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CHARACTERIZATION OF COMPOUNDS OBTAINED BY CHEMICAL OXIDATION OF CAFFEIC ACID IN ACIDIC CONDITIONS

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Key Word Index—Caffeic acid; caffeic acid *o*-quinone; tetrahydrofuran derivatives; sodium periodate oxidation.

Abstract—Sodium periodate oxidation of caffeic acid was investigated in aqueous solutions with pH ranging from 2 to 7. Products formed from quinone evolution were monitored by HPLC. It was found that they were affected both qualitatively and quantitatively by the conditions of the reaction. The rate and the yield of the reaction increased with the pH. In particular, two products obtained at pH values lower than 4.6 approximately, were analysed and isolated by reverse phase HPLC. By using ^1H and ^{13}C NMR and mass spectrometries, these compounds were shown to be two stereoisomers of 2,5-(3′,4′-dihydroxyphenyl)tetrahydrofuran 3,4-dicarboxylic acid.

INTRODUCTION

The oxidative browning of polyphenols in plant derived foods and beverages generally results in a loss of nutritional value and appearance of undesirable brown colours [1]. The first step in browning is the oxidation of *o*-diphenols to the corresponding highly reactive *o*-quinones [2, 3]. Enzymic oxidation is the most important reaction in fresh fruits and juices when polyphenoloxidase is present [4, 5]. Non-enzymic autoxidation can also take place and cause damage in the presence of oxygen, more especially when the medium is alkaline [6]. *O*-Quinones can react along different pathways according to their electrophilic and oxidative properties. As electrophiles, they form adducts with different nucleophilic substrates including amino derivatives and thiols [2, 7], as well as water [2, 8]. As oxidants, *o*-quinones oxidize other products with lower redox potentials, for instance other phenolic compounds [2, 3, 8–11], and are reduced to the original phenol. In the absence of other substrates, the *o*-quinone may condense with the corresponding hydroquinone, either through a Michael type addition [12–14], or through a mechanism involving semiquinone radical intermediates [14, 15] and polymerize. Caffeic acid derivatives, such as chlorogenic (caffeoylquinic) acid in apples, caftaric (caffeoyltartaric) acid in grapes, are among the major phenolics acting as good substrates in oxidative browning. Some studies using enzymic [16–19] or chemical [6, 14, 15, 20] oxidation are available but few products obtained in this kind of reaction have been identified, probably because they are usually unstable. However, recent results have been reported on caffeic acid model systems [15, 20] and chemical oxidation, in alkaline aqueous solutions. The structures of several dimeric compounds were established and a mechanism involving radical coupling and intramolecular nucleophilic attack was proposed.

Using caffeic acid as a model, the purpose of our work was to select chemical oxidation conditions giving a set of products as close as possible to those of enzymic oxidation and then to study the influence of different factors on the condensation reaction of the quinone, especially in acidic conditions.

RESULTS AND DISCUSSION

Oxidation of caffeic acid using heterogenous conditions seems to be the most convenient method to prepare a solution of its quinone. Different reagents were tested in various conditions: cerium ammoniacal nitrate [21] and *o*-chloranil [22] were shown to give poor yields with our substrate and the best results were achieved with periodate supported on a macroporous anion exchange resin (Amberlite IRA 904) [23], in acetonitrile. Thus, solutions of pure quinone were obtained by filtering off the resin. The purity of these solutions was checked by NMR spectrometry and the quinone characterized from corresponding ^1H NMR data.

The disappearance of the caffeic acid quinone was studied in different conditions, following its specific absorption at 406 nm. In the same way, the various products formed from the quinone were monitored by HPLC and compared both qualitatively and quantitatively according to the pH and the solvent in which they were prepared.

