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REGULAR ARTICLE

On the free radical scavenging mechanism of protocatechuic acid, regeneration of the catechol group in aqueous solution

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Abstract The free radical scavenging activity of protocatechuic acid has been studied in aqueous and lipid solutions, using the density functional theory. It was found to be a moderately good protector in non-polar environments (lipid), while in aqueous solution it is predicted to be an excellent peroxyl radical scavenger. In such media, the pH has an important role in the free radical scavenging activity of protocatechuic acid. At physiological pH, after the first peroxyl radical is scavenged, and in the presence of a good electron-donor species, such as the superoxide radical anion, the latter is consumed and protocatechuic acid is regenerated. This means that, under such conditions, it has the ability of scavenging several radical equivalents, two per cycle. An equivalent cyclic process can be assumed as possible also for other scavengers with the catechol moiety. If this assumption is confirmed, the role of compounds with a catechol moiety as free radical scavengers might be even more important that what has been assumed so far.

Keywords Kinetics · Hydrolysis · Water-assisted · Acid–base equilibria · Peroxy radicals

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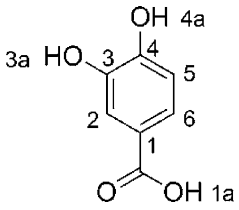
1 Introduction

Protocatechuic acid (3,4-dihydroxybenzoic acid, H_3Prc, Scheme 1) is a natural catechol-type phenol found in a wide variety of fruits and vegetables such as olive oil, white grape wine, raspberries, chinese oranges, mulberries, mango, guava, and onions [1–7]. It has also been identified as one of the main metabolites of more complex phenols such as anthocyanins [8], which are usually at high concentrations in human diet. It has been reported that protocatechuic acid has several beneficial health effects including antibacterial [9], antimutagenic [10], anticoagulatory [6], anti-inflammatory [6], and antihyperglycemic [11] activities. It has also been identified as an efficient antioxidant [6, 12–16]. This is a particularly appealing property since it makes protocatechuic acid a good protector against oxidative stress (OS), which constitutes a major health problem currently associated with the development of numerous diseases such as cancer [17–20], cardiovascular disorders [21–24], atherosclerosis [25–28], fetal growth restriction and preeclampsia [29–31], and several degenerative neurological disorders including Parkinson's and Alzheimer's diseases [32–34]. This chemical stress is triggered by an excess of reactive oxygen species (ROS) and often involves reactions between free radicals and molecules of high biological importance such as lipids, proteins, and DNA. Thus, dietary products that behave as good radical scavengers can help counteracting the detrimental and cumulative effects of OS in humans. Moreover, the study of their mechanisms of action as well as their scavenging ability is of vital importance for understanding their protective activity and for designing efficient strategies against OS.

In the particular case of protocatechuic acid, despite the available evidence proving its protective effects against

Scheme 1 Structure of protocatechuic acid, and site numbering



OS, there is still a large lack of information about the chemical processes involved in such action. There is no quantitative kinetic data on the reactions of this compound with ROS. There is little information on its reactivity depending on the polarity of the environment and related to the influence of pH in aqueous solution [35]. Regarding the mechanism, or mechanisms, involved in its radical scavenging activity, there are five previous studies for the reaction of H_3Prc with DPPH. Kawabata et al. [36] proposed that, in acetone solution, it is rapidly oxidized to the corresponding quinone, via two protons and two electrons transfer. The reactivity of this quinone upon further oxidation was found to be slow. In a following paper, Saito et al. [37] found that H_3Prc is rapidly converted to protocatechuquinone 3-methyl hemicacetal and protocatechuquinone during the reaction with DPPH, when it takes place in methanol solution. Later, Saito and Kawabata [38] proposed that the high radical scavenging activity of catechols in alcohol solutions is due to the nucleophilic addition of an alcohol molecule to the quinone, which would regenerate the catechol structure. They also proposed that such regenerations would be highly influenced by the electro-withdrawing character of the substituent in the phenolic moiety. In the same work, the necessity of further studies regarding the reactions of protocatechuic acid with peroxyl radicals in aqueous solution was pointed out. Saito and Kawabata [39] also found that the DPPH scavenging activity of H_3Prc is lower than that of its esters in alcoholic solvents. They justified this finding based on the dissociation of the carboxylic function, which decreases the electron-withdrawing character of the substituent, and therefore the reactivity of the quinone toward the nucleophilic attack of the solvent molecules. Additionally, Saito et al. [40] found that when reacting with DPPH in non-alcoholic solvents (acetone or acetonitrile), H_3Prc and its esters scavenge 2 equivalents of radical, while in alcoholic solvents protocatechuates scavenge more than 4 equivalents of radical. They also pointed out that further studies aiming to elucidate the oxidation mechanisms of these compounds in biological aqueous systems are needed.

Accordingly, it can be stated that the mechanism of the DPPH reaction with H_3Prc in alcoholic solvents has been exhaustively studied. However, there is no information in aqueous solution or in non-polar solvents resembling the lipid environment. There are no previous reports on the amount of radical equivalents that can be scavenged by H_3Prc under such conditions. Moreover, there are no previous investigations on the reaction mechanisms involved in the peroxyl radical scavenging activity of H_3Prc. The details on how fast the first oxidation step takes place to yield the quinone constitute another missing piece of information. Therefore, providing new physiochemical insights on these aspects is the main goal of the present work.

