# Effects of pH and Ferric Ions on the Antioxidant Activity of Olive Polyphenols in Oil-in-Water Emulsions

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**ABSTRACT:** The effects of four phenolic compounds occurring in olives and virgin olive oil, namely, oleuropein, hydroxytyrosol, 3,4-dihydroxyphenylethanol-elenolic acid (3,4-DHPEA-EA) and 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (3,4-DHPEA-EDA), on the oxidative stability of stripped olive oil-in-water emulsions were studied at three pH values in the presence or absence of ferric chloride at 60°C. In the stability test, the addition of phenolic compounds in emulsions at pH 5.5 significantly extended the induction time of lipid oxidation, and the activities in decreasing order were 3,4-DHPEA-EA > 3,4-DHPEA-EDA > hydroxytyrosol > α-tocopherol ~ oleuropein >> control. The effect of concentration, iron, and pH on the antioxidant activity of the phenolic compounds in stripped olive oil-in-water emulsions was analyzed by response surface methodology. Oleuropein and hydroxytyrosol enhanced the prooxidant effect of ferric chloride at pH 3.5 and pH 5.5 but not at pH 7.4. The 3,4-DHPEA-EDA reduced the prooxidant effect of ferric chloride at pH 5.5 and pH 7.4, but at pH 3.5 prooxidant effects were evident at higher phenol concentration. The 3,4-DHPEA-EA reduced the prooxidant effect of ferric ions at all pH values tested. Differences in activity of the phenols may be explained by consideration of their free radical scavenging activity and ferric reducing capacity.

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**KEY WORDS:** Antioxidants, 3,4-DHPEA-EA, 3,4-DHPEA-EDA, emulsions, hydroxytyrosol, *Olea europaea,* oleuropein, olive oil, olive oil polyphenols, secoiridoids.

Virgin olive oil contains a large number of phenolic compounds including phenyl alcohols, namely, 3,4-dihydroxyphenylethanol (3,4-DHPEA, or hydroxytyrosol) and *p*-hydroxyphenylethanol (*p*-HPEA, or tyrosol) as well as phenyl acids. Derivatives of 3,4-DHPEA, in particular the dialdehydic form of elenolic acid linked to 3,4-DHPEA (3,4-DHPEA-EDA), an isomer of oleuropein aglycone (3,4-DHPEA-EA), and the dialdehydic form of elenolic acid linked to *p*-HPEA (*p*-HPEA-EDA) have been identified as the major terpenoid (secoiridoid) compounds of virgin olive oil (1–3). Polyphenol content is important for the quality of virgin olive oil, and its contribution to the oxidative stability of the oil is widely accepted. Total phenols and derivatives of 3,4-DHPEA have been correlated ($r = 0.97$) with the oxidative stability of virgin olive oil (2), whereas tocopherols have shown a poor correlation ($r = 0.05$). When tested in oil, 3,4-DHPEA and its derivatives have shown much stronger antioxidant activity than α-tocopherol (2,4).

Virgin olive oil is used by the food industry in the manufacture of sauces and mayonnaise, which are products with pH values in the acid range. Antioxidant behavior is more complex in emulsions than in bulk oil because more variables influence lipid oxidation, including emulsifiers (5,6) and pH (4,6,7). The presence of the aqueous phase often decreases the activity of antioxidants because hydrogen-bonded complexes formed with water are ineffective in scavenging lipid radicals by hydrogen donation (8). Lipids in food emulsions exist as lipid dispersions in an aqueous matrix that may contain a variety of water-soluble components including transition metals. Among the transition metals, iron may be the most important prooxidant for lipid oxidation owing to its higher concentration than copper, which is a more effective prooxidant at equal concentration. Phenols may chelate transition metal ions, hence reducing metal-induced oxidative reactions (9), but they also reduce $Fe^{3+}$ to $Fe^{2+}$. Since $Fe^{2+}$ is a relatively active prooxidant by catalyzing the decomposition of peroxides into free radicals (10), the metal-reducing properties of polyphenols can increase oxidative reactions. No research that systematically evaluates antioxidant activity of olive oil phenolics with respect to the interactions between these variables has been reported previously. The aim of this study was to evaluate the antioxidant activity of hydroxytyrosol, 3,4-DHPEA-EA, 3,4-DHPEA-EDA, and oleuropein on the basis of their ability to inhibit lipid oxidation in stripped olive oil-in-water emulsions. The influence of antioxidant concentration, pH, and the presence of iron was studied.