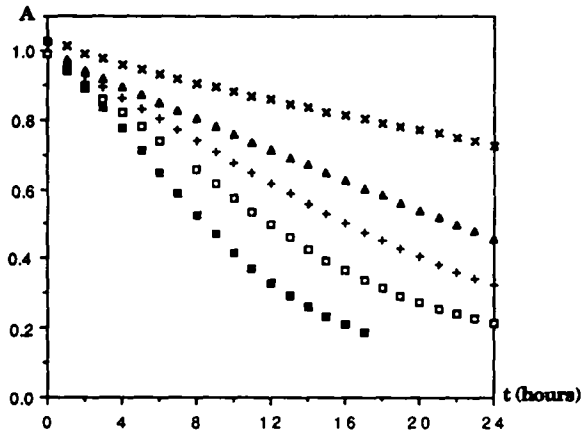


Fig. 1. Effect of solvent on the kinetics of quinone disappearance. [quinone] = 0.48 mM; $T = 20°$; A: maximum absorbance of quinone visible band; × MeCN, Δ20% H_2O, +30% H_2O, □40% H_2O, ■50% H_2O.

Solvent effect

The stability of the quinone was determined in acetonitrile, water and thereafter in mixtures of both solvents. The results (Fig. 1) show that the quinone disappears more rapidly as the amount of water in the mixture increases. They are in good agreement with previous reports which emphasized the particular instability of quinones in aqueous solutions [24–26]. We suggest that the highly polar protic water molecules might speed up the reaction by stabilizing transitory species upon solvation, in particular. Alternatively, water could act as a nucleophile towards the quinone, giving hydroxylated adducts. This latter hypothesis is widely accepted [25, 27] but as yet, has not been directly proved. Moreover, it was observed that the number of products arising from quinone evolution is greater in water and aqueous mixtures than in pure acetonitrile or other organic solvents such as tetrahydrofuran, for instance (Fig. 2).

pH Effect

Kinetics of the quinone disappearance were investigated over the pH range of 2–7 in buffer solutions containing citric acid, disodium hydrogen phosphate and potassium chloride, at an ionic strength of 0.5. It was found that the rate of reaction does not greatly alter in the pH range 3–5, but increases drastically over pH 5 (Fig. 3). This pH dependency may indicate the involvement of reactive phenolate ions in the quinone evolution [6]. In order to further account for the pH dependency of the apparent rate constant associated with the disappearance of quinone, the possibility of a base catalysis was investigated. Kinetic runs were carried out at pH 2, 4 and 5.6, providing conditions under which one of the citric acid species is predominant. The plot of experimental rate constant against total citric acid concentration, at different pH values, gives two good linear relationships, the slopes of which reflect the effect of buffer concentration (Fig. 4). At pH 2, the neutral form of citric acid prevails and no significant effect by the buffer is observed. At higher pH values, the experimental rate constant increases with the increase of buffer concentration, indicating that the mono-anionic and di-anionic forms of citric acid may enhance the reactivity of the quinone. This finding may be of interest in the case of juices and other beverages where carboxylic acids such as citric acid are naturally occurring.

The set of products arising from the quinone spontaneous evolution depends greatly upon the pH. HPLC chromatograms exhibit some major products and several

