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# Antioxidant Mechanisms of Polyphenolic Caffeic Acid Oligomers, Constituents of *Salvia officinalis*

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**ABSTRACT**

Caffeic acid, rosmarinic acid and oligomers of caffeic acid with multiple catechol groups are all constituents of *Salvia officinalis.* Their antioxidant potential was investigated with regard to their radical scavenging activity and the stability and structure of the intermediate radicals. Pulse-radiolytic studies revealed very high rate constants with hydroxyl radicals. Evidence from kinetic modeling calculations suggested an unusual complex behavior due to the presence of both $O_4$- and $O_3$-semiquinones and formation and decay of a hydroxyl radical adduct at the vinyl side chain. The radical structures observed by EPR spectroscopy after autoxidation in slightly alkaline solutions were only partially identified due to their instability and generally represented dissociated $O_4$-semiquinones. Hybrid density-functional calculations of the potential radical structures showed distinct differences between the resonance stabilization of the $O_4$- and $O_3$-semiquinones of caffeic and dihydrocaffeic acids, reflected also in the considerably faster decay of the $O_3$-semiquinone observed by pulse radiolysis. No evidence was found for dimerization reactions via Cβ radicals typical for lignin biosynthesis.

**Key words:** Caffeic acid oligomer, catechol semiquinone, electron paramagnetic resonance, hydroxyl radical, kinetic modeling, pulse radiolysis, *Salvia officinalis*.

**Abbreviations:** B3LYP: Becke's three parameter hybrid functional using Lee, Yang, and Parr correlation functional; DFT: density-functional theory; DPPH: 2,2-diphenyl-1-picryl hydrazyl; HRP: horseradish peroxidase; PCM: polarized continuum model.

## INTRODUCTION

*Salvia officinalis* (sage) dried leaves are commonly used as a culinary spice for flavoring and seasoning. The name *Salvia* comes from the Latin meaning 'to heal' and points to the popularity of this plant in traditional medicine (1, 2). Sage is reputed to be one of the richest sources of potent antioxidants (3-6), being rosmarinic acid, carnosic acid and their derivatives the best-known examples (4, 7). Recent studies showed that various *Salvia* species including *Salvia officinalis* (6, 8-10) contain a surprisingly high number of diverse catechol compounds, most comprising oligomers of caffeic acid. The caffeic acid oligomers have been shown to be very effective scavengers of 2,2-diphenyl-1-picryl hydrazyl (DPPH) and superoxide anion (10,11). For a more detailed study of their antioxidant mechanisms, we selected five minor constituents of *Salvia officinalis*, all with multiple catechol moieties (see Scheme I). Pulse radiolysis (12-14) was employed to study the radical scavenging effectivity and stability of the semiquinone structures. EPR spectroscopy (15) was used to identify likely radical target sites of the individual catechol structures and hybrid density-functional theory (DFT) calculation (16, 17) was used to confirm the experimentally obtained isotropic coupling constants and to assign the respective hydrogen atoms.

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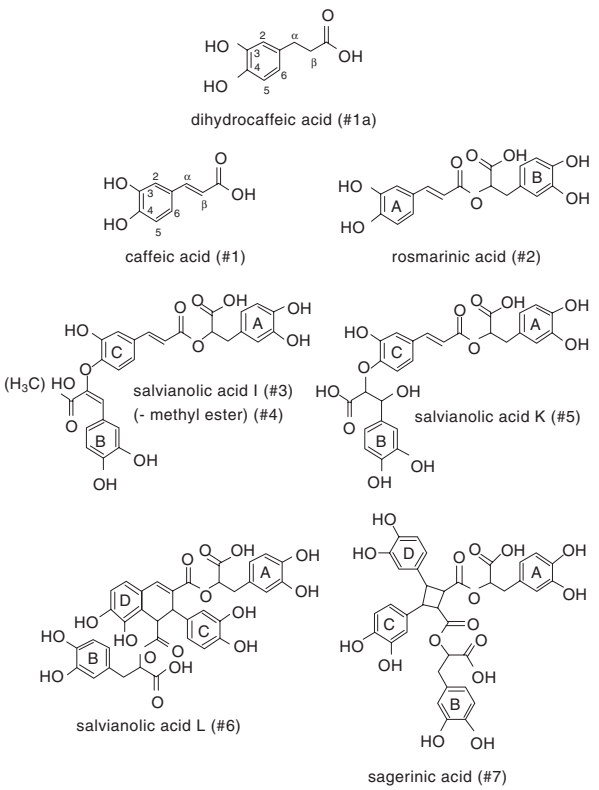