2 Computational details

Geometry optimizations and frequency calculations have been carried out using the M05-2X functional [41] and the 6-311+G(d,p) basis set, in conjunction with the SMD continuum model [42] using pentyl ethanoate and water as solvents to mimic lipid and aqueous environments, respectively. The M05-2X functional has been recommended for kinetic calculations by their developers [41], and it has been also successfully used by independent authors for that purpose [43–46]. It is also among the best performing functionals for calculating reaction energies involving free radicals [47]. SMD is considered a universal solvation model, due to its applicability to any charged or uncharged solute in any solvent or liquid medium for which a few key descriptors are known [42].

Unrestricted calculations were used for open-shell systems and local minima and transition states were identified by the number of imaginary frequencies (NIMAG = 0 or 1, respectively). In the case of the transition states, it was verified that the imaginary frequency corresponds to the expected motion along the reaction coordinate, by intrinsic coordinate calculations (IRC). All the electronic calculations were performed with the Gaussian 09 package of programs [48]. Thermodynamic corrections at 298.15 K were included in the calculation of relative energies, which correspond to 1 M standard state. For those reactions that directly involve water molecules, the relative energies have also been corrected for the standard state of liquid water, that is, 55.55 M. In addition, the solvent cage effects have been included according to the corrections proposed by Okuno [49], taking into account the free volume theory [50].

The rate constants (k) were calculated using the conventional transition state theory (TST) [51–53]. Reaction path degeneracies and tunneling corrections have been taken into account. The tunneling corrections, defined as the Boltzmann average of the ratio of the quantum and the classical probabilities, were calculated using the zero curvature tunneling corrections (ZCT) [54]. For the electron transfer reactions the barriers were estimated using the Marcus theory [55–57]. In addition, some of the calculated

rate constants (k) are close to the diffusion limit; thus, the apparent rate constant (k_{app}) cannot be directly obtained from TST calculations. In the present work, the Collins–Kimball theory is used for that purpose [58]:

$$k_{app} = \frac{k_D k_{act}}{k_D + k_{act}} \quad (1)$$

where k_{act} is the thermal rate constant, obtained from TST calculations, and k_D is the steady state Smoluchowski [59] rate constant for an irreversible bimolecular diffusion-controlled reaction:

$$k_D = 4\pi R D_{AB} N_A \quad (2)$$

where R denotes the reaction distance, N_A is the Avogadro number, and D_{AB} is the mutual diffusion coefficient of the reactants A (free radical) and B (scavenger). D_{AB} has been calculated from D_A and D_B according to Ref. [60], D_A and D_B have been estimated from the Stokes–Einstein approach [61, 62]:

$$D = \frac{k_B T}{6\pi\eta a} \quad (3)$$

where k_B is the Boltzmann constant, T is the temperature, η denotes the viscosity of the solvent, in our case water ($\eta = 8.91 \times 10^{-4}$ Pa s) and pentyl ethanoate ($\eta = 8.62 \times 10^{-4}$ Pa s); and a is the radius of the solute.

Since spin contamination is an issue that can affect the accuracy of energies and structures of open-shell systems, the spin-squared values for all the open-shell species, before and after annihilation of the first spin contaminant have been checked; as well as their percent errors with respect to the expected value. In all cases, the deviations from the ideal value ($\langle S^2 \rangle = 0.75$) were lower than 4 and 0.12 % before and after annihilation of the first spin contaminant. It has been established that for differences within 10 % error the obtained results can be trusted [63, 64]. Therefore, the spin contamination is negligible for all the radicals species studied in this work and their energy values are reliable.

3 Results and discussion

The experimental values of the pKas of H_3Prc are 4.38, 8.74, and 10.67 [65], with the first deprotonation involving the carboxylic group. To assess which phenolic OH is involved in the second deprotonation both processes were investigated. It was found that the Gibbs free energy of the deprotonation from site 4a is 1.22 kcal/mol lower than that of the deprotonation from site 3a. Therefore, the deprotonation sequence is that shown in Scheme 2, in agreement with that proposed by Hatzipanayioti et al. [35]. The molar fractions of the different species were estimated from the above-mentioned pKa values, and it was found that at physiological pH (7.4) the dominant form of protocatechuic acid is the carboxylate mono-anion (H_2Prc^-), with a population equal to 95.5 %. For the di-anion ($HPrc^{2-}$) and the neutral species (H_3Prc) the populations are 4.4 and 0.1 %, respectively; while the population of the fully deprotonated species (Prc^{3-}) is negligible (Fig. 1). Accordingly, H_3Prc, H_2Prc^-, and $HPrc^{2-}$ are included in the present study for the reactions in aqueous solution. H_3Prc and $HPrc^{2-}$ are taken into account despite their lower population under physiological conditions to investigate the influence of the pH on the radical scavenging activity of protocatechuic acid. On the other hand, in non-polar (lipid) media only the neutral form is used, since such media do not promote the necessary solvation to stabilize the ionic species.

3.1 Fist stage

The first step involved in the free radical scavenging activity of protocatechuic acid can take place though a variety of reaction mechanisms, as it is the case for many other scavengers [66–73]. Those considered in this work are radical adduct formation (RAF), hydrogen transfer (HT), and single electron transfer (SET) in aqueous solution, while in non-polar solution only RAF and HT have been considered. They have been modeled for the reactions of the different species of protocatechuic acid with the hydroperoxyl radical ($HOO^{\cdot}$):