## MATERIALS AND METHODS

Hydroxytyrosol was synthesized from 3,4-dihydroxyphenylacetic acid (Sigma-Aldrich Quimica-S.A., Madrid, Spain) according to the procedure of Baraldi *et al*. (11). Oleuropein was purchased from Extrasynthese (Genay, France) or extracted from olive leaves according to the procedure of Gariboldi *et al.* (12). The aglycone 3,4-DHPEA-EA was obtained from oleuropein by enzymatic reaction using β-glycosidase (Fluka, Buchs, Switzerland) according to the procedure of Limiroli *et al.* (13). The olive oil component 3,4-DHPEA-EDA was obtained from olive leaves according to the proce-

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dure of Paiva-Martins and Gordon (14). α-Tocopherol, Tween 20, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical were supplied by Sigma-Aldrich. All other reagents were analytical grade or purer.

Olive oil stripped of natural tocopherols and phenols was prepared from commercial virgin olive oil by washing with 0.5 M NaOH (Merck, Darmstadt, Germany) solution and passing twice through an aluminum oxide column (Merck). Complete removal of tocopherols was confirmed by HPLC, according to IUPAC Method 2.432 (15).

*Determination of radical scavenging activity*. The effect of each antioxidant on DPPH radical concentration was estimated according to the procedure described by Brand-Williams *et al.* (16). Details of the procedure were reported by Gordon *et al.* (4) and by Paiva-Martins and Gordon (14).

*Ferric-reducing antioxidant potential (FRAP) assay*. The ferric reducing capacity of each phenolic compound was determined by the procedure described by Benzie and Strain (17) with some modifications. A suitable buffer solution (3 mL) was mixed with ferric tripyridyltriazine FRAP reagent (0.1 mL) and methanolic phenol solution (1000 μM, 0.1 mL) at 37°C, and absorbance at 593 nm was determined against a blank after 6 min reaction time. The reducing capacity was determined at pH 3.5, 5.5, and 7.4, with each of the buffer solutions used in emulsion preparation. Standard curves were prepared at each pH with freshly prepared solutions of ferrous sulfate (100–1000 μM) for calibration of the FRAP assay. Reaction of Fe(II) involves a one-electron exchange reaction and is taken as unity. The blank-corrected signal given by a 100 μM solution of Fe(II) is equivalent to a FRAP value of 100 μM. Absorbance changes observed for each phenolic test sample were converted into FRAP value (in μM) by the equation:

$$\text{FRAP value} = (\text{change in absorbance at 593 nm in test sample at 6 min/change in absorbance at 593 nm in standard at 6 min}) \times \text{FRAP value of Fe(II)} \quad [1]$$

FRAP values were determined in triplicate.

*Emulsion samples*. Oil-in-water emulsions (30%, 33 g) were prepared in 100-mL Erlenmeyer flasks. Olive oil (10 g), stripped of natural tocopherols and phenols, was mixed with each additive at the required concentration according to Table 1. Tween 20 (0.66 g) was dissolved in the required buffer solution (22.3 g), and the mixture was sonicated for 10 min in an ice bath.

Buffer solutions used were acetate buffer 0.05 M, pH 3.5; acetate buffer 0.05 M, pH 5.5; and 3-*N*-morpholinopropanesulfonic acid 0.05 M, pH 7.4.

*Oxidation experiments*. Samples were oxidized in the dark at 60°C. The antioxidant activity of each phenol was determined in 25 emulsions prepared on the same day, and each phenol was studied in an independent experiment. Isolation of oil from emulsions for analysis was by freezing, thawing,

**TABLE 1**
**Time (d) for Emulsions to Achieve a Conjugated Diene (CD) Content of 0.4% and a *p*-Anisidine Value (AV) of 10 During Storage at 60°C**

