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Effect of the Simultaneous Interaction among Ascorbic Acid, Iron and pH on the Oxidative Stability of Oil-in-Water Emulsions

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ABSTRACT: The objective of this study was to demonstrate how different factors can simultaneously influence the oxidative stability of an oil-in-water emulsion, and how these factors can be used to enlarge the variation range of oxidation markers, expressed as peroxide value (PV) and TBARS. Initially, a Plackett−Burman design was used to screen seven factors (temperature, pH, and iron, copper, ascorbyl palmitate, ascorbic acid, and sodium chloride concentrations). A temperature elevation of 30 to 60 °C reduced PV and TBARS, a pH change from 3.0 to 7.0 increased PV and reduced TBARS, and the presence of ascorbic acid (1 mmol/L) had no significant effect on PV but increased TBARS ($p < 0.05$). Thus, the temperature was fixed at 30 °C, and an emulsion was formulated with different combinations of ascorbic acid, iron, and pH according to a central composite rotatable design. Regression models were fitted to PV and TBARs responses and optimized to get the higher values of both markers of oxidation. The optimized emulsion contained 1.70 mmol/L AH (ascorbic acid) and 0.885 mmol/L $FeSO_4 \cdot 7H_2O$ (1.0 mmol/L Fe^{2+}) at pH 5.51 and 30 °C. The range of variation observed for oxidation markers in the optimized emulsion model (PV, 0−4.27 mequiv/L; TBARS, 0−13.55 mmol/L) was larger than the variation observed in the nonoptimized model (PV, 0−1.05 mequiv/L; TBARS, 0−1.00 mmol/L). The antioxidant activity of six compounds (Trolox, α-tocopherol, caffeic acid, gallic acid, catechin, and TBHQ) was evaluated using the optimized emulsion conditions. After application of the Tukey HSD post hoc statistical test, the samples that were not different ($p < 0.05$) in the nonoptimized emulsions showed a significant difference in the optimized emulsions. Considering the importance of the interactions on oxidation studies, our model represents a significant improvement in a direct methodology that can be applied to evaluate natural compounds under different combination of factors.

KEYWORDS: emulsion, oxidation, ascorbic, metal, pH, factorial design

INTRODUCTION

Lipid oxidation is one of the major causes of shelf life reduction of food emulsions. Several infant formulas consist of oil-in-water emulsions that contain polyunsaturated fatty acids supplemented with ascorbic acid and iron to meet some nutritional requirements.[1,2] Besides infant formulas, meat emulsions rich in polyunsaturated fatty acids also contain a significant amount of iron (as free metal or in heme form) and sodium erythorbate, an ascorbic acid analogue used as color fixer.[3] The prooxidant effect of ascorbic acid, when combined with iron at a specific concentration, is well-known because of its capacity to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}).[4−6] However, this behavior in a food emulsion depends on numerous factors such as pH, temperature, presence of other compounds, oil-droplet interface area, thickness and permeability of the interfacial area, oil concentration, surfactant ionic charge,[7] and especially the molar ratio between ascorbate and the iron.[8]

Because emulsions are highly susceptible to oxidation, considerable effort has been expended to develop strategies for improving the oxidative stability of these products.[9−11] Although much progress has been achieved in the past few years and has brought relevant information to food manufacturers, most of the previous studies have evaluated one factor at a time. However, oxidation in food emulsions is a dynamic process in which all factors constantly interact with each other.[9] Thus, the contributions of studies that investigate factors with fixed variation ranges would be much greater if the interactions could be simultaneously evaluated, especially in those involving natural antioxidants.

The acceleration of the oxidative reaction is useful to screen the efficacy of potential antioxidants,[4] and although antioxidants should be evaluated on the food itself, it is difficult to standardize the accelerated oxidation using foods as substrates. In this case, direct methods that contain an oxidizable substrate can be applied if the basic chemical principles can be deduced.[12] A notable number of studies have reported the antioxidant action of artificial and natural compounds measured by indirect methodologies.[13−15] However, many of these studies have reported controversial results, even for the same material determined by different assays in different laboratories.[16] When these new compounds are applied to systems that contain an oxidizable substrate, such as triacylglycerols or phospholipids, the results can differ from those obtained using indirect methods.[13,17] Many factors have been suggested to justify these differences.[13,12] Among them, the

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Table 1. Plackett−Burman (PB) Design Applied for the Initial Screening of Significant Factors That Influence the Oxidation Rate

	factors[a] (coded values)						
	temp (°C)	Fe^{2+} (mmol/L)	Cu^{2+} (mmol/L)	AP (mmol/L)	AH (mmol/L)	NaCl (%)	pH
assay							
1	+1	−1	+1	−1	−1	−1	+1
2	+1	+1	−1	+1	−1	−1	−1
3	−1	+1	+1	−1	+1	−1	−1
4	+1	−1	+1	+1	−1	+1	−1
5	+1	+1	−1	+1	+1	−1	+1
6	+1	+1	+1	−1	+1	+1	−1
7	−1	+1	+1	+1	−1	+1	+1
8	−1	−1	+1	+1	+1	−1	+1
9	−1	−1	−1	+1	+1	+1	−1
10	+1	−1	−1	−1	+1	+1	+1
11	−1	+1	−1	−1	−1	+1	+1
12	−1	−1	−1	−1	−1	−1	−1
13	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0
true values[b]							
(−1)	30	0.00	0.0	0.0	0.0	0.0	3.0
0	45	0.25	0.5	0.5	0.5	0.5	5.0
(+1)	60	0.50	1.0	1.0	1.0	1.0	7.0

[a] Factors are designated as temperature (temp), concentration of iron (Fe^{2+}), copper (CU^{2+}), ascorbyl palmitate (AP), ascorbic acid (AH), and sodium chloride (NaCl), and pH. Coded values: (+1), (0), and (−1) correspond to the highest, intermediate, and lowest values of each factor. [b] Corresponds to the values adopted for each factor in the emulsion formulation. For example, assay 1 was performed under the following conditions: 60 °C, iron absence, 1.0 mmol/L copper, ascorbyl palmitate absence, ascorbic acid absence, salt absence, and pH 7.0.

narrow variation in the oxidation markers can mask potential antioxidant effects, particularly when phenolic compounds that exhibit very similar molecular structures are being evaluated in bulk oils or emulsions.[18] The simultaneous addition of iron and ascorbate is currently used to accelerate the oxidation[4] and could also be used to promote the amplification of the oxidation markers, depending on the type of emulsifier, temperature, pH, and presence of other compounds. Our hypothesis is that better discrimination could be achieved among the samples if more lipids could be oxidized in the model.

