[h] Probability value obtained from one-way ANOVA

[i] Values expressed as means ± SD ($n = 2$)

Lipid Oxidation Measurements

Ascorbyl palmitate was added directly to the oil according to the concentration described in Table 1 and 2 (original range). Iron (II) sulfate heptahydrate was first diluted in a mixture of water and Tween 20 (1.0%, w/w) (27.8 mg/5.0 mL) and then added to the oil (250 µL/5.0 g oil). The oil samples (0.5 mL) were transferred to 1.5-mL, opaque, unsealed vials and stored in the dark at a fixed temperature (40 °C) in an oven (L.S. 1.0 A, Logen Scientific, Diadema, SP) for twelve days. At different time intervals, two vials from each assay were taken to evaluate the chemical markers of oxidation. All assays were performed in duplicate.

Lipid hydroperoxide concentrations were determined according to procedures of Shanta and Decker [16] with some modifications. Oil samples (50 µL) were mixed with 1.75 mL of an iso-octane/2-propanol solution (3:1, v/v), which resulted in a final volume of 1.80 mL. The mixture was vortexed three times for 10 s, and 20 µL of the mixture was added to a 2.98 mL solution of methanol/1-butanol (2:1, v/v). The final volume of the sample was 3.0 mL. A thiocyanate/ferrous solution was prepared by mixing 500 µL of 3.94 M thiocyanate solution with 500 µL of 0.072 M Fe^{2+} solution. The 0.072 M Fe^{2+} solution was obtained from the supernatant of a mixture of 1.5 mL 0.144 M $FeSO_4$ and 1.5 mL 0.132 M $BaCl_2$ in 0.4 M HCl. The thiocyanate/ferrous solution (30 µL) was added to the methanol/1-butanol mixture (3.0 mL), vortexed and incubated at room temperature for 20 min. Following the incubation period, the samples' absorbance readings were measured at 510 nm using a UV–Vis mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan). Due to the high concentrations of hydroperoxides, some samples were diluted in a methanol/1-butanol mixture prior to reading, with dilutions ranging from 1:10 to 1:20 (v/v). The hydroperoxide content was determined using a standard curve prepared with known concentrations of cumene hydroperoxide. Concentrations were expressed as meq kg^{-1} oil. Lipid hydroperoxide concentrations were measured for 12 days. During this time, all assays showed an increase, followed by a reduction in their hydroperoxide concentration. The values obtained during the ascendant phase were considered for analysis. Based on these data, a cubic-order polynomial function was fitted for each sample. The maximum hydroperoxide concentration ($LOOH_{max}$) and the area under the curve (AUC_{LOOH}) were selected as markers of the primary products of the oxidation. AUC

values were calculated using MATHEMATICA v.7 software (Wolfram Research Inc., Champaign, IL).

The amount of thiobarbituric acid reactive substances (TBARS) in the samples was determined using a modified method from McDonald and Hultin [17]. The oil (0.02 mL) was diluted with 0.98 mL of an iso-octane/2-propanol mixture (3:1, v/v), mixed with 2.0 mL of TBA reagent (15% w/v trichloroacetic acid and 0.375% w/v thiobarbituric acid in 0.25 M HCl, mixed with 2% BHT in ethanol) in test tubes and placed in a boiling water bath for 15 min. The tubes were cooled at room temperature for 10 min then centrifuged at 1,000*g* for 15 min. The absorbances were read at 532 nm using a UV–Vis mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan). Due to the high concentrations of TBARS, some samples were diluted in an iso-octane/2-propanol mixture (3:1, v/v), with dilution factors ranging from 1:10 to 1:40 (v/v). The concentrations of TBARS were determined based on a standard curve prepared using 1,1,3,3-tetraethoxypropane (TEP). The concentrations were expressed as mmol kg^{-1} oil. The TBARS concentration was followed for 12 days and only the ascendant part of the curve was analyzed. The maximum TBARS concentration ($TBARS_{max}$) was selected as a marker of secondary products from oxidation.

