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Metal-Catalyzed Oxidation of a Structured Lipid Model Emulsion

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The effects of temperature, time, metal, citric acid, and tocopherol contents on the oxidation stability of a model oil-in-water emulsion prepared with enzymatically synthesized menhaden oil−caprylic acid structured lipid were evaluated by response surface methodology. The emulsions were stabilized by whey protein isolate. Oxidation was monitored by measuring lipid hydroperoxides and thiobarbituric acid reactive substances (TBARS). Cupric sulfate and ferrous sulfate were used to study the effect of metal concentration and type. A statistical model was developed to determine the relationships between all variables considered. The relationships differed depending on the type of metal catalyst used. For both metal types, the metal concentration had the highest positive effect on peroxide value. Citric acid had the highest negative effect on peroxide value for iron-containing emulsions, while tocopherol had the highest negative effects for copper-containing emulsions. Results from the TBARS test did not vary significantly enough to yield an acceptable model.

KEYWORDS: Caprylic acid; fish oil; response surface methodology; structured lipid; oxidation; whey protein isolate

INTRODUCTION

In the past few years there has been a flurry of research on various structured lipids (SLs) and their potential health benefits (*1*−*4*). Among the fatty acids considered for SL synthesis are medium-chain and polyunsaturated fatty acids, because of their potential health benefits. Medium-chain fatty acids have higher plasma clearance and are readily metabolized and utilized as fuel and energy (*5*). Polyunsaturated fatty acids, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have several health benefits, including reduction of thrombotic tendencies, hypertriacylglycerolemia (*6*), and prevention of coronary heart disease (*7*). Fish oils are a good source of polyunsaturated fatty acids and as such have been coupled with medium-chain fatty acids to obtain even healthier SLs (*8*).

Despite the purported potential health benefits, the use of highly unsaturated oils is limited by their high susceptibility to oxidation. This problem is exacerbated in complex food systems, where pro-oxidants such as transition metals may exist. This is especially true in iron-fortified foods. Transition metals decrease the oxidative stability of foods through their ability to decompose lipid peroxides into free radicals (*9*). In biological systems, lipid peroxide stability is improved in the absence of metals (*9*). In lipid emulsions, other factors, such as droplet size, surfactant type, and concentration, also affect lipid oxidation (*10*−*12*). In food systems, free radicals are usually generated in the aqueous phase, and these radicals have important implications for the oxidation of emulsified oils (*9*).

Response surface methodology (RSM) may be used in systems where the objective is to locate a feasible treatment combination for which the mean response is maximized or minimized (*13*). One of the main advantages of RSM is the reduced number of experimental runs needed to obtain sufficient information for statistically acceptable results.

The objective of the research described in this paper was to examine the relationship between five factors, temperature, time, metal concentration, citric acid concentration, and α-tocopherol concentration, in influencing the oxidation properties of our fish oil-based SL model emulsions during our specified time period. The study was divided into two parts of five factors, each with metal type being the distinguishing factor. RSM was used to evaluate oxidation as affected by the relationships between the different factors and their levels.

MATERIALS AND METHODS

Materials. Immobilized Lipozyme IM from *Rhizomucor miehei* was purchased from Novo Nordisk Biochem North America, Inc. (Franklinton, NC). Refined, bleached, and deodorized (RBD) menhaden oil was obtained from Omega Protein, Inc. (Reedville, VA). Caprylic acid (99% pure), citric acid, α-tocopherol, thiobarbituric acid, cupric sulfate, and ferrous sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Commercially available whey protein isolate, *Bi*PRO, was provided by Davisco Foods International, Inc. (Le Sueur, MN). Trichloroacetic acid and organic solvents were purchased from J.T. Baker, Inc. (Phillipsburg, NJ). Cadmium acetate and potassium iodide were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI) and Fisher Scientific (Fair Lawn, NJ), respectively.