To evaluate this hypothesis, an initial design (Plackett−Burman) was performed to identify the factors (temperature, pH, and iron, copper, ascorbyl palmitate, ascorbic acid, and sodium chloride concentrations) that influenced the selected oxidation markers in our study. From these results, pH, iron and ascorbic acid were selected, and their values were optimized in the same model to increase the content of the oxidation markers. Finally, six compounds (Trolox, α-tocopherol, caffeic acid, gallic acid, catechin, and TBHQ) were evaluated for their antioxidant activities in the optimized and nonoptimized models.

Thus, the first objective of this study was to demonstrate how three different factors (pH, iron, and ascorbic acid) can simultaneously influence the oxidative stability of an oil-in-water emulsion. The second objective was to optimize the combination of these factors to enlarge the range of variation of the oxidation markers.

MATERIALS AND METHODS

Materials. Isooctane, 2-propanol, methanol, hexane and 1-butanol were obtained from Merck (Whitehouse Station, NJ, USA). Flaxseed oil (stored in the dark at 4 °C), ammonium thiocyanate, barium chloride, iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), silicic acid, activated charcoal, 1,1,3,3-tetraethoxypropane (TEP), imidazole, sodium dodecyl sulfate (SDS), copper(II) sulfate, sodium chloride, ascorbic acid, 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.

Emulsion Preparation and Lipid Oxidation Measurements. A 1% (v/v) oil-in-water emulsion was prepared using 10.0 mL of flaxseed oil stripped of its minor components, according to the methodology proposed by Khan and Shahidi[19] and modified by Waraho et al.,[20] and 90.0 mL of sodium acetate−imizadole buffer solution (10 mmol/L each, pH 7.0) containing 35 μmol/L of SDS emulsifier. The oil was added to the SDS solution and homogenized in four passages (35 MPa pressure) using a high-pressure valve homogenizer (A-10, Alitec, São Paulo, Brazil). After each pass, the emulsion was cooled in an ice bath to room temperature. Next, the pH of each emulsion was measured and adjusted using 0.01 M HCl or 0.1 M NaOH, and the emulsion was homogenized again. The samples were protected from light and heat. To measure emulsions' particle size, one drop of sample was placed over a glass lamina with the assistance of a capillary tube and then covered with a coverslip. Laminas with samples were analyzed on a polarized-light microscope (BX-50, Olympus, Center Valley, PA, USA) connected to a digital video camera (Media Cybernetics, Bethesda, MD, USA). The pictures were enhanced 40× using the application Image Pro-Plus v. 4.5.1.22 for Windows (Media Cybernetics, Bethesda, MD, USA). The analyses were performed in quadruplicate, and the particle sizes were determined from the

Table 2. Central Composite Design Containing Three Factors Selected by the PB Design at Three Variation Levels and the Oxidative Oxidation Markers Observed in Each Assay

	factors[a]			oxidation markers[b]		
	Fe^{2+} (mmoL/L)	AH (mmoL/L)	pH	LOOH (mequiv/L)	TBARS (mmoL/L)	T_{LOOH} (h)
assay						
1	−1.00	−1.00	−1.00	3.59	5.69	50.0
2	1.00	−1.00	−1.00	3.09	9.89	74.0
3	−1.00	1.00	−1.00	2.94	6.04	98.0
4	1.00	1.00	−1.00	2.57	12.59	36.1
5	−1.00	−1.00	1.00	5.11	4.35	48.0
6	1.00	−1.00	1.00	5.35	6.48	78.2
7	−1.00	1.00	1.00	4.06	8.00	96.4
8	1.00	1.00	1.00	4.37	12.45	40.1
9	−1.68	0.00	0.00	4.45	4.73	96.0
10	1.68	0.00	0.00	4.29	12.00	70.1
11	0.00	−1.68	0.00	5.26	6.40	70.0
12	0.00	1.68	0.00	3.88	11.72	78.2
13	0.00	0.00	−1.68	1.52	9.05	42.0
14	0.00	0.00	1.68	4.48	7.50	42.3
15	0.00	0.00	0.00	4.56	14.31	89.9
16	0.00	0.00	0.00	4.50	14.23	89.8
17	0.00	0.00	0.00	4.55	14.30	90.2
pooled SD[b]				1.00	3.47	22.0
true values						
−1.68	0.00	0.00	3.00			
−1.00	0.20	0.40	3.81			
0.00	0.50	1.00	5.00			
+1.00	0.80	1.60	6.19			
1.68	1.00	2.00	7.00			

[a] Factors are designated as iron concentration (Fe^{2+}), ascorbic acid (AH) and pH. [b] Values are means ($n = 2$) followed by the pooled standard deviation. Oxidation markers were measured in the (1%) emulsions at 30 °C.

images. The mean particle diameter in the emulsions ranged from 63.2 ± 24.0 μm to 92.5 ± 37.1 μm. Each emulsion was separated into several vials (1 mL) and kept in an oven (L.S. 1.0 A, Logen Scientific, São Paulo, Brazil) under different temperatures (30−60 °C). Every 2 h, samples were collected to determine the oxidation markers of oxidation. Near the peaks, the assay was repeated over shorter time intervals. Lipid hydroperoxide concentrations were determined according to procedures in Shantha and Decker.[21] The amount of thiobarbituric acid reactive substances (TBARS) in the samples was determined according to the method proposed by McDonald and Hultin.[22] Measurements were taken in duplicate, and the values were expressed as mequiv/L and mmol/L of emulsion for hydroperoxides and TBARS, respectively. The results of LOOH and TBARS concentrations used in this study correspond to the maximum value observed in each assay. In addition, in the second design, the time necessary to achieve the hydroperoxide peak was also determined and expressed as hours.