**Scheme I.** Structures of sage phenolic acids (caffeic acid and oligomers).

## MATERIALS AND METHODS

Caffeic acid (3,4-dihydroxycinnamic acid) and its oligomers were isolated from *Salvia officinalis* (8-10); in addition, caffeic acid and dihydrocaffeic acid (3,4-dihydroxydihydrocinnamic acid) were purchased from Fluka (Taufkirchen, Germany) and rosmarinic acid from ICN Biochemicals (Eschwege, Germany). Horseradish peroxidase (type VI), hydrogen peroxide (perhydrol), and sodium azide were also obtained from Fluka (Taufkirchen, Germany).

Pulse-radiolytic experiments were carried out with hydroxyl (•OH) radicals as electrophilic species in slightly alkaline solutions as described earlier (12-14). For this purpose, solutions were prepared with Milli-Q water and saturated with $N_2O$. The transient kinetics were evaluated both by linear regression analyses of pseudo-first and second order reactions and, owing to the deviations from these simple first- or second-order kinetics, by kinetic modeling of probable sequences of reactions (12,18).

EPR spectra were recorded after oxidation of the substances with horseradish peroxidase/ hydrogen peroxide ($HRP/H_2O_2$) in alkaline solution (pH 8.7-9.8), using a Bruker ESP-300 instrument, X-band, modulation amplitude 0.4 Gauss, gain $5x10^3$, microwave power 20mW, and scan speed of 0.6 Gauss/second (15).

Calculations based on hybrid density-functional theory (DFT) at the B3LYP level of theory were carried out with Gaussian98 (19). Three-dimensional structures were geometrically optimized with ChemOffice MOPAC restricted Hartree-Fock (CambridgeSoft) and the Z-matrix imported into the Gaussian program. Using the model compound caffeic acid and its possible radical structures, various basis sets were assayed for their correlation with the experimental data, taking into consideration the solvent water using the polarized continuum model (PCM). The best correlations between experimentally and theoretically derived coupling constants were obtained with the basis sets 6-311G* and 6-311G**.

## RESULTS

### *Pulse radiolysis*

Despite the fact that all compounds contain at least one and up to 4 catechol structures within the molecule, as seen in Figure 1, the transient spectra depict quite considerable differences. The figure is divided into two sections to highlight the difference between the first four substances with apparent bleaching of the parent compounds and that of compounds #5 and #7, which do so only minimally.

Comparing the literature values for caffeic acid, the only compound studied before, they showed a good agreement with the value we obtained with linear regression analysis: $7.4x10^9$ at 385nm vs. $5.5x10^9$ at 400nm (20) or $7.4x10^9$ at 470nm (21), all dimensions $M^{-1}s^{-1}$. Yet, almost consistently we encountered biphasic build-up and decay kinetics as deviations from simple pseudo-first order build-up and second-order decay, and therefore resorted to a kinetic modeling approach (12, 18). As listed in Table I, optimal fits of the