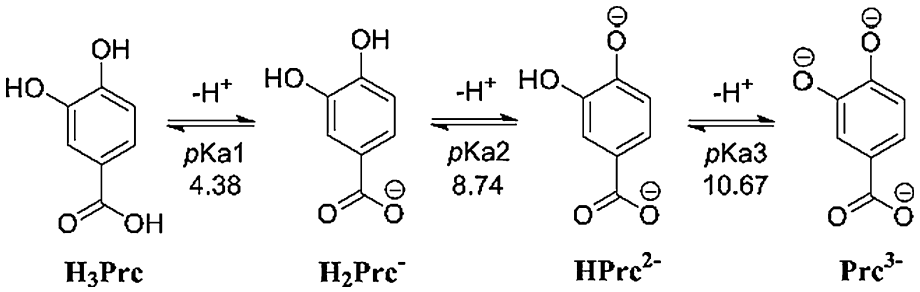
$$\text{RAF}: \ H_nPrc^q + HOO^{\cdot} \rightarrow [H_nPrc - OOH]^{q\cdot}$$

$$\text{HT}: \ H_nPrc^q + HOO^{\cdot} \rightarrow H_{(n-1)}Prc^{q\cdot} + HOOH$$

$$\text{SET}: \ H_nPrc^q + HOO^{\cdot} \rightarrow H_nPrc^{(q+1)\cdot} + HOO^-$$

This radical has been chosen for this study because it is the simplest of the peroxyl radicals ($ROO^{\cdot}$), which are among the free radicals of biological relevance that can be effectively scavenged to retard OS [74]. This is because they have not too short half-lives, which is required for efficient interception by phenolic compounds [75]. Therefore, $ROO^{\cdot}$ have been proposed as major reaction partners for the protective action of this kind of compounds [75]. They are formed within living organisms, where they are involved in DNA cleavage and protein backbone modification [76]. $ROO^{\cdot}$ are also involved in the oxidation of lipoproteins and biological membranes and had been held responsible for microvascular damage [77]. It has even been proposed that the main antioxidant function of phenolic compounds is to trap $ROO^{\cdot}$ [78, 79]. In the particular case of $HOO^{\cdot}$, it has been suggested to be central to the toxic side effects of aerobic respiration [80]. It has also been pointed out that more information on the reactivity of this species is needed [80]. In addition,

Scheme 2 Deprotonation sequence and *p*Ka [65] values



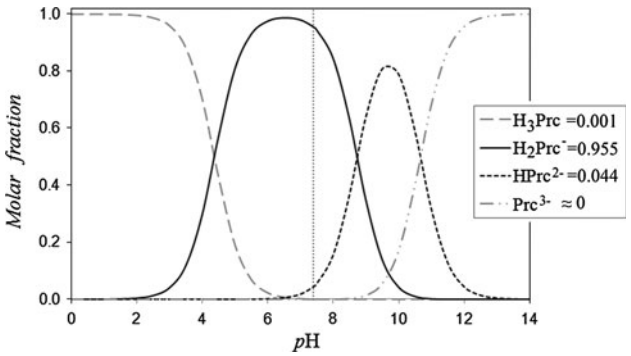


Fig. 1 Distribution diagram and molar fractions of neutral (H_3Prc), mono-anionic (H_2Prc^-), di-anionic ($HPrc^{2-}$), and tri-anionic (Prc^{3-}) forms of protocatechuic acid at physiological pH

radicals of intermediate to low reactivity have been recommended for studying the relative scavenging activity of different compounds [81, 82]. This is because when reacting with highly reactive radicals, such as $^{\cdot}OH$, there is a large variety of compounds that would react at similar rates (close to the diffusion limit). Thus, comparisons based on such reactions might lead to miss-conclude that all the analyzed compounds are equally efficient as antioxidants even when that might not be the case for a wider spectrum of free radicals. Moreover, it has been proposed that such highly reactive radicals cannot be intercepted in biological systems with reasonable efficiency [80].

In non-polar environments, the SET mechanism is not expected to contribute to the overall reactivity of H_3Prc toward free radicals since such environments do not promote the necessary solvation of the intermediate ionic species yielded by this mechanism. However, just to prove this point, the reaction Gibbs energy (ΔG^0) for the SET process was calculated and found to be equal to 84.4 kcal/mol (Table 1). This value definitively rules out the viability of SET in lipid media. In aqueous solution, SET from H_3Prc and H_2Prc^- to $HOO^{\cdot}$ remains significantly endergonic, while SET involving $HPrc^{2-}$ is only slightly endergonic ($\Delta G^0 = 3.26$ kcal/mol). This process actually corresponds to a sequential proton electron transfer mechanism (SPET) involving the dominant species at physiological pH (H_2Prc^-). It should be noted that the products yield via SPET are identical to those yield by HT from the phenolic OH in H_2Prc^-. In addition, since the population of $HPrc^{2-}$ is low but not negligible under physiological conditions (4.4 %), its contributions to the overall reactivity of protocatechuic acid toward $HOO^{\cdot}$ could be significant, provided that it reacts fast enough.

The Gibbs free energies (ΔG) for the different reactions are provided in Table 1 and the enthalpies of reaction (ΔH) in Table 1S (Electronic Supplementary Material, EMS). All the reaction paths corresponding to RAF processes were found to be endergonic, with ΔG values ranging from 7.8 to 20.4 kcal/mol (Table 1). Thus, the RAF mechanism has been ruled out as viable, regardless of the solvent polarity and also of the reacting species in aqueous solution (H_3Prc, H_2Prc^-, or $HPrc^{2-}$). With respect to the HT mechanism, the path involving the carboxylic group in H_3Prc (path 1a, i.e., the reaction path involving site 1a) was found to be endergonic by 27.7 and 13.2 kcal/mol in aqueous and pentyl ethanoate solutions, respectively. On the other hand, the HT reactions from the phenolic OH groups were found to be exergonic in all the studied cases. The thermochemical viability of such processes was found to increase when going from non-polar to polar environments. In addition, in aqueous solution the exergonicity increase as the deprotonation degree of the protocatechuic species acting as H donor increases. For H_2Prc^-, which is the prevailing form under physiological conditions, the ΔG^0 values were found to be around -5 kcal/mol for both phenolic sites; while for H_3Prc they are -2.4 and -1.7 kcal/mol for sites 3a and 4a, respectively. Based on the computed thermochemical data, it can be established that in non-polar (lipid) environment the $HOO^{\cdot}$ scavenging activity of protocatechuic acid takes place exclusively by HT from the phenolic OH groups. In aqueous solution, HT is also expected to take place, but SET from $HPrc^{2-}$ need to be further analyzed, based on kinetic considerations. Other authors have proposed that in aqueous solution the radical forms of protocatechuic acid can be involved in further reactions with different species [83]. However, this point escapes the purposes of the present work.