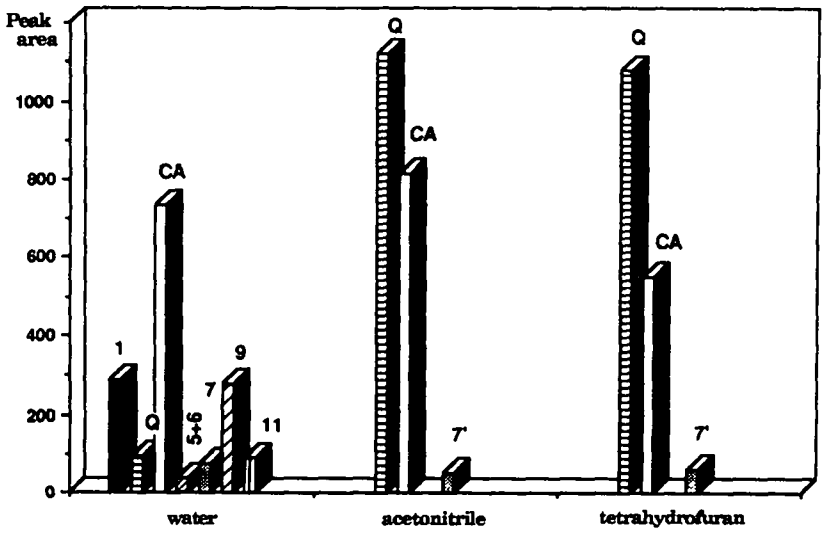


Fig. 2. Set of major products from caffeic acid oxidation and relative ratios on HPLC chromatograms, obtained after 1 hr, in varied solvents. CA: caffeic acid; Q: caffeic acid *o*-quinone; 1, 5, 6, 7, 7', 9 and 11: reaction products (numbered according to their HPLC retention times).

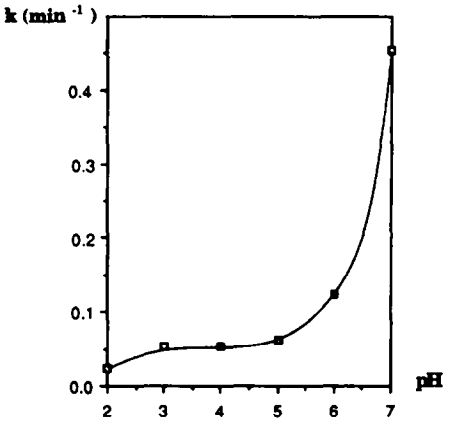


Fig. 3. Effect of pH on the experimental rate constant k of quinone disappearance, in buffer solutions. Ionic strength = 0.5; [quinone] = 1.0 mM; $T = 20°$.

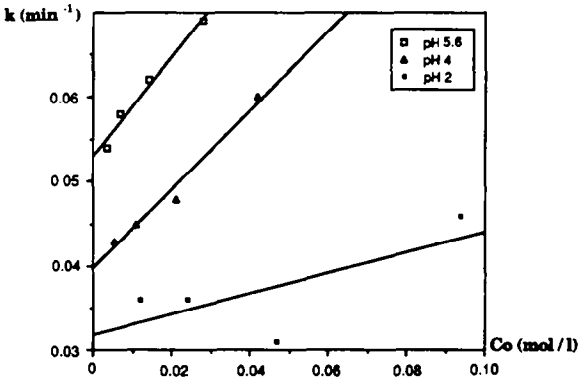


Fig. 4. Effect of total citric acid concentration C_0 on the experimental rate constant k of quinone disappearance, in buffer solutions. Ionic strength = 1; [quinone] = 1.0 mM; $T = 20°$.

minor compounds. Most are less polar than caffeic acid, according to their retention times on reverse phase systems. The chromatographic profile of the major products is represented in Fig. 5: compound **7** is present at all pH values, whereas **6** and **9**, two of the most important products obtained at pH 3.6, disappear above pH 4.6. With increasing pH, the total amount of substances increases and the major products are quite different both qualitatively and quantitatively. At pHs above 5.6, **1** completely disappears whereas a coloured product Q' accumulates; from close inspection of its UV-visible spectrum, Q' appears to be a new quinone arising from cross-oxidation of a phenolic compound by the caffeoylquinone.

Different products of non-enzymic autoxidative phenolic reactions, in caffeic acid solutions were recently characterized [15]. It is claimed that the oxidation of caffeic acid at different pHs (above pH 4) leads to similar chromatographic profiles (especially pHs 5–8), except for the proportions of some products. Our results show that other products are formed in more acidic conditions, namely **6** and **9** as major products.

Structural elucidation of compounds **6** *and* **9**

After separation by HPLC, detection being carried out at 280 nm, UV-visible spectra were recorded from 260 to 500 nm. It was found that products **6** and **9** displayed a single absorption spectrum with λ_{max} at 284 nm. The lack of absorption above 300 nm indicates the disappearance of the ethylenic bond of the caffeic moiety. Moreover, **9** was shown to be stable in acetonitrile, whereas in aqueous acidic solution it was partly converted into **6**. This suggests that these compounds are isomers. They were isolated by semi-preparative HPLC and investigated by NMR and mass spectrometries.