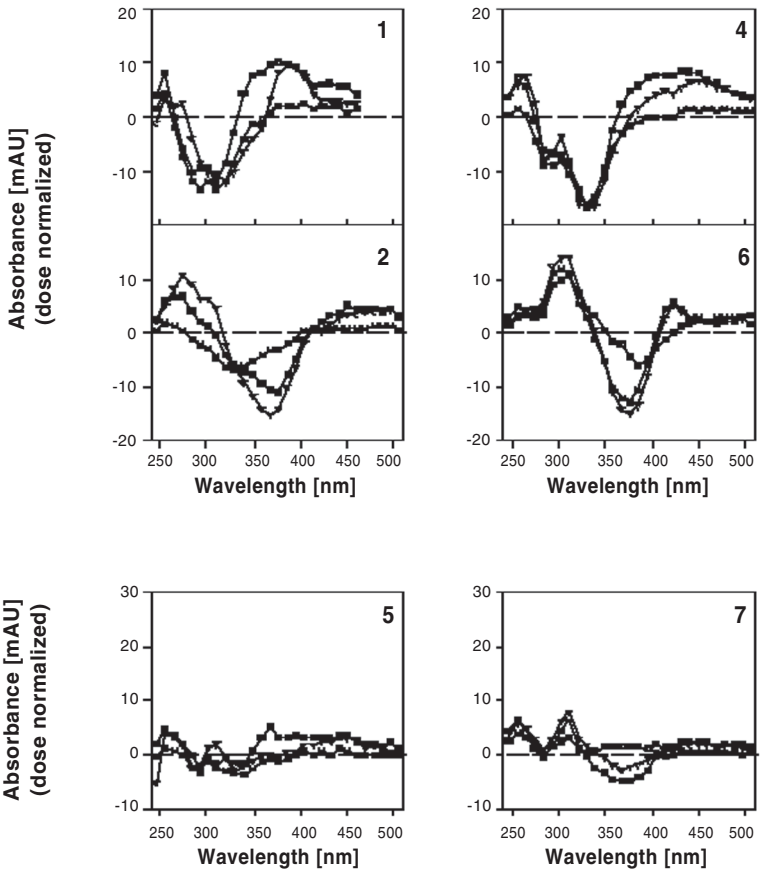



**Figure 1.** Pulse-radiolytic transient spectra of caffeic acid oligomer semiquinones generated with hydroxyl radicals dose-normalized. All initial transient spectra - solid circles - observed at 5 μs after the pulse; solid triangles and diamonds depict intermediate observation times varying according to the optical changes; solid squares, depict the final observation period.
Figure 1a: **1** - caffeic acid: 44.4 μmoles, pH 8.5; **2** - rosmarinic acid: 47.8 μmoles, pH 8.4; **3** - salvianolic acid I: 36.3 μmoles, pH 8.7-8.8; **4** - salvianolic acid I methyl ester: 35.0 μmoles, pH 8.6-8.7; **6** - salvianolic acid L: 43.0 μmoles, pH 8.7.
Figure 1b: **5** - salvianolic acid K: 43.6 μmoles, pH 8.5; **7** - sagerinic acid: 32.8 μmoles, pH 8.5.

TABLE I

Results of kinetic modeling in hydroxyl radical system

| Substance | λ | $k_{1p}$ ($x10^9$ $M^{-1}s^{-1}$) | $k_{2m}$ ($x10^9$ $M^{-1}s^{-1}$) | $2k_{3p}$ ($x10^6$ $M^{-1}s^{-1}$) | $2k_{4m}$ ($x10^8$ $M^{-1}s^{-1}$) | $k_5$ ($x10^5$ $M^{-1}s^{-1}$) | n |
|---|---|---|---|---|---|---|---|
| caffeic acid (#1) | 385 | 10.2 | 4.05 | 11.8 | 2.95 | - | 9 |
| rosmarinic acid (#2) | 455 | 3.85 | 6.05 | 17.9 | 12.4 | - | 9 |
| salvianolic acid I (#3) | 440 | 2.85 | 5.85 | 6.40 | 11.8 | - | 9 |
| salvianolic acid I ester (#4) | 440 | 8.65 | 3.05 | 2.95 | 17.2 | - | 8 |
| salvianolic acid K (#5) | 450 | 3.45 | 4.50 | - | 3.05 | 5.60 | 6 |
| sagerinic acid (#7) | 450 | 2.50 | 6.90 | 1.40 | 5.70 | - | 7 |

experimental digitized data points with the theoretical curves were achieved by the model where we assumed synchronous formation of *ortho*-semiquinones with the principal radical site in either $O_3$- (3-PhO•) or $O_4$- (4-PhO•) position (22) - reactions [1, 2]:

[1] •OH + PhO- → 4-PhO• + $H_2O$
[2] •OH + PhO- → 3-PhO• + $H_2O$
[3] 4-PhO• + 4-PhO• → PhO- + Ph=O
[4] 3-PhO• + 3-PhO• → PhO- + Ph=O
[5] Ph=O → product

Both semiquinone mesomers are assumed to decay by second order to the same corresponding quinones and parent catechols (reactions [3, 4]), albeit at quite different rate constants with the $O_3$-semiquinone being far more unstable. Accounting for the bimolecular recombination of the •OH radicals was required because of the non-stoichiometric scavenging at the low concentrations of the substrates. Modified kinetic schemes, taking into account the addition of •OH radicals either at the aromatic ring (23) or at the vinyl side chain (24), gave optimal results only in two cases (data not shown).