For the kinetic study, we have not included the channels of reaction described above as endergonic because, even if they take place at a significant rate, they would be

Table 1 Gibbs free energies of reaction (ΔG^0, kcal/mol), at 298.15 K

	H_3Prc (I)	H_3Prc (II)	H_2Prc^- (II)	$HPrc^{2-}$ (II)
SET	84.40	36.88	29.68	3.26
HT				
Site 1a	27.72	13.17		
Site 3a	−1.86	−2.36	−5.16	−12.97
Site 4a	−1.57	−1.66	−5.12	
RAF				
Site 1	20.37	19.02	14.09	13.23
Site 2	14.35	11.75	12.09	15.55
Site 3	13.91	12.69	11.02	7.80
Site 4	12.97	9.93	9.44	13.47
Site 5	17.70	16.87	15.21	11.37
Site 6	16.46	13.53	10.39	12.88

(*I*) pentyl ethanoate solution, (*II*) aqueous solution

reversible and therefore the formed products will not be observed. However, they might still represent significant channels if their products rapidly react further. This would be particularly important if these later stages are sufficiently exergonic to provide a driving force, and if their barriers of reactions are low. That can be the case for the SET mechanism involving $HPrc^{2-}$, since the formed radical ($HPrc^{\cdot 2-}$) can easily donate one electron yielding the quinone carboxylate. Therefore, SET from $HPrc^{2-}$ has been included in the kinetic calculation. The subsequent electron transfer reaction will be analyzed later when the following stages in the radical scavenging activity of protocatechuic acid are considered.

The fully optimized geometries of the transition states (TS) are shown in Figs. 1S and 2S (Electronic Supplementary Material, ESM). No intramolecular interactions between the reacting fragments were found. These figures show that the TSs become earlier in aqueous solution than in non-polar media for both reaction sites (3a and 4a). This suggests that the reactivity of protocatechuic acid in aqueous solution should be increased, with respect to non-polar solutions. In addition, the TSs involving H_2Prc^- are earlier than those involving H_3Prc, which indicates that the reactivity of protocatechuic acid may be influenced by the pH. In the particular case of HT from site 3a in $HPrc^{2-}$, there is no transition state. This is because the *p*Ka value of $HOO^{\cdot}$ (4.69) [84], which is significantly lower than the *p*Ka of H_2Prc^- (8.74) [65]. Thus, $HOO^{\cdot}$ transfer a proton to $HPrc^{2-}$ (yielding H_2Prc^- and $O_2^{\cdot -}$) as they approach each other.

The Gibbs free energies of activation ($\Delta G^{\neq}$) are reported in Table 2. The lowest value was found for the SET process involving $HPrc^{2-}$. However, as expected based on their large endergonicities, the SET reactions involving the other forms of protocatechuic acid (H_3Prc and H_2Prc^-) have very high barriers. Regarding the HT mechanism, in non-polar media (I) the barrier of path 3a was found to be 1.23 kcal/mol lower than that of path 4a. A similar trend was found for these paths in aqueous solution when $HOO^{\cdot}$ is reacting with H_3Prc, with a difference in $\Delta G^{\neq} = 1.63$ kcal/mol. On the other hand, when the reacting species is H_2Prc^- the barriers of paths 3a and 4a become very similar (0.18 kcal/mol different), which indicates that the selectivity of H_2Prc^- is lower than that of H_3Prc. In addition, the barriers of both HT paths are lower for H_2Prc^- than for H_3Prc, suggesting that the reactivity of the mono-anion toward oxygenated free radicals is higher than that of the neutral protocatechuic acid.

The rate constants for the different channels of reaction (k_{app}), as well as the total values for each reacting species of protocatechuic acid (k_{tot}), and the overall rate coefficients ($k_{overall}$) in pentyl ethanoate (I) and aqueous solution (II) are reported in Table 3. The k_{app} values have been calculated as described in the Sect. 2, and the values of k_{tot} and $k_{overall}$ as follows:

$$k_{overall}^{(I)} = k_{tot}^{(I)} = k_{app}^{(I)}(3a) + k_{app}^{(I)}(4a) \quad (4)$$

$$k_{overall}^{(II)} = p^N k_{tot}^N + p^A k_{tot}^A + p^{A2} k_{tot}^{A2} \quad (5)$$

where p^N, p^A, and p^{2A} account for the molar fractions in aqueous solution of H_3Prc, H_2Prc^-, and $HPrc^{2-}$, respectively, at pH = 7.4, and were obtained from the experimental *p*Ka values. The total contributions of each form (k_{tot}^N, k_{tot}^A, and k_{tot}^{2A}) have been estimated by summing up the rate constants of the different reaction paths as:

$$k_{tot}^N = k_{app}^N(\text{SET}) + k_{app}^N(\text{HT}, 3a) + k_{app}^N(\text{HT}, 4a) \quad (6)$$

$$k_{tot}^A = k_{app}^A(\text{SET}) + k_{app}^A(\text{HT}, 3a) + k_{app}^A(\text{HT}, 4a) \quad (7)$$

$$k_{tot}^{2A} = k_{app}^{2A}(\text{SET}). \quad (8)$$

It seems worthwhile to insist on the fact that the product of the $HPrc^{2-}$ reaction via SET is identical to that yield by HT from site 4a in H_2Prc^-. In addition, this product and its tautomer (the radical yield by HT from site 3a in H_2Prc^-) are almost isoergonic with a difference in Gibbs free energy equal to 0.04 kcal/mol. Consequently, both are expected to coexist via tautomeric equilibrium.