Experimental Design and Statistical Proceedings. This study was divided into three parts, adopting a sequential design strategy as described by Rodrigues and Iemma.[23] First, the effects of seven factors (temperature, pH, and iron, copper, ascorbyl palmitate, ascorbic acid, and sodium chloride concentrations) on the oxidative stability of the emulsions were checked using a Plackett−Burman (PB) design, as described in Table 1. From the results obtained in this first design, three factors were selected for the second design (central composite rotatable design, CCRD) to estimate the simultaneous interaction among the factors within the variation range (Table 2) and also to determine the level of each factor that would maximize the emulsion oxidation markers. Optimization was carried out using the Derringer and Suich[24] method. Afterward, six compounds with different polarities were evaluated according to their antioxidant activity using the optimized and non-optimized emulsions.

Evaluation of the Compounds' Antioxidant Activity. Trolox, α-tocopherol, caffeic acid, gallic acid, catechin, and TBHQ (1 mmol/L) were added to 1% stripped flaxseed oil emulsions containing Fe^{2+} (0.885 mmol/L $FeSO_4 \cdot 7H_2O$) and ascorbic acid (1.70 mmol/L AH) at pH of 5.5. The antioxidants were added directly after adjustment of the pH. The solutions were protected from light and heat. Vials containing 1.0 mL of the solutions were kept at 30 °C for 36 h. Hydroperoxide (LOOH) and TBARS concentrations were determined for all samples following the previously described methodologies.

Statistical Analysis. The data obtained in this study was initially checked according to the homogeneity of variances using the Hartley test. The main effects of the seven factors on the oxidation reaction were determined based on the results from the PB design considering an alpha value (p) of 5%.

For optimization, three factors (x_1, x_2 and x_3) selected by the PB design were applied in a CCRD. Data were submitted to ANOVA and sequentially fitted to the response-surface regression procedure according to the following second-order polynomial equation:

$$\hat{y} = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_{11} + b_{22}x_{22} + b_{33}x_{33} + b_1b_2x_1x_2 + b_1b_3x_1x_3 + b_2b_3x_2x_3$$

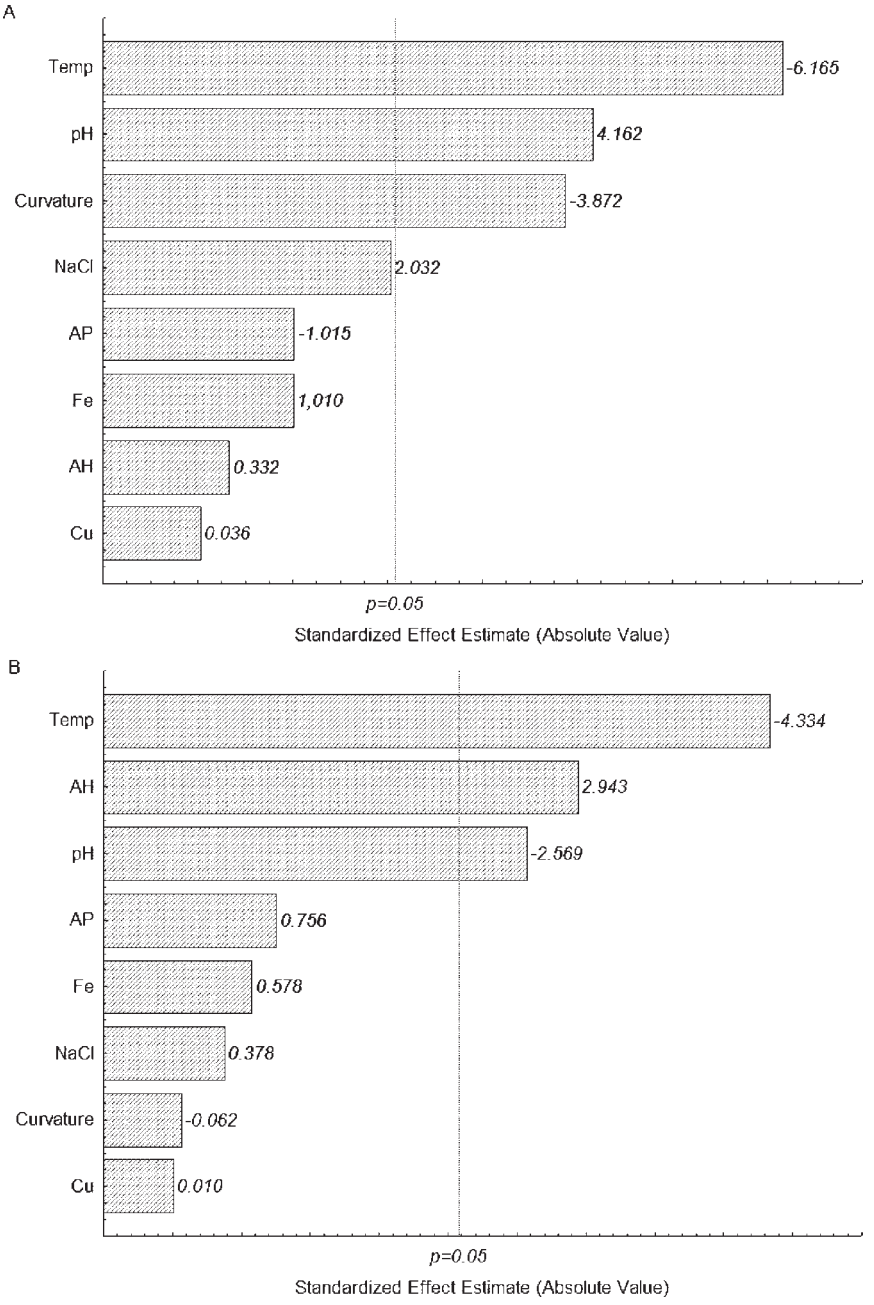

Figure 1. Pareto chart of standardized effects observed in 1% emulsions: (A) LOOH); (B) TBARS.