Evaluation of the Natural and Synthetic Compounds using the Optimized Model

Trolox (0.0, 2.0, 4.0, 6.0 and 8.0 mg), α-tocopherol (0.0, 2.0, 4.0, 6.0 and 8.0 mg), caffeic acid (0.0, 2.0, 4.0, 6.0 and 8.0 mg), gallic acid (0.0, 2.0, 4.0, 6.0 and 8.0 mg), catechin (0.0, 2.0, 4.0, 6.0 and 8.0 mg) and TBHQ (0.0, 2.0, 4.0, 6.0 and 8.0 mg) were dissolved in 2.0 mL methanol, and 25 µL of each solution was mixed with 0.5 mL stripped flaxseed oil, Fe^{+2} (1.47 mmol/L) and ascorbyl palmitate (1.54 mmol/L). The solutions were protected from air and light by keeping them covered and adding nitrogen inside the tubes. Vials containing 0.5 mL of the solutions were kept at 40 °C for 8 days. Hydroperoxide and TBARS concentrations were determined for all samples following the methodologies described above. A second-degree polynomial model was adjusted to the "oxidation products concentration x compound concentration" and was used to determine the IC_{50} (half maximal inhibitory concentration) value for each compound.

HPLC Analysis of Tocopherols

The flaxseed oil tocopherol content was determined using a modified method of Gliszczynska-Swiglo and Sikorska [18]. All analyses were performed using an HPLC (Agilent Technologies 1200 series, Santa Clara, CA, USA), equipped with a Zorbax Reverse-phase C18 column (150 mm x 4,6 mm; 5 µm) with pre-column LC8-D8 (Phenomenex AJ0-1287, Torrance, USA). For the determination of α, (β + γ) and δ-tocopherols in the oils, a mobile phase consisting of 50% acetonitrile (solvent A) and 50% methanol (solvent B) was used with a flow rate of 1 mL/min and an isocratic method under a constant pressure of 34 bar. The injection volume was 20 µl and a rheodyne-type injector was used. The eluate, containing 0.125 mL oil sample plus 1.125 mL isopropanol, was detected using a diode array detector (DAD) set at 292 nm. Tocopherols (α, β + γ and δ) were identified by comparing their retention times with those of corresponding standards. Results were expressed as mg kg^{-1} oil. To validate tocopherol results, soybean oil was also subjected to the stripping process and tocopherol analysis.

Results and Discussion

A tocopherol analysis of both full and stripped flaxseed oil showed an absence of α-, β + γ- and δ-tocopherols. Values from 0.0 to 5.0 mg kg^{-1} oil and from 100.0 to 150 mg kg^{-1} oil were expected for α- and β + γ-tocopherol, respectively [12]. We attribute the differences between our experimental values and the expected values to the processing (refining) applied on our samples or to the possibility that tocopherol oxidization could have occurred during transportation. Soybean oil was also evaluated, containing α- (156.81 ± 6.65 mg/kg), β + γ- (676.15 ± 3.18 mg/kg) and δ- (244.59 ± 1.81 mg/kg) tocopherols. In contrast, no tocopherols were found in the stripped form of soybean oil. This pattern suggests that the stripping method used in this assay was suitable to remove tocopherols from oils. Although no tocopherols were found in the flaxseed oil used in these experiments, the stripping process was important for removing all other minor components that might interfere in the oxidation process. Flaxseed oil contains approximately 53% linolenic acid, so it is a suitable substrate to produce malondialdehyde, which was measured as TBARS.

Oxidation of stripped flaxseed oil in $2^{(3-0)}$ and $3^{(2-0)}$ full-factorial designs (shown in Tables 1 and 2, respectively) were analyzed by measurement of the maximum concentrations of hydroperoxides ($LOOH_{max}$), the time taken to reach the $LOOH_{max}$ (TIME), the maximum concentrations of thiobarbituric acid reactive substances ($TBARS_{max}$) and the area calculated by the integration of the peroxide value curve from time zero to the time when $LOOH_{max}$ was reached (AUC_{LOOH}). The significant differences observed among the assays in all four parameters ($p < 0.001$) allowed the adjustment of the models to the experimental data.