| | | | | Oleuropein | | Hydroxytyrosol | | 3,4-DHPEA-EDA[a] | | 3,4-DHPEA-EA[b] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample | pH | Iron conc. ($mg \cdot kg^{-1}$) | Phenol (mM) | Time to CD = 0.4% | Time to AV = 10 | Time to CD = 0.4% | Time to AV = 10 | Time to CD = 0.4% | Time to AV = 10 | Time to CD = 0.4% | Time to AV = 10 |
| 1 | 3.5 | 0 | 0 | 1.91 | 3.15 | 2.26 | 3.32 | 2.24 | 3.20 | 1.76 | 3.10 |
| 2 | 3.5 | 0 | 0.8 | 8.87 | 8.28 | 6.91 | 8.14 | 28.02 | 29.89 | 17.46 | 19.15 |
| 3 | 3.5 | 0.4 | 0.4 | 0.93 | 1.56 | 0.97 | 1.65 | 1.17 | 1.47 | 1.30 | 2.16 |
| 4 | 3.5 | 0.4 | 1.2 | 0.86 | 1.17 | 0.90 | 1.04 | 2.93 | 2.82 | 5.16 | 5.03 |
| 5 | 3.5 | 0.8 | 0 | 1.05 | 1.92 | 1.14 | 0.91 | 1.17 | 2.10 | 1.12 | 2.10 |
| 6 | 3.5 | 0.8 | 0.8 | 0.78 | 1.11 | 0.87 | 0.93 | 0.90 | 1.25 | 2.08 | 2.20 |
| 7 | 3.5 | 1.2 | 0.4 | 0.84 | 1.32 | 1.04 | 1.65 | 0.95 | 1.65 | 1.11 | 1.45 |
| 8 | 3.5 | 1.2 | 1.2 | 0.82 | 0.87 | 0.81 | 0.87 | 1.00 | 1.21 | 2.46 | 2.35 |
| 9 | 5.5 | 0 | 0.4 | 4.87 | 8.02 | 6.46 | 6.54 | 24.21 | 25.89 | 14.17 | 15.60 |
| 10 | 5.5 | 0 | 1.2 | 27.99 | 29.15 | 29.43 | 30.78 | 43.12 | 45.35 | 41.24 | 43.09 |
| 11 | 5.5 | 0.4 | 0 | 1.6 | 3.09 | 2.25 | 3.34 | 2.14 | 2.85 | 2.14 | 3.55 |
| 12 | 5.5 | 0.4 | 0.8 | 1.13 | 1.03 | 1.71 | 2.25 | 4.63 | 5.10 | 6.42 | 6.60 |
| 13 | 5.5 | 0.8 | 0.4 | 1.24 | 2.3 | 1.93 | 2.76 | 1.97 | 3.12 | 4.45 | 4.37 |
| 14 | 5.5 | 0.8 | 1.2 | 1.16 | 2.03 | 1.92 | 1.74 | 4.12 | 4.63 | 8.10 | 8.10 |
| 15 | 5.5 | 1.2 | 0 | 1.45 | 2.8 | 2.25 | 2.89 | 1.91 | 3.25 | 1.95 | 3.40 |
| 16 | 5.5 | 1.2 | 0.8 | 1.15 | 2.16 | 1.45 | 1.95 | 2.15 | 3.48 | 7.08 | 7.55 |
| 17 | 7.4 | 0 | 0 | 4.88 | 5.37 | 7.04 | 8.25 | 7.67 | 7.70 | 7.03 | 7.40 |
| 18 | 7.4 | 0 | 0.8 | 23.42 | 24.82 | 22.56 | 23.56 | 27.68 | 29.30 | 27.13 | 29.15 |
| 19 | 7.4 | 0.4 | 0.4 | 7.02 | 7.76 | 6.85 | 7.51 | 5.42 | 7.24 | 10.51 | 11.30 |
| 20 | 7.4 | 0.4 | 1.2 | 10.34 | 11.87 | 10.34 | 11.65 | 17.12 | 18.32 | 16.97 | 17.45 |
| 21 | 7.4 | 0.8 | 0 | 2.65 | 4.58 | 4.31 | 4.18 | 3.26 | 5.03 | 4.26 | 5.25 |
| 22 | 7.4 | 0.8 | 0.8 | 5.46 | 7.2 | 6.16 | 7.01 | 5.31 | 6.41 | 10.82 | 11.65 |
| 23 | 7.4 | 1.2 | 0.4 | 5.80 | 6.31 | 4.31 | 4.39 | 4.39 | 5.48 | 8.21 | 9.20 |
| 24 | 7.4 | 1.2 | 1.2 | 7.53 | 8.98 | 7.08 | 8.09 | 6.80 | 7.97 | 10.10 | 11.03 |
| 25 | 5.5 | 0 | 0 | 2.79 | 3.88 | 2.35 | 2.99 | 2.8 | 4.32 | 2.79 | 3.70 |

[a]3,4-DHEA-EDA, the dialdehydic form of elenolic acid linked to 3,4-dihydroxyphenylethanol.
[b]3,4-DHPEA-EA, an isomer of oleuropein aglycone.

and centrifugation. Progress of oxidation was monitored by determination of the conjugated dienes (CD) (AOCS Official Method Ti 1a-64) and *p*-anisidine value (AV) (AOCS Official Method Cd 18-90) (18).