In penthyl ethanoate solution (I), HT from site 3a was found to be 5.34 times faster than that corresponding to HT from site 4a. Therefore, it is predicted to be the major channel of reaction in the lipid environment. Under such conditions, path 3a was found to represent 84.2 % of the overall reactivity of H_3Prc toward $HOO^{\cdot}$ (Table 2S, ESM). The overall rate constant in lipid solution was found to be equal to 5.14×10^3 $M^{-1}s^{-1}$. According to this value and taking into account that the rate constants corresponding to the $HOO^{\cdot}$ damage to polyunsaturated fatty acids are in the range 1.18–3.05 $\times$ 10^3 M^{-1} s^{-1} [75], it can be stated that

Table 2 Gibbs free energies of activation ($\Delta G^{\neq}$, kcal/mol), at 298.15 K

	H_3Prc (I)	H_3Prc (II)	H_2Prc^- (II)	$HPrc^{2-}$ (II)
SET		42.24	31.60	5.89
HT				
Site 3a	14.99	15.65	14.63	[a]
Site 4a	16.22	17.28	14.45	

(*I*) pentyl ethanoate solution, (*II*) aqueous solution

[a] See the discussion in the text on the TS search

Table 3 Rate constants for every reaction path and overall rate coefficient (at pH = 7.4, in $M^{-1}s^{-1}$), at 298.15 K

	H_3Prc (I)	H_3Prc (II)	H_2Prc^- (II)	$HPrc^{2-}$ (II)
SET		6.19E−22	4.05E−11	1.26E+07
HT				
Site 3a	4.33E+03	4.88E+00	1.32E+04	
Site 4a	8.10E+02	7.80E−01	2.34E+04	
Total	5.14E+03	5.66E+00	3.66E+04	1.26E+07
Overall	5.14E+03		1.26E+07	

Including the molar fraction of the different forms of protocatechuic acid at pH = 7.4

(*I*) pentyl ethanoate solution, (*II*) aqueous solution

protocatechuic acid can act as a moderate $HOO^{\cdot}$ scavenger in such medium. In addition, its peroxyl scavenging activity, in non-polar environments, was found to be lower than that of carotenes ($\sim 10^5$–10^6 M^{-1} s^{-1}) [85, 86], dopamine (8.2×10^5 M^{-1} s^{-1}) [87], canolol (6.8×10^5 M^{-1} s^{-1}) [88], hydroxytyrosol (6.4×10^5 M^{-1} s^{-1}) [89], sesamol (3.3×10^4 M^{-1} s^{-1}) [90], and sinapinic acid (1.7×10^4 M^{-1} s^{-1}) [91]; similar to that of α-mangostin (7.8×10^3 M^{-1} s^{-1}) [92]; and higher than that of tyrosol (7.1×10^2 M^{-1} s^{-1}) [89], melatonin (3.1×10^2 M^{-1} s^{-1}) [93], and caffeine (3.2×10^1 M^{-1} s^{-1}) [73].

In aqueous solution, it was found that the SET from $HPrc^{2-}$ is by far the fastest of the studied reaction paths, considering all the acid–base forms of protocatechuic acid (Table 3). Despite the fact that its molar fraction is relatively low at pH = 7.4, and it has been included in the calculations, the contribution of this reaction path to the overall reactivity of protocatechuic acid toward $HOO^{\cdot}$ is >99 % (Table 2S). It should be noted that the SET from $HPrc^{2-}$ corresponds to a SPET process from H_2Prc^-, which is the dominant form of protocatechuic acid under physiological conditions. Analyzing the k_{tot} values of the different forms of this compound it becomes evident that as the deprotonation degree increases so does the efficiency of protocatechuic acid for scavenging $HOO^{\cdot}$ and probably other alkyl or alkenyl peroxy radicals. Accordingly, the peroxyl radical scavenging activity of protocatechuic acid is predicted to be highly dependent on the pH of the environment, decreasing as it becomes more acid. This is mainly due to the importance of the SET path from $HPrc^{2-}$. However, the HT rate constants are also significantly influenced by the form of protocatechuic acid involved in the process. They are considerably increased when the carboxyl group is deprotonated, that is, when the reacting species is H_2Prc^-.

At physiological pH, in aqueous solution, the overall rate constants was found to be 1.26×10^7 $M^{-1}s^{-1}$. This means that under such conditions the protective effects of protocatechuic acid against OS are expected to be much higher than that of melatonin (2.0×10^1 M^{-1} s^{-1}) [93], caffeine (3.3×10^{-1} M^{-1} s^{-1}) [73], allicin (7.4×10^3 M^{-1} s^{-1}) [94], and thioacrolein (2.9×10^4 M^{-1} s^{-1}) [94]; higher than that of α-mangostin (1.4×10^6 M^{-1} s^{-1}) [92] and dopamine (2.2×10^5 M^{-1} s^{-1}) [87]; similar to that of 2-propenesulfenic acid (2.6×10^7 M^{-1} s^{-1}) [94] and glutathione (2.7×10^7 M^{-1} s^{-1}) [95]; and lower than that of sesamol (2.4×10^8 M^{-1} s^{-1}) [90]. Based on these results, it can be stated that protocatechuic acid is among the best peroxyl radical scavengers in aqueous solution, at physiological pH, and this is considering only its efficiency for scavenging the first radical. However, it seems to be possible that protocatechuic acid scavenges more than that.

