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LC-MS Investigation of Oxidation Products of Phenolic Antioxidants

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Two oxidation systems were examined for the oxidation of three groups of phenolic antioxidants; five cinnamic acids, two benzoic acids, and two phenols characteristic of olive fruits. Periodate oxidation, which is reported to produce products similar to polyphenol oxidase, was contrasted with the reactivity of the Fenton system, an inorganic source of hydroxyl radicals. Reaction products were identified as various quinones, dimers, and aldehydes, but the nature of the products differed between the two oxidation systems. Structure−activity effects were also observed for the different phenols. All cinnamic acids in this study reacted with the Fenton reagent to produce benzaldehydes as the main products, with the exception of 5-caffeoylquinic acid. In contrast, periodate oxidation gave no reaction with some of the cinnamic acids. Quinone formation was observed for the two compounds, caffeic acid and 5-caffeoylquinic acid, possessing *o*-hydroxy groups. Caffeic acid was unusual in that dimer formation was the main initial product of reaction. Benzoic acids were readily oxidized by both systems, but no identifiable products were isolated. Oleuropein was oxidized by both oxidants used in this study, resulting in quinones in each system, whereas little or no oxidation of tyrosol was observed. This highlights the importance of conjugation between the alkene double bond and the hydroxy group. The results question the validity of many existing methods of testing antioxidant activity.

KEYWORDS: Phenols; oxidation; LC-MS; antioxidant

INTRODUCTION

The chemistry of life is the chemistry of oxidation. Paradoxically, oxidation reactions are now believed (*1*−*3*) to contribute to the process of aging and to be involved in the etiology of diseases such as atherosclerosis and cancer. Antioxidants (*4*) play a key role in mediating the balance between beneficial and deleterious oxidations. Participants at the Saas Fee conference on free radical and antioxidant biology and preventive medicine were in no doubt of the value of antioxidants. The Saas Fee Declaration (*5*) that has been signed by many hundreds of researchers states inter alia that "There is now general agreement that there is a need for further work at the fundamental scientific level...The major objective...is the prevention of disease. This may be achieved by use of antioxidants which are natural physiological substances." The types of questions that are now being asked "at the fundamental scientific level" are the following: How are antioxidants absorbed by the body and transported to where they are needed? Can they be manipulated by changing dietary patterns to enhance and prolong life? What makes a good antioxidant, and how do they work (*6*)? Answers to the first two questions are gradually being supplied by medical researchers. The last two questions are really chemical questions, ones that need to be addressed by chemists who have the appropriate skills and cross-disciplinary focus. Historically, antioxidant studies have been dominated by medical researchers and food scientists who have worked independently. Hence, there are gaps in the literature that must be filled by the sound application of chemical principles and techniques.

Fruits and vegetables are important sources of antioxidants (*7*), and their effectiveness as antioxidants is demonstrated by structure−activity relationships. Plant phenols are highly reactive as witnessed by their role in enzymatic browning as substrates for a number of oxidoreductases, in nonenzymatic browning reactions, and in oxidative nonbrowning reactions. Each of these involves oxidation of the phenols but via different mechanisms. Enzymatic browning involves an initial highly specific enzymatic hydroxylation of monophenols into *o*-diphenols and *o*-diphenols into quinones. The exact mechanism of nonenzymatic browning is not well understood (*8*), whereas recent research has been driven by the involvement of phenols in oxidative nonbrowning reactions. It is in this role that phenols function as antioxidants; the first step in this action is believed to involve abstraction of the phenolic-hydrogen atom.

This process is important in two situations—in the food sciences, where the term antioxidant is often implicitly restricted to chain-breaking inhibitors of lipid peroxidation; and in humans,

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where free radicals generated in vivo damage many other targets besides lipids. However, the processes involved in foods and in physiological situations are fundamentally the same. The measurement of antioxidant activity is achieved indirectly by the effects of the antioxidant in controlling the extent of oxidation. The features of an oxidation are a substrate, an oxidant and an initiator, intermediates, and final products; measurement of any one of these can be used to assess antioxidant activity. For instance, in monitoring antioxidant activity in a food, potential measurements (*9*) include peroxide value (PV), thiobarbituric acid reactive substances (TBARS), iodine value, free fatty acid content, polymer content, viscosity, absorption at 232 and 268 nm, color, fatty acid composition, and ratio of unsaturated to saturated fatty acids (e.g., C18:2/C16:0). Physiological activity can be assessed by in vitro measurements such as the susceptibility of isolated low-density lipoproteins (LDL) to oxidation (*10*) or, preferably, in vivo measurement of LDL oxidation products such as hydroxy-fatty acids or oxysterols or indirect indicators of lipid oxidation (e.g., F-2-isoprostanes) (*11*−*13*). Alternatively, the immunological response to antigenic lipid oxidation products can be measured. These methods show extreme diversity, but the interesting feature is that direct measurement of the antioxidants themselves has not been applied. Additionally, the discrepancies in the reported efficacy and mechanisms of antioxidant action "have confused the understanding of the molecular mechanisms of antioxidant reactivity" (*14*).

Studies in which the antioxidant molecules were measured directly during oxidation reactions (*15*) are generally restricted to the synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). For legislative reasons, the breakdown products were systematically investigated with the aim to identify chemical structures and evaluate their potential to act as carcinogens. What was revealed was that these artificial antioxidants condense with each other and form larger, more complex molecules that are potentially carcinogenic. Other studies of plant phenols (*16*, *17*) have also identified condensation products during the "breakdown" of the naturally occurring antioxidant caffeic acid. Interestingly, the breakdown products identified by Fulcrand et al. (*18*) appear to be antioxidants themselves. Thus, there is potential for a single antioxidant molecule to undergo many oxidations before it loses antioxidant activity. Again, this aspect of antioxidant chemistry has not been explored (*19*) but may explain some of the inadequacies we uncovered in the established methodology.

Nevertheless, questions such as "what makes good antioxidants?" and "how do they work?" would best be answered by studying the antioxidant molecules as they undergo reactions in which they protect a substrate by being preferentially oxidized. However, because these reactions may be incredibly complex, there is a need to understand the basic oxidative chemistry of phenolic antioxidants in the absence of a substrate. The development of a comprehensive scheme for the mechanism of antioxidant action requires this knowledge. In this study, oxidation of various phenols was examined using two systems—periodate and Fenton oxidations, both of which are standard procedures for measuring antioxidant activity. Three general kinds of phenolic antioxidants were selected for examination: five cinnamic acids, two benzoic acids, and two phenols important in olives.