SL Synthesis and Composition. SLs from menhaden oil and caprylic acid were synthesized using a packed-bed column bioreactor as described by Xu et al. (*14*). SL products were purified using a KDL-4 (UIC Inc., Joliet, IL) short-path distillation unit under conditions previously described (*15*). Fatty acid composition was determined by

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Table 1. Operating Variables, Levels, and Experimental Design for Peroxide Values[a]

expt no.	time (h)	temp (°C)	Cit (μM)	Toc (μM)	Met (μM)	PV(copper) (mmol/L)	PV(iron) (mmol/L)
1	0	5	0	0	300	0.00013	8.9×10^{-5}
2	48	5	0	0	0	3.9×10^{-5}	6.3×10^{-5}
3	0	25	0	0	0	5.9×10^{-5}	8.2×10^{-5}
4	48	25	0	0	300	8.8×10^{-5}	7.6×10^{-5}
5	0	5	300	0	0	5.6×10^{-5}	6.9×10^{-5}
6	48	5	300	0	300	0.00035	4.5×10^{-5}
7	0	25	300	0	300	0.00016	7.6×10^{-5}
8	48	25	300	0	0	3.2×10^{-5}	7×10^{-5}
9	0	5	0	300	0	5.4×10^{-5}	7×10^{-5}
10	48	5	0	300	300	0.00010	8×10^{-5}
11	0	25	0	300	300	0.00010	0.00013
12	48	25	0	300	0	0.00015	3.5×10^{-5}
13	0	5	300	300	300	7.8×10^{-5}	9.4×10^{-5}
14	48	5	300	300	0	4.1×10^{-5}	5.6×10^{-5}
15	0	25	300	300	0	4.2×10^{-5}	5.1×10^{-5}
16	48	25	300	300	300	0.00011	3.6×10^{-5}
17	0	15	150	150	150	7.6×10^{-5}	6.3×10^{-5}
18	48	15	150	150	150	0.00017	7.5×10^{-5}
19	24	5	150	150	150	0.00013	9.4×10^{-5}
20	24	25	150	150	150	0.00012	6.3×10^{-5}
21	24	15	0	150	150	0.00010	8.8×10^{-5}
22	24	15	300	150	150	8.7×10^{-5}	9.6×10^{-5}
23	24	15	150	0	150	7.5×10^{-5}	9.5×10^{-5}
24	24	15	150	300	150	5.2×10^{-5}	0.0001
25	24	15	150	150	0	4.3×10^{-5}	5.5×10^{-5}
26	24	15	150	150	300	9.7×10^{-5}	7.3×10^{-5}
27	24	15	150	150	150	8.4×10^{-5}	7.3×10^{-5}
28	24	15	150	150	150	8.5×10^{-5}	0.0001
29	24	15	150	150	150	9.65×10^{-5}	9.7×10^{-5}

[a] Abbreviations: expt no., experiment number; temp, temperature; Cit, citric acid; Toc, α-tocopherol; Met, metal; PV, peroxide value.

gas chromatography according to the method described by Fomuso and Akoh (*16*).

Experimental Design. An experimental design was created with computer software for design of experiments and optimization, Modde 5.0 (Umetri, Umea, Sweden). A three-level and five-factor fractional factorial design was used in this study. The factors and their levels are shown in **Tables 1** and **2**. Experiments were conducted in a randomized order. The study was divided into copper- and iron-containing emulsions.

Emulsion Preparation. Ten percent SL oil-in-water model emulsions were prepared in 10 mM phosphate buffer solutions (pH 7.0). Whey protein isolate was used as the emulsifier for all emulsions. Separate emulsions were prepared for the different experimental units generated by Modde 5.0 for response surface analysis. Whey protein isolate concentration was maintained at 1% for all emulsions. This level was based on previous experiments performed to determine the effects of emulsifier concentration on oxidation properties. SL oil-in-water model emulsions were prepared using a high-pressure valve homogenizer (Emulsiflex, C5, Avestin, CA) at 50 MPa. All emulsions were passed through the homogenizer six times. The resulting emulsions were stored at specified temperature and time treatments and analyzed without further storage.

Evaluation of Lipid Oxidation. Oxidation was monitored over a 48-h period. Progress was monitored by measuring lipid peroxides and thiobarbituric acid reactive substances (TBARS) (*17*).

Data Analysis and Optimization. Data were analyzed using Modde 5.0 (*18*). Second-order coefficients were generated by regression analysis with backward elimination. The quadratic response surface model was fitted to the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii}X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij}X_iX_j$$

where Y is the response variable, β_0 is the intercept, β_i are first-order model coefficients, β_{ii} are quadratic coefficients for the ith variable, β_{ij} are interaction coefficients for the interaction of variables i and j, and X_i and X_j are independent variables.