In general, an increase in temperature accelerated the oxidative deterioration of the primary products and increased the volatility of the secondary products. This trend resulted in reduced concentrations of primary and secondary reaction products, as shown by lower $LOOH_{max}$, $TBARS_{max}$ and AUC_{LOOH} values (Tables 1, 3). Since our objective was to obtain the maximum content of oxidation products, the temperature value was fixed at 40 °C for all the subsequent evaluations. The presence of Fe^{2+} also accelerated the oxidative reaction, increasing hydroperoxide and TBARS formation, as well as decreasing the time taken for these substances to reach their peak amounts. The presence of ascorbyl palmitate resulted in both antioxidant and prooxidant effects depending on its concentration and interactions with other compounds, such as iron. Table 3 presents the coefficients of the polynomial models for the $(2^{(3-0)})$ full-factorial design, which was adjusted to the four oxidation end-points. The block effect, the p value relative to "lack-of-fit" and the adjusted coefficient of determination (R^2_{adj}) were analyzed. The polynomial models fit the data well as shown by their non-significant lack-of-fit value, non-significant block effect and high R^2_{adj} values (>0.94).

Table 4 presents the coefficients of the polynomial models for the $3^{(2-0)}$ full-factorial design, which were adjusted to the four oxidation end-points. The block effect, the p value relative to the "lack-of-fit" and the adjusted coefficient of determination (R^2_{adj}) were also analyzed. Similar to the first design, the polynomial models also fit the data well as shown by the non-significant lack-of-fit value, the non-significant block effect and high R^2_{adj} values (> 0.98). Figure 2 shows the response surface plots obtained from the second $3^{(2-0)}$ full-factorial design, which were applied to $LOOH_{max}$, time, $TBARS_{max}$ and AUC_{LOOH}. The optimization using all four parameters suggested that maximum oxidation could be achieved with stripped flaxseed oil containing Fe^{+2} at a concentration of 1.47 mmol/L and ascorbyl palmitate at a concentration of 1.58 mmol/L. These results demonstrate a clear additive effect between these two factors. Figure 3 presents the observed and estimated results obtained at three randomized points, which had been evaluated with the same experimental procedures used for building the models. No statistical differences were observed between the experimental and estimated points in all four parameters for all three randomized points, thereby suggesting the high quality of the models used in this study.

Oil oxidative stability evaluation is a complex process that involves the use of least two different analytical techniques to obtain an adequate description of the process [4]. The choice of chemical markers or end-points to describe the process depends on many factors, including fatty acid compositions, presence of minor polar compounds, temperature, light exposition and surface area. Furthermore, small variations on these factors can deeply change the chemical markers' values, becoming difficult to compare the antioxidant action of new compounds. With this limitation in mind, we proposed to develop a model to evaluate oil oxidation using very simple chemical markers at their maximum levels of variation. Our model was

Table 3 Coefficients of the polynomial models adjusted to the primary and secondary products observed in the first $2^{(3-0)}$ full factorial design and quality evaluation of the model fitness

Regression coefficients	$LOOH_{max}$	Time	$TBARS_{max}$	AUC_{LOOH}
Mean (β_0)	605.37 ($p < 0.001$)	7.48 ($p < 0.001$)	17.20 ($p < 0.001$)	1.996.07 ($p < 0.001$)
Temperature (β_1)	−75.44 ($p = 0.001$)	−1.03 ($p = 0.001$)	−3.11 ($p = 0.002$)	−602.40 ($p < 0.001$)
Fe^{2+} (β_2)[a]	+27.87 ($p = 0.010$)	−0.78 ($p = 0.001$)	+1.36 ($p = 0.008$)	−220.32 ($p < 0.001$)
Asc (β_3)[b]	−30.23 ($p = 0.009$)	+0.48 ($p = 0.002$)	+0.98 ($p = 0.015$)	+361.10 ($p < 0.001$)
Temp × Fe^{2+} (β_{12})	–	–	−1.77 (p = 0.005)	+77.36 ($p = 0.001$)
Temp × Asc (β_{13})	−33.18 ($p = 0.007$)	−0.48 ($p = 0.003$)	−1.55 ($p = 0.006$)	−215.81 ($p < 0.001$)
Fe^{2+} × Asc (β_{23})	–	−0.78 ($p = 0.001$)	–	−67.70 ($p = 0.001$)
Temp × Fe^{2+} × Asc (β_{123})	–	−0.77 ($p = 0.001$)	−1.04 ($p = 0.014$)	− 114.06 ($p < 0.001$)
p (replicate)[c]	0.112	0.684	0.102	0.016
p *(lack of fit)*[d]	0.205	0.069	0.400	0.050
Adjusted R^2	0.946	0.985	0.979	0.998

[a] Iron (II) sulfate heptahydrate (Fe^{2+})

