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Interactions of Ferric Ions with Olive Oil Phenolic Compounds

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The ferric complexing capacity 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), and their stability in the presence of ferric ions were studied. At pH 3.5, all compounds formed a reversible 1:1 complex with ferric ions, but hydroxytyrosol could also form complexes containing >1 ferric ion per phenol molecule. At pH 5.5, the complexes between ferric ions and 3,4-DHPEA-EA or 3,4-DHPEA-EDA were relatively stable, indicating that the antioxidant activity of 3,4-DHPEA-EA or 3,4-DHPEA-EDA at pH 5.5 is partly due to their metal-chelating activity. At pH 7.4, a complex containing >1 ferric ion per phenol molecule was formed with hydroxytyrosol. Oleuropein, 3,4-DHPEA-EA, and 3,4-DHPEA-EDA also formed insoluble complexes at this pH. There was no evidence for chelation of Fe(II) by hydroxytyrosol or its derivatives. At all pH values tested, hydroxytyrosol was the most stable compound in the absence of Fe(III) but the most sensitive to the presence of Fe(III).

KEYWORDS: Antioxidants; hydroxytyrosol; *Olea europaea*; oleuropein; iron; polyphenols; olive oil; 3,4-DHPEA-EA; 3,4-DHPEA-EDA

INTRODUCTION

The presence of metals in edible oils occurs through natural and environmental contamination and by introduction during processing. Their potential deleterious effect is the enhancement of the oxidation rate of edible oils by increasing the rate at which free radicals are generated from fatty acids, hydroperoxides, or from secondary oxidation products, including alcohols or aldehydes. Lipids and emulsifiers used in food emulsions normally contain small quantities of lipid peroxides (*1*, *2*), and oxidative decomposition of these peroxides to form lipid radicals is effectively accelerated by transition metals such as iron. Antioxidant behavior is more complex in emulsions than in bulk oil because more variables influence lipid oxidation, including emulsifiers (*2*, *3*) and pH (*1*, *2*, *4*). The presence of the aqueous phase often decreases the activity of antioxidants because hydrogen bonded complexes formed with water are less effective in scavenging lipid radicals by hydrogen donation (*5*). Since transition metals, especially iron, are also common contaminants of water and normal constituents of foods, it would be expected that water-soluble metal ions could catalyze the oxidation of dispersed lipids at the oil−water interface. In transition metal−free lipid systems, phenolics act as antioxidants by acting as hydrogen donors, with the formation of aryloxy radicals. In the presence of transition metal ions, both radical scavenging and metal chelation contribute to the antioxidative effects of phenols. Phenols may chelate transition metals ions, hence reducing metal-induced oxidative reactions (*6*), but they also reduce Fe^{3+} to Fe^{2+}. Since Fe^{2+} is more active than Fe^{3+} at catalyzing the decomposition of hydroperoxides into free radicals (*7*), the metal-reducing properties of polyphenols can increase oxidative reactions.

Virgin olive oil is used in a wide variety of Mediterranean dishes as bulk oil and is used in the manufacture of sauces and mayonnaise, which are emulsions with pH values in the acid range. 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) components in virgin olive oil (*8*−*10*). The phenol content is important for the quality of virgin olive oil, and the contribution of these components to the oxidative stability of the oil is widely accepted. Total phenols and derivatives of 3,4-DHPEA were correlated ($r = 0.97$) with the oxidative stability of virgin olive oil (*9*), whereas tocopherols showed a poor correlation ($r = 0.05$). When tested in oil, 3,4-DHPEA and its derivatives showed much stronger antioxidant activity then α-tocopherol (*4*, *9*).

Previous work has reported the antioxidant effects of pure olive oil phenolic compounds (hydroxytyrosol, hydroxytyrosol acetate, oleuropein, 3,4-DHPEA-EA, and 3,4-DHPEA-EDA)

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assessed by the diphenylpicrylhydrazyl (DPPH) assay, the ferric reducing antioxidant potential (FRAP) assay, storage in oil, and storage in emulsions (both with and without ferric ions) (*4*, *11*, *15*). According to the literature, the compounds showing the best antioxidant activity in O/W emulsions in the presence of iron were DHPEA-EA and DHPEA-EDA, which did not show prooxidant activity in contrast to hydroxytyrosol and oleuropein. However, when the radical scavenging activity was measured for these compounds, DHPEA-EA showed a much higher activity then DHPEA-EDA, and this suggests that chelation of iron is of major significance in determining the antioxidant activity of these compounds in the presence of iron and water.

The aim of this study was to evaluate the interactions of ferric ions with hydroxytyrosol, 3,4-DHPEA-EA, 3,4-DHPEA-EDA, and oleuropein and understand their significance for the effects of the phenolic compounds in bulk olive oil stripped of antioxidants, in stripped olive oil-in-water emulsions, and in processed olives.

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. (*12*). Oleuropein was purchased from Extrasynthese (Genay, France) or extracted from olive leaves according to the procedure of Gariboldi et al. (*13*). Aglycone 3,4-DHPEA-EA was obtained from oleuropein by enzymatic reaction using β-glycosidase (Fluka, Buchs, Switzerland) according to the procedure of Limirioli et al. (*14*). The olive oil component 3,4-DHPEA-EDA was obtained from olive leaves according to the procedure of Paiva-Martins and Gordon (*15*). All other reagents were analytical grade or purer.