Table 2. Operating Variables, Levels, and Experimental Design Used for TBARS[a]

expt no.	time (h)	temp (°C)	Cit (μM)	Toc (μM)	Met (μM)	TBARS (copper) (μmol/L)	TBARS (iron) (μmol/L)
1	0	5	0	0	300	1×10^{-6}	1.0×10^{-6}
2	48	5	0	0	0	1.3×10^{-6}	1.3×10^{-6}
3	0	25	0	0	0	1.5×10^{-6}	7.1×10^{-7}
4	48	25	0	0	300	1.7×10^{-6}	1.5×10^{-6}
5	0	5	300	0	0	1.5×10^{-6}	9.9×10^{-7}
6	48	5	300	0	300	1.7×10^{-6}	1.3×10^{-6}
7	0	25	300	0	300	1.7×10^{-6}	9.2×10^{-7}
8	48	25	300	0	0	1.2×10^{-6}	1.9×10^{-6}
9	0	5	0	300	0	1.3×10^{-6}	7.5×10^{-7}
10	48	5	0	300	300	1×10^{-6}	7.3×10^{-7}
11	0	25	0	300	300	1×10^{-6}	6.5×10^{-7}
12	48	25	0	300	0	4×10^{-7}	6.3×10^{-7}
13	0	5	300	300	300	1.3×10^{-6}	1×10^{-6}
14	48	5	300	300	0	4.6×10^{-7}	3.7×10^{-7}
15	0	25	300	300	0	1.2×10^{-6}	1.2×10^{-6}
16	48	25	300	300	300	1.7×10^{-6}	1.2×10^{-6}
17	0	15	150	150	150	8.2×10^{-7}	7.1×10^{-7}
18	48	15	150	150	150	1.7×10^{-6}	1.2×10^{-6}
19	24	5	150	150	150	1.2×10^{-6}	1.2×10^{-6}
20	24	25	150	150	150	1.7×10^{-6}	1.3×10^{-6}
21	24	15	0	150	150	1.2×10^{-6}	1.0×10^{-6}
22	24	15	300	150	150	1.3×10^{-6}	9.9×10^{-7}
23	24	15	150	0	150	1.3×10^{-6}	1.1×10^{-6}
24	24	15	150	300	150	9.3×10^{-7}	1.0×10^{-6}
25	24	15	150	150	0	5.2×10^{-7}	4.1×10^{-7}
26	24	15	150	150	300	1.3×10^{-6}	1.1×10^{-6}
27	24	15	150	150	150	2.1×10^{-6}	1.3×10^{-6}
28	24	15	150	150	150	1.6×10^{-6}	1.2×10^{-6}
29	24	15	150	150	150	1.9×10^{-7}	1.6×10^{-6}

[a] Abbreviations: expt no., experiment number; temp, temperature; Cit, citric acid; Toc, α-tocopherol; Met, metal; TBARS, thiobarbituric acid reactive substances.

RESULTS AND DISCUSSION

The best-fitting quadratic models by multiple linear regression and backward elimination were obtained for peroxide values of both copper- and iron-containing emulsions. The models and their corresponding coefficients are shown in **Tables 3** and **4**. R^2 values for both models were 0.97 and 0.85 for copper- and iron-containing emulsions, respectively. Both models showed no significant lack of fit. On the other hand, models obtained from TBARS values (results shown in **Table 2**) displayed significant lack of fit and could not be fitted by multiple linear regression. Primary oxidation products (hydroperoxides) are intermediary oxidation products that decompose to form secondary oxidation products (TBARS). The lack of a significant TBARS model may be because aldehydes formed in the system did not respond well in the TBARS test or could have undergone further reaction.