MATERIALS AND METHODS

Reagents. Reagents were obtained as follows and were used without further purification: oleuropein from Extrasynthase; sodium periodate, Amberlite IRA-900 resin, caffeic, 5-caffeoylquinic, 3-hydroxycinnamic, *p*-coumaric, ferulic, gallic, and syringic acids, 4-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, and tyrosol from Sigma-Aldrich Chemical Co.; 3-hydroxybenzaldehyde, iron(II) sulfate, HPLC grade methanol, and acetonitrile from BDH; vanillin from Unilab. Grade 1 water (ISO3696) purified through a Milli-Q water system was used for all chromatographic analyses and sample and standard preparation.

A stock solution of the phenolic compounds was prepared in CH_3CN/H_2O (5:1) at a concentration of 16 mM. The dilution of the stock solution for each of the reaction systems meant the final solvent composition was 72% water and 28% acetonitrile.

Periodate Oxidation. This was based on modifications to the method reported by Fulcrand et al. (*18*). In our hands, exclusion of oxygen made little difference to the outcome of the reaction and, therefore, reactions were performed in atmosphere. The periodate anion exchange resin was prepared using a modified procedure from Harrison and Hodge (*20*). Initially, Amberlite (25 g) was stirred overnight with $NaIO_4$ (20 g) in water (200 mL). The liquid was then decanted, and a further 20 g of $NaIO_4$ in 200 mL of H_2O was added and stirred for 6 h. The resin was then filtered off, washed with water (500 mL), tetrahydrofuran (THF) (100 mL), and finally ethyl acetate (100 mL). The resin was then dried overnight in a vacuum oven at 30 °C.

The stock solution of the respective phenolic compound was diluted by the addition of 2 mL of water to give the same concentration of phenol as used for the Fenton reactions. To this was added 80 mg of the IO_4^- form of the Amberlite as prepared above. This mixture was allowed to react with occasional stirring for 10 min, whereupon the liquid was decanted from the resin. LC-MS injections were made from this decanted solution. The reaction times used in the results refer to the period elapsed between the addition of the IO_4^-/Amberlite and the time of the injection. It includes the 10 min the phenolic compound was in contact with the IO_4^-/Amberlite.

Fenton Oxidation (Fe^{2+}/EDTA/H_2O_2). Oxidation was performed by the addition of an aqueous solution (1 mL) of 100 μM Fe^{2+}/EDTA (1:1) to the phenolic compound (1 mL) followed by 3% H_2O_2 (1 mL). The combination of Fe^{2+}/EDTA/H_2O_2 will be referred to as the "Fenton reagent" or the "Fenton system" below. The ratio of phenol/Fe^{2+}/EDTA/H_2O_2 used in this study was found to produce products at a rate detectable by HPLC analysis over 24 h. When the concentration of Fe^{2+}/EDTA was 10 μM, no oxidation was observed in 24 h. An Fe^{2+}/EDTA concentration of 10 mM led to complete removal of all peaks in the HPLC chromatogram, indicating that the phenols had been completely oxidized to non-UV-absorbing products. The complete "mineralization" of phenolic compounds by Fenton oxidation has been recently reported (*21*).

Liquid Chromatography—Mass Spectrometry (LC-MS). Samples were analyzed using a Beckman (Fullerton, CA) liquid chromatograph, consisting of a 126 pump and a 168 diode array detector. A Quattro II, triple-quadrupole mass spectrometer (Micromass) was used for mass spectrometric analysis. The HPLC column used was an SGE (Ringwood, Australia) Wakosil, C18 column (150 × 2 mm, 5 μm). This was used with gradient elution with the solvents being 0.05% aqueous formic acid (solvent A) and methanol (solvent B). Data were acquired by both the Masslynx data system for the mass spectrometer and the Beckman data system for the diode array; two wavelengths from the diode array were also recorded by the Masslynx system to allow aligning of the data. These wavelengths were 280 and 325 nm.

During LC-MS, scans were performed for both positive and negative ions. Cone voltage fragmentation was also used in both ion modes. The LC gradient used was from 10% solvent B to 90% solvent B in 20 min, held for 5 min at 90% solvent B, and then from 90% solvent B to 10% solvent B in 5 min. The flow rate was 0.2 mL min^{-1}. A splitter system was used on the solvent flow from the HPLC that allowed ~50 μL of the flow to the electrospray source.

RESULTS

LC-UV/MS Monitoring of Products. Reactions for both oxidation systems were monitored over time by liquid chromatography coupled to UV−vis diode array and ESI-MS detection, and the main products identified are summarized in **Table 1**. Structures of the phenolic compounds used in this study

Table 1. Products of the Oxidation of Phenolic Compounds in the Periodate and Fenton Systems (Numbers in Parentheses Correspond to Compound Numbers in **Figure 1**)

	periodate oxidation		Fenton oxidation	
compd	**initial main product**[a] other products	**main product at 24 h** other products	**initial main product** other products	**main product at 24 h** other products
caffeic acid (**1**)	**dimer (6)** quinone (**5**)	none detected as precipitate formed after 120 min	**3,4-dihydroxybenzaldehyde (10)** **dimer (6)**	**3,4-dihydroxybenzaldehyde (10)** dimer (**6**) all peaks had decreased in intensity
3-hydroxycinnamic acid (**2**)	**no reaction**	**no reaction**	**3-hydroxybenzaldehyde (11)** caffeic acid (**1**) 3,4-dihydroxybenzaldehyde (**10**) dilactone dimers[b] (**7**)	**3-hydroxybenzaldehyde (11)** caffeic acid (**1**) 3,4-dihydroxybenzaldehyde[c] (**10**) dilactone dimers[b] (**7**)
p-coumaric acid (**3**)	**no products detected**	**4-hydroxybenzaldehyde (12)**	**4-hydroxybenzaldehyde (12)** caffeic acid (**1**)[b]	**4-hydroxybenzaldehyde (12)** caffeic acid (**1**)[c] 3,4-dihydroxybenzaldehyde (**10**)
ferulic acid (**4**)	**vanillin (13); dimer (9)**	**vanillin (13); dimer (9)**	**vanillin (13)** compound MW 226; dimer (**9**)	**vanillin (13)** compound MW 226; dimer (**9**)
5-caffeoylquinic acid (**14**)	**quinone (15)**	**no single main product**	**quinone (15)** compound MW 308 (**16**); dimer 3,4-dihydroxybenzaldehyde (**10**)	**no single main product**
tyrosol (**17**)	**no reaction**	**no reaction**	no major products (see text)	no major products (see text)
oleuropein (**18**)	**quinone (19)** quinone isomer	**quinone (19)** quinone isomer compounds MW 680	**quinone (19)** quinone isomer compound MW 538	**no single main product**
gallic acid (**20**)	**no single main product**	**no single main product**	this reaction not performed	
syringic acid (**21**)	**radical (22/23)** coeluting compounds MW 334, 352, 366, 446 compound MW 334	**radical (22/23)** coeluting compounds MW 334, 352, 366, 446	**radical (22/23)** compound MW 230 coeluting compounds MW 214, 450 coeluting compounds MW 352, 366, 446 compound MW 334	**radical (22/23)**[d] compound MW 230[c] coeluting compounds MW 214, 450[c] coeluting compounds MW 352, 366, 446

[a] Usually after 10 min, unless otherwise specified in text. [b] In trace quantities. [c] This had increased in concentration. [d] Monitored at 5 h.