*EPR spectroscopy*

Generating radicals in the EPR cavity by oxidation with HRP/$H_2O_2$ in alkaline solutions (15) resulted in signals of the principal radicals which should all represent the *ortho*-semiquinone structures (Fig 2). While there is some apparent similarity between the spectra for #1, #2 and #7, on the one hand, and #6, #5 on the other hand, it has to be emphasized that this similarity is only superficial and does not allow to deduct analogous coupling constants. Furthermore, basically all EPR spectra are more or less unstable resulting in overlapping spectra which prevented quantitative evaluation for substances #4, #6, and #7. Dihydrocaffeic acid (#1a) was included only for the EPR (and DFT) studies in view of the presence of both saturated and unsaturated side chains in compounds #2-5.

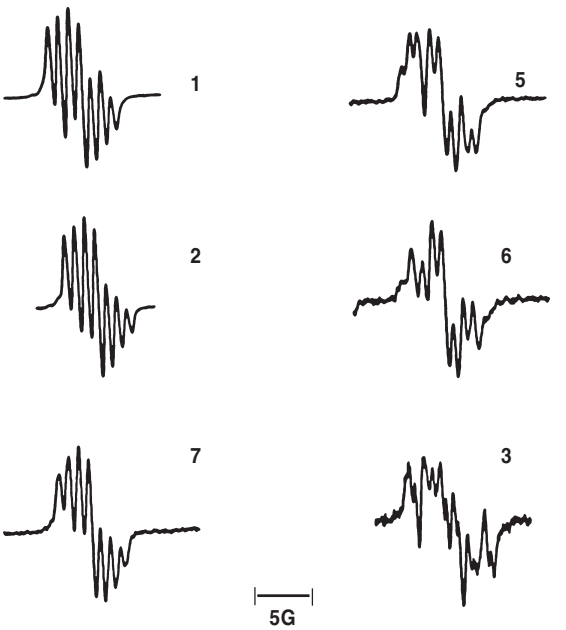



**Figure 2.** EPR spectra of caffeic acid oligomer radicals horseradish peroxidase, 10 µL of stock solution, 7 µmoles, in 1 mL substrate solution, the reaction was started by adding 40 µL hydrogen peroxide of a stock solution, 87 mmoles;
**1** - caffeic acid, 2.67 mmoles, pH 9.8; **2** - rosmarinic acid, 1.34 mmoles, pH 9.5; **6** - salvianolic acid L, 0.7 mmoles, pH 8.7; **7** - sagerinic acid, 0.69 mmoles, pH 8.9; **3**, - salvianolic acid I, 0.89 mmoles, pH 9.5; **5**, - salvianolic acid K, 0.92 mmoles, pH 9.2.

In Figure 3, the EPR spectra of caffeic (a) and dihydrocaffeic acids (b) at pH 9.6 and at different times are compared in the abscence and presence of zinc acetate. In the absence of $Zn^{2+}$ (left panels) only the $O_4$-semiquinone radical anion exists. In the presence of $Zn^{2+}$ the so-called 'spin-stabilization' effect (25,26) is apparent only for caffeic acid, reflected in the considerable change of the peak height ratios when comparing the initial and the 50 seconds spectra. Since all spectra decay over time, this 'spin stabilization' is actually a misnomer and should better be phrased 'spin enhancement' since the formation of the $Zn^{2+}$ complexes causes a considerable increase of the signals (factor of two for caffeic acid and five for