[b] Ascorbyl palmitate

[c] Probability value observed for the replicates (blocks)

[d] Probability value obtained from one-way ANOVA

[e] For example, the regression should be read as: $\hat{y}_{LOOH} = 605.37 - 75.44x_1 + 27.87x_2 - 30.23x_3 - 33.18x_1x_3$

Table 4 Coefficients of the polynomial models adjusted to the primary and secondary products observed in the second $3^{(2-0)}$ full factorial design and quality evaluation of the model fitness

Regression coefficients	$LOOH_{max}$	Time	$TBARS_{max}$	AUC_{LOOH}
Mean (β_0)	740.57 ($p < 0.001$)	9.05 ($p < 0.001$)	26.99 ($p < 0.001$)	2925.75 ($p < 0.001$)
Linear Fe^{2+} (β_1)[a]	+55.02 ($p = 0.003$)	−1.47 ($p = 0.001$)	+6.07 ($p < 0.001$)	−868.42 ($p = 0.002$)
Quadratic Fe^{2+} (β_1^2)	−58.58 ($p = 0.007$)	−0.58 (p = 0.011)	−2.24 ($p = 0.010$)	−369.28 ($p = 0.036$)
Linear Asc (β_2)[b]	−81.87 ($p = 0.001$)	+1.50 ($p = 0.001$)	+9.81 ($p < 0.001$)	+491.73 ($p = 0.007$)
Quadratic Asc (β_2^2)	−122.08 ($p = 0.002$)	−0.60 ($p = 0.010$)	+1.45 ($p = 0.024$)	−753.91 ($p = 0.009$)
Fe^{2+} (β_1) x Asc (β_2)	−13.96 ($p = 0.019$)	−0.25 ($p = 0.009$)	−1.51 ($p = 0.003$)	–
p (replicate)[c]	0.278	1.000	0.567	0.854
p (lack of fit)[d]	0.323	0.376	0.157	0.634
Adjusted R^2	0.983	0.992	0.996	0.982

[a] Iron (II) sulfate heptahydrate (Fe^{2+})

[b] Ascorbyl palmitate

[c] Probability value observed for the replicates (blocks)