It has been established by Saito et al. [37–40] that protocatechuic acid and its esters scavenge two radicals while converted into the corresponding quinone in a wide variety of solvents. They also found that in alcoholic solutions protocatechuates scavenge more than 4 equivalents of radical and explained this finding by the regeneration of the catechol moiety via nucleophilic addition of a solvent alcohol molecule. These authors have also pointed out the necessity of investigating if such behavior is also possible in aqueous solution.

3.2 Second stage

For that purpose, we have first considered the possible fate of the radical formed after the reaction of protocatechuic acid with $HOO^{\cdot}$ in aqueous solution (**I1**, Scheme 3). Two different routes have been modeled arising from electron and proton transfers. The first route (**A**) leads to the formation of the quinone (**Q**, Scheme 3) and consist of two steps. The first one (**2A**) is the deprotonation of **I1**, through acid–base equilibrium with the surrounding water molecules. The second one (**3A**) is the electron transfer to a radical in the environment. For this step, we have considered the same radical that for the initial step ($HOO^{\cdot}$). The second route (**B**) also comprises two steps. The first one (**2B**) is the electron transfer from an electron donor to **I1**. Since the anion superoxide ($O_2^{\cdot-}$) is the conjugate base of

$HOO^{\cdot}$, we have consider $O_2^{\cdot-}$ as the electron donor in this step. It is followed by the protonation of the intermediate **I2B** regenerating the dominant form of protocatechuic acid at physiological pH (H_2Prc^-). The Gibbs energies of reaction, the Gibbs energies of activation, and the rate constants for the different steps are reported in Table 3S (ESM). The site from which the initial HT took place has been explicitly specified in the table.

According to the values in Table 3S, step **2A** is endergonic by ~8.5 kcal/mol. However, it should be noticed that this is an acid–base equilibrium:

$$(\mathbf{2A})I1 \rightleftharpoons I2A + H^+$$

Therefore, it is governed by the pH of the environment. The values in Table 3S corresponds to the standard conditions (pH = 0), that is to the following equilibrium constant

$$K_{2A} = \frac{[I2A][H^+]}{[I1]} = e^{-\Delta G^0_{2A}/RT} \quad (9)$$

However, we are interested on this process when it takes place under physiological conditions, that is, with the pH buffered to a value of 7.4. Therefore, since under such conditions $[H^+]$ remains unchanged ($[H^+] = 10^{-pH} = 3.98 \times 10^{-8}$ M), a conditional equilibrium constant (K') can be defined [96–99], according to:

$$K'_{2A} = \frac{K_{2A}}{[H^+]} = \frac{e^{-\Delta G^0_{2A}/RT}}{10^{-pH}} = e^{-\Delta G'_{2A}/RT} \quad (10)$$

As a result, the conditional Gibbs energy of reaction at each particular buffered pH would be:

$$\Delta G'_{2A} = \Delta G^0_{2A} - 2.303RT(pH) \quad (11)$$

which means that as the pH increases so does the thermochemical viability of reaction **2A**. At pH ≥ 6.2, it becomes exergonic, with $\Delta G'_{2A} = -1.6$ kcal/mol at pH = 7.4. Thus, under physiological conditions reaction **2A** is feasible (Fig. 2). This deprotonation process takes place without barrier and is only controlled by the $[H^+]$ of the surroundings. For reaction **3A**, which is the rate controlling step, the barrier is low (5.25 kcal/mol) which means that the reaction is very fast, with a rate constant (k_{app}) equal to 6.53×10^8 $M^{-1}s^{-1}$. This reaction yields the quinone (**Q**, Scheme 3) and the anion HOO^-, and is slightly endergonic ($\Delta G^0 = 1.61$ kcal/mol). However, hydrogen peroxide (H_2O_2) has a *p*Ka in aqueous solution equal to 11.75 [100], which means that at pH values below 11.75 HOO^- would be spontaneously protonated. The ΔG^0 of this process, obtained from the experimental *p*Ka value, is −16.03 kcal/mol under standard conditions and −6.37 kcal/mol at pH = 7.4. This means that the whole variation in ΔG associated with step **3A** would be negative at this pH. Therefore, the formed quinone is stable enough and might be involved in subsequent reaction steps. Its hydrolysis would be analyzed in the following section, since it is the equivalent process to the nucleophilic addition of solvent molecules proposed for alcohols.

Regarding route **B**, the electron transfer from $O_2^{\cdot-}$ (reaction **2B**) was found to be significantly exergonic (Table 3S). The Gibbs energies of reaction are −17.36 and −18.63 kcal/mol when the species accepting the electron is the intermediate formed by HT from site 3a and 4a, respectively. Reaction **2B** was found to be basically barrier-less, with $\Delta G^{\neq} = 0.01$ kcal/mol in both cases. Consequently, it is a diffusion-controlled process, with $k_{app} = 2.6 \times 10^9$ M^{-1} s^{-1}. The following step (**3B**) was also found to be significantly exergonic with $\Delta G^0 = -20.11$ and −18.88 kcal/mol depending on the radical that initiates the route (3a or 3b). Since this process is a protonation:

$$(\mathbf{3B})I2B + H^+ \rightleftharpoons H_2Prc^-$$

it takes place without a barrier and controlled by the pH of the surroundings. Its conventional equilibrium constant is:

$$K_{3B} = \frac{[H_2Prc^-]}{[HPrc^{2-}][H^+]} = e^{-\Delta G^0_{3B}/RT} \quad (12)$$