From the P values shown in **Table 3**, it can be seen that copper content had the strongest positive influence on peroxide value and tocopherol concentration had the strongest negative impact. For iron-containing emulsions, the strongest positive influence was also exerted by iron concentration (**Table 4**), but unlike with copper, the strongest negative influence was from time. The main effect of each factor under consideration is defined as the change in response obtained by a change in the level of the factor, averaged over the levels of the other factors. In observing the main effects, temperature appeared to be

Table 3. Regression Coefficients[a] and *P* Values for Copper-Catalyzed Oxidation, $R^2 = 0.97$

variables	peroxide value (mmol/L)	*P* value ($\alpha = 0.05$)
intercept (β_0)	9.3×10^{-5}	1.8×10^{-8}
time (β_1)	1.8×10^{-5}	0.0014
temp (β_2)	-5.6×10^{-6}	0.2097
Cit (β_3)	6.4×10^{-6}	0.1543
Toc (β_4)	-1.4×10^{-5}	0.0080
Met (β_5)	3.8×10^{-5}	3.3×10^{-6}
time × time (β_{11})	3.2×10^{-5}	0.0181
temp × temp (β_{22})	3.0×10^{-5}	0.0201
Toc × Toc (β_{44})	-3.1×10^{-5}	0.0192
Met × Met (β_{55})	-2.3×10^{-5}	0.0596
time × temp (β_{12})	-1.2×10^{-5}	0.0242
time × Cit (β_{13})	9.5×10^{-6}	0.0575
time × Met (β_{15})	6.8×10^{-6}	0.1531
temp × Cit (β_{23})	-1.6×10^{-5}	0.0043
temp × Toc (β_{24})	2.3×10^{-5}	0.0004
temp × Met (β_{25})	-1.8×10^{-5}	0.0021
Cit × Toc (β_{34})	-2.7×10^{-5}	0.0001
Cit × Met (β_{35})	2.6×10^{-5}	0.0002
Toc × Met (β_{45})	-2.8×10^{-5}	9.3×10^{-5}

[a] Results obtained after backward elimination. See **Table 1** for abbreviations.

Table 4. Regression Coefficients[a] and *P* Values for Iron-Catalyzed Oxidation, $R^2 = 0.85$

variables	peroxide value (mmol/L)	*P* value ($\alpha = 0.05$)
intercept (β_0)	8.5×10^{-5}	3.05×10^{-13}
time (β_1)	-1.0×10^{-5}	0.0013
temp (β_2)	-2.3×10^{-6}	0.4029
Cit (β_3)	-8.6×10^{-6}	0.0053
Toc (β_4)	-1.2×10^{-6}	0.6655
Met (β_5)	8.2×10^{-6}	0.0072
time × time (β_{11})	-1.3×10^{-5}	0.0639
Toc × Toc (β_{44})	1.6×10^{-5}	0.0287
Met × Met (β_{55})	-1.8×10^{-5}	0.0149
time × Toc (β_{14})	-4.7×10^{-6}	0.1112
time × Met (β_{15})	-6.5×10^{-6}	0.0351
temp × Toc (β_{24})	-5.4×10^{-6}	0.0747
Cit × Met (β_{35})	-7.5×10^{-6}	0.0174
Toc × Met (β_{45})	7.9×10^{-6}	0.0133

[a] Results obtained after backward elimination. See **Table 1** for abbreviations.

insignificant. Also, citric acid was insignificant for copper-catalyzed oxidation and tocopherol for iron-catalyzed oxidation. In the presence of significant interactions between factors, the significance of main effects is often masked (*13*). To draw any meaningful conclusions, it is preferable to examine the effects of the treatment combinations or interactions. From contour plots, we can observe the different interactions between parameters and their effects on the response. Predictions can also be made about response values within the limits of the region studied. Contour plots were studied with all the other factors held at their center values.

Copper(II) Sulfate-Catalyzed Study. Figure 1A shows the effect of varying storage time and temperature on peroxide value. The lowest peroxide values were obtained at median temperatures and times. At high temperature and long time, peroxide values increased, with some incidences of high peroxide value at short time and high temperature. This is probably a result of less hydroperoxide stability at high temperature, rather than slower lipid oxidation rates. It has been shown (*19*) that lipid peroxides stored at higher temperatures (37 °C) decompose faster than those stored at lower temperatures (4 °C); this was confirmed by a corresponding increase in carbonyl value at higher temperature (*19*). A combination of properties such as temperature stability of hydroperoxides and their rate of formation and decomposition at various temperatures may be responsible for this observation. The amount of a given decomposition product at a given time during the autoxidation process is determined by the net balance between the effect of several factors (*20*). Among them are hydroperoxide structure, temperature, degree of saturation, and the stability of decomposition products (*20*).