Electrospray mass spectrometry analyses in negative mode gave similar spectra for **6** and **9** with major peaks at m/z: 393, 357, 313 and 269. Since the molecular weight of caffeic acid is 180, these results indicate that **6** and **9** are dimers. That hypothesis is confirmed by two fragments at m/z: 313 and 269 obtained from the fragment at m/z 357 by successive loss of mass 44, thought to be CO_2.

The ^{13}C NMR spectrum of **9** was run in acetonitrile solution (Table 1). Only nine peaks were observed, hence the dimeric structure is symmetrical. This result is supported by the ^1H NMR spectrum of **9** (Table 1) where only one 1,3,4-trisubstituted aromatic proton system was present in the field between δ6.74 and 6.85. The lack of both vinylic protons and corresponding ^{13}C confirms the loss of the conjugated ethylenic bond in the caffeic acid moiety. Elsewhere, two complex signals were observed at δ5.68 and 3.80, respectively. Decoupling experiments indicate that the corresponding protons are coupled and moreover the proton at δ5.68 was also coupled with aromatic protons. These data, together with the ^{13}C signals at δ82.9 and 49.3, agree with a symmetrical tetrasubstituted tetrahydrofuran structure [28]. The coupling constants in the AA'XX' spin system of the tetrahydrofuran ring could not be measured exactly but their small values support *trans* coupling for these protons, allowing us to propose the structures **9a** and **9b** (Fig. 6).

The ^1H NMR spectrum of **6** (Table 2) exhibited six protons in the aromatic region, between δ6.7 and 6.9, and four signals (1H each one) consistent with an ABXY spin system [28], as in 2,5-(3',4'-dihydroxyphenyl) tetrahydrofuran 3,4-dicarboxylic acid. The interconversion of **9** into **6**, in acidic aqueous solutions can easily be explained by an epimerization at C-2 or C-5 after ring-opening as shown in Scheme 1. Two possible structures **6a** and **b** can occur from isomerization of **9a** and **b**, respectively. Only **6a** is consistent with the observed coupling constant values, thus **9a** was confirmed for compound **9**. Furthermore, from the mass spectra, the two peaks m/z 393 and 357 can now be assigned at $[M+18-H]^-$ and $[M-18-H]^-$, respectively, according to Scheme 2.

The mechanism for the formation of **6** and **9** involves the dimerization of caffeic acid. In agreement with previous reports [15, 20], this dimerization may occur from a

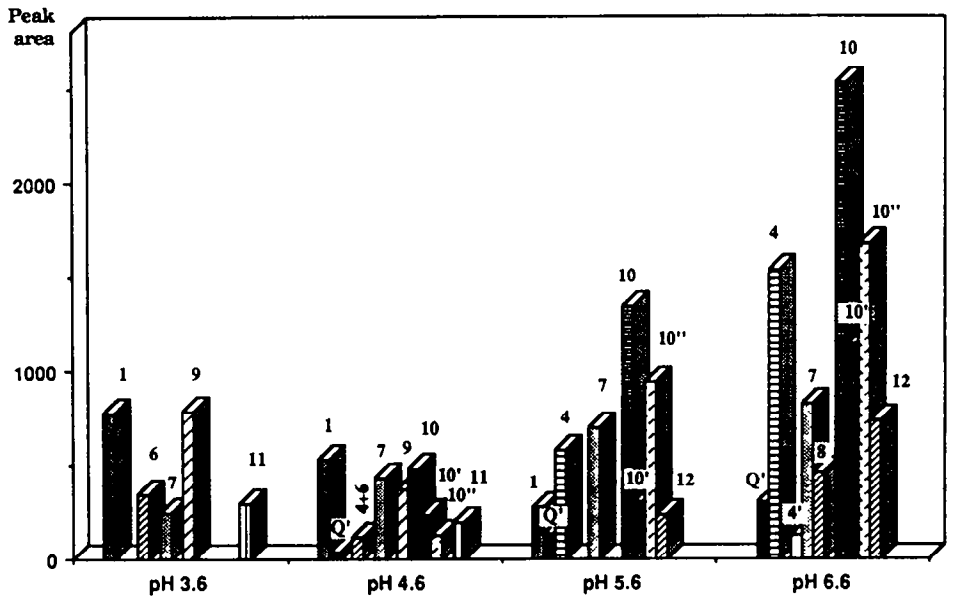
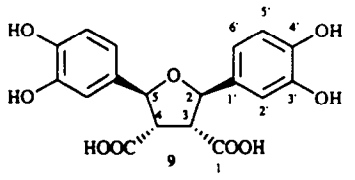