And the conditional equilibrium constant:

$$K'_{3B} = K_{3B}[H^+] = e^{-\Delta G^0_{3B}/RT}\left(10^{-pH}\right) = e^{-\Delta G'_{3B}/RT} \quad (13)$$

This means that, based on the conditional Gibbs free energy:

$$\Delta G'_{3B} = \Delta G^0_{3B} + 2.303RT(pH) \quad (14)$$

increasing the pH has the opposite effect that what was described above for reaction **2A**, that is, as the pH increases the thermochemical viability of reaction **3B** decreases. The turning point was found at pH = 13.8; while $\Delta G'_{3B} = -8.79$ at pH = 7.4. At this pH, and in the presence of the pair $HOO^{\cdot}/O_2^{\cdot-}$, route **B** is predicted to take place very fast in a cascade way. Therefore, in the aqueous phase, under physiological conditions, the catechol moiety is predicted to be efficiently regenerated after scavenging two free radicals (one $HOO^{\cdot}$ and one $O_2^{\cdot-}$). Therefore, unless a reaction with other species in the surroundings consume H_2Prc^-, or some of the intermediates, this scavenger would be "recycled" as it traps two free radicals per cycle. This can be a plausible explanation supporting its ability for scavenging more than 4 radical equivalents in aqueous solution, at least at pH around 7.4. Moreover, since an equivalent cyclic process seems to be possible for other scavengers with the catechol moiety, further investigations on other compounds with such structural feature are

Scheme 3 Mechanisms for the peroxyl scavenging activity of protocatechuic acid, in aqueous solution at physiological pH

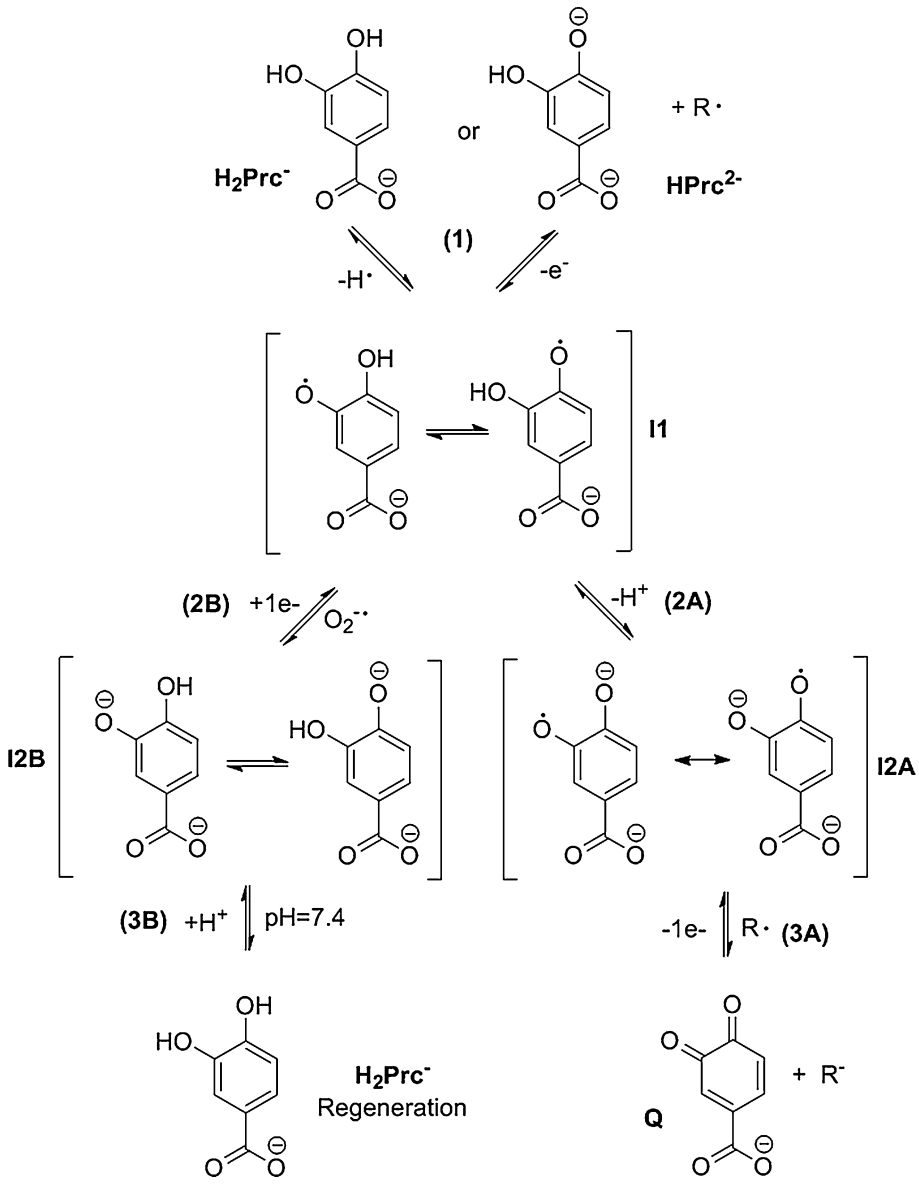
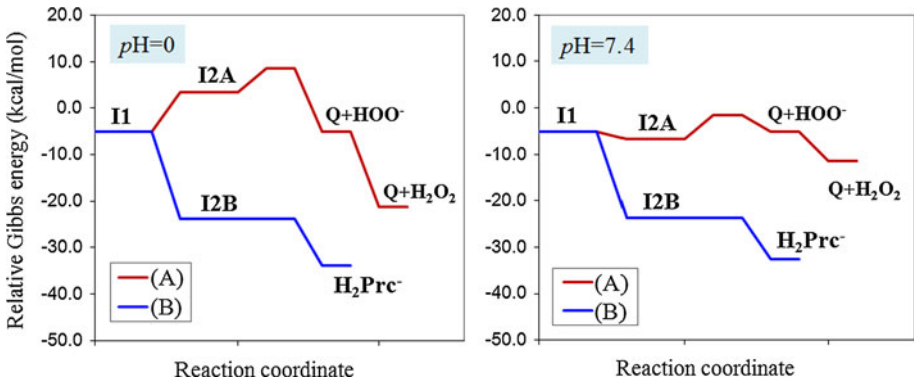