Ferric and Ferrous Ion Chelating Activity of Phenolic Compounds at pH 3.5, 5.5, and 7. Chelation of Ferric Ions. The formation of metal complexes was investigated in acetate buffer (0.05 M) at pH 3.5 and 5.5 and in MOPS (3*N*-morpholinopropanesulfonate) buffer (0.05 M) pH 7.4. Each phenolic compound was dissolved in methanol at a concentration of 4 mM, and 1 mL of each solution was then diluted in the appropriate buffer to give the final concentration of 400 μM. UV/vis spectra of the pure compounds were determined at room temperature in the region of 210−750 nm. Solutions of ferric chloride in acetate buffer pH 3.5 were also prepared and diluted such that, after introduction of a 20 μL aliquot of each dilution into the assay, ferric ions were in a ratio iron/phenol of 0.25, 0.5, 0.75, 1, 2, 3, 4, and 5. These solutions were kept in PVC vials during manipulation. Phenolic compound solutions (3 mL) and ferric chloride solutions were added directly to quartz cuvettes, mixed by inverting, and their absorbance was immediately measured. EDTA solutions were prepared and diluted such that, after introduction of a 20 μL aliquot of each dilution into the assay, the ratio iron/EDTA was 1:1.25. Spectra were run immediately after the addition of EDTA solution and after 15 or 30 min depending on the observed color of the solutions.

Chelation of Ferrous Ions. The chelation of ferrous ions by phenolic compounds was also investigated. Solutions of ferrous sulfate in degassed acetate buffer (0.05 M, pH 3.5) were prepared and diluted such that after addition of a 20 μL aliquot of each dilution into the assay, ferrous ions were in the ratio iron/phenol of 0.5 and 1. Phenolic compound solutions at pH 3.5, 5.5, and 7.4 (3 mL, 400 μM) and ferrous sulfate solutions were also added directly to quartz cuvettes, mixed by inverting, and their absorbance was immediately measured. Changes in the spectra were not observed in the first minute but after a while, the color (blue) and spectra similar to those recorded with ferric ions were obtained. All solutions were kept and stored in the dark as much as possible during manipulation. A blank containing only buffer and ferrous ions was run at the same time, and formation of ferric ions, given by an absorbance in the UV region, was not observed during the assay for the blank. To understand if the blue color was due to a slow chelation of ferrous ions or due to the chelation of ferric ions obtained by oxidation of ferrous ions by air, a new assay was performed under argon. A hydroxytyrosol solution (3 mL) was placed in a glass vial closed by a septum. Argon was passed through the solution by means of a thin needle to purge all the oxygen with a second needle for exit of argon from the vial. A ferrous sulfate solution (20 μL) was introduced into the vial by means of a syringe and mixed. This solution was then stored in the dark for 30 min under argon, and no blue color was observed after this time. The vial was then opened to the air for another 30 min in the dark, and a blue color was then observed.

Stability of Phenolic Compounds at pH 3.5, 5.5, and 7.4 in the Presence and Absence of Ferric Chloride. The stability of a mixture of hydroxytyrosol, oleuropein, 3,4-DHPEA-EA, and 3,4-DHPEA-EDA (0.6 mM each component) in a 65% ethanol/water solution at pH 3.5, 5.5, and 7.4 was investigated in the presence and absence of Fe(III) (0.6 mM). The solution containing a mixture of phenolic compounds was prepared in 10 mL volumetric flasks by dilution with ethanol/water (13:7). Each solution (4 mL) was placed in two different vials (1.5 cm × 4 cm), and 50 μL of a solvent mixture was added to one vial, and 50 μL of a ferric chloride solution (final concentration of 0.3 mM) was added to the other vial. The vials were vortexed for 30 s and stored at 37 °C in the dark. Periodically, solutions in the vials were agitated on a vortex mixer and sonicated for 1 min before convenient amounts of solution (120 μL) were taken and stored at −30 °C.

The changes in each component during storage were monitored by HPLC. Conditions were the same as described previously.[15] Clear samples were injected directly into the HPLC apparatus without previous treatment. Samples containing precipitates were filtered through a Whatman 0.2 μm PVDF filter before injection.

Nephelometry. Nephelometry experiments were performed in a HACH 2100N laboratory turbidimeter. The optical apparatus was equipped with a tungsten filament lamp with three detectors: a 90° scattered-light detector, a forward-scatter light detector, and a transmitted light detector. Previous calibration was performed using a Gelex Secondary Turbidity Standard Kit (HACH), which consists of stable suspensions of a metal oxide in a gel. This analytical method requires ideal conditions where all particles are small and identical. The phenol solutions were those used in the metal chelation study. Each solution (2 mL) was mixed with the ferric solution in a test tube and the mixture was shaken and stored at room temperature. The EDTA solution was then added in a ratio iron/EDTA of 1:1.25. The results were expressed in nephelos turbidity units (NTU).

RESULTS AND DISCUSSION

It is known that the stability of complexes formed between ligands and metals contributes to the lower reducing capacity of the former compounds. To understand the different reducing capacity of these compounds, their chelating properties were analyzed by spectrophotometry.