where $\hat{y}$ is the estimated response, b_0 represents the main value, and b_i, b_{ii}, and b_{ij} are the linear, quadratic, and interaction terms of the model. The models' quality of fit was determinate by R^2 and p values relative to the lack of fit. Because all assays were repeated twice, the block effect was also evaluated in each model. Validation of the regressions was estimated by the relative error to compare the observed values to five randomized combinations with a fixed value estimated by the adjusted polynomial equation. The statistical software package Statistica v.9.0 (Statistica Inc., Tulsa, OK) was used to perform all analyses and to plot the graphs of the response surfaces. An alpha value of 5% was adopted to reject the null hypothesis in this study. However, it is plausible to use a p of 10% in biochemical processes' screening using PD designs, as so to avoid neglecting significant factors due to a too severe alpha value, as stated by Haaland.[25]

RESULTS

Screening Design. The effects of each factor on the oxidative stability of the emulsions, as measured by LOOH and TBARS concentrations and using a PB design, are shown in Figure 1A and and Figure 1B, respectively. This analysis, which only considered the principal effects and curvature, was performed to screen the variables for the second step. The temperature was the most important factor for both markers (LOOH and TBARS). An increase of the temperature (from 30 to 60 °C) significantly ($p < 0.001$) reduced both the LOOH (SE = −6.165) and the TBARS (SE = −4.334) concentrations. Because the objective of this study was to achieve higher values for the oxidation markers, the temperature was fixed at the lowest level (30 °C) for the second step. The LOOH concentration increased

Table 3. Effect Estimates (± Standard Error) and Quality Evaluation of the Models Adjusted to the Three Oxidation Markers (LOOH, TBARS, and T_{LOOH})

	markers of oxidation reaction		
effect estimates (±SE)	LOOH (mequiv/L)	TBARS (mmoL/L)	T_{LOOH} (h)
mean	4.54 ± 0.01	14.28 ± 0.02	89.96 ± 0.07
Fe^{2+} (linear, x_1)	−0.09 ± 0.01	4.33 ± 0.02	−15.76 ± 0.07
Fe^{2+} (quadratic, x_1^2)	−0.15 ± 0.01	−4.20 ± 0.02	−4.82 ± 0.08
AH (linear, x_2)	−0.81 ± 0.01	3.17 ± 0.02	5.01 ± 0.07
AH (quadratic, x_2^2)	−0.00 ± 0.01	−3.17 ± 0.02	−11.22 ± 0.08
pH (linear, x_3)	1.71 ± 0.01	−0.81 ± 0.02	0.73 ± 0.07
pH (quadratic, x_3^2)	−1.12 ± 0.01	−4.26 ± 0.02	−33.82 ± 0.08
Fe^{2+} × AH (linear, x_1x_2)	0.05 ± 0.02	1.17 ± 0.02	−43.09 ± 0.09
Fe^{2+} × pH (linear, x_1x_3)	0.35 ± 0.02	−1.04 ± 0.02	2.91 ± 0.09
AH × pH (linear, x_2x_3)	−0.21 ± 0.02	1.64 ± 0.02	0.06 ± 0.09
blocks	0.01 ± 0.01	0.01 ± 0.01	0.06 ± 0.06
probability value (lack of fit)	0.259	0.222	0.071
determination coefficient (R^2)	0.99861	0.99973	0.99972