[d] Probability value obtained from one-way ANOVA

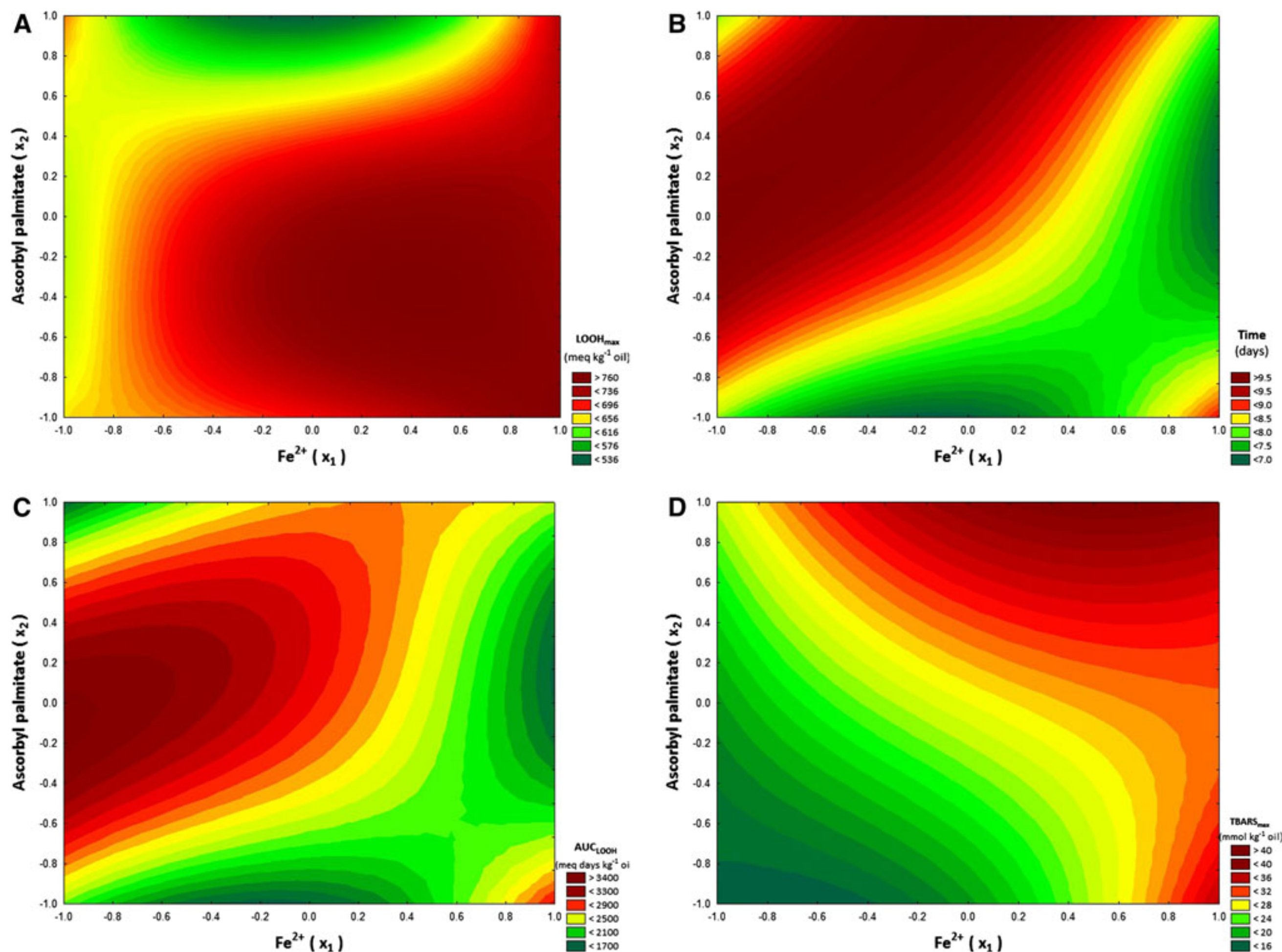

Fig. 2 Surface plots of the second $3^{(2-0)}$ full factorial design showing the effects of Fe^{2+} and 6-O-palmitoyl-L-ascorbic acid on stripped flaxseed oil oxidation (**a** $LOOH_{max}$, **b** time, **c** AUC_{LOOH} and **d** $TBARS_{max}$)