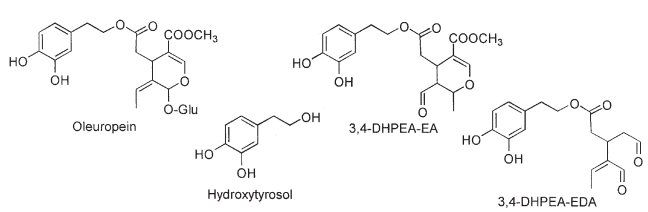
*Experimental design*. The influence of iron, pH, and phenol concentration on the stability of the emulsions was studied by response surface methodology. SPSS (Statistical Package for the Social Sciences, SPSS, Inc.; Chicago, IL) software was used for data analysis. Response surface methodology uses an experimental design to fit a model by least squares analysis. Initial concentrations of phenolic compounds ranged from 0 to 1.2 mmol·kg$^{-1}$ of oil phase, iron concentration was in the range 0 to 1.2 mg·kg$^{-1}$, and pH values were 3.5, 5.5, and 7.4. A half-replicate fractional factorial design was used. In this design, alternate combinations are omitted so that the number of combinations of three parameters, iron concentration, phenol concentration, and pH, with four, four, and three levels, respectively, is reduced by half from 48 possible to 24 studied for each phenol, but an additional sample was included to provide a control at pH 5.5 (Table 1).

*Statistical analysis*. Statistical analysis to determine significant differences in antioxidant activity of all phenolic compounds involved plotting CD against time to determine times to certain values and then applying one-way ANOVA with Tukey's honestly significant difference multiple comparison to determine differences significant at the 5% level.

The effects of pH, iron concentration, and phenol concentration on the oxidative stability of emulsions were analyzed by univariate ANOVA for log (time) and performed with SPSS 10.0 software. Statistical differences between FRAP values and between the concentrations required for 50% radical scavenging activity ($EC_{50}$ values) from the DPPH test were assessed by one-way ANOVA with the level of significance set at $P < 0.05$. FRAP tests were performed in triplicate and DPPH tests in quadruplicate.

## RESULTS AND DISCUSSION

*Oxidative stability of olive oil-in-water emulsions containing olive phenolics.* Virgin olive oil contains a polar fraction including total polyphenol content of 50–800 mg·kg$^{-1}$ (19), which corresponds to total concentration in the range 0.17–2.7 mmol·kg$^{-1}$ if the mean relative molecular mass is taken as 300. These data were used to select suitable concentrations of polyphenols to study. Pure olive polyphenols (hydroxytyrosol, oleuropein, 3,4-DHPEA-EA, and 3,4-DHPEA-EDA) (Scheme 1) added to stripped olive oil-in-water emulsions (pH 5.5) at a concentration of 0.8 mmol·kg$^{-1}$, based on the oil phase, were very effective in stabilizing emulsions stored at 60°C, with hydroxytyrosol, 3,4-DHPEA-EDA, and 3,4-DHPEA-EA being much more effective then α-tocopherol (Fig. 1). Oleuropein showed an activity similar to α-tocopherol when assessed by the time for emulsions to achieve a CD content of 0.4% (Table 1). The order of antioxidant activity was in accordance with the order for olive phenolic compounds added to oil-in-water emulsions at a concentration of 0.3 mmol·kg$^{-1}$ reported by Gordon *et al.* (4) except that α-tocopherol was more effective than oleuropein at the lower concentration. At the higher concentration, α-tocopherol showed some prooxidant activity in the early stages of autoxidation, as already described in bulk oils (20,21). Although oleuropein was the least effective olive polyphenol, it showed an increase in antioxidant activity with concentration in a way similar to hydroxytyrosol (Fig. 2). According to the polar paradox (22), less polar antioxidants are effective in emulsions because they are concentrated at oil–water interfaces. More polar antioxidants are less effective in emulsions because they are mainly present in the aqueous phase. The order of polarity of the olive polyphenols based on the partition between octanol and water is hydroxytyrosol > oleuropein > DHPEA-EDA > 3,4-DHPEA-EA > α-tocopherol (Ref. 4 and, for DHPEA-EDA, unpublished data). The results at a concentration of 0.8 mmol·kg$^{-1}$ were in accordance with the polar paradox except for α-tocopherol and oleuropein. Comparing the chemical structures of oleuropein and hydroxytyrosol (Scheme 1), it is clear that oleuropein is a glucoside but it also has some additional nonpolar features, which offset the increase in polarity owing to the sugar. In the case of oleuropein, the antioxidant activity in the emulsions was similar to that of hydroxytyrosol in the absence of iron. This can be seen from the data for sample 2 (pH 3.5), sample 10 (pH 5.5), and sample 18 (pH 7.4) in Table 1. According to