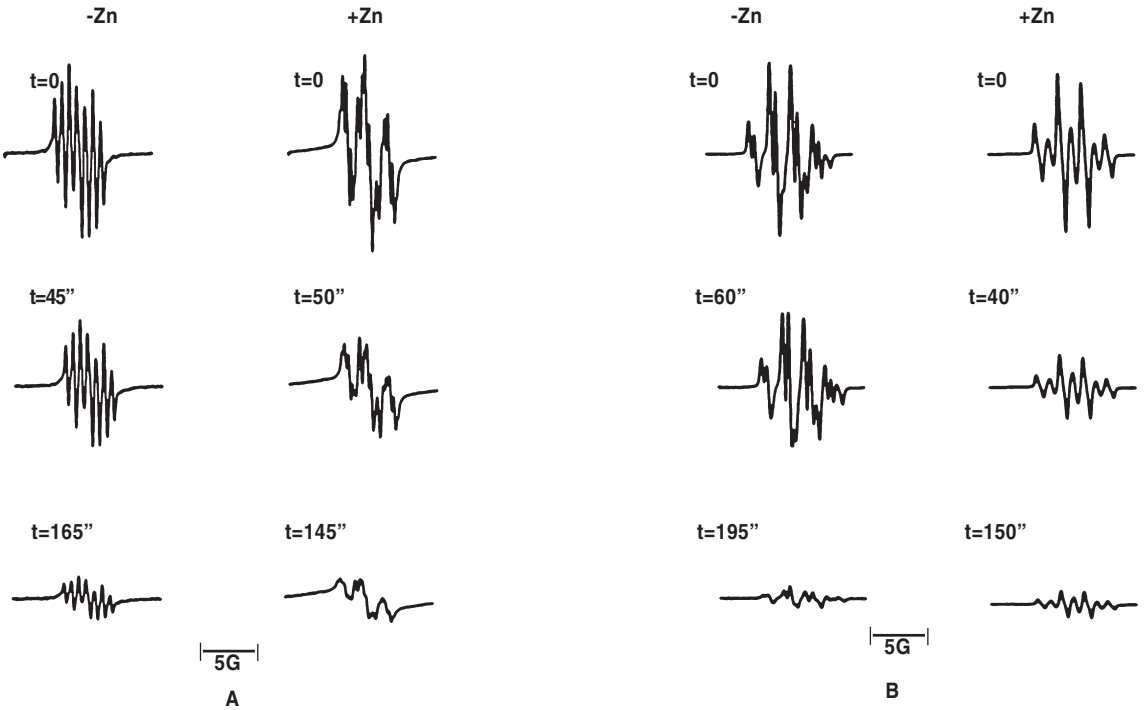



**Figure 3.** EPR spectra of caffeic and dihydrocaffeic acid radicals anions in the absence and presence of zinc acetate. Horseradish peroxidase, 10 μL of stock solution, 7 μmoles, in 1 mL substrate solution, pH 9.6, the reaction was started by adding 40 μL hydrogen peroxide of a stock solution, 87 mmoles; times given are delay times after the first measurement.
(a) caffeic acid (5.9 mmoles), left panel without $Zn^{2+}$, arbitrary ordinate scale ±1700; right panel with $Zn^{2+}$ (46 mmoles), arbitray ordinate scale ±3000;
(b) dihydrocaffeic acid (6.8 mmoles), left panel without $Zn^{2+}$, arbitrary ordinate scale ±3000; right panel with $Zn^{2+}$ (46 mmoles), arbitray ordinate scale ±15000.

dihydrocaffeic acid, arbitrary ordinate scale). In any case, the $Zn^{2+}$-stabilized spectra of caffeic acid thus verify the formation of both $O_3$- and $O_4$-semiquinones in analogy to the studies of Rettori et al. (22) and the kinetic modeling calculations (see above).

In Table II, we compared experimentally obtained coupling constants both from the literature (27-31) and from our own studies (14) for caffeic and dihydrocaffeic acids with theoretical values obtained from density-functional calculations. The calculations also resulted in isotropic coupling constants for radical species (i.e. the $O_3$-semiquinones of caffeic acid and dihydrocaffeic acid, the •OH adduct radicals of #1) which were too unstable to be observable with EPR spectroscopy, yet yielded quite different data indicative of distinct radical structures. What needs to be emphasized is the fact that due to the large size of the molecules of caffeic acid oligomers, DFT calculation of their radicals was limited to smaller model structures, representing the most likely radical sites (14, 17). Table III lists the experimentally derived isotropic coupling constants for the caffeic acid oligomers for which an evaluation was possible. They are compared with those theoretically obtained values, which most closely correspond to the experiments and whose structures are shown in the lower part of the table.