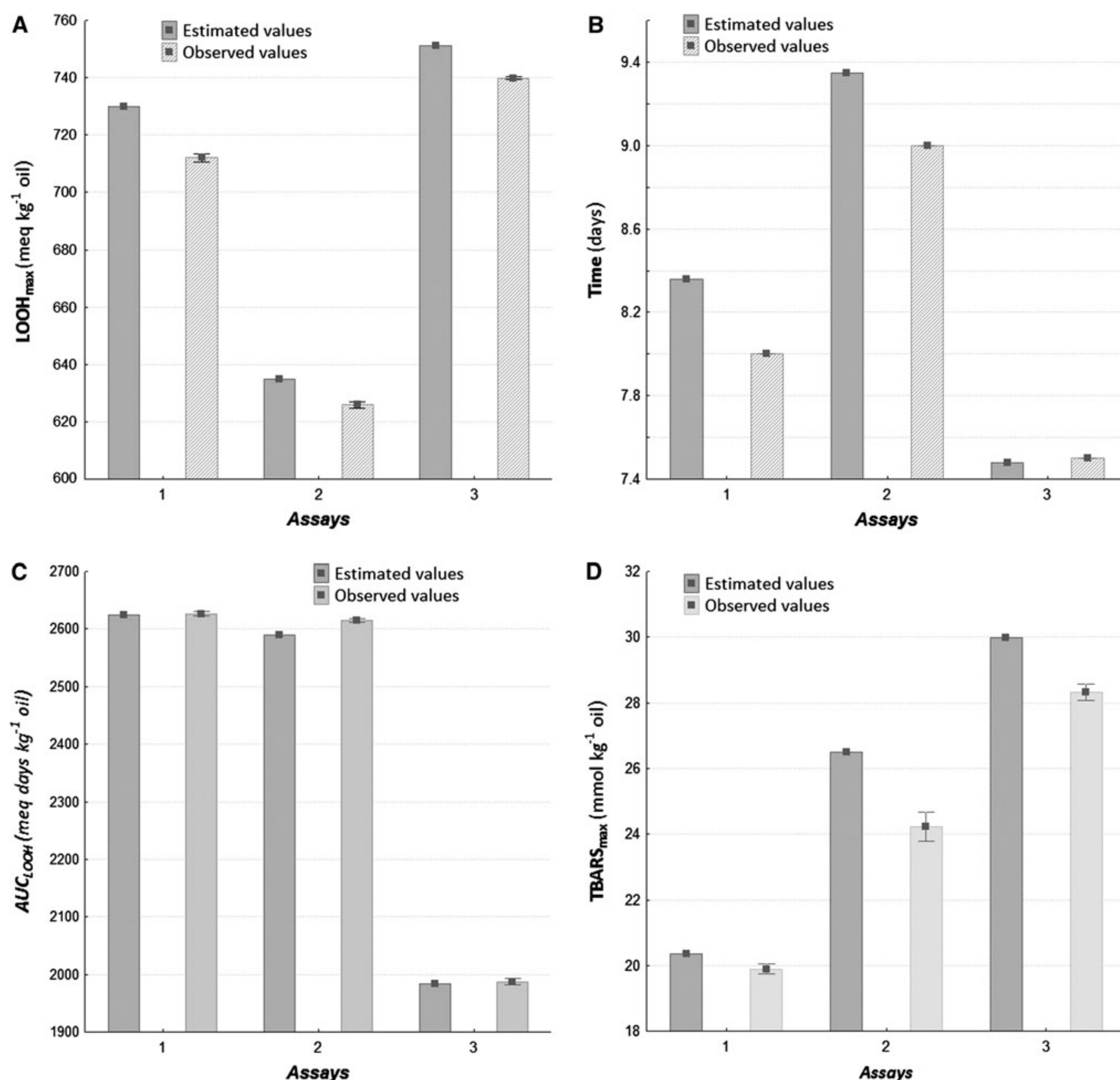

Fig. 3 Validation of the optimized system based on the second $3^{(2-0)}$ full factorial design expressed as mean values ($n = 2$). **a** $LOOH_{max}$ (Chi-square = 1.469549; $df = 5$; $p < 0.916556$); **b** time (Chi-square = 0.0567427; $df = 5$; $p < 0.999960$); **c** AUC_{LOOH} (Chi-square = 0.5341780; $df = 5$; $p < 0.990818$); and **d** $TBARS_{max}$ (Chi-square = 0.6056443; $df = 5$; $p < 0.987743$)

obtained through use of flaxseed oil stripped of its tocopherols added Fe^{2+} (1.47 mmol/L) and ascorbyl palmitate (1.54 mmol/L) at 40 °C for 8 days. Four markers were originally used to evaluate the oil oxidation in our study. However, as observed in Fig. 2, it was evident that the AUC_{LOOH} (Fig. 2c) was very similar to the time to reach $LOOH_{max}$ (Fig. 2b). Thus, our discussion was based on the effects of these three markers: $LOOH_{max}$, the time to reach $LOOH_{max}$ and TBARS.

The increase in temperature from 40 to 60 °C significantly decreased the LOOH ($\beta_1 = -75.44$), but had minor effects on TBARS concentration ($\beta_1 = -3.11$) (Table 3). Lipid oxidation reactions have high activation energies (16.2 kcal mol^{-1}). Thus, there is an increase in autoxidation of oils and decomposition of hydroperoxides as the temperature increases [2, 19]. The effect of temperature on the oxidation of oils containing high amounts of polyunsaturated fatty acids is known and can be estimated by the Arrhenius equation [20]. The clear influence of temperature on the formation of hydroperoxides was reported by the observation of increases in LOOH values (from 0 to 600 mmol/kg) in stripped rapeseed oil stored between