UV−vis spectral properties are due to differences between the ground state and the excited states of molecules. Ligands such as phenols possess characteristic absorption bands that are normally in the ultraviolet region of the spectrum. These bands remain in the spectra of the complexes but may be shifted somewhat from their original position. However, strongly oxidizing cations, such as Fe^{3+}, often have ligand-to-metal charge-transfer bands that are low enough in energy to encroach into the visible region. Thus, Fe(III) forms many colored complexes with ligands including phenolic compounds that act as good electron donors. These transitions take place between molecular orbitals of which one is largely a metal orbital and the other is largely a ligand orbital. They thus correspond to the transfer of an electron from the ligand to the metal caused by the absorption of light. One problem associated with interpreting these charge-transfer transitions is the lack of certainty about which orbital from which the electron came.

On interaction with ferric ions, new peaks and shifts in the original maximum wavelength will be expected and assumed to be characteristic of an iron−phenol complex, and the reversibility of the reaction would confirm this. However, olive

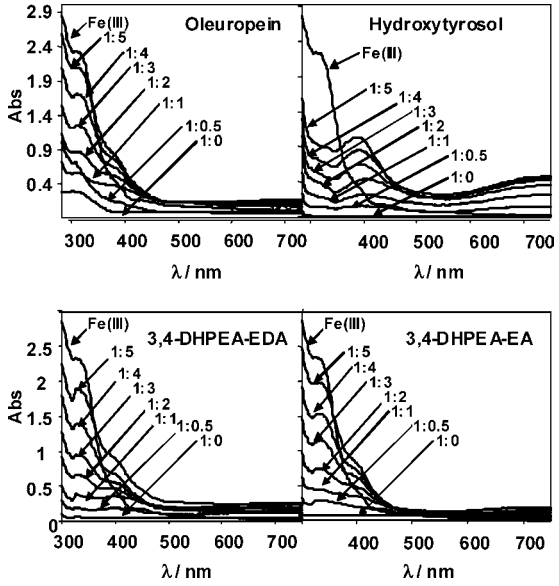

Figure 1. Chelate formation of phenolic compounds at 400 μM in the presence of increasing ferric ion concentrations at pH 3.5. Fe(III) is solution of ferric cloride at 2000 μM.

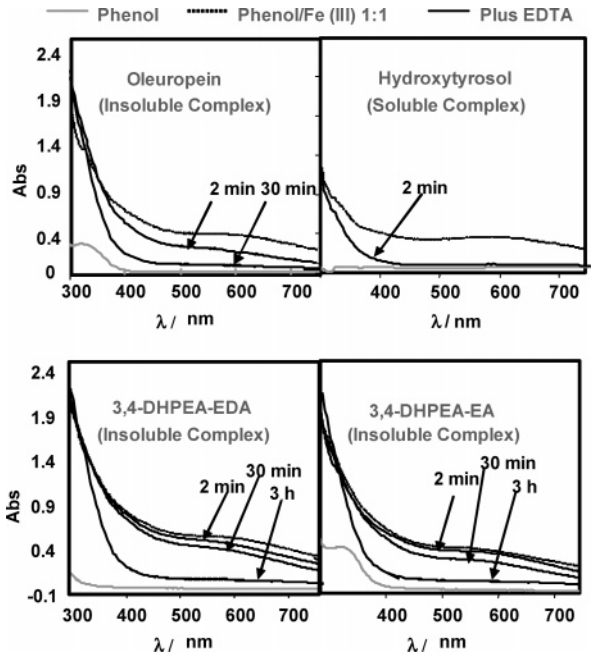

Figure 2. Effect of EDTA on ferric ion chelate formation with phenolic compounds at 400 μM, at pH 5.5, after different times of addition.

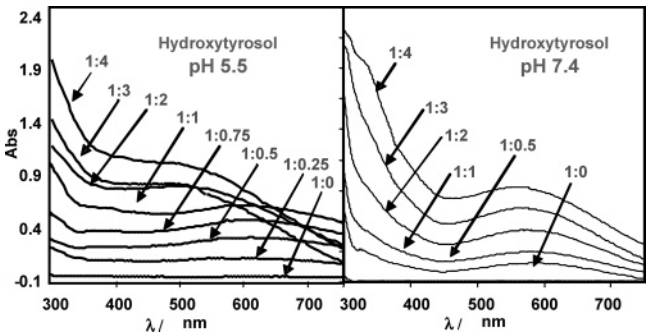

Figure 3. Ferric ion complex formation with hydroxytyrosol at 400 μM and pH 5.5 and 7.4 with increasing ferric ion concentrations.

oil phenolic compounds did not show evident changes in their spectra by this method. Nevertheless, formation of chelates with olive oil phenolic compounds may not produce significant changes in the UV/vis spectra of the compounds, and their charge-transfer bands may have low intensities. In general, more ionic transition metal complexes have smaller absorption intensities than covalent analogues, whatever the symmetry (*16*), and this study needed to be performed with a concentration higher (400 μM) than usually is reported for other compounds (30 μM). However, at these higher concentrations, EDTA, EDTA chelates, and iron solutions interfered greatly in the UV region, and only the visible spectra are reported.