Significant interactions were shown in the model between temperature and citric acid, metal, and α-tocopherol (**Tables 3** and **4**). As the contour plots show (**Figure 1B**), peroxide values were lowest at intermediate temperatures when citric acid levels varied. Higher levels of citric acid led to an increase in peroxide value, with the lowest values again being at median temperature levels. There was a more rapid increase in peroxide values at lower temperatures than at higher temperatures. When peroxide values were observed as a function of α-tocopherol and temperature (**Figure 1C**), the lowest peroxide values were obtained at high α-tocopherol levels and middle to low temperatures. Peroxides increased with an increase in copper concentration but even more so at lower temperatures than at high temperatures (**Figure 1D**). Mancuso et al. (*21*) found that iron's increased ability to decompose lipid peroxides prevented peroxide accumulation and thus led to lower peroxide values. It is likely that at high copper concentration and high temperature, peroxide values may be lower because of increased hydroperoxide decomposition. The estimated main effect of temperature is not significant because of the presence of significant interactions. When its effect was examined at different levels of time, citric acid, metal, and α-tocopherol, we observed that temperature is indeed significant ($P < 0.05$).

There was also significant interaction between citric acid and α-tocopherol. Contour plots show that at high levels of α-tocopherol, increasing levels of citric acid led to a decrease in peroxide values (**Figure 1E**). Meanwhile, at low levels of tocopherol, high levels of citric acid had a pro-oxidant effect. This is an example of synergism in antioxidants, where α-tocopherol may be considered the primary antioxidant since it appears to be the more effective of the two when used on its own. One of the more common types of synergism includes the combined action of a free radical acceptor such as α-tocopherol and a metal chelator such as citric acid (*22*, *23*). Hydrophilic antioxidants are generally less effective than lipophilic antioxidants in protecting lipids in oil-in-water emulsions (*24*, *25*). Others have shown lower antioxidant activity for lipophilic antioxidants such as α-tocopherol in the presence of water (*26*, *27*). From the contour plots, varying metal and citric acid (**Figure 1F**), and metal and α-tocopherol (**Figure 1G**), it can be seen that α-tocopherol has a lowering effect on peroxide values, while citric acid appears to have a pro-oxidant effect. Modde 5 generated optimal conditions for minimum and maximum peroxide values. The conditions for obtaining minimum hydroperoxides for copper-catalyzed oxidation are shown in **Table 5**. Peroxide values vary depending on the levels of first-order variables. More than one combination can lead to the same peroxide value (**Table 5**).

Iron(II) Sulfate-Catalyzed Study. In the iron-containing emulsions, there were fewer significant ($P < 0.05$) interactions than in the copper-catalyzed emulsions studied (**Table 4**). Variations in peroxide value were not as great, and R^2 was also lower (0.85). The rate of hydroperoxide decomposition depends on the concentration, chemical state, and type of metal. **Figure**