An unexpected result of the DFT calculations was the fact that for the caffeic acid dianion radical, eight different mesomeric structures (depicted in the first

TABLE II

Correlation of coupling constants of sage phenolic acid and model radicals obtained from EPR experiments and DFT calculations

| Substance/structure[a] | size[b] | $a_2$ | $a_5$ | $a_6$ | $a_\alpha$ | $a_\beta$ | ref | comments |
|---|---|---|---|---|---|---|---|---|
| **caffeic acid radical (#1)** | | | | | | | | |
| Experimental results | 18 | **1.20** | **1.35** | **2.75** | **-** | **2.45** | 14 | HRP/$H_2O_2$, pH 9.5 |
| -"- | " | **1.20** | **1.35** | **2.75** | **0.15** | **2.45** | 14 | alkaline autoxidation, pH 9.5 |
| -"- | " | 0.15 | 1.35 | 2.75 | 1.20 | 2.45 | 27 | $a_{H2}/a_{H\alpha}$ wrongly assigned |
| -"- | " | 0.30 | 1.29 | 2.90 | - | 2.40 | 28 | -"– |
| -"- | " | 0.28 | 1.23 | 2.83 | 1.27 | 2.36 | 29 | cc not assigned |
| *Calculations* | | | | | | | | |
| Monoanion $O_4$ semiquinone | 19 | *2.15*$_5$ | *3.14*$_5$ | - | *1.71*$_5$ | *5.46* | | PCM, 6-311G*/** |
| Dianion $O_4$ semiquinone | 18 | ***1.29*** | ***1.29***$_5$ | ***2.79*** | ***0.78*** | ***2.05*** | | PCM, 6-311G*/** |
| " all 8 semiquinones | " | ***1.31***$_5$ | ***1.32*** | ***2.78***$_5$ | ***0.88*** | ***2.01***$_5$ | | PCM, 6-311G*/** |
| "$O_3$ semiquinone [c] | 18 | *0.18* | *0.78* | *3.14*$_5$ | *0.85* | *2.38* | | PCM, 6-311G*/** |
| **dihydrocaffeic acid radical (#1a)** | | | | | | | | |
| *Experimental results* | 20 | **-** | **0.95** | **3.60** | **3.60 (2)** | - | 14 | *HRP/$H_2O_2$ at pH 9.5* |
| **-"-** | " | - | **0.70** | **4.0** | **4.0 (2)** | - | 14 | stable $Zn^{2+}$ complex at pH 9.5 |
| $O_3$-semiquinone (?) | " | 0.22 | 0.88 | 3.73 | 3.56 (2) | - | 30 | UV photolysis, pH 7,11 |
| -"- | " | - | 0.62 | 4.02 | 4.02 (2) | - | 30 | $Zn^{2+}$ complex at pH 6.6 |
| $O_4$-semiquinone | " | 0.46 | 1.04 | 3.70 | 3.30(2) | - | 28 | alkaline autoxidation |
| -"- | " | 0.3 | 0.83 | 3.73 | 3.40(2) | - | 31 | $Tl^+$ complex in MTHF |
| *Calculations* | | | | | | | | |
| Monoanion ($O_4$ semiquinone) | 21 | *2.18*$_5$ | *3.58* | - | *17.24 (2)* | *0.11 (2)* | | PCM, 6-311G*/** |
| -"- ($O_3$ semiquinone)[c] | " | *3.11*$_5$ | *2.37* | *8.28* | *0.37 (2)* | *0.40 (2)* | | PCM, 6-311G*/** |
| Dianion ($O_4$ semiquinones (5)) | 20 | ***0.79*** | ***1.34***$_5$ | ***3.48*** | ***3.08 (2)*** | ***0.28 (2)*** | | PCM, 6-311G*/** |
| " ($O_3$ semiquinones (3))[c] | " | *0.82* | *1.66*$_5$ | *3.27* | *5.03*$_5$ *(2)* | *0.19 (2)* | | PCM, 6-311G*/** |

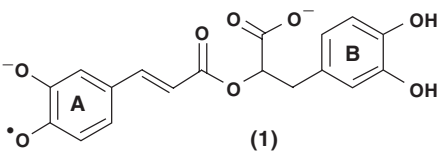
[a] compound structures see Scheme I, radical structures see Scheme II; bold typeface - own EPR experiments, normal font - literature values, italics - DFT calculations, bold italics - best correlation of DFT data with experimental coupling constants;
[b] size denotes the number of atoms in the molecule;
[c] very unstable radical, not observable by EPR.