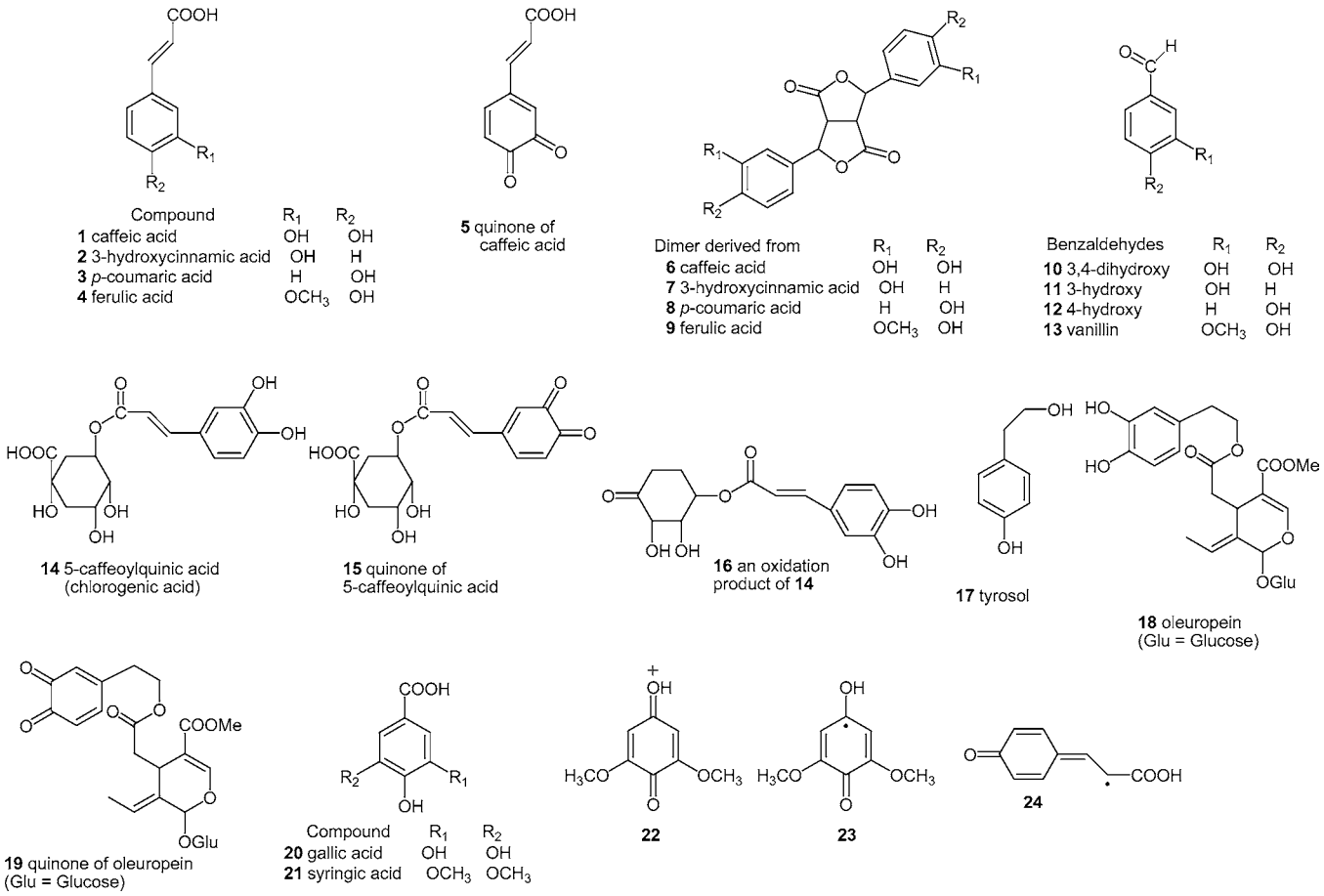

Figure 1. Phenolic compounds used in this study and some proposed oxidation products.

and some proposed oxidation products are shown in **Figure 1**. Chromatographic profiles were qualitatively similar when monitored at 280 nm or by ESI total ion chromatogram (TIC). Most compounds produced molecular ions in both the positive and negative ion modes, which assisted in the confirmation of molecular weight. In the instances where distinct peaks were not observed in the TIC, extracted ion chromatograms (EICs; **Figure 2**) were used to monitor likely reaction products. We have recently used this method of systematically searching EICs to monitor phenolic metabolism in olives (*22*). Retention and spectral data are summarized in **Table 2** for starting materials and oxidation products.

Effect of Solvent on Oxidation of Phenolic Compounds. Our previous investigations of antioxidant activity (*23*) used phenolic compounds and olive extracts dissolved in 50:50 CH_3OH/H_2O. On the other hand, Fulcrand et al. (*18*) used a number of solvent systems while investigating periodate oxidation of caffeic acid, with the majority of their preparative work carried out in aqueous acetonitrile. Initial experiments involved methanol/water as the solvent; however, it appeared that there was some methylation of oxidation products (*24*), particularly for oleuropein. To avoid the complication of the solvent taking part in the reactions, a solvent system of acetonitrile/water was used for all oxidations.

Caffeic Acid (1). *Periodate Oxidation.* The 280 nm chromatogram (**Figure 3A**) of the solution obtained after 20 min of reaction between caffeic acid and periodate showed a main peak due to unreacted **1** and two smaller peaks due to the quinone of caffeic acid (**5**) and a dimer (**6**). The UV spectrum of **5** is shown in **Figure 3B**, with an absorption maximum at 403 nm characteristic of other quinones observed in this study (see below). The three major identifiable products correspond to those reported by Fulcrand et al. (*18*) albeit with different relative proportions due to different reaction conditions. However, we did not observe a peak to lower retention time than the quinone, denoted "**1**" in Fulcrand et al. (*18*). This peak had the same intensity as the dimer in Fulcrand et al.'s study but was not identified by them.