Fig. 5. Set of major products from caffeic acid oxidation and relative ratios on HPLC chromatograms obtained after 4 hr, at different pH values, in buffer solutions. Q': coloured compound; 1–12: reaction products (numbered according to their HPLC retention times).

Table 1. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectral data of **9** in CD_3CN

H	δ (ppm)	J (Hz)
2	5.68 *m*	not measured
3	3.80 *m*	not measured
2'	6.84 *d*	2.3
5'	6.85 *d*	8.2
6'	6.74 - 6.77 *dd*	8.2 ; 2.3



C	δ (ppm)
1	176.1
2	82.9
3	49.3
1'	131.4
2'	113.7
3'	145.9
4'	146.2
5'	116.5
6'	118.7

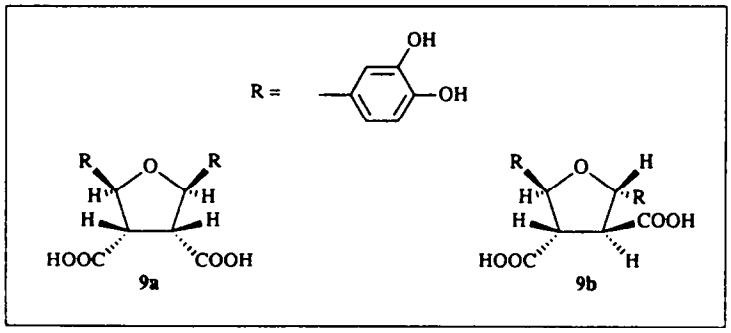
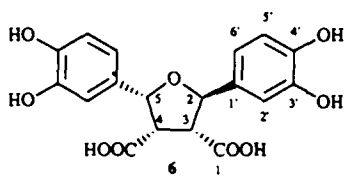


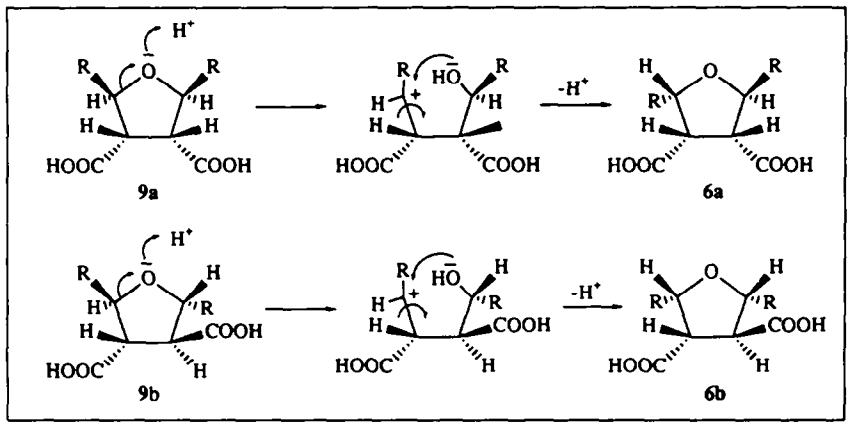
Fig. 6. Possible isomeric structures of compound **9**.