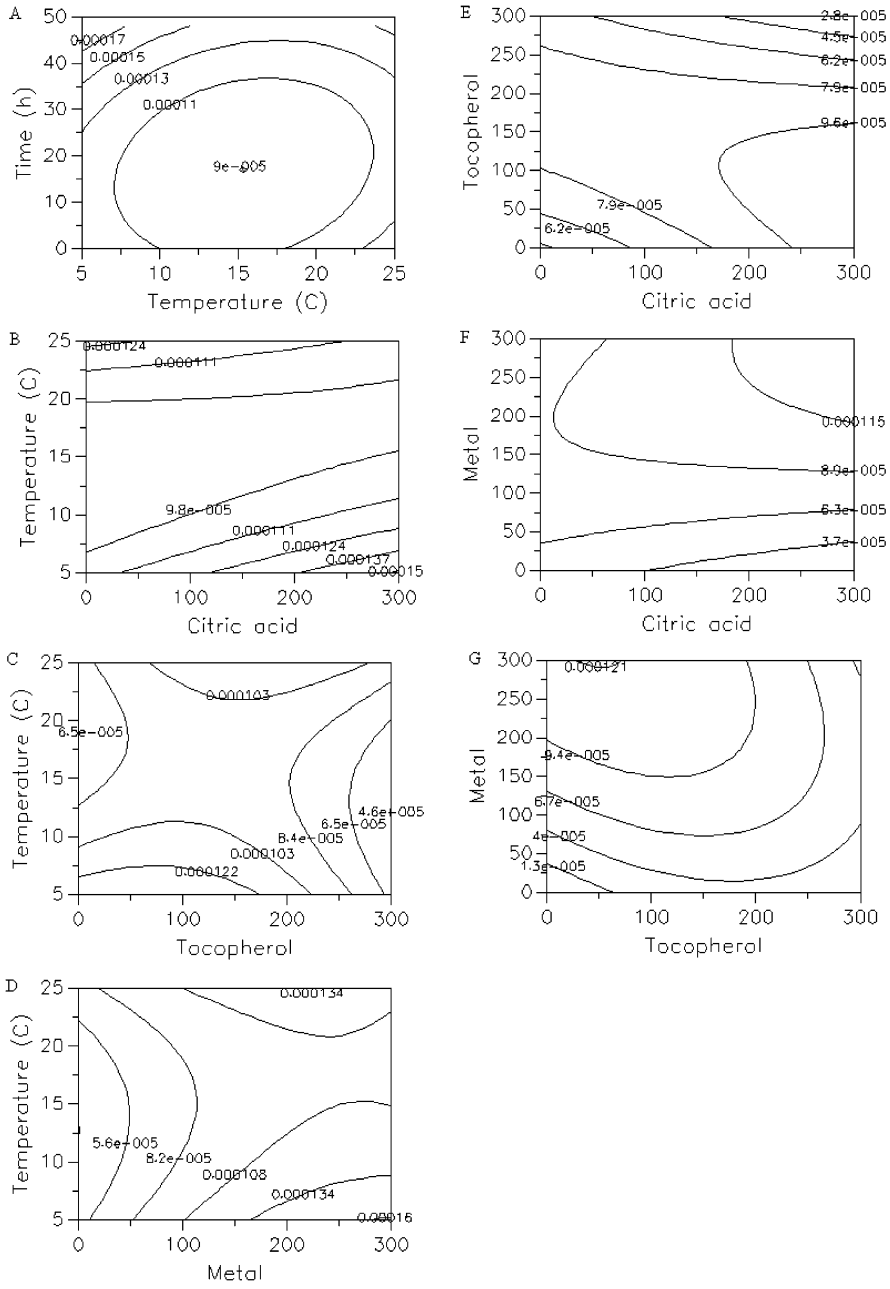

Figure 1. Contour plots for peroxide values of significant interactions for copper catalyzed study with all other factors [temperature (°C), metal concentration (μM), α-tocopherol (μM), citric acid (μM), and time (h)] held at their center values.

Table 5. Optimal Conditions for Minimal Peroxide Values As Generated by Modde 5.0 for the Copper-Catalyzed Study[a]

time (h)	temp (°C)	Cit (μM)	Toc (μM)	Met (μM)
24	25	150	0	0
27	14	9	0	0
24	5	150	300	0
11	17	259	299	300
9	10	0	69	0
38	25	300	0	0
9	25	300	300	0
24	15	210	150	0

[a] See **Table 1** for abbreviations.

2A shows that while increasing iron content increased the peroxide value, increasing incubation time brought about a decrease in the peroxide value. We can attribute this to hydroperoxide decomposition rates being greater than formation rates. Transition metal/hydroperoxide interactions will both decompose peroxides and produce free radicals, and changes in peroxide concentrations in the presence of metals actually represents a balance between peroxide formation and decomposition (*28*). From the plot of iron and citric acid concentrations (**Figure 2B**), we noted that peroxide values are highest at low citric acid concentrations and high metal concentrations. This is very likely a result of the metal-chelating power of citric acid. In general, metal chelators partly deactivate trace metals in emulsions by removing both added and endogenous transition metals from emulsion droplet surfaces (*29*). Unlike the case in the copper study, citric acid was a more effective antioxidant than α-tocopherol, because at higher concentrations of α-tocopherol and metal, the peroxide value again increased (**Figure 2C**). This observation could be a result of more rapid oxidation of α-tocopherol, which led to higher peroxide levels. It is also likely that Fe^{2+} decomposes hydroperoxides more rapidly than Cu^{2+}. Generally, a pro-oxidant effect of α-tocopherol occurs