A number of dimeric compounds, of molecular weight 358, formed from the coupling of two caffeoyl radicals have been reported in the literature (*17*, *25*). Of these, only one structure, a dehydrodicaffeic acid dilactone [this nomenclature was used by Lacki and Duvnjak (*26*) to describe the analagous dimer formed from ferulic acid], also reported as a "furofuran-type lignan" (*17*), has lost conjugation between the aromatic ring and the alkene double bond (**6**). This would result in a blue shift in the UV absorbance spectrum as observed in the UV spectrum of the dimer formed under our conditions (**Figure 3C**). We have thus assigned the dimer formed in our system to the "dehydrodicaffeic acid dilactone", **6**.

After a reaction period of 120 min, **6** had become the major product. There was also an increase in the baseline of the chromatogram indicating the formation of a wide rage of compounds including other dimers reported in the literature (*17*) as indicated by their UV spectra. After this time, the reaction mixture started to produce a precipitate that was insoluble in acetonitrile, methanol, or water. This was probably due to the formation of polymeric species. The reaction was not monitored further.

Fenton Oxidation. After 10 min, the reaction of **1** and the Fenton reagent produced two main products observable as peaks in the 280 nm chromatogram. There were also a number of smaller product peaks together with intensity under the baseline that stretched from the void volume to >20 min. The earlier

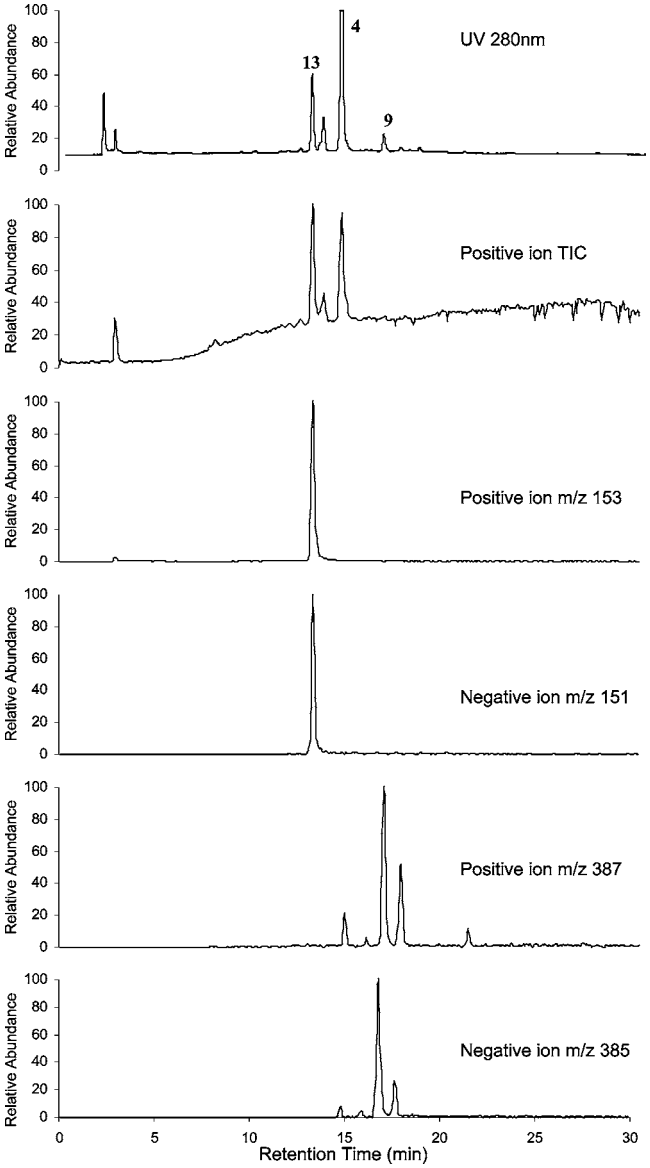

Figure 2. Chromatograms from the reaction of **4** with the Fenton reagent after 1 h of reaction. Numbering of peaks corresponds to compounds in **Figure 1**. Extracted ion chromatograms (EICs) are shown for **13**, *m*/*z* 153 positive ion mode and *m*/*z* 151 negative ion mode; and **9**, *m*/*z* 387 positive ion mode and *m*/*z* 385 negative ion mode.

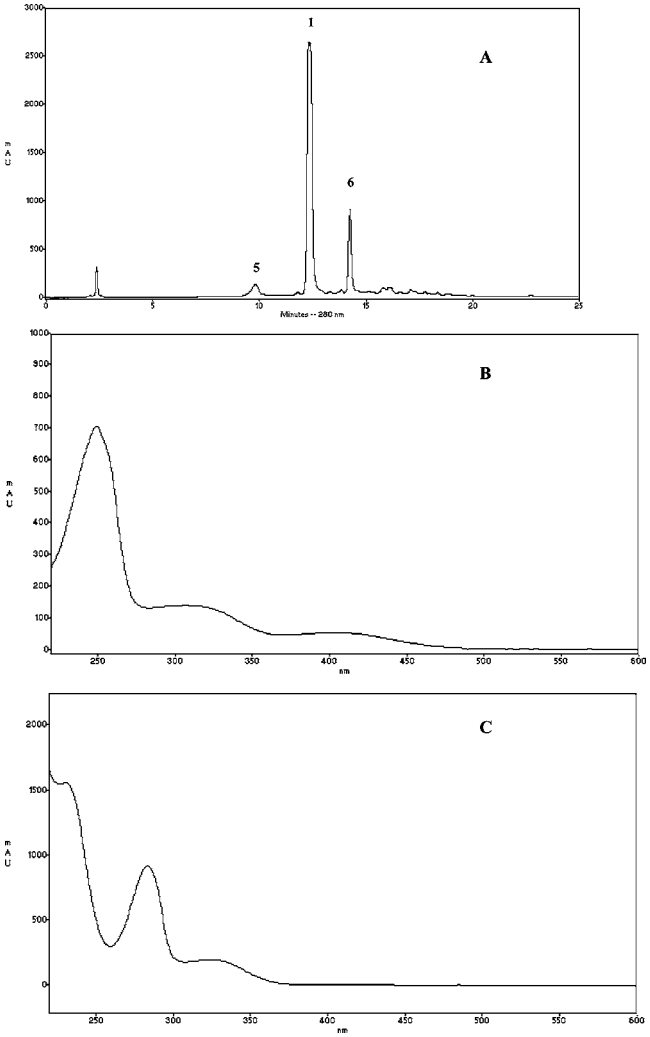

Figure 3. (**A**) UV chromatogram at 280 nm of the periodate oxidation of **1**. Reaction time = 20 min. Numbering of peaks corresponds to compounds in **Figure 1**. (**B**) UV spectrum of **5**. (**C**) UV spectrum of **6**.

eluting product was 3,4-dihydroxybenzaldehyde, **10**, which was characterized by the retention time (10.0 min) and mass spectrum of an authentic sample. The later eluting product had positive and negative ion mass spectra identical to those reported for **6** from the periodate oxidation, above. With Fenton oxidation, **5** was not observed. Over time, the peak due to unreacted **1** decreased in intensity, until after 23 h it had all but disappeared from the 280 nm chromatogram. There was an increase in the intensity under the baseline during this time. From the 280 nm chromatogram it was difficult to follow the change in concentrations of **6** and **10**; however, EICs revealed that the concentrations of these species also decreased with time.