coupling reaction of two semiquinone radicals. Although caffeic acid is not a good nucleophile, the dimer may also result from the reaction between caffeic acid and the electrophilic *o*-quinone, especially at low pH. In spite of some unsuccessful assays to detect the presence of radicals from electron spin resonance (ESR) spectroscopy, the radical mechanism cannot be ruled out and further investigations are currently under way. Several isomers may arise from the nucleophilic attack of water on the exocyclic double bond of the quinone methide, followed

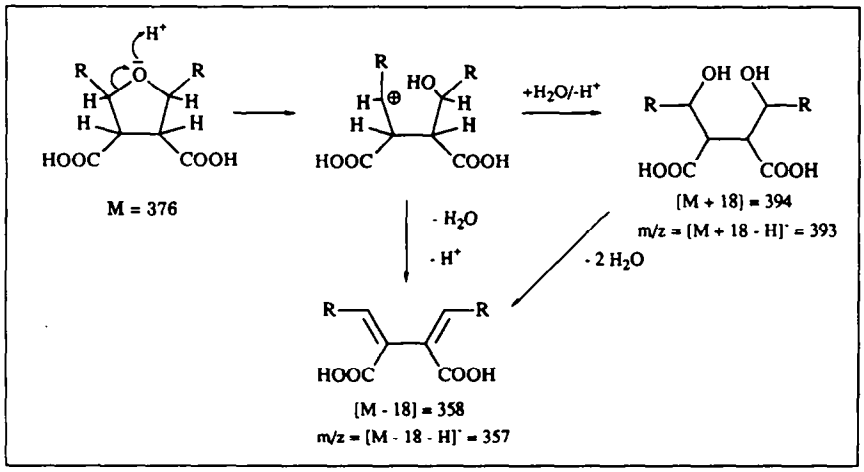
Table 2. ^{1}H NMR spectral data of **6** in CD_3CN at 400 MHz

H	δ (ppm)	J (Hz)
2	5.60 *d*	3.4
3	3.70 - 3.73 *dd*	3.4 ; 10.0
4	4.00 - 4.07 *dd*	8.6 ; 10.0
5	5.79 *d*	8.6
2'; 5'; 6'	6.7 - 6.9 *m*	not measured





Scheme 1. Epimerization reactions of compounds **9a** and **b**.

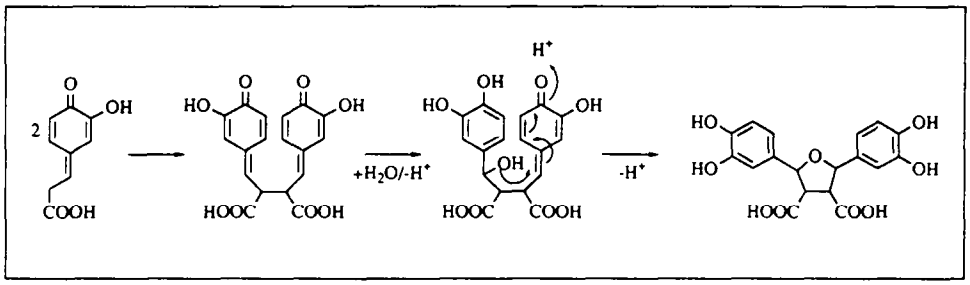


Scheme 2. Secondary reactions occurring in the course of mass spectrometry experiments.

by the formation of the tetrahydrofuran ring (Scheme 3) depending on the configuration of the asymmetric carbon atoms in the ring.

It was observed that **9** is formed prior to **6**. It could not be proved, however, whether **6** is formed directly or if it only arises from isomerization of **9**. Aqueous solutions of **9** evolve to an equilibrium with approximately 70% of **9** and 30% of **6**, while in acetonitrile, **9** is stable and **6** is quantitatively converted into **9**. Thus **9** seems to be both the kinetic and thermodynamic product of the reaction. Finally, we compared the different species obtained in the enzymic oxidation of caffeic acid, using the same solvent and pH conditions, with the species obtained in this work. From HPLC analyses, it was shown that the major products (e.g. compounds **6**, **7**, **9** and **11**) appear under both enzymic and chemical oxidations.