TABLE III

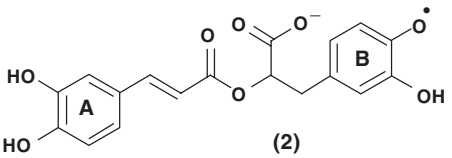
Isotropic coupling constants of semiquinones of caffeic acid oligomers from experiments and DFT calculations

| Substance[a] | size[b] | $a_{H2}$ | $a_{H5}$ | $a_{H6}$ | $a_{H\alpha}$ | $a_{H\beta}$ | $A_{H\gamma}$ | comment |
|---|---|---|---|---|---|---|---|---|
| Rosmarinic acid | | | | | | | | |
| Experimental | 39 | 2.58 | 1.26 | - | - | 3.85 | - | $HRP/H_2O_2$ |
| Calculated (1) | " | 2.50 | 1.27 | 2.14 | 0.67 | 2.08 | 0.26 | PCM 6-311G* |
| Salvianolic acid I | | | | | | | | |
| Experimental | 40 | 2.0 | 3.8 | 0.8 | 4.0 | - | - | $HRP/H_2O_2$ |
| Calculated (2) | " | 1.89 | 3.84 | 0.35 | 4.06 (2) | 0.41 | - | PCM 6-311G* |
| Salvianolic acid K | | | | | | | | |
| Experimental | 40 | 3.9 | 1.8 | 8.1 | - | 1.8 | - | $HRP/H_2O_2$ |
| Calculated (3) | " | 4.0 | 1.58 | 9.57 | 0.69 | 0.98 | 3.27 | PCM 6-311G* |

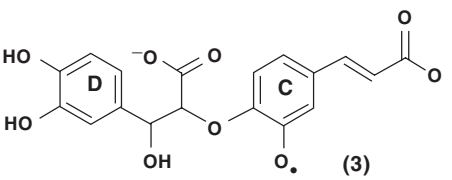
[a] compound structures see Scheme I
[b] size denotes the number of atoms in the molecule.




(1)




(2)

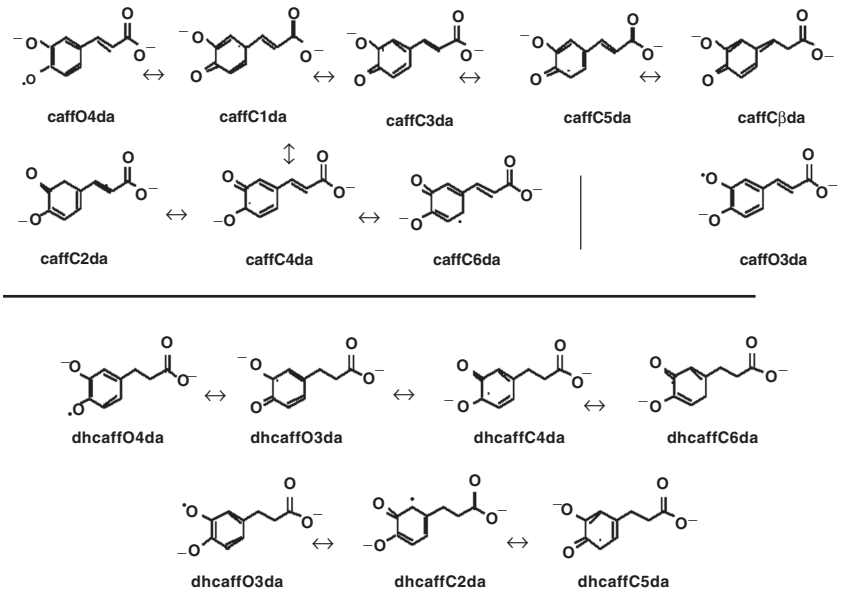



(3)

two lines in Scheme II) have basically the same coupling constants as the $O_4$-semiquinone itself and only the $O_3$-semiquinone deviates. In the case of the dihydrocaffeic acid dianion radical, as shown in the lower two lines of the scheme, four mesomeric structures for the $O_4$-semiquinone stand opposed to three for the $O_3$-semiquinone.

## DISCUSSION

Reactivity with •OH radicals generated during pulse-radiolytic experiments demonstrated the highly effective radical-scavenging potential of the caffeic acid oligomers, with rate constants at diffusion-controlled limits. The complex and diverse kinetic behavior, as revealed by biphasic




**Scheme II.** Relevant mesomeric and isomeric structures of caffeic acid and dihydrocaffeic acid radicals used for DFT calculations.

build-up and decay of individual transient absorptions, could optimally be modeled by the kinetic scheme presented above. From the rate constants listed in Table II, it is apparent that the ratio of reactions [1] and [2] governs both the initial build-up as well as the mono- or biphasic decay. While in general build-up kinetics reflect pseudo-first order attack of the primary radicals, decay occurs by second order disproportionation of the semiquinones to the same parent compound and respective quinone. Monophasic behavior occurs whenever one reaction dominates strongly over the others. Potential dimerization (24, 32, 33) instead of disproportionation - with the focal point being the 'CaffCβda' mesomeric structure (top right in Scheme II) - would not be apparent in the kinetic model as both are second-order processes. As shown in Table III, the DFT calculations support the distinction between the different stabilities of the mesomers, with a far superior electron delocalization (hence resonance stabilization of the radical) of the respective $O_4$-semiquinones.