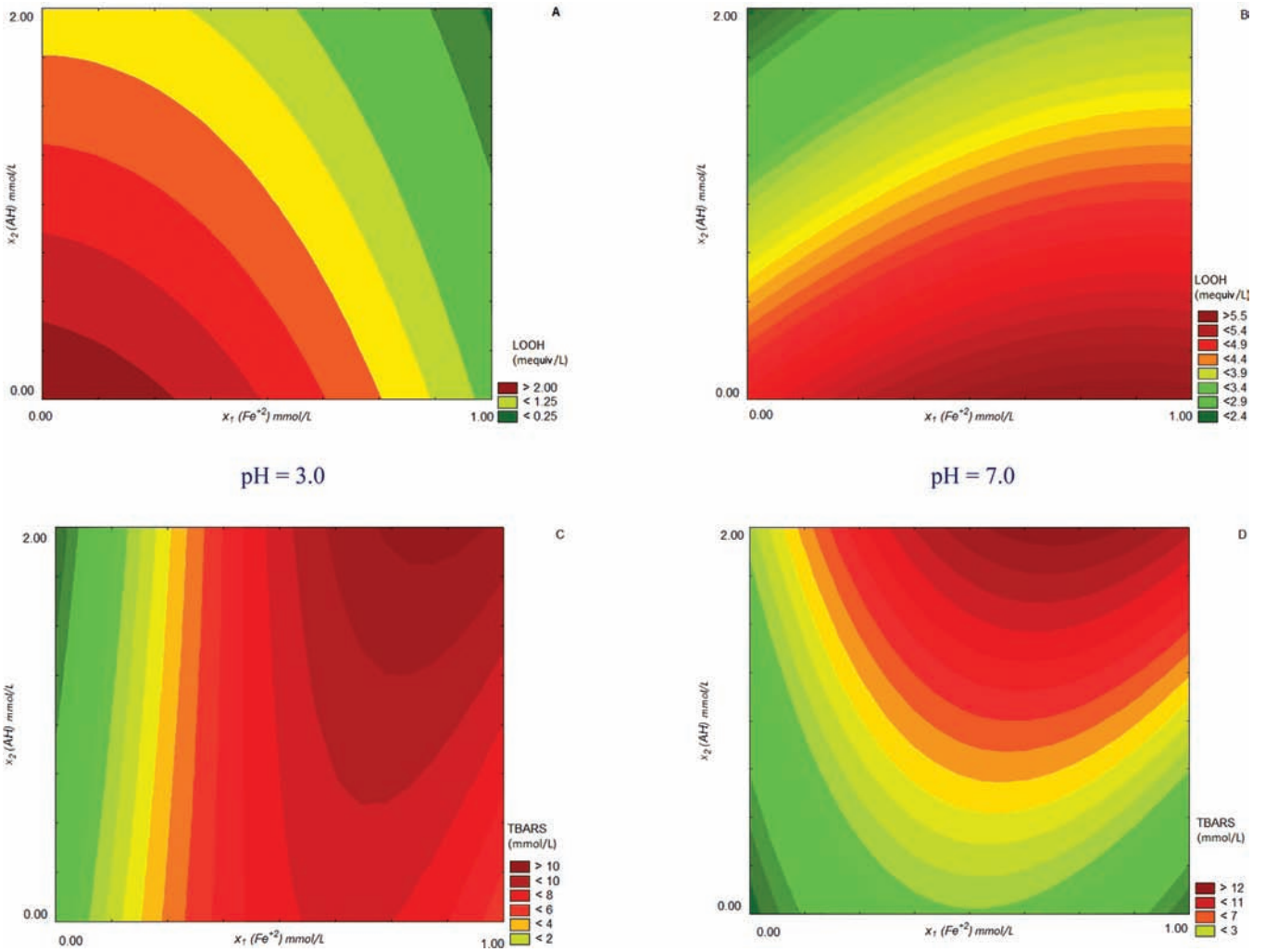

Figure 2. Fitted surface of the responses LOOH and TBARS measured in the (1%) emulsions at pH 3.0 and pH 7.0 based on the interaction between Fe^{2+} and AH: (A) LOOH at pH 3.0; (B) LOOH at pH 7.0; (C) TBARS at pH 3.0; (D) TBARS at pH 7.0.

Table 4. Validation of the Selected Model Using Five Randomized Combinations of the Three Factors

	additional assays				
factors and markers	1	2	3	4	5
Fe^{2+} (mmol/L)	0.885	0.247	0.375	0.732	0.967
AH (mmol/L)	1.700	1.792	1.155	0.083	0.446
pH	5.51	4.37	3.61	4.63	6.24
LOOH					
pred[a]	4.24	3.51	2.80	4.65	5.32
obsd[b]	4.22 ± 0.04	3.47 ± 0.01	2.79 ± 0.03	4.67 ± 0.02	5.21 ± 0.02
rel error (%)[c]	−0.47	−1.15	−0.36	0.43	−2.11
TBARS					
pred[a]	13.31	7.90	7.60	4.21	10.28
obsd[b]	13.27 ± 0.03	7.94 ± 0.04	7.65 ± 0.04	4.26 ± 0.02	10.30 ± 0.01
rel error (%)[c]	−0.30	0.50	0.65	1.17	0.19
T_{LOOH}					
pred[a]	36.0	108.4	72.6	89.0	80.4
obsd[b]	36.0	108.0	72.0	88.0	80.0
rel error (%)[c]	0	−0.37	−0.83	−1.14	−0.50

[a] Predicted values obtained by the respective polynomial models. [b] Observed mean values (n = 2) followed by the standard deviation (SD). [c] Relative error (%) = $((y_{obs} - y_{pred})/y_{obs}) \times 100$. Values $-5\% < x < 5\%$ indicate there are no significant differences between the observed value and the value predicted by the model.

(SE = +4.162) when the pH was changed from 3.0 to 7.0, and no other significant effect was observed for the other six variables on this marker. In this model, the statistically significant curvature reduces the standard error, indicating that other significant variables are not masked by the higher standard error caused by the central-point variation.

In addition to temperature, the pH level and the presence of ascorbic acid (AH) showed significant effects on the TBARS marker. An increase of the pH from 3.0 to 7.0 reduced the TBARS concentration (SE = −2.569), whereas the presence of AH (1 mmol/L) showed the opposite result in that the TBARS concentration increased (SE = +2.943). No influence was observed on the TBARS response for the other four variables. Thus, only three variables affected the emulsion oxidation within the evaluated range: temperature, pH, and ascorbic acid (AH). As temperature was fixed at the lowest value, pH and AH were the variables selected for the next step. In function of the well-known redox interaction reported in the literature for systems containing iron and AH, we opted to include this third variable (Fe^{2+}) in the CCRD and amplify the variation range of AH from 0.0 to 1.0 to 0.0−2.0 mmol/L.

Central Composite Rotatable Design. In the second step, a CCRD was applied to model the oxidative behavior of the emulsion when pH, AH, and iron concentrations were changed within a predefined range of variation (Table 2). Analysis of the LOOH and TBARS concentrations was performed in the 17 assays shown in Table 2. In addition to these two oxidation markers, the time to achieve the maximum concentration of hydroperoxides was also evaluated (T_{LOOH}). The estimates of the effects and the quality evaluation of the models adjusted to the three responses (LOOH, TBARS, and T_{LOOH}) are shown in Table 3. All of the models exhibited a good quality of fit to the experimental data. For this reason, contour curves were produced based on these models. The objective of performing a CCRD was to check the interactions between the factors and also to optimize the oxidation conditions.

Regarding the interactions, a number of possibilities can be evaluated in a system containing three independent variables (x_1, x_2, and x_3). In this study, the interaction between AH and iron (Fe^{2+}) as a function of pH variation on the LOOH and TBARS (Figure 2) responses was evaluated. At pH 3.0, higher values of LOOH (Figure 2A) were obtained when Fe^{2+} and AH were present at the minimum concentration. However, at pH 7.0 (Figure 2B), the formation of LOOH was less dependent on the Fe^{2+}concentration and increased only when the AH concentration was less than 1.0 mmol/L. At acidic pH levels (Figure 2C), higher TBARS values were observed when Fe^{2+} and AH were present at their maximum concentrations. Under neutral pH conditions (Figure 2D), higher TBARS values were practically independent of the Fe^{2+} concentration and increased only when the AH concentration was higher than 1.0 mmol/L.