5 and 60 °C [21]. However, Yin and Sathievel [21] reported that menhaden oil stored at 50 °C had lower rates of hydroperoxide formation than when it is stored at room temperature. The authors attributed this fact to the higher decomposition of primary hydroperoxides at 50 °C. Considering that our objective was to achieve maximum LOOH values, the temperature of 40 °C was obviously chosen for the second step of optimization. Still in the first experimental design, the prooxidant effect of the Fe^{2+} (x_2) at 1.0 mmol/L and the antioxidant effect of the ascorbyl palmitate (x_3) at 1.5 mmol/L were evident when the LOOH concentration ($\beta_2 = +27.87$ and $\beta_3 = -30.23$) and time to achieve the maximum LOOH level ($\beta_2 = -0.78$ and $\beta_3 = +0.48$) were observed (Table 3). Transition metals such as iron and copper promote the formation of free radicals by decomposing LOOH through redox reactions (Fig. 1). The increase observed in LOOH formation when Fe^{2+} was present was caused both by the reducing effect of Fe^{2+} on the LOOH scission that produces $LO^{\cdot}$ (alkoxyl radical) + OH^{-}, and the oxidant effect of the Fe^{3+} that forms $LOO^{\cdot}$ (peroxyl radical) + H^{+} (Fig. 1), exponentially raising the oxidation rates. On the other hand, until the concentration of Fe^{2+} was raised to 1.5 mmol/L, ascorbyl palmitate showed only antioxidant action. Ascorbic acid and ascorbyl palmitate are powerful reducing agents that are able to inactive metals and, thus, reduce the rate of initiation and decomposition of LOOH, to reduce LOOH to stable alcohols and to scavenge oxygen [19]. However, according to Niki and Noguchi [22], ascorbic acid acts like a prooxidant when the ascorbic acid/Fe^{3+} ratio is low. No significant interactions (β_{23}) were observed when Fe^{2+} and ascorbyl palmitate were added together to the $LOOH_{max}$ and $TBARS_{max}$ responses. A prooxidant effect has been extensively reported when mixtures of ascorbic acid and Fe^{2+} are added to oils [23]. For this reason, the second factorial design was performed using a wider range of variation for these two factors.

Fixing the temperature at 40 °C, the maximum LOOH concentration at the minimum time associated to the maximum values of TBARS were achieved when Fe^{+2} was added near its highest concentration (1.47 mmol/L; range 0–2 mmol/L) and with ascorbyl palmitate at an intermediate level (1.54 mmol/L; range 0–3 mmol/L). This effect can be easily visualized in Fig. 2. For example, without Fe^{2+}($x_2 = -1$), ascorbyl palmitate exerts an antioxidant action (green and yellow surfaces; clearer surfaces) (Fig. 2a). However, when Fe^{2+} increases from $x_2 = -1$ to $+1$, the LOOH concentration achieves its maximum value (red surface; darker surface) at the intermediate concentration of ascorbyl palmitate ($x_3 = 0$). The LOOH concentration reduces again when ascorbyl palmitate is closer to its highest level ($x_3 = +1$). These trends show that the prooxidant effect of the combination of ascorbyl palmitate and Fe^{2+} is strongly dependent on their relative concentrations. Although this observation is largely known, this is the first time that it has been graphically shown.

After decomposing hydroperoxides (LOOH), iron remains in its oxidized form (Fe^{3+} (ferric ion)), which slowly reacts with other LOOH. Ferrous ion (Fe^{2+}) acts 100 times faster than ferric ion (Fe^{3+}) [2]. Ascorbyl palmitate reduces Fe^{3+} to Fe^{2+}, increasing the oxidation rate and LOOH decomposition. This explains the synergistic oxidant effects observed between Fe^{2+} and ascorbyl palmitate in our model, which caused a decrease in the peak time ($\beta_{23} = -0.78$). Let et al. [24] reported that, at high concentrations of ascorbyl palmitate (300 mg/kg), the regeneration of metal ions might override the antioxidant properties, resulting in prooxidant effects in salad dressing enriched with fish oil. Micciché et al. [25], investigating iron-based alkyd paints driers using methyl linoleate as substrate, observed that the molar ratio of 2:1 (ascorbyl palmiate:iron-2-ethylhexanoate) reduced the lag time to zero, resulting in a LOOH of 600 mmol/kg after 100 h. They also observed that the oxidation occurred slower when the proportion of ascorbyl palmitate was above 2:1, which might be attributable to the antioxidant properties of ascorbyl palmitate above this critical concentration.