With regard to the EPR spectra, we mainly observed the same 4-PhO• species at least part-time, since we obtained the best agreement of experimental, literature values and theoretical coupling constants for caffeic acid (27, 28) and dihydrocaffeic acid $O_4$-semiquinone dianions (28, 30, 31), including all possible mesomeric structures. In view of the unreflected depiction of both the $O_4$- (28, 31) and $O_3$-semiquinones (30) as probable radical structures, as well as the obviously wrongly assigned $a_{H2}/a_{H\alpha}$ for the caffeic acid radical (26, 28), the theoretical studies could thus be used as final arbiters of questionable radical structures.

Following the same approach as Cadenas and coworkers (22), the spin-stabilization studies using an excess of zinc acetate (25, 26, 30) in moderately alkaline solutions (pH 9.6-9.8) clearly reveal the presence of more than one radical species, whereas in the absence of $Zn^{2+}$ only one radical is seen. The results thus support our assumption of the simultaneous formation of both $O_3$- and $O_4$-semiquinones in the kinetic modeling calculations, where the $O_3$-semiquinone is too unstable to be seen in the absence of the bivalent ion, but becomes stabilized in its presence.

Consistent with earlier observations (16, 17), we always achieved better correlations between experimental and theoretical calculations for the coupling constants of the aromatic as compared to the side-chain

hydrogen atoms. In fact, comparing the theoretical coupling constants of the neutral and dissociated catechol radicals (the carboxyl group is always considered dissociated), the participation of the excess electron from the phenolate group obviously enhances the overall resonance stabilization (data not shown). Since application of this theoretical approach for more complex structures is still in its infancy, the only comparable studies were carried out for the calculations of the spin densities of three undissociated semiquinone structures of caffeic acid (34) and calculations of bond-dissociation energies of phenolic protons (35, 36).

## CONCLUSIONS

Using pulse radiolysis, EPR spectroscopy and the ancillary theoretical approaches of kinetic modeling and hybrid density-functional theory calculations, over the years we have studied the antioxidant mechanisms of various natural polyphenolic compounds. Based upon these observations and confirming evidence from the literature, we can distinguish between three different types of mechanisms:

- flavonoids proper, especially 3-hydroxy flavonols, have the preferred site of attack in the catechol structure of the B-ring, with the initially formed semiquinone stabilized by the 2,3-double bond (37, 38); the quinones and quinone methides, formed after disproportionation of the semiquinones, are potential pro-oxidants either due to futile redox cycling or nucleophilic attack at macromolecules (39, 40);
- proanthocyanidins (condensed tannins) as well as gallo- and ellagitannins (hydrolyzable tannins) likewise form semiquinones after initial radical attack and quinones/quinone methides by subsequent disproportionation; however, in this case, the quinones may combine with the parent catechol structures by phenolic coupling, thereby doubling the size of the molecule; this oligomerization in effect causes an enhancement of the antioxidant potential of these compounds (13, 41);
- multiple catechol structures in the caffeic acid oligomers are again the major target sites during their antioxidant function; in this case, however, complex kinetics due to parallel attack at various sites (simultaneous formation of both a $O_3$- and $O_4$-semiquinone, attack at aliphatic vinyl side chains) obscure the overall picture (14). While the eventually formed quinones are stable within the pulse-radiolytic time frame, evidence from the literature suggests that slow dimerization reactions occur via various mesomeric semiquinone structures (24, 32, 33).

An interesting aspect of these mechanisms is the close similarity with potential biosynthetic pathways. Both types of tannins form oligomers in chemical syntheses with structures resembling natural compounds, yet the underlying enzymatic reactions are still unknown (42-44). With regard to the dimerisation of caffeic acid (24, 32, 33), similar radical-induced reactions seem to take place during the biosynthesis of lignins (45-47).

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