Optimization and Validation. These oxidation markers were taken into account to build the desirability function in order to maximize values of LOOH and TBARS and minimize values of T_{LOOH}. The following combination of coded variables was suggested: 1.29 (Fe^{2+}), 1.18 (AH), and 0.43 (pH). This combination achieved approximately 85% of the desirability function and corresponded to the following true values: 0.885 mmol/L $FeSO_4 \cdot 7H_2O$ (1.0 mmol/L Fe^{2+}), 1.700 mmol/L AH, and pH of 5.51. The range of variation of the oxidation markers observed in the optimized model (PV, 0−4.27 mequiv/L; TBARS, 0−13.55 mmol/L) was larger than those observed in the nonoptimized model (PV, 0−1.05 mequiv/L; TBARS, 0−1.00 mmol/L). Four additional points were examined to validate the models that were adjusted for LOOH, TBARS, and T_{LOOH} responses. No significant differences were observed between the estimated and the experimental results for all three responses (Table 4). Finally, the antioxidant activity of six compounds was determined on a molar basis using the emulsion in which the oxidation conditions were optimized and nonoptimized by the response surface methodology (Figure 3). The comparison between the two emulsion systems for the LOOH (Figures 3A) and

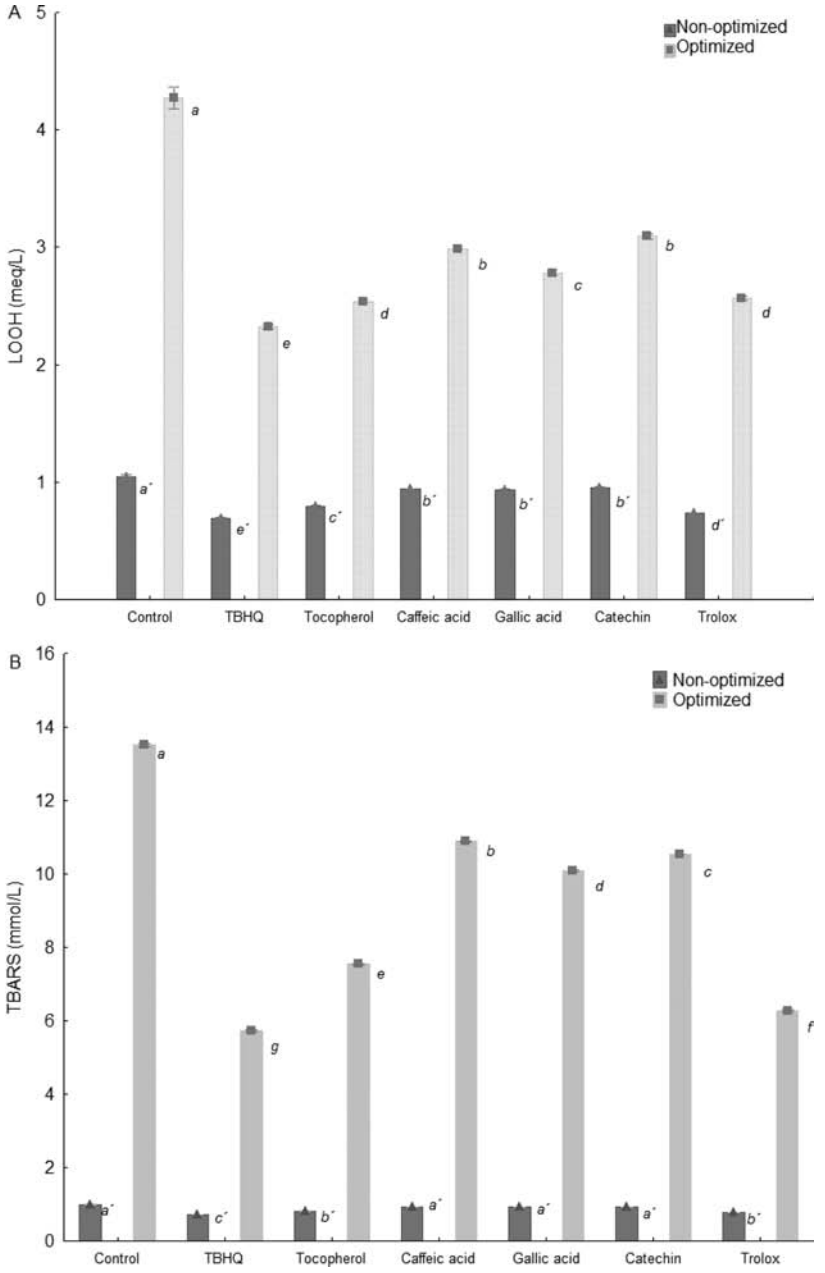

Figure 3. Oxidation markers measured before and after optimization: (A) LOOH and (B) TBARS. Optimized: 30 °C, 1% stripped oil, 1. 7 mmol/L AH, 0.885 mmol/L Fe^{2+}, pH 5.5. Nonoptimized: 30 °C, 1% stripped oil, 0.0 mmol/L AH, 1.000 mmol/L Fe^{2+}, pH 3.0.

TBARS values (Figures 3B) indicated that a better discrimination of these markers could be achieved in the optimized system.

DISCUSSION

The first objective of our study was to evaluate the simultaneous action of different factors on the oxidative stability of the emulsions over a range of variation instead of at fixed values for each factor. In this case, all possible interactions between ascorbic acid (AH) and iron (Fe^{2+}) under acidic and neutral conditions were evaluated using a CCRD. Our model followed the recommendations proposed by AOCS,[26] including the use of purified oil stripped of its minor components, mild temperatures, and analysis of primary and secondary products of the reaction. The chemical interpretation of the responses presented in the contour curves is summarized in Figure 4.

Higher concentrations of LOOH were observed in the emulsions prepared at neutral pH than in those prepared at acidic pH levels. At pH 3.0, higher Fe^{2+} concentrations reduced the hydroperoxide concentrations (LOOH), and this behavior was more pronounced when AH was also present at higher concentrations. In a study reported by Jacobsen et al.,[27] lower levels of LOOH were observed upon addition of AH, and the effect was greatest at low pH values. The authors attributed this result to the increased breakdown of LOOH at low pH in the presence of iron, as evidenced by the increase in the total volatiles. Polyunsaturated fatty acids placed in the inner position of the lipid globule (Figure 4) are oxidized by the radicals present in the emulsion