**SCHEME 1**

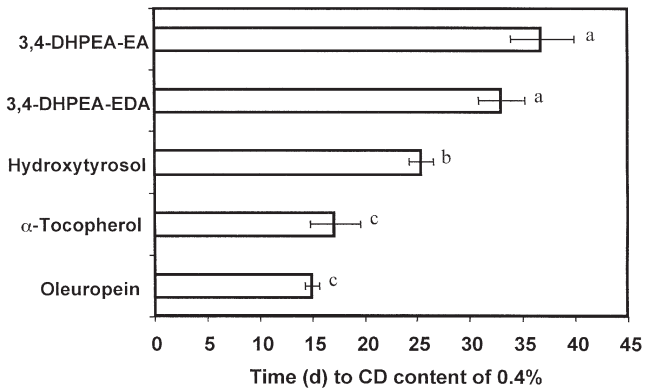



**FIG. 1.** Time for stripped olive oil-in-water emulsion samples (pH 5.5) containing additives (0.8 mmol/kg of oil phase) to reach a conjugated diene content of 0.4%. Letters indicate samples that were significantly different ($P < 0.05$). 3,4-DHPEA-EDA, the dialdehydic form of elenolic acid linked to 3,4-dihydroxyphenylethanol; 3,4-DHPEA-EA, an isomer of oleuropein aglycone.