The antioxidant activities of the compounds chosen to test the model were evaluated by LOOH (hydroperoxide) concentration and TBARS concentration, expressed as IC_{50} values (Table 5). In this study, IC_{50} was the concentration of the antioxidant required for a 50% oxidation inhibition compared with the control without antioxidant. All compounds showed non-significant heterogeneity of variances ($p > 0.5$) and significant differences ($p < 0.001$), indicating that the model was suitable for evaluating natural and artificial compounds. The IC_{50} values were also reported in molar concentrations. Except for two samples analyzed by TBARS (α-tocopherol and gallic acid), statistically significant differences ($p < 0.001$) were observed among all of the compounds. The six compounds chosen to test our model have different molecular structures, solubilities, numbers of hydroxyl groups and numbers of aromatic rings, which contribute to their different antioxidant performances using in vitro methodologies [7, 8]. However, when these same compounds are applied in bulk oils or emulsions, taking the polar paradox into account, the differences of the results observed in the in vitro methodologies are often not confirmed. For example, in our study, TBHQ showed the best antioxidant activity, which is in agreement with the results reported by Bera et al. [5], whereas the phenolic compounds, Trolox and tocopherol had less antioxidant activity, although their antioxidant activities measured against free radicals have been reported to be high in other in vitro evaluations [26, 27]. According to Maqsood and Benjakul [7], caffeic and ferulic acids had

Table 5 Antioxidant activity of the compounds evaluated by IC_{50} to the LOOH and TBARS concentration, using the optimized system

Compounds[a]	IC 50 (mg/kg) to LOOH	IC 50 (mg/kg) to TBARS	IC 50 (mmol/L) to LOOH	IC 50 (mmol/L) to TBARS
α-Tocopherol	$170.00^{a} \pm 0.01$	$78.25^{a} \pm 1.06$	$0.39^{a} \pm 0.01$	$0.19^{a} \pm 0.01$
Trolox	$52.95^{d} \pm 0.06$	–	$0.21^{d} \pm 0.01$	–
Caffeic acid	$46.88^{e} \pm 0.11$	$49.25^{b} \pm 1.06$	$0.26^{c} \pm 0.01$	$0.27^{b} \pm 0.01$
Gallic acid	$56.10^{c} \pm 0.14$	$30.47^{d} \pm 0.76$	$0.33^{b} \pm 0.01$	$0.18^{d} \pm 0.01$
Catechin	$59.75^{b} \pm 0.00$	$35.15^{c} \pm 0.49$	$0.21^{e} \pm 0.01$	$0.20^{c} \pm 0.01$
TBHQ	$28.73^{f} \pm 0.01$	$24.95^{e} \pm 0.21$	$0.17^{f} \pm 0.01$	$0.15^{e} \pm 0.01$
P (Hartley)[b]	0.510	0.780	0.450	0.721
P (ANOVA)[c]	< 0.001	< 0.001	< 0.001	< 0.001

[a] Values expressed as means ± SD ($n = 2$)

[b] Probability value obtained from Tests of Homogeneity of Variances (Hartley)

[c] Probability value obtained from ANOVA. Values in the column followed by the same upper script letter are not statistically different

statistically significant differences in their antioxidant activity when measured by lipoxygenase inhibition, ABST radical scavenging, FRAP and methyl chelating activity, while similar effects were not observed on the prevention of the formation of hydroperoxides in fish oil-in-water emulsions ($p > 0.05$).

Our hypothesis is that, in some situations, the discrepancy observed between in vitro methods and when the compounds are directly applied in oils and emulsions could be caused by the narrow range of oxidation markers variation, causing the measured results to be too close to allow statistically significant differences. For example, in our study, the range of LOOH variation prior to optimization was 0.0–458 meq kg^{-1} oil, whereas after optimization the LOOH values ranged from 0.0 to 751.13 meq kg^{-1} oil. In the optimized condition, the separation of the antioxidant effects is greater than in the non-optimized one. Although other factors are also involved on the differences observed between the results obtained by in vitro methods and those found directly in the food matrix, the maximization of the end-points range can surely contribute to better describe these differences. The model developed in our study was built using most of the conditions recommended by Frankel and Finley [12], including the use of a suitable substrate (flaxseed oil), relatively mild conditions of oxidation (below 60 °C), initial low levels of primary products (LOOH = 3.09 ± 0.75 meq kg^{-1} oil) and secondary products (TBARS = 0.02 ± 0.01 mmol kg^{-1} oil), and the inclusion of initiation and early stages of the propagation phases of oxidation (8 days).

Conclusions

Response Surface Methodology allowed us to maximize the oxidation of bulk oil by considering several chemical markers at the same time. This study presents a simple, practical and easily reproducible model to evaluate the antioxidant potential effect of natural and synthetic compounds in bulk oils. In addition, the models present predictive capacities, allowing other combinations among the factors to be further assessed.

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