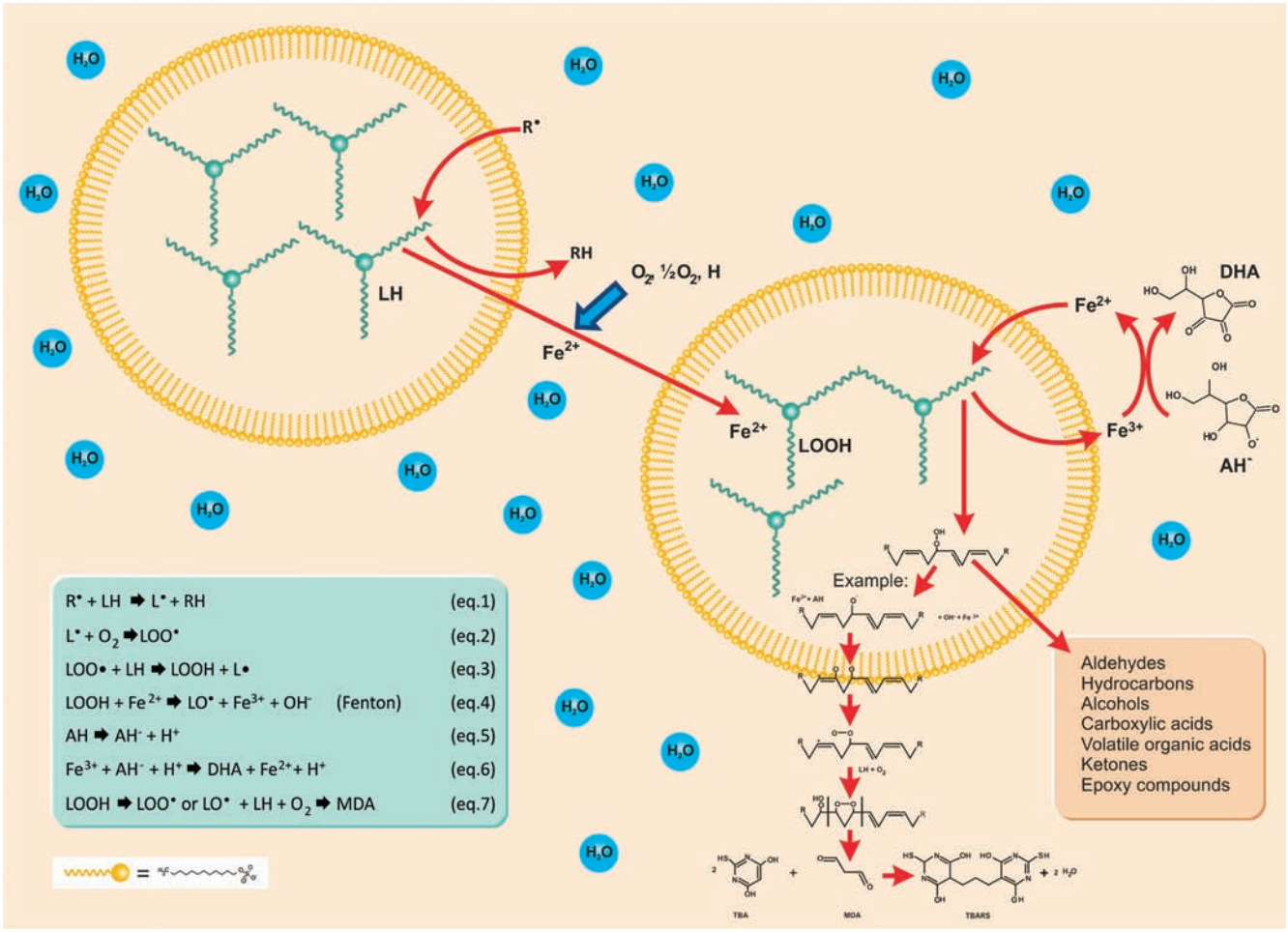

Figure 4. Scheme of lipid oxidation based on the oil-in-water emulsion applied as model.

($R^{\bullet}$) to form alkyl radicals ($L^{\bullet}$) (Figure 4, eq 1). Under aerobic conditions, oxygen is added to the alkyl radical to form peroxyl radicals ($LOO^{\bullet}$) (Figure 4, eq 2).[9] Hydrogen is subsequently attracted to the lipid radicals to produce hydroperoxides (LOOH) (Figure 4, eq 3). In oil-in-water emulsions, one of the major mechanisms of lipid oxidation is the metal-promoted decomposition of lipid hydroperoxide to a free radical (Figure 4, eq 4).[28] A reduction of pH increases Fe^{2+} solubility, contributing to the decomposition of LOOH, catalyzed by Fe^{2+}.[17] According to Choe and Min,[29] ferrous ion (Fe^{2+}) acts 100 times faster in decomposing hydroperoxides than ferric ion (Fe^{3+}). Consequently, the capacity of AH to reduce Fe^{3+} to Fe^{2+} (Figure 4, eqs 5 and 6) may explain why lower LOOH values were observed when Fe^{2+} and AH were present at their maximum concentrations, although the rate of Fe(III) reduction by AH is not proportional to the concentration of AH.[2] According to Mei et al.,[30] iron-promoted lipid oxidation rates were highest in SDS-stabilized emulsion droplets at lower pH values,[3−5] because the emulsifier SDS is strongly negative at pH 3.0, attracting more metals to the droplet surface. The activity of iron for this decomposition reaction increases significantly when ascorbic acid is added and the pH decreases from 7.0 to 5.5.[28] Boon et al.[31] observed that lycopene degraded faster at pH 3.0 than at pH 7.0 in a model emulsion system that contained ferric and ferrous species. However, according to Xie et al.,[17] methyl linoleate micelles at pH 6.8 exhibited faster rates of oxidation than micelles at pH 3.0.

Contradictory conclusions can result from differences in the emulsion preparation, such as concentration and ionic charge of the emulsifier, globule size, type of lipid substrate, composition of the aqueous phase, the method by which the oxidation is induced, the emulsion stability during the assay, and the oxidation markers selected to follow the reaction, among other factors. In our study, the lower LOOH concentration at pH 3.0 was caused by faster decomposition in the presence of AH and Fe^{2+}, and not because of its reduced formation. This fact was confirmed when AH was present without Fe^{2+} (Figure 2A). In this situation, high LOOH values were observed (>2.0 mequiv/L), which suggests the lack of an antioxidant effect of the AH. According to the "polar paradox" reported by Porter,[32] polar antioxidants such as AH are more effective in bulk oils than in emulsions. This effectiveness results from their greater affinity for the aqueous phase, which keeps these types of molecules far from the interface where the oxidation occurs.[33] However, it is important to consider that the polar paradox does not take into account the interactions between pH, iron, and antioxidants.