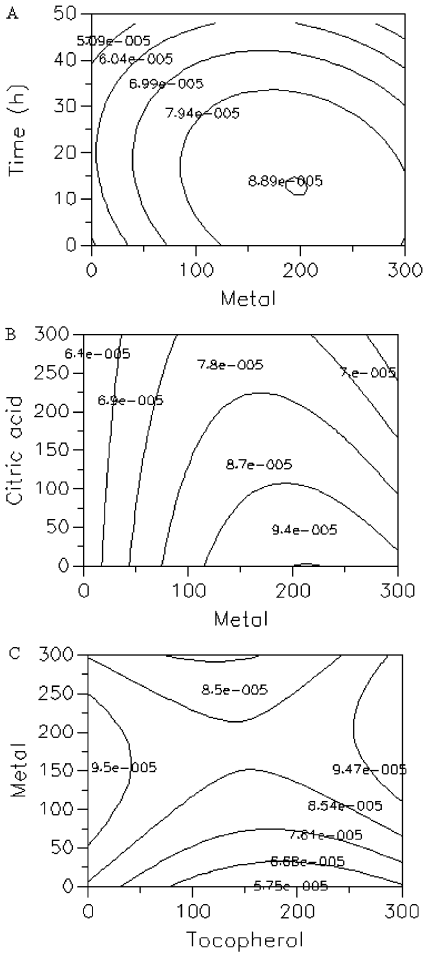

Figure 2. Contour plots for peroxide values of significant interactions for iron-catalyzed study with all other factors [temperature (°C), metal concentration (μM), α-tocopherol (μM), citric acid (μM), and time (h)] held at their center values.

Table 6. Optimal Conditions for Minimal Peroxide Values As Generated by Modde 5.0 for the Iron-Catalyzed Study[a]

time (h)	temp (°C)	Cit (μM)	Toc (μM)	Met (μM)
48	25	300	202	0
0	25	134	196	0
48	14	300	139	300
48	16	296	138	300
48	5	300	60	300
48	25	0	240	0
48	25	300	240	300

[a] See **Table 1** for abbreviations.

when it is present in relatively high concentrations. This occurs via radical formation according to the reaction (*20*)

$$\mathrm{ROOH} + \mathrm{TH_2} \rightarrow \mathrm{RO^{\bullet}} + \mathrm{TH^{\bullet}} + \mathrm{H_2O}$$

where ROOH is a hydroperoxide, TH_2 is α-tocopherol, $RO^{\bullet}$ is a peroxy radical, and $TH^{\bullet}$ is the α-tocopherol radical. Concentrations of α-tocopherol may be higher in iron-catalyzed oxidation because formation of hydroperoxides is not as rapid as in copper-catalyzed oxidation. Oxidation, therefore, does not progress rapidly enough to use up α-tocopherol to the same degree. Conditions for obtaining minimum hydroperoxides for iron-catalyzed oxidation are shown **Table 6**. Low oxidation values can be obtained under different combinations of first-order variables (**Table 6**).

Table 7. Verification of Predicted and Experimental Peroxide Values

	copper-catalyzed study	iron-catalyzed study
predicted (expt no. 25)	3.1×10^{-5}	5.9×10^{-5}
experimental (expt #no. 25)	4.3×10^{-5}	5.5×10^{-5}
predicted[a]	7.3×10^{-5}	4.1×10^{-5}
experimental[a]	6.6×10^{-5}	5.2×10^{-5}

[a] Conditions used were as follows: time, 48 h; temp, 15 °C; citric acid, 150 μM; α-tocopherol, 300 μM; and metal, 0 μM.

Model Validation. The adequacy of the model was examined by comparing experimental data with the predicted values and by performing independent validation experiments at specified conditions. The predicted values were obtained by substituting the predicted model variables with the experimental synthetic conditions (**Table 7**). The results obtained showed no significant difference between predicted and experimental results at $\alpha = 0.95$.

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