At pH 3.5, on the addition of ferric ions to hydroxytyrosol solutions, an intensification of a green color could be observed with the increase in iron concentration accompanied by a rise in the absorption at 380−415 and at 600−750 nm. The spectrum changed for ratios of Fe(III)/hydroxytyrosol >2 (**Figure 1**) accompanied by an increase in the absorbance at 735 nm. On the addition of 0.5−5 mM EDTA solutions, the original spectrum was recovered immediately after addition for all ratios (**Figure 1**). The huge decrease in the intensity of the band at 330 nm in the Fe(III) spectrum (**Figure 1**), together with the progressive increase in the intensity of the band at 405 nm with the Fe(III) concentration, shows that at least two ferric ions per phenol molecule and probably more were involved in complex formation at pH 3.5.

At pH 3.5, oleuropein formed a green complex after the addition of iron with a rise in the absorption at 410 nm. This absorbance increased up to the ratio oleuropein/Fe(III) of 1, but no increase was observed for further additions of iron (**Figure 1**). It therefore appears that oleuropein only chelated one ferric ion per molecule, but the iron solution also interfered in this region, and this conclusion was not very clear. Nevertheless, the increases in the absorbance at 410 nm for ratios >1 seemed to be due to the increase of the ferric chloride solution absorbance rather than any change in the complex formed. Visually, it could also be observed that the green color became more yellowish with further additions of iron, which probably means increases in the concentration of the $[Fe(H_2O)_5(OH)]^{2+}$ ions, which are yellow. On the addition of EDTA solution, the broad peak at 410 nm disappeared immediately after the addition, and colorless solutions were obtained, proving the reversibility of complex formation (**Figure 1**).

At pH 3.5, 3,4-DHPEA-EDA and 3,4-DHPEA-EA solutions showed similar colors and similar spectra in the presence of ferric ions (**Figure 1**), which were very similar to the spectra obtained for oleuropein mixed with ferric ions. As already discussed for oleuropein, these compounds only chelate one ferric ion per phenol molecule at pH 3.5. On the addition of EDTA solutions, the initial spectra were immediately recovered in the visible region (**Figure 1**).

Hydroxytyrosol solutions added to iron at a ratio of 1: 0.25, 1:0.5, 1:0.75, 1:1, 1:2, 1:3, and 1:4 exhibited a blue grayish color at pH 5.5 (**Figures 2 and 3**) without any precipitation. For ratios up to 1, an increase in the absorbance at all visible wavelengths was observed with a broad peak occurring at 620 nm. At ratios >1, the visible spectrum of hydroxytyrosol changed, with the disappearance of the broad peak at 620 nm and an increase of absorbance between 480 and 580 nm. On addition of 0.5−5 mM EDTA solutions, the original spectrum was recovered as shown for the ratio of 1:1 in **Figure 2**. The recovery of the spectrum was immediate after the addition of EDTA for ratios up to one but was slower (15 min) at hyty/Fe^{3+} ratios >1. These results could also be confirmed visually by the disappearance or decrease in intensity of the blue color.

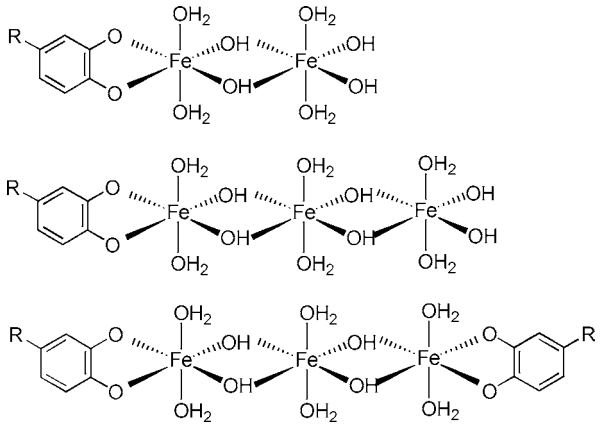

Figure 4. Possible ferric ion complexes formed in solutions with ratios iron(III)/phenol over 1. Large aggregates may be formed.

Apparently, new complexes were formed for ratios of hyty/Fe^{3+} >2, but several complexes may have contributed to the spectra. In addition to the mononuclear hydroxo-species (*17*), binuclear cations, in which the iron atoms are bridged by OH groups, may have formed (**Figure 4**).

Oleuropein solutions containing Fe^{3+} at a ratio of 1:1 exhibited a blue−gray color at pH 5.5, with a rise in the absorption at all visible wavelengths with a broad peak at 570 nm. On the addition of the EDTA solution, the original spectra were recovered for oleuropein solutions at pH 5.5 after 20 min (**Figure 3**). These results could be confirmed visually by the intensity of the blue color. Solutions with the ratio oleuropein/Fe^{3+} of 1:2 were also analyzed. A blue−gray solution was also obtained, but the solution became cloudy, and a fine precipitate was formed leading to the impossibility of measurements in a spectrophotometer. Nevertheless, EDTA was added, and an almost clear colorless solution was obtained after 1 h (**Figure 3**). Apparently, oleuropein was able to chelate more than one ferric ion, but insoluble complexes were formed at this concentration, and the intensity of the color could not be measured. The possibility of the nonchelated iron forming insoluble gelatinous hydroxides, $Fe(OH)_3 \cdot nH_2O$, reported by some authors at pH 5.5 (*16*) was investigated. It was found that 1200 μM ferric ion solution in the same acetate buffer at pH 5.5 did not show any precipitation, which is in accordance with the pK_{a3} for ferric ions ($pK_{a3} = 6.5$). It can therefore be concluded that the precipitate observed with iron and oleuropein was not due to gelatinous ferric hydroxide.