3-Hydroxycinnamic Acid (2). *Periodate Oxidation.* The 280 nm chromatogram of the solution obtained 10 min after **2** had been reacted with periodate did not show any new peaks, suggesting no reaction. There was no increase in baseline intensity as observed for **1**. After 18 h, the 280 nm chromatogram had not changed. Attempts were made to force oxidation by increasing the amount of periodate and allowing longer reaction time, but no products could be detected.

Fenton Oxidation. The reaction between **2** and the Fenton reagent was monitored at 1 and 23 h. Unlike the periodate oxidation system, oxidation with the Fenton reagent resulted in products, and after 1 h, the main product had a molecular weight of 122, assigned to 3-hydroxybenzaldehyde (**11**), on the basis of the retention time (12.4 min) and mass spectrum of an authentic standard. Eluting with a very similar retention time was **1**. This was formed by 4-hydroxylation of **2**. A minor product at 1 h was 3,4-dihydroxybenzaldehyde (**10**), presumably formed by oxidation of **1** as observed above. After 23 h, there was a small decrease in **2**, virtually no change in **1** or **11**, and a small increase in **10**. EICs at 1 and 23 h showed traces (100 times less than other products) of the dilactone dimers of **1** and **2**, that is, **6** and **7**, respectively.

***p*-Coumaric Acid (3).** *Periodate Oxidation.* The solution obtained from reaction of **3** with periodate did not show any new peaks in the 280 nm chromatogram after 10 min, nor was there any increase in baseline intensity as observed for **1**. After 18 h, the dominant peak in the 280 nm chromatogram was still due to **3**; however, a trace of 4-hydroxybenzaldehyde (**12**, retention time = 11.9 min) had appeared (confirmed with a standard; ≪1% of **3**). Under more strenuous oxidation condi-

Table 2. Retention and Spectral Data for Starting Compounds and Their Oxidation Products

compd no. (refer to **Figure 1**)	name	retention time (min)	absorption maxima (nm)	mol wt
1	caffeic acid	12.2	240, 303(s), 323	180
2	3-hydroxycinnamic acid	14.8	231, 277, 316(s)	164
3	*p*-coumaric acid (4-hydroxycinnamic acid)	13.8	227, 310	164
4	ferulic acid	14.0	236, 297(s), 323	194
	oxidation product of ferulic acid	12.8	270 (broad)	226
5	quinone of caffeic acid	9.9	249, 307, 403	178
6	dimer of caffeic acid	14.2	230. 283, 326	358
7	dimer of 3-hydroxycinnamic acid		weak	326
8	dimer of *p*-coumaric acid		weak	326
9	dimer of ferulic acid	16.4	234, 281, 323	386
10	3,4-dihydroxybenzaldehyde	10.0	228, 279, 310	138
11	3-hydroxybenzaldehyde	12.4	coelution with caffeic acid	122
12	4-hydroxybenzaldehyde	11.9	281(br)	122
13	vanillin	12.0	228, 280, 310	152
14	5-caffeoylquinic acid	11.0	248, 302(s), 327	354
15	quinone of 5-caffeoylquinic acid	9.1	249, 306, 400	352
16	ketone oxidation product of 5-caffeoylquinic acid	12.8	236, 317(br)	308
	dimer from 5-caffeoylquinic acid	12.8	weak	706
17	tyrosol	10.0	276	138
18	oleuropein	15.7	233, 281, 319	540
	oleuropein isomer	17.0	236, 281, 319	540
19	quinone of oleuropein	14.0	240, 392	538
	quinone of oleuropein isomer	15.4	237, 393	538
	oxidation product of oleuropein	17.0	232, 280, 327	680
	oxidation product of oleuropein	18.3	234, 280, 329	680
	oxidation product of oleuropein	17.7	weak	538
20	gallic acid	6.2	272	170
21	syringic acid	12.3	276, 363	198
22/23	oxidation product of syringic acid	9.8	290, 400	see Results
	oxidation products of syringic acid	15.8	coelution	334, 352, 366, 446
	oxidation product of syringic acid	17.4	268	334

tions, such as a greater amount of periodate or longer reaction time (results not shown), the concentration of dilactone dimer, **8**, could be up to ~10% of **3**.

Fenton Oxidation. The reaction between **3** and the Fenton reagent produced one major product, **12**, confirmed by retention time, UV spectrum, and mass spectrum of an authentic standard. There was evidence from EICs of a trace of **1** but not the corresponding benzaldehyde, **10**. No trace of any dimers was found by EICs. After 22 h, **12** was still the major product and had more than doubled in concentration. EICs revealed no dimers. The concentration of **1** had more than doubled, and the peak due to **10** had grown. The peak due to **3** had decreased by approximately half.

Ferulic Acid (4). *Periodate Oxidation.* Compound **4** contains an electron-donating methoxy group, which in the 3-position may affect reactivity by an inductive rather than resonance effect. The 280 nm chromatogram of the reaction solution of ferulic acid with periodate showed, after 30 min, a main peak due to unreacted **4** with small peaks due to a dimer, **9**, and vanillin, **13**. The UV spectrum of **9** was consistent with a dilactone dimer as discussed for **1**, above. The identity of **13** was confirmed with a standard. After reaction for 16 h, the peaks due to **9** and **13** had increased in intensity, with **9** the dominant product. There was an increase in baseline intensity as observed for **1**, together with numerous small peaks that were not identified.

Fenton Oxidation. Three major product peaks were observed in the 280 nm chromatogram 1 h after **4** had reacted with the Fenton reagent (**Figure 2**). The main product was vanillin, **13**. A second major product had a molecular weight of 226, corresponding to the insertion of two oxygen atoms to ferulic acid (194 + 32 = 226). The UV spectrum for this compound indicates that conjugation between the aromatic ring and the alkene bond may have been lost. The third major product was the dilactone dimer, **9**.

After 21 h, the peak due to **4** decreased slightly (80% of original concentration) whereas that due to **13** increased ~2-fold. The peak due to **9** had increased slightly, whereas the peak due to dihydroxylated ferulic acid (MW 226) had increased by a factor of 2. In contrast to the periodate oxidation, for which **9** was the main product, with the Fenton oxidant, **13** was the main product.