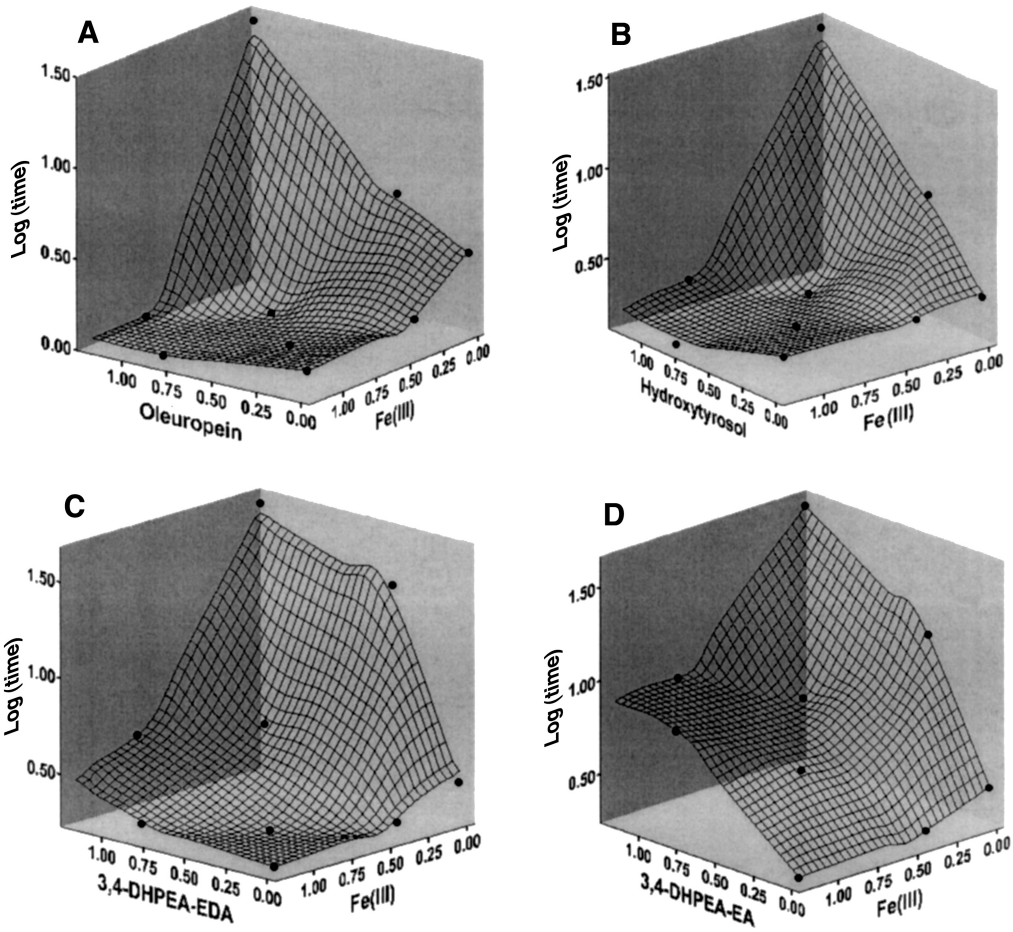



**FIG. 2.** Effect of phenolic compound concentration (mM) and ferric ion concentration ($mg \cdot kg^{-1}$) on the oxidation of stripped olive oil TAG-in-water emulsions at pH 5.5, stored at 60°C, assessed by the logarithm of the time (d) for emulsions to reach a conjugated diene content of 0.4%. (A) Oleuropein; (B) hydroxytyrosol; (C) 3,4-DHPEA-EDA; (D) 3,4-DHPEA-EA. For abbreviations see Figure 1.

the CD and AV values, oleuropein was slightly more stable than hydroxytyrosol at pH 3.5 and 7.4 but slightly less stable at pH 5.5. Although oleuropein has a higher partition coefficient in an octanol/water system than hydroxytyrosol (4), the difference is small and glycosides commonly have antioxidant activity that corresponds to less polar molecules than would be predicted according to their polarity (23). AV determinations confirmed the order of antioxidant activity (Table 1). The time to AV = 10 was slightly longer (normally up to 1.5 d) than the time to CD = 0.4%, and there was no evidence that there were specific effects on formation of secondary oxidation products that were not present in the times to the CD end point, which mainly reflects hydroperoxide formation.

*The effect of concentration, ferric ion, and pH on the antioxidant activity of phenolic compounds in stripped olive oil-in-water emulsions.* The effects of pH, iron concentration, and phenol concentration on the stability of emulsions were studied by preparing and storing 25 emulsions. Table 1 shows the results for each sample.

Since stability decreased very strongly with low levels of iron, it was found that the use of log (time) for samples to reach a certain value of CD or AV gave the best correlation for mathematical models (general linear model) describing the effects on stability of pH, iron concentration ($mg \cdot kg^{-1}$) in the emulsion, and concentration ($mmol \cdot kg^{-1}$) of each phenolic compound based on the oil phase.

The equation for time to CD of 0.4% in the presence of oleuropein was:

$$\begin{aligned}\log(\text{time}) = {} & 1.094 - 0.316\,(\text{pH}) \\ & - 1.995\,(\text{iron}) + 0.212\,(\text{phenol}) \\ & + 0.12\,(\text{pH})(\text{iron}) + 0.0559\,(\text{pH})(\text{phenol}) \\ & - 0.534\,(\text{iron})(\text{phenol}) + 0.0353\,(\text{pH})^2 \\ & + 1.0\,(\text{iron})^2 + 0.045\,(\text{phenol})^2\end{aligned} \quad [2]$$

The corresponding equation for other phenols to reach CD = 0.4% or to reach an AV of 10 can be deduced by substituting the coefficients given in Table 2 into the equation. The coefficients for each variable in the first-order form are $\beta_1$, $\beta_2$, and $\beta_3$, whereas $\beta_{12}$, $\beta_{13}$, and $\beta_{23}$ are the coefficients for each interaction among the variables, and $\beta_1^2$, $\beta_2^2$, and $\beta_3^2$ are the coefficients for each variable in the second-order form.