3,4-DHPEA-EA and 3,4-DHPEA-EDA solutions added to ferric chloride at a molar ratio of 1:0.5 and 1:1 exhibited a blue−gray color at pH 5.5 without any precipitation in the first seconds after mixing. However, after 1 min, these solutions became cloudy, but dark precipitates were not observed. Despite this cloudiness, spectra were run and can be observed in **Figure 2**. On addition of EDTA solution (0.5 mM, final concentration), the original spectra were only slightly recovered for 3,4-DHPEA-EDA and 3,4-DHPEA-EA solutions at pH 5.5 even 30 min after addition (**Figure 2**). These results could also be confirmed visually by the limited decrease of the blue color intensity.

All other olive oil phenolic compounds may chelate metals as demonstrated for hydroxytyrosol at pH 5.5, but since precipitates appeared, the phenomenon could not be studied by UV/vis spectrophotometry. Therefore, the turbidity of these solutions was measured by nephelometry (**Table 1**). The turbidity of solutions at molar ratios of 1:1 increased with time until they reached a stable value after 15 min. When more ferric ions were added, the turbidity of solutions increased quickly to values out of the range of the scale due to the formation of polynuclear complexes. On addition of EDTA, the turbidity disappeared after a period of time according to the stability of complexes (**Table 1**) formed. At a molar ratio of Fe(III)/phenol of 0.5, the turbidity fell in the order 3,4-DHPEA-EA and 3,4-DHPEA-EDA > oleuropein, and the lower turbidity of the oleuropein sample was also reflected in the shorter time during which turbidity remained after the addition of EDTA as compared to the other two phenols.

Table 1. Turbidity of Mixtures Containing Phenolic Compounds and Ferric Ions at pH 5.5[a]

	turbidity/NTU				
	ratio phenol/Fe(III)		ratio phenol/Fe(III) 1:2		
compound	1:1	1:2	plus EDTA	plus EDTA 1 h	plus EDTA 2 h
hydroxytyrosol	0 (±0)[b]	0	0	0	0
oleuropein	33 (±2)	>99	42 (±6)	1 (±1)	1 (±1)
3,4-DHPEA-EDA	36 (±2)	>99	>99	33 (±3)	7 (±3)
3,4-DHPEA-EA	35 (±1)	>99	>99	32 (±4)	17 (±3)

[a] EDTA solution was added in a ratio iron/EDTA of 1:1.25, and the turbidity of the mixtures was measured immediately and after 1 and 2 h of addition. The results were expressed in nephelometry turbidity units (NTU). [b] Mean (standard deviation in parentheses) of triplicate determinations.

At pH 7.4, hydroxytyrosol formed soluble dark blue complexes, at least up to ratios of Fe(III)/hydroxytyrosol of 4 (**Figure 3**). If chelation of all ferric ions added had not occurred, the precipitation of $Fe(OH)_3$ should have been observed at this pH. On the addition of EDTA solutions, the unchelated spectrum was partially recovered immediately after mixing, but this change continued over time, and after 30 min, the spectrum of hydroxytyrosol was almost completely restored in the visible range. At pH 7.4, these observations are consistent with hydroxytyrosol forming only one kind of complex with ferric ions since a significant change in the spectrum with an increase of iron concentration was not observed. These spectra were very similar to the spectra obtained at pH 5.5 for ratios of iron/phenol higher than 2 (**Figure 3**). Probably this is due to the greater formation of polynuclear ferric ions in this more alkaline solution, which would decrease the effective ratio of ferric ions/phenol. According to the pK_a for ferric ions, different hydroxy complexes are formed in dilute solutions, and their percentage in solution will depend on the pH

$$Fe^{3+} + H_20 \rightarrow [Fe(H_2O)_6]^{3+}$$

$$[Fe(H_2O)_6]^{3+} + H_2O \rightarrow [Fe(H_2O)_5(OH)]^{2+} \ (pK_{a1} = 2.9)$$

$$[Fe(H_2O)_5(OH)]^{2+} + H_2O \rightarrow [Fe(H_2O)_4(OH)_2]^{+} \ (pK_{a2} = 3.9)$$

$$[Fe(H_2O)_4(OH)_2]^{+} + H_2O \rightarrow [Fe(H_2O)_4(OH)_3] \ (pK_{a3} = 6.5)$$

In addition to these reactions, the formation of the binuclear cations occurs by the reaction known as oleation

$$2[Fe(H_2O)_6]^{3+} + H_2O \rightarrow [Fe(H_2O)_8(OH)_2]^{4+} \ (pK_a = 2.9)$$

In the presence of base, further proton donation by this ion, followed by oleation, leads to larger aggregates. Hydroxytyrosol may chelate preformed aggregates (**Figure 4**).