5-Caffeoylquinic Acid (14). *Periodate Oxidation.* This compound contains a caffeic acid moiety esterified to quinic acid; thus, quinone and dimer formation would be expected on the basis of the reactions above. The 280 nm chromatogram of the solution obtained 10 min after reaction of **14** with periodate showed one major product peak. This peak (retention time = 9.1 min) was assigned to the quinone, **15**, from its molecular weight and UV spectrum. After 5 h, the 280 nm chromatogram showed a number of broad peaks of low intensity along with an increase in baseline intensity between 7.5 and 17.0 min. EICs showed that there was still some **15** present at this time. It is likely that the quinic acid moiety is reactive and that oxidation/polymerization reactions lead to a large number of very similar products.

Fenton Oxidation. The 280 nm chromatogram obtained after 10 min of reaction between the Fenton reagent and **14** showed the predominant peak (>90% of total intensity) was due to unreacted **14**, with a number of smaller peaks. EICs showed a trace of **10** and a small peak at 8.9 min, which had the same UV spectrum as **15**, above. A peak at 12.8 min had a molecular weight of 308, corresponding to loss of 46 amu from the starting material. This may be due to oxidative decarboxylation of the acid group on quinic acid to give the ketone, **16**. Coeluting with this peak was a smaller peak (~10 times less intense) with

molecular weight 706, consistent with dimer formation. Given the absolute lack of dimer in the periodate reaction, it is unlikely to be a dilactone dimer. There was not the same level of intensity under the baseline as observed in the periodate oxidation.

After 24 h, there were no discernible peaks in the 280 nm chromatogram, just broad intensity under the baseline from the void volume to 15 min. It is possible that lower molecular weight compounds are formed with the Fenton oxidation than with periodate oxidation as the intensity occurs at longer retention times in the periodate system (see above). EICs showed that some unreacted **14** was still present (<1% of starting concentration), along with some **10** at about the same concentration as at 10 min, but there were no peaks due to the dimer or **15**.

Tyrosol (17). *Periodate Oxidation.* The reaction between **17** and periodate did not result in any detectable change to the 280 nm chromatogram for up to 20 h after mixing. Therefore, it would appear that **17** is not oxidized to any significant extent by periodate.

Fenton Oxidation. Only very small peaks in the 280 nm chromatogram were detected after 6 h of reaction. One "peak" was very broad (7−9 min, centered on ~8 min). The other peak was sharper at 11.8 min. EICs for hydroxylation and other oxidation products provided no conclusive evidence for the identity of these peaks, although some of the intensity of the 8 min peak could be due to hydroxytyrosol and/or 4-hydroxyphenylacetaldehyde. The peak at 11.8 min was due to a compound with an apparent molecular weight of 122 (EICs at *m*/*z* 123 positive and 121 negative ion modes showed a single peak at this retention time), and this combination of retention time and molecular weight is consistent with that reported for **12**, above.

Little change was observed in the 280 nm chromatogram after 27.5 h of reaction. The peak due to **17** had decreased slightly, whereas that due to **12** showed a slight increase. The Fenton reagent produced a slow reaction to products unlike the periodate system, for which no reaction was detected.

Oleuropein (18). *Periodate Oxidation.* The products of the oxidation of **18** by periodate were found to be significantly influenced by the solvent used. Furthermore, the reaction between oleuropein and periodate was somewhat complicated by the presence of two isomers of **18** in the starting material (detected in the 280 nm chromatogram). The predominant isomer (15.7 min) was assumed to be the one shown in **Figure 2** and cited in the literature (e.g., ref *27*). In the 280 nm chromatogram, the peak due to the second isomer (17.0 min) was initially 20% of the intensity of the main peak. The mass spectrum for this compound (17.0 min) was almost identical to that of the main isomer; however, there were slight differences in the fragmentation of the glycone, indicating that the isomerism may have arisen from attachment of the glycone to the secoiridoid ring.

After 10 min of reaction, the main peak in the 280 nm chromatogram was attributed to the quinone of oleuropein, **19**, with a retention time of 14.0 min. The UV spectrum shows an absorption maximum around 392 nm, consistent with the quinones observed for the cinnamic acids, above. The positive and negative ion mass spectra indicate a compound with a molecular weight of 538, also consistent with this compound being the quinone. An isomeric form of this compound, presumably due to oxidation of the oleuropein isomer, gave a peak at 15.4 min.

The UV chromatogram at 280 nm after 20 h of reaction showed **19** was still the largest peak. There were now a larger number of other peaks formed, together with considerable intensity under the baseline. Of the new peaks to grow during this time, the predominant one had a retention time of 17.0 min and a molecular weight of 680, but no structure can be assigned to it at this stage. A smaller peak at 18.3 min appears to be isomeric on the basis of UV and mass spectra.

Of the compounds tested in this study, oleuropein showed the greatest solvent dependence to the outcome of periodate oxidation. When oxidation was performed in methanol/water, the main reaction products, formed subsequently from the quinone, were due to methylation of the hydroxyl groups on the hydroxytyrosol moiety.

Fenton Oxidation. Ten minutes after mixing, the 280 nm chromatogram showed the main product peak at 14.0 min, assigned to **19** on the basis of the UV spectrum and mass spectral fragmentation pattern, as discussed above. Two other peaks with the same molecular weight at 15.4 and 17.7 min were also observed at this time. On the basis of fragmentation patterns, the peak at 15.4 min was due to a compound oxidized at the elenolic acid moiety, whereas the compound eluting at 17.7 min appears to be formed by oxidation of the glucose. Two peaks for unreacted oleuropein could be found at 15.7 and 17.0 min. There was more intensity under the baseline and more smaller peaks than in the periodate system.

After 22 h, there were virtually no discernible peaks in the 280 nm chromatogram, just intensity under the baseline from 10 to 22 min. EICs showed trace amounts of **19** and some unreacted **18**.

Gallic Acid (20). *Periodate Oxidation.* When gallic acid was reacted with periodate for 10 min, the 280 nm chromatogram showed a very broad area of baseline intensity stretching from the void volume at 1.5 min to 18 min. There was a large, broad peak near the void volume (centered on 2.3 min) and three smaller peaks eluting after 14 min. However, there were no discernible peaks in the positive or negative ion TICs. EIC searches for dimers, quinones, and ellagic acid (a known oxidation product of gallic acid) gave no assignable products. Over the next 20 h, no product peaks emerged.

Fenton Oxidation. This analysis was not considered to be likely to produce any useful information, on the basis of the absence of discernible products from the periodate oxidation system. Therefore, Fenton oxidation was not undertaken.