**TABLE 2**
**Regression Coefficients, $R^2$, and Probability ($P$)[a] of $F$ Values for the Equations Relating log (time to CD = 0.4%) or log (time to AV = 10) to Iron, pH, and Phenol Concentration for Each Phenolic Compound**

| | Oleuropein | | Hydroxytyrosol | | 3,4-DHPEA-EDA | | 3,4-DHPEA-EA | |
|---|---|---|---|---|---|---|---|---|
| | CD | AV | CD | AV | CD | AV | CD | AV |
| Intercept | 1.094 | 1.683** | 0.342 | 0.411 | 0.08638 | 0.157 | –0.676 | –0.0579 |
| $\beta_1$[b] | –0.316 | –0.452 | –0.09420 | –0.0519 | 0.131 | –1.687 | 0.322 | 0.214 |
| $\beta_2$ | –1.995* | –0.399* | –1.385* | 0.405* | –1.885* | 0.170* | –1.293* | –1.336* |
| $\beta_3$ | 0.212 | –1.925 | 0.456 | –1.341 | 0.786 | 0.541 | 1.443* | 0.839** |
| $\beta_{12}$ | 0.120 | 0.128 | 0.04398 | 0.05179 | 0.08067 | 0.06858 | 0.07168 | 0.08663 |
| $\beta_{13}$ | 0.05590 | 0.09555 | –0.08844 | 0.00580 | 0.02565 | 0.05394 | –0.04636 | 0.01054 |
| $\beta_{23}$ | –0.534** | –0.387 | -0.646* | –0.592* | –0.694* | –0.716* | –0.422* | –0.478* |
| $\beta_1^2$ | 0.03532 | 0.03805 | 0.02407 | 0.01762 | –0.00417 | –0.00972 | –0.01624 | –0.0122 |
| $\beta_2^2$ | 1.000* | 0.854* | 0.902* | 0.814* | 0.976* | 0.964* | 0.609* | 0.613* |
| $\beta_3^2$ | 0.04506 | 0.267 | 0.110 | 0.111 | –0.0649 | –0.0757 | –0.3164 | –0.167 |
| $R^2$ | 0.885 | 0.869 | 0.902 | 0.902 | 0.918 | 0.903 | 0.941 | 0.930 |
| $P$ of $F$ values | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

[a]See Table 1 for other abbreviations.
[b]1, pH; 2, iron; 3, phenolic compound. *Significant at the 0.01 level. **Significant at the 0.05 level.

The fit of the data into the equation was tested by the $R^2$ values, which were greater than 0.9 for both chemical attributes, CD and AV, except for oleuropein, where the $R^2$ values were greater then 0.86. The relation between phenol concentration, iron concentration, and log (time) at pH 5.5 is shown in Figures 2A–D for each of the four phenols studied. Similar figures can be drawn for pH 3.5 and 7.4. Further statistical tests, checking the probability of $F$ values ($P < 0.0005$), and the plot of residuals against predicted values showed, for all compounds tested, that the models were a satisfactory summary of the observations. It is clear that 3,4-DHPEA-EDA was similar to 3,4-DHPEA-EA at pH 5.5 in having a sharp rise in stability with low concentrations of phenol with a less steep rise at higher concentrations. Hydroxytyrosol showed a less steep increase in stability with concentration at low concentrations, and oleuropein showed an even less steep increase with concentration at low phenol concentrations. The reduction in stability by Fe(III) was severe for hydroxytyrosol and oleuropein and still quite strong for 3,4-DHPEA-EDA, but it was much less severe for 3,4-DHPEA-EA. From the statistical analysis, ferric ion concentration and the interaction between ferric ions and phenols were the most important factors affecting the stability of emulsions for all compounds.

In the absence of ferric ions, the four compounds showed a marked increase in antioxidant activity with an increase in phenol concentration at all pH values. However, hydroxytyrosol and oleuropein showed a marked prooxidant effect in the presence of ferric ions at pH 3.5 (Table 1) and 5.5 (Figs. 2A and 2B), with the effect increasing with phenol concentration. However, at pH 7.4 an antioxidant effect for both compounds could be observed. Ferric ion solubility is very low at alkaline pH owing to the formation of ferric hydroxide, so it is not surprising that the activity of the antioxidants is not reduced so markedly in the presence of ferric ions at pH 7.4. The polyphenol 3,4-DHPEA-EDA also showed prooxidant effects at pH 3.5 in the presence of ferric ions but not at pH 5.5 (Fig. 2C) or at pH 7.4. However, 3,4-DHPEA-EA showed antioxidant activity in the presence of ferric ions at all three pH values, with the magnitude depending greatly on both phenol and ferric ion concentrations (Fig. 2D).