At pH 7.4, the formation of gray−blue solutions with dark precipitates was observed a few seconds after mixing for oleuropein, 3,4-DHPEA-EA, and 3,4-DHPEA-EDA for all the ratios of iron/phenol tested. Nevertheless, spectra were recorded, and increases in absorbance in the visible wavelength range were accompanied by an intensified dark blue color with formation of a precipitate. On addition of EDTA solutions, the original spectra were partly recovered for oleuropein but not for the other compounds, even after 30 min at pH 7.4.

In lipid systems where the ferric ion concentration is much lower than the phenolic compound concentration, the high stability of chelates contributes to the antioxidant properties of compounds. In the presence of phenolic compounds, the main chelates expected to be formed at pH 3.5 and 5.5 for ratios of phenol/iron >1 are [with $Ph(OH)_2$ representing an olive oil phenolic compound]

$$\text{at pH 3.5: } [Fe(H_2O)_5(OH)]^{2+} + Ph(OH)_2 \rightarrow [Fe(H_2O)_3(OH)Ph(O)_2] + 2\,H_3O^+$$

$$\text{at pH 5.5: } [Fe(H_2O)_4(OH)_2]^{+} + Ph(OH)_2 \rightarrow [Fe(H_2O)_2(OH)_2Ph(O)_2]^{-} + 2\,H_2O^+$$

The slower the rate of recovery of phenols from their $[Fe(H_2O)_2(OH)_2Ph(O)_2]^-$ chelates on addition of EDTA, the more negative the reduction potential, so they are more resistant to reduction and better metal chelators. At pH 5.5, metal chelation would be in the order 3,4-DHPEA-EA and 3,4-DHPEA-EDA > oleuropein > hydroxytyrosol. Hydroxytyrosol complexes have the worst resistance to reduction followed by oleuropein. However, all chelates of $[Fe(H_2O)_3(OH)Ph(O)_2]$ showed less stability at pH 3.5 then the chelates of $[Fe(H_2O)_2(OH)_2Ph(O)_2]^-$ at pH 5.5; therefore, ferric ions would be reduced more effectively at the former pH. The stability of the different chelates may also be different at pH 3.5 but since EDTA has a high formation constant with Fe(III) ($\log \beta = 25$), these differences could not be observed.

The chelating activity of ferrous ions by phenolic compounds was also investigated. Changes in the spectra from 200 to 750 nm for all compounds at pH 3.5 and 5.5 were not observed in the first minutes after mixing but after a while, at pH 5.5, the color (blue) and spectra similar to those recorded after the addition of ferric ions were obtained if air, Fe^{2+}, and a phenol were present but not for samples lacking one of these components. These data indicated that the phenolic compounds were not chelating ferrous ions but were chelating ferric ions formed by aerial oxidation of ferrous ions. Free ferric ions formed by oxidation of ferrous ions should be chelated by phenolic compounds in solution, and the oxidation reaction would be shifted in the direction of increased formation of ferric ions. However, the rate of oxidation of Fe^{2+} was dependent on the phenolic compound. The rate of oxidation was in the ratio of 1:1:5:10 for 3,4-DHPEA-EA, 3,4-DHPEA-EDA, oleuropein, and hydroxytyrosol.

The stability of a mixture of hydroxytyrosol, oleuropein, 3,4-DHPEA-EA, and 3,4-DHPEA-EDA at 37 °C and 0.6 mM concentration in 65% ethanol/water solution at pH 3.5, 5.5, and 7.4 was investigated in the presence and absence of Fe(III) (0.6 mM). The changes in each component during storage were monitored by HPLC. **Figure 5** shows the changes in concentration of phenolic compounds over time in the presence and absence of iron (III).

At pH 3.5 in the absence of iron, all compounds showed remarkable stability except 3,4-DHPEA-EDA. However, in the presence of iron, 3,4-DHPEA-EA was the most stable compound, and hydroxytyrosol was the compound most affected by the presence of iron, followed by 3,4-DHPEA-EDA.

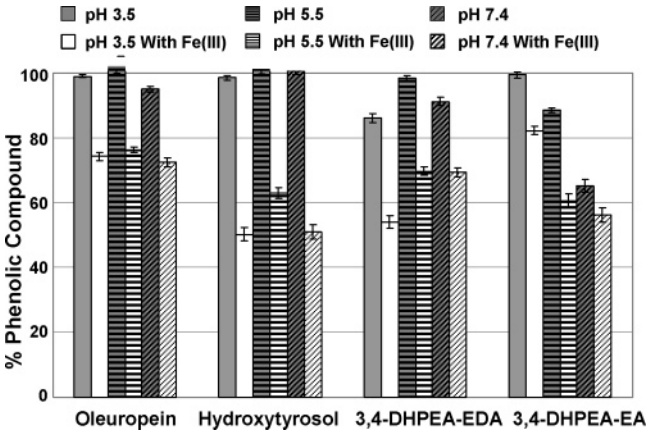

Figure 5. Changes in phenolic compound concentrations after 48 h when stored as a mixture in aqueous ethanolic solutions at pH 3.5, 5.5, and 7.4 in the presence and absence of ferric ions. Mean (error bars represent range) of two determinations for each duplicate.