Syringic Acid (21). *Periodate Oxidation.* It was considered that using **21** would allow some products to be identified, because methylation of the 3- and 5-hydroxy groups of gallic acid would limit reactivity of the phenol (cf. caffeic and ferulic acid in which ferulic acid reactions were much "cleaner"). After 1 h, the 280 nm chromatogram showed three main product peaks, but the peak with the largest intensity (>90% of total intensity) was unreacted **21**. A peak with a retention time of 9.8 min displayed unusual MS behavior. The positive ion spectrum gave a peak at *m*/*z* 169 consistent with **22**; however, the negative ion spectrum gave a peak at *m*/*z* 168, which is not $[M - H]^-$, but could be due to the radical anion of **22**, reduced in the ESI source. There was some evidence of redox activity in the ESI source for these species. A peak at *m*/*z* 170 in the positive ion spectrum was 30% of the *m*/*z* 169 peak and therefore would not likely be solely due to the ^{13}C isotopomer (expected to be ~8% for a C_8 compound), but could be due to the protonated form of the hydroquinone radical **23**. Assignment of this peak as **23** is supported by the UV spectrum, which shows an absorbance at 400 nm.

Two other product peaks occurred at 17.4 and 15.8 min in the 280 nm chromatogram. The peak at 17.4 min had a MW of

334, and EICs at *m*/*z* 335 (positive ion mode) and 333 (negative ion mode) showed another peak with this apparent MW of 334 at 15.8 min. The two peaks had a ratio of approximately 2:1, respectively. However, the mass spectra derived from the 15.8 min peak also showed molecular ions at 352, 366, and 446 amu, indicating coelution of four compounds. No structures have been assigned to these as yet.

After 19 h, two main product peaks were identified in the 280 nm chromatogram with retention times of 9.8 and 15.8 min, although unreacted **21** was still the predominant peak. The peak at 9.8 min was slightly more intense than the peak at 15.8 min. The peak at 9.8 min in this chromatogram had positive and negative ion mass spectra identical to those obtained in the 1 h chromatogram. The peak at 15.8 min also had the same mass spectra as at 1 h; that is, it appeared that there were four coeluting compounds with MWs of 334, 352, 366, and 446 amu. There was very little intensity under the baseline compared to some of the other compounds (gallic acid, caffeic acid, chlorogenic acid, and oleuropein).

Fenton Oxidation. After 10 min of reaction, the main peak in the 280 nm chromatogram was due to unreacted syringic acid. As in the periodate oxidation, the main product peak occurred at 9.8 min and had identical mass spectra as observed, above. Peaks at 15.8 and 17.4 min, corresponding to the same products as in the periodate reaction, were also present; however, their intensity was opposite to the above. That is, in the 280 nm chromatogram the 15.8 min peak was twice the intensity of the 17.4 min peak. EICs also revealed differences. Whereas with periodate there appeared to be two compounds of MW 334 eluting at 15.8 and 17.4 min, with Fenton oxidation, only the 17.4 min peak had this apparent molecular weight. As with periodate oxidation, the 15.8 min peak was made up of coeluting compounds with MWs of 352, 366, and 446 amu.

Two extra product peaks could be resolved in the 280 nm chromatogram of the Fenton oxidation of **21** compared to the periodate oxidation. A peak at 10.9 min had an apparent MW of 230 amu, which corresponded to addition of two oxygen atoms to syringic acid. A peak at 12.9 min corresponded to two coeluting compounds with apparent MWs of 450 and 214 amu. A mass of 214 amu equates to the addition of one oxygen atom to syringic acid. It is not known at this stage whether oxygenation occurred on the methoxy group or the aromatic ring. For the 214 amu compound, a fragment ion in the positive ion mode at *m*/*z* 197 corresponded to loss of H_2O ($[M + H]^+$ $215 - 18 = 197$), perhaps indicating that the methoxy group was oxygenated.

DISCUSSION

This study is intended as a broad survey of the oxidation reactions of a number of different classes of phenolic compounds with two types of oxidants. This is in contrast to other studies in which the emphasis has been on the reaction of one compound with one oxidant but with analysis in detail (X-ray diffraction, 2D-NMR, etc.) of the product(s) formed. Therefore, in this study structural assignments have been made on the basis of the available evidence—including UV−vis spectra, mass spectra, and, when possible, authentic standards—without attempting to isolate pure compounds and subject them to more rigorous structural analysis by 2D-NMR methods. Moreover, given our interest in antioxidant activity of phenolic compounds in foods (*9*, *23*, *28*, *29*), the dimeric compounds formed by the cinnamic acids, above, will be of less importance than monomeric products such as quinones and aldehydes.

Three classes of phenolic compounds with the common structural feature of a single aromatic ring were chosen for this study. Oxidation of phenolic compounds with two or more aromatic rings (e.g., flavonoids) was expected to produce an even wider array of products (e.g., for quercetin, *30*). Cinnamic acid derivatives were investigated because these compounds (especially **1**) have been the subject of numerous and ongoing studies on the reaction products of oxidation, often in different contexts. For example, Cilliers and Singleton (*25*) investigated the oxidation of **1** in the context of nonenzymatic browning in foods. More recently (*17*) the oxidation of **1** was investigated in relation to a better understanding of lignan biosythesis. As well as the cinnamic acids, we investigated two phenolic compounds—tyrosol and oleuropein—of importance to olives as there has recently been much interest in the antioxidant and other properties of olives and olive products (*23*, *31*). And third, we investigated several benzoic acid phenols because compounds such as gallic acid have been reported to possess potent antioxidant activity (*32*).

The choice of oxidants was based on several criteria. The use of periodate is well-established in the literature (*17*, *18*) and is reported to produce products similar to polyphenol oxidase (*18*). On the other hand, reactive oxygen species (ROS), including hydroxyl and peroxyl radicals, have been implicated in oxidative damage in lipids and in oxidative processes in vivo. It was therefore of interest to contrast the reactivity of the phenolic compounds listed above with ROS. We chose an inorganic source of hydroxyl radicals, the Fenton system, to eliminate side reactions that may have occurred if an organic source of ROS were used [e.g., 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH)]. This oxidant has been used recently to investigate oxidative damage of DNA (*33*).

Oxidation of Cinnamic Acids. The reaction of **1** with periodate produced the quinone (**5**) and dimer (**6**) as the initial main products consistent with other studies (*17*, *18*). The formation of these products can be rationalized by the structural features of **1**. The ortho phenolic groups favor quinone formation. Dimer formation appears to be related to the fact that the alkene double bond is conjugated with the aromatic ring, and conjugation with the 4-hydroxyl group may also be important. By systematically altering the structure of the cinnamic acid nucleus, further insights into structure−reactivity relationships may be gained. The structural variations investigated in this study were as follows: monohydroxylated cinnamic acids, where the hydroxy group was conjugated (**3**) or not conjugated (**2**) with the alkene double bond; methylation of the 3-hydroxy group of **1** (**4**); and esterification of the carboxylic acid group of **1** (**14**).