In conclusion, our results show that the set of molecular species arising from caffeic acid *o*-quinone evolution is



Scheme 3. Mechanism for the formation of tetrahydrofuran derivatives.

solvent and pH dependent. Two products, isolated from acidic aqueous solutions (pH 3.6) were characterized as two isomers of 2,5-(3',4'-dihydroxyphenyl) tetrahydrofuran 3,4-dicarboxylic acid. Their formation demonstrated that water acts as a nucleophile towards the quinone methide at pH lower than 4.6 while this reaction is not observed at higher pH values. Our results are consistent with previous reports on lignin biosynthesis models [29, 30].

EXPERIMENTAL

Materials. Sodium periodate and Amberlite IRA 904 were purchased from Fluka and caffeic acid was obtained from Lancaster.

Preparation of polymer-supported periodate. The periodate form of Amberlite IRA 904 was prepared according to the procedure described in ref. [23].

Oxidation of caffeic acid. Caffeic acid (18 mg) in MeCN (10 ml) was stirred with the periodate form of Amberlite IRA 904 (37.5 mg, e.g. 2 equivalents of oxidant) for 4 min, at 20°, under an Ar atmosphere. The resin was filtered off and the quinone solution diluted with appropriate solvent [31]. The evolution of these solutions was then followed either by spectrometry or by HPLC.

UV-visible spectrometry. Visible absorption spectra were recorded using a Hewlett Packard diode-array spectrometer, fitted with a thermostated quartz cell ($d = 1$ cm). Absorbance values were measured at the caffeic acid quinone visible λ_{max} (around 400 nm, depending on the solvent and pH of the medium), at 20°. All solutions were purged with argon before measurements. Kinetic runs were performed with a quinone concentration of 1 mM. The pH value was fixed by various buffer solutions. The pH range investigated (2–7), was obtained by mixing citric acid, Na_2HPO_4 and KCl according to the procedure cited in ref. [32]. Buffer solutions at pH 2, 4 and 5.6 with controlled concentration of citric acid, were prepared from 0.1 M solns of citric acid and NaOH, and 1 M KCl solution, in different mixtures.

HPLC analyses. A Spectra-Physics LC system including a SP8800 Ternary Proportioning Pump with a Hewlett Packward 1040M diode array detector was used for analytical and semi-prep. sepns. Analyses were monitored at 280 nm, and the UV-visible spectra of oxidation products were recorded from 260 to 500 nm. The column was a Lichrosorb RP-Select B column (5 μm packing, 250 × 4.6 mm i.d.). All sepns were carried out at room temp. The mobile phase was a gradient with starting mobile phase A as 0.1% HCO_2H in H_2O. Mobile phase B was 80% MeCN (containing 0.1% HCO_2H) and 20% mobile phase A. Linear gradients were applied: from 5 to 20% B in 20 min and from 20 to 40% B in 20 min.

Isolation of compounds **6** *and* **9**. Caffeic acid (181 mg) in MeCN (100 ml) was stirred with the periodate form of Amberlite IRA 904 (1.3 g) for 4 min, at 20°, under Ar atmosphere. The resin was filtered off and the quinone solution diluted (5 ×) with potassium hydrogen tartrate solution (2.5 $g\,l^{-1}$), containing caffeic acid (2.5 mM). The reaction mixture was allowed to react for 6 hr at room temp., concd under red. pres. at 40° and finally freeze-dried. Compounds **6** and **9** were sepd and isolated on a SFCC Nucleosil (10 μm packing, 250 × 10 mm i.d.) column.

NMR spectrometry. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectrometric measurements were carried out at a constant temp. of 25°. All spectra were recorded in CD_3CN.

Electrospray mass spectrometry in negative mode. Samples were dissolved in an aqueous solution of MeCN–H_2O (1:1) and analysed on a Bio-Q quadrupole mass spectrometer (Fisons, Manchester, U.K.). The extraction cone voltage was usually 55 V. Data acquisition was performed in the multi-channel acquisition mode (MCA).

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