*DPPH scavenging test and FRAP assay*. The radical scavenging activity of the phenolic compounds, assessed by the antioxidant concentration required for 50% reduction in DPPH radical concentration in 15 min ($EC_{50}$) (Table 3), decreased in the order: of 3,4-DHPEA-EA >> hydroxytyrosol > oleuropein > α-tocopherol > 3,4-DHPEA-EDA. The 3,4-DHPEA-EA showed the highest radical scavenging activity of the compounds tested, which could explain the antioxidant activity at all pH values even in the presence of ferric ions. However, the 3,4-DHPEA-EDA had a lower radical scavenging activity than hydroxytyrosol, although it showed a better antioxidant activity in the presence of ferric ions.

In order to understand the behavior of these compounds in the presence of ferric ions, the ferric-reducing ability of the phenolic compounds was evaluated by the FRAP assay. The ferric-reducing activity of antioxidants is important for their antioxidant activity in emulsions containing ferric ions, because Fe(II) is a much more effective catalyst of autoxidation than Fe(III) (10). Owing to the solubility characteristics of these compounds, they were dissolved in methanol for the FRAP assay. To avoid any possible influence of the methanol

**TABLE 3**
**DPPH Radical Scavenging Effects**

| Compound | $EC_{50}$[a] | No. of reduced DPPH |
|---|---|---|
| 3,4-DHPEA-EDA | 0.28[a] (± 0.01) | 1.8 |
| α-Tocopherol | 0.25[b] (± 0.01) | 2.0 |
| Oleuropein | 0.22[c] (± 0.01) | 2.2 |
| Hydroxytyrosol | 0.19[d] (± 0.01) | 2.7 |
| 3,4-DHPEA-EA | 0.12[e] (± 0.01) | 4.3 |

[a]Roman superscripts within a column indicate samples that were significantly different ($P < 0.05$). $EC_{50}$ values (concentrations required for 50% radical scavenging activity) expressed as mol of antioxidant/mol of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Mean (SD in parentheses) of four determinations. See Table 1 for abbreviations.

**TABLE 4**
**FRAP Values[a] for Phenolic Compounds**

| Compound | pH 3.5 | pH 5.5 |
|---|---|---|
| Hydroxytyrosol | 1751.5 (± 21.3)[a] | 1795.0 (± 26.0)[a] |
| α-Tocopherol | 1727.2 (± 7.5)[a] | 1776.5 (± 28.5)[a] |
| Oleuropein | 1466.2 (± 8.6)[b] | 1504.8 (± 19.4)[b] |
| 3.4-DHPEA-EA | 1221.6 (± 9.4)[c] | 1233.5 (± 47.1)[c] |
| 3.4-DHPEA-EDA | 996.5 (± 20.4)[d] | 1013.9 (± 34.5)[d] |

[a]Mean (SD in parentheses) of triplicate determinations. Roman superscripts indicate samples that are significantly different ($P < 0.05$). FRAP, ferric-reducing antioxidant potential; for other abbreviations see Table 1.

(100 μL) in the reaction mixture (3.1 mL), standard curves with $FeSO_4$ were also prepared with the addition of 100 μL of methanol. Table 4 shows the FRAP values of the compounds calculated using the calibration curves obtained with the appropriate buffer solution. The ferric reducing ability at pH 3.5 and 5.5 decreased in the following order: hydroxytyrosol, α-tocopherol > oleuropein > 3,4-DHPEA-EA > 3,4-DHPEA-EDA. The FRAP value of each compound was independent of pH when tested at pH 3.5 and 5.5. Iron solubility is very low at alkaline pH owing to the formation of ferric hydroxide, so the FRAP test did not show any ferric-reducing activity at pH 7.4. These results help to explain the high antioxidant activity of 3,4-DHPEA-EA in the presence of iron because this compound has the highest radical scavenging activity and a relatively low iron-reducing capacity. Hence, the more active prooxidant Fe(II) is formed to a lesser extent with 3,4-DHPEA-EA than with other polyphenols. Lower radical scavenging activity was exhibited by 3,4-DHPEA-EDA, but this compound retained antioxidant activity in iron-catalyzed oxidation owing to its low iron reducing capacity.

This work has shown that the behavior of phenolic compounds in emulsions can be explained by radical scavenging activity, pH, the presence of metals and the reducing capacity, as well as the partition coefficients for partitioning between the oil and water phases. Phenolic components of olive oil show high antioxidant capacity in the pH range 3.5–7.4, but their activity is reduced or they may even be prooxidant in the presence of ferric ions. Since food emulsions containing olive oil including mayonnaise and sauces normally have an acidic pH, it is important that contamination with ferric ions be avoided or that an effective metal-chelating agent be included in the formulation.

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