At pH 5.5 in the absence of ferric ions, all compounds except DHPEA-EA showed very good stability. Nevertheless, hydroxytyrosol was again the compound whose stability was most reduced by the presence of ferric ions (**Figure 5**).

At pH 7.4, hydroxytyrosol showed a remarkable stability in the absence of Fe(III) when compared with other phenols, with DHPEA-EA being the most affected by the alkaline pH with a decrease to about 63% of the original concentration after 48 h (**Figure 5**). In the presence of iron, the stability of hydroxytyrosol was drastically reduced, and it had degraded even more than 3,4-DHPEA-EA after 48 h (**Figure 5**).

Apparently, metal chelation, oxidation, and oxidation followed by polymerization were the main reactions occurring during storage of solutions. Hydrolysis, if it occurred, was not observed since hydroxytyrosol was not detected by HPLC analysis in 3,4-DHPEA-EA or 3,4-DHPEA-EDA solutions in the presence or absence of iron.

In the presence of Fe(III), at pH 3.5, compounds oxidized but without further reactions that could lead to brown pigments. However, at pH 5.5 and 7.4, oxidized phenolic compounds reacted further to give brown pigments, probably by polymerization. Although no reducing activity was observed for these phenolic compounds at pH 7.4, it was found by other authors that $Fe^{III}(OH)_3$ promoted polymerization of phenolic compounds (*18*). At pH 7.4, some phenolate ions may exist, and their autoxidation catalyzed by iron is extremely easy since the product of a single-electron oxidation of a phenolate ion is a semiquinone free-radical stabilized by the same resonance delocalization of the unpaired electron around the ring as the phenolate ion. For hydroxytyrosol and its derivatives, which are derivatives of 1,2-dihydroxybenzene, oxidative removal of a second electron leads to an *o*-quinone. The reactive electrophilic *o*-quinone intermediate can readily undergo attack by nucleophiles such as other phenols, and loss of phenolic compounds in the presence of iron may also occur by the polymerization of phenols to dark pigments.

The oxidative browning of polyphenols in foods generally results in a loss of nutritional value and the appearance of undesirable brown colors. However, in some processed food such as black tea, plums, and ripe olives, these reactions are part of desirable changes essential to the product. The effect of

pH and iron salts in the flesh of ripe olives processed for table olive production has been widely studied by several authors (*19*−*23*). However, these studies concerned the darkening of whole olives mainly at high pH values (>7). Ionic interactions in the fruits between iron and other compounds such as proteins, polysaccharides, or a combination of iron with tannins to form iron tannate, also black in color, are also important for color formation and fixation in the ripe olives The darkening process has been mainly related to enzyme-catalyzed oxidation by polyphenol oxidase or chemical browning reactions, involving the oxidation of natural polyphenols in olives to quinones, followed by the transformation of quinones into different dark compounds. In processed foods, with the enzyme removed or inactivated, substantial nonenzymatic autoxidation takes place.

According to recent reports (*22*), a huge decrease in oleuropein with an increase in hydroxytyrosol and oleuropein aglycones, especially 3,4-DHPEA-EA, was observed during brine storage of olives. However, during the following aerobic alkali treatment, a great decrease in oleuropein-derived aglycones together with an increase of hydroxytyrosol concentration occurred together with the development of the surface fruit color. A sharp decrease of hydroxytyrosol was observed only in the final step, after addition of ferrous ions. These observations are in accordance with the present work. Hydroxytyrosol showed a remarkable stability at pH 7.4, when in a mixture with other compounds, and the worst stability when ferric ions were present. However, 3,4-DHPEA-EA oxidizes very easily at this pH, which may contribute to the darkening process during alkaline treatment. Since olive phenolic compounds, namely, hydroxytyrosol and oleuropein derivatives, vary significantly in stability at different pH values, it is not surprising that the dark color of ripe olives and its homogeneity will also depend on the initial composition of the phenolic fraction.

The low stability of hydroxytyrosol in the presence of ferric ions can be now explained by its chelating properties. The higher chelating capacity and the lower stability of complexes formed by hydroxytyrosol would provoke a faster turn-over of ferric ions in the phenolic solution containing ferric ions, leading to a higher consumption of hydroxytyrosol in the reduction of further ferric ions.

It is also important to point out that olive oil is used widely in the preparation of many dishes and sauces in Mediterranean countries. Roast and stewed meat may, together with the added water, contribute to a high concentration of free ferric ions in prepared food. Since DHPEA-EDA is the most concentrated antioxidant compound found in the phenolic extract, it is interesting to see that this compound has good antioxidant behavior even in the presence of ferric ions. According to these results, when food is processed with olive oil in the presence of water, olive phenolic extracts with higher quantities of DHPEA-EDA and DHPEA-EA would be better then olive oil extracts with higher quantities of hydroxytyrosol, despite the higher antioxidant activity of hydroxytyrosol in bulk oil.

LITERATURE CITED

(1) Huang, S.; Frankel, E. N.; Schwarz, K.; German, B. Effect of pH on Antioxidant Activity of α-Tocopherol and Trolox in Oil-in-Water Emulsions. *J. Agric. Food Chem.* **1996**, *44*, 2496−2502.

(2) Mancuso, J. R.; McClements, D. J.; Decker, E. A. The Effects of Surfactant Type, pH, and Chelatores on the Oxidation of Salmon Oil-in-Water Emulsions. *J. Agric. Food Chem.* **1999**, *47*, 4112−4116.