These structural variations produced different outcomes in reactions with periodate: **2** did not react with this oxidant, whereas **3** gave a trace of the benzaldehyde, **12**, or, under more forcing conditions, the dimer, **8**. This suggests that conjugation of the 4-hydroxy group with the alkene double bond is important for reaction with this oxidant. Similarly, **4** has this pattern of conjugation, and a dimer, **9**, and an aldehyde, **13**, were identified as products. However, having a methoxy group in the 3-position led to an increase in the rate of dimer formation (detectable after 10 min), relative to **3**. Dimer formation with **4**, however, was slower than for **1**. Previous studies (*17*, *18*) with **1** have indicated that formation of an *o*-quinone occurs prior to dimer formation. For both **3** and **4**, the lack of *o*-hydroxy groups means that quinones (analogous to **5**) are unable to form and that the routes to dimers (**8**, **9**) and/or aldehydes (**12**, **13**) may proceed through semiquinone radical intermediates (e.g., **24**). Such radicals are likely to be highly reactive and were not detected under our conditions. With oxidation of **14**, the quinone, **15**,

was detected after 10 min, but no dimers were detected. It is likely that the quinic acid moiety sterically interferes with the dimerization process.

All cinnamic acids in this study reacted with the Fenton reagent to produce benzaldehydes as the main products, with the exception of **14**, for which the main initial product was **15**, but only in low concentrations. Quinones were not observed for any of the other cinnamic acids. The formation of benzaldehydes from the chemical oxidation of cinnamic acids has not been widely reported. This reaction has not been observed for **1**, **2**, or **3**. There are two accounts of the oxidation of **4** to vanillin using O_2 (*34*) or H_2O_2 (*35*) as oxidants. However, Pan et al. (*36*) found that virtually no oxidation occurred between H_2O_2 and **4** under alkaline conditions, with only trace amounts of homovanillic acid formed. On the other hand, the microbial conversion of **4** to vanillin is well-known (*37*).

Although similar products were observed for the oxidation of cinnamic acids, they differed in the extent to which they reacted with the oxidants. No reaction could be detected between **2** and periodate, whereas the Fenton reagent was able to oxidize this compound, albeit slowly. At the other extreme, **14** was almost completely oxidized by periodate and was totally oxidized by the Fenton reagent. Across both oxidation systems an order of reactivity may be given: **14** > **1** > **4** ≈ **3** > **2**. On the basis of these results, it would appear that phenolic compounds with *o*-hydroxy groups would make better antioxidants, as is widely reported in the literature. However, when comparing antioxidant activity using the phycoerythrin assay (*38*), we found that at low phenol concentrations, **3** was a better antioxidant than both **1** and **14**, and at high concentrations the order of antioxidant activity was **14** > **3** ≫ **1** (*23*). On the other hand, in a lipid system the order of antioxidant activity was found to be **1** ≫ **14** ≈ **3** (*23*). It would thus appear that the ability of a compound to act as an antioxidant is heavily dependent on the actual system in which oxidation is taking place.

Oxidation of Olive-Derived Phenols. Because of our interest in the phenolic composition and antioxidant activity of olives (*22*, *23*, *39*−*41*), we also investigated two of the more common phenolic compounds found in olives and olive oil—tyrosol (**17**) and oleuropein (**18**). Recently, there has been much interest in potential health benefits from oleuropein (*31*), a compound that contains an *o*-dihydroxy functional group esterified to a secoiridoid glycone. Tyrosol provides a contrasting system in that it contains a monohydroxy aromatic group without the secoiridoid moiety.

Compound **17** contains a hydroxy group in the 4-position of the aromatic ring and is thus structurally analogous to *p*-coumaric acid, **3**. However, a comparison of the reactivity of the two compounds highlights the importance of conjugation between the alkene double bond and the hydroxy group. In **17**, where this conjugation does not occur, little (or no) oxidation is observed with both oxidants, whereas oxidation of the conjugated **3** is observed in both systems. In agreement with these results, **17** was found to have little antioxidant activity in the aqueous phycoerythrin assay (*23*) but, interestingly, had activity intermediate between **1** and **14** in a lipid oxidation system (*23*).

Compound **18** was found to be oxidized by both oxidants used in this study, resulting in quinones in each system. There was evidence (mass and UV spectra) that the glucose moiety is susceptible to oxidation. Like the other *o*-diphenols used in this study, **18** had completely reacted with the Fenton reagent after 24 h. Compound **18** has been evaluated to be an effective antioxidant in the aqueous phycoerythrin assay (*23*) and, in fact, is more potent than both **1** and **14**. On the other hand, it had little antioxidant activity in a lipid oxidation system (*23*).

Oxidation of Benzoic Acids. Compound **20** was found to be highly reactive to periodate oxidation and was completely oxidized within 10 min. No readily identifiable products were able to be determined, and it can be concluded from the broad area of baseline intensity that a large number of very similar products have been formed. Substitution of methyl groups at the 3- and 5-positions, giving **21**, resulted in fewer products being formed, as expected, with very little baseline intensity. However, structures were not easily deduced for any of the peaks in the 280 nm chromatogram. Further work is needed to characterize these compounds and, by using milder conditions (lower temperature, less oxidant) it may be possible to isolate some of the early oxidation products of **20**.

Conclusion. A broad range of phenolic compounds with the common structural feature of a single aromatic ring have been studied using two oxidation systems: resin-immobilized periodate and the Fenton reagent. Systematic structural variation of the phenols was found to influence their reactivity and the oxidation products formed with some general trends observed. Formation of a distinct quinone compound is related to the presence of *o*-hydroxy groups on the benzene ring, that is, **1**, **14**, and **18**. Conjugation of the quinone to an alkene double bond facilitates dimer formation (unless steric interference occurs, **14**) and enhances reactivity. The latter point is illustrated by the complete reaction of **1** and **14** with periodate in <5 h, whereas periodate oxidation of **18** results in the quinone **19** as the major product after 20 h. Fenton oxidation of cinnamic acid derivatives gave aldehydes as the primary products, with some dilactone dimer formation also evident. Significantly, the most reactive compounds in this study, **1** and **14**, were not always found to have the highest activity when functioning as antioxidants (*23*), and conversely a compound with relatively low reactivity, **17**, can be an effective antioxidant in a different system (*23*). This calls into question antioxidant test methods based on simplistic methodology (*9*, *42*).

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