When pH is increased from 3.0 to 7.0, the iron solubility reduces, and the LOOH formation becomes faster than its decomposition, which increases the LOOH stability in the emulsions.[34] It is known that as the pH increases, the rate of Fe(III) reduction by AH decreases.[2] The reaction at neutral pH in our study was independent of Fe^{2+} concentration and almost exclusively dependent on AH concentration (Figure 2B). When

the AH concentration is low, LOOH suffers less decomposition; however, when the AH concentration is increased above 1 mmol/L, the reaction in eq 6 (Figure 4) is accelerated. This increased rate of reaction favors the reduction of the sparingly soluble Fe^{3+}, which promotes a strong prooxidant effect in the emulsion. In addition, at neutral pH, AH ($pK_a = 4.04$) is present predominantly as the monoanion (AH^-), which facilitates the electron transfer to Fe^{3+}.[35] A more rapid increase in the concentration of malondialdehyde (MDA) in micelles at pH 6.8 than in micelles at pH 3.0 was observed by Xei et al.,[17] who have attributed this result to the low solubility or precipitation of transition metals in the continuous phase.

Regarding Figure 2C, higher TBARS concentrations were obtained when AH and Fe^{2+} were present at their maximum concentrations at pH 3.0. After decomposition, the carboxylic acid end of the fatty acid is esterified to the glycerol chain of the triacylglycerol or phospholipid, unless it undergoes further decomposition to a low-molecular-weight compound. The methyl terminal undergoes different reactions to form a number of volatile products, including MDA[9] (Figure 4, eq 7). The main secondary products of linolenate oxidation are aldehydes, carboxylic acids, alcohols, and hydrocarbons. Most of these products are volatile and are responsible for the off-flavor in oxidized oils.[29] The measurement of MDA, by its derivatization to TBARS, is a common method for following the oxidation of fatty acids that contain three or more double bonds.[36] Although TBARS measurement is considered a nonspecific oxidation marker, proteins and Maillard reaction products, which are typical interfering substances in TBARS results, were not present in the emulsion used in our study. Figure 2C suggests that AH does not exert any antioxidant effect toward the MDA formation, here expressed as TBARS, because this marker at pH 3.0 does not change in either the presence or absence of AH. However, these results do not indicate whether AH is capable of reducing the formation of secondary products of oxidation other than MDA.

LOOH decomposition represents the first step to MDA formation (Figure 4, eq 7), for which $LO^•$ and $LOO^•$ both serve as precursors. Because the lipid radicals are more soluble than the original lipid, AH may be able to reduce these molecules as a function of their relative reduction potentials (for example, $E^{o\prime}$ = 0.28 V for ascorbate and $E^{o\prime}$ = 1.0 V for peroxyl radicals).[9] However, this effect was not observed in our emulsion. Oxygen is involved in the formation of MDA from LOOH. Even though AH is able to react directly with oxygen, thereby excluding it from the emulsion,[9] this effect was also not verified by our results. According to Frankel et al.,[33] hydrophilic antioxidants become diluted to the point that they cannot adequately protect the oil in the oil−water interface. Miccichè et al.[8] observed a reduction of the catalytic activity of the ascorbate/iron combination at a molar ratio greater than 2/1 and attributed this result to a possible antioxidant effect of the ascorbyl palmitate. Similar results might have been observed in our study if a molar ratio greater than 2/1 had been tried and ascorbyl palmitate had been used instead of ascorbic acid.

When the pH is increased from 3.0 to 7.0 (Figure 2D), an AH concentration greater than 1 mmol/L exerts a strong prooxidant effect, which increases the TBARS values from around 3 to 12 mmol/L, independent of the concentration of Fe^{2+}. The same explanation used for LOOH can be applied to this situation. Considering the limited solubility of Fe^{2+} in the emulsion at neutral pH compared to that at acidic pH, any contribution of the AH to the reduction of Fe^{3+} has an important impact on the LOOH decomposition and consequent TBARS formation. Yen et al.[37] have observed similar results. Using TBARS as a marker in the deoxyribose model, the authors observed that TBARS formation increased with increasing concentration of AH and reached a maximum value when the concentration of AH was 1.65 mmol/L. In practical terms, the application of AH or erythorbate in emulsions containing iron cannot be an alternative for controlling the oxidation rate, unless other antioxidants or metal chelators are present. A similar conclusion was reached by Jacobsen et al.,[27] who reported that, in the presence of AH and iron, oxidation in fish oil-enriched mayonnaise is increased via the decomposition of LOOH at the oil−water interface.

The second objective of this study was to apply the optimized method to evaluate the antioxidant activity of compounds with different molecular structures and polarities. According to Miccichè et al.,[8] the combination of ascorbyl palmitate and iron(II) perchlorate hydrate at a molar ratio of 2.0/1.0 reached its optimal catalytic activity, as determined by measurements of lag time. In our study, a molar ratio of 1.9/1.0 was identified as the optimal proportion, in agreement with the results reported by Miccichè et al.,[8] although the models applied in these two studies were different. A better degree of differentiation among the compounds was achieved in the optimized method than in the nonoptimized method (Figure 3). For example, after application of the Tukey HSD post hoc statistical test, samples that were not different ($p < 0.05$) in the nonoptimized emulsions showed a significant difference in the optimized emulsions. In our study, the optimized oxidation conditions, including the combination of ascorbate/iron (2:1) at pH 5.5 and 30 °C, combined with the use of SDS as the emulsifier, and a stripped oil as substrate were shown to be an interesting model to measure the antioxidant activity of different compounds with similar molecular structures. However, it is recommended that further studies evaluate additional secondary products, because antioxidants may have different effects on the formation of other volatile oxidation products besides MDA.

In summary, the factorial design used in this study allowed the observation of the prooxidant behavior of infinite combinations of Fe^{2+} and ascorbic acid in the pH range of 3.0 to 7.0. Moreover, the model's optimization was extremely useful to discriminate the antioxidant activity effects of different compounds. This study presents a system that details how initial factors can be selected for further optimization to increase the formation of lipid oxidation products. This system can also be applied to improve direct methodologies for evaluating the antioxidant activity of natural compounds.

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