(3) Mei, L.; McClements, D. J.; Decker, E. A. Lipid Oxidation in Emulsions as Affected by Charge Status of Antioxidants and Emulsion Droplets. *J. Agric. Food Chem.* **1999**, *47*, 2267−2273.

(4) Gordon, M. H.; Paiva-Martins, F.; Almeida, M. Antioxidant Activity of Hydroxytyrosol Acetate as Compared With Other Olive Oil Polyphenols. *J. Agric. Food Chem.* **2001**, *49*, 2480−2485.

(5) Roginsky, V. A. Effectiveness of Lipid- and Water-soluble Phenolic Antioxidants in the autoxidation of polyunsaturated Fatty Acids Esters in Microheterogeneous solutions. *Biol. Membr.* **1990**, *4*, 437−451.

(6) Moran, J. F.; Klucas, R. V.; Grayer, R. J.; Abian, J.; Becana, M. Complexes of Iron, with Phenolic Compounds from Soybean oxidant Properties. *Free Radical Biol. Med.* **1997**, *22*, 861−870.

(7) Dunford, H. B. Free Radicals in Ion Containing Systems. *Free Radical Biol. Med.* **1987**, *3*, 405−421.

(8) Montedoro, G. F.; Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Maccchioni, A. Simple and Hydrolyzable Compounds in Virgin Olive Oil. 3. Spectroscopic Characterizations of the Secoiridoid Derivatives. *J. Agric. Food Chem.* **1993**, *41*, 2228−2234.

(9) Baldioli, M.; Servili, M.; Perreti, G.; Montedoro, G. F. Antioxidant Activity of Tocopherols and Phenolic Compounds of Virgin Olive Oil. *J. Am. Oil Chem. Soc.* **1996**, *73*, 1589−1593.

(10) Brenes, M.; García, A.; García, P.; Rios, J. J.; Garrido, A. Phenolic Compounds in Spanish Olive Oils. *J. Agric. Food Chem.* **1999**, *47*, 3535−3540.

(11) Paiva-Martins, F.; Gordon, M. H. Effects of pH and ferric ions on the antioxidant activity of olive polyphenols in oil-in-water emulsions. *J. Am. Oil Chem. Soc.* **2002**, *79* (6), 571−576.

(12) Baraldi, P. G.; Simoni, D.; Manfredini, S.; Menziani, E. Preparation of 3,4-Dihydroxy-1-benzeneethanol: A Reinvestigation. *Liebigs Ann. Chem.* **1983**, 684−686.

(13) Gariboldi, P.; Jommi, G.; Verotta, L. Secoiridoids From *Olea europaea*. *Phytochemistry* **1986**, 865−869.

(14) Limiroli, R.; Consonni, R.; Ranalli, A.; Bianchi, G.; Zetta, L. ^{1}H NMR Study of Phenolics in Vegetation Water of Three Cultivars of *Olea europaea*: Similarities and Differences. *J. Agric. Food Chem.* **1996**, *44*, 2040−2048.

(15) Paiva-Martins, F.; Gordon, M. H. Isolation and Characterization of the Antioxidant Component 3,4-Dihydroxyphenylethyl 4-formyl-3-formylmethyl-4-hexenoate from Olive (*Olea europeia*). *J. Agric. Food Chem.* **2001**, *49*, 4214−4219.

(16) Shriver, D. F.; Atkins, P. W.; Langford, C. H. In *Inorganic Chemistry*, 2nd ed.; Shriver, D. F., Atkins, P. W., Langford, C. H., Eds.; Oxford University Press: Oxford, 1998; p 199.

(17) Deiana, S.; Gessa, C.; Marchetti, M.; Usai, M. Phenolic Acid Redox Properties: pH Influence on Iron(III) Reduction by Caffeic Acid. *Soil Sci. Soc. Am. J.* **1995**, *59*, 1301−1307.

(18) Shindo, H.; Huang, P. M. Catalytic polymerization of hydroquinone by primary minerals. *Soil Sci.* **1985**, *139*, 505−511.

(19) Brenes, M.; García, P.; Garrido, A. Phenolic Compounds Related to the Black Color Formed during the Processing of Ripe Olives. *J. Agric. Food Chem.* **1992**, *40*, 1192−1196.

(20) García, P.; Brenes, M.; Vattan, T.; Garrido, A. Kinetic Study at Different pH values of the oxidation process to Produce Ripe Olives. *J. Sci. Food Agric.* **1992**, *60*, 327−331.

(21) Brenes, M.; García, P.; Garrido, A. Effect of pH on the Color Formed by Fe−Phenolic Complexes in Ripe Olives. *J. Sci. Food Agric.* **1995**, *67*, 35−41.

(22) Marsilio, V.; Campestre, C.; Lanza, B. Phenolic compounds change during California-style ripe olive processing. *Food Chem.* **2001**, *74*, 55−60.

(23) Garcia, P.; Romero, C.; Brenes, M.; Garrido A. Effect of Metal Cations on the Chemical Oxidation of Olive *o*-Diphenols in Model Systems. *J. Agric. Food Chem.* **1996**, *44*, 2101−2105.

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