Fig. 2 Reaction profiles of routes *A* and *B*, at pH = 0 (standards conditions) and at pH = 7.4 (physiological pH)



recommended, in particular those aiming for estimating the number of radical equivalents that they can scavenge. It seems worthwhile to emphasize that if this route is confirmed the role of compounds with a catechol moiety as free radical scavengers might be even more important that what has been assumed so far.

3.3 Third stage

Based on the mechanism proposed by Saito et al. [37–40] for the nucleophilic addition of a solvent molecule to **Q**, in alcoholic solutions, we have first considered the exact equivalent reaction route for the hydrolysis of this compound (Scheme 4). It should be noted, however, that this is expected to be a very slow, or even non-noticeable, process at pH $\geq$ 4.38 because carboxylate is a very poor electron-withdrawing group, and according to Saito et al. [37–40], this dramatically decreases the susceptibility of the **Q** toward the nucleophilic attacks of solvent molecules. In fact, in alcoholic solutions these authors found that such process is slow but feasible for the esters while it was not observed for the acid. However, since there is no information about it in aqueous solution, we have tested its viability for this solvent.

Before start discussing the results, it seems worthwhile to clarify the meaning of the acronyms used to identify the different species. The first letter in the acronyms means I = intermediate or P = final product. The numbers in the acronyms refer to the sites involved in the reactions. The first one corresponds to the addition of OH^- from water, the second one to the addition of the H^+ from water, for those with a third number it corresponds to site at which the other proton, originally in the site at which the OH^- addition takes place, migrates. The names of the paths were chosen to facilitate rapid identification with respect to the initial sites of the water addition, though the letter *a* corresponding to the involved O atom has not been included in the paths' acronyms for the sake of simplicity.

To investigate the hydrolysis of the quinone we have tested different approaches: (1) including only the reacting water molecule, that is, without any other solvent molecule assisting the process, (2) with one water molecule assisting the first step, that is, the nucleophilic addition, (3) with one solvent molecule assisting all the reaction steps, and (4) with one solvent molecule assisting the water addition and two water molecules assisting the proton migration. This step (proton migration) is possible for paths Q-54 and Q-23, but not for paths Q-33 and Q-44. The results for path Q-54 are shown in Table 4S (ESM) and Fig. 3. It was found that including the assistance of other solvent molecules in the modeling is crucial. The first important change is that the number of individual steps decreases (Scheme 5) when the reactions are water-assisted. The second one, but equally important, is that the reaction barriers lower and the number of endergonic steps decreases (Fig. 3). The red and blue arrows in Scheme 5 represent endergonic and exergonic steps, respectively.

It is important to notice that the intermediates and products for each reaction step were identified by performing IRC calculations and optimizing the final points to minima, without any restrictions. Following this strategy, it was found that when the water addition is assisted by another solvent molecule the yielded intermediate is different (**I-5**, **3a**) and lower in energy by 19.8 kcal/mol than the originally proposed (**I-5**, **4a**) (Table 4S). This causes that this step, which is almost isoergonic when it yields **I-5**, **4a**, becomes significantly exergonic for the formation of **I-5**, **3a**. In addition, the Gibbs energy of activation decreases by 3.2 kcal/mol for the water-assisted addition. Accordingly, it is proposed that in aqueous solution the nucleophilic addition is actually assisted by other solvent molecules. For all the used approaches, (**i**)–(**iv**), the water addition was found to be the rate determining step (Table 4S) of the **Q** hydrolysis. However, the importance of the water assistance is also present in the proton migration (PM). Analyzing only the PM reactions, it was found that the slowest of them becomes faster as the number of assisting water molecules increases from 0 to 2. Thus, based on the behavior of the different used approaches we have chosen approach (**iv**) for the analysis of the four hydrolysis paths.

The structures of the TS are shown in Fig. 4. All of them present ring-like structures involving both the reacting and the assisting water molecules. The minimum energy paths (MEP) for both steps: water addition (WA) and PM for path **Q-54** are provided as ESM (Figs. 3S and 4S). They were obtained from extended IRC calculations using up to 60 points in the products direction with step size equal to 0.1 bohr. The MEP for the other hydrolysis paths are not shown because all of them have the same behavior.

It was found that WA is almost isoergonic for all the hydrolysis paths, with ΔG^0 values ranging from -1.93 to 0.72 kcal/mol, while the PM reactions are significantly exergonic (Table 4). The Gibbs energies of activation were found to be relatively low for the PM reactions, $\sim$9.6 kcal/mol. On the other hand, they were found to be significantly high for the WA reactions. For this step, the $\Delta G^{0\neq}$ values were found to be larger than 20 kcal/mol for all the hydrolysis paths, with the lowest values corresponding to paths Q-33 and Q-44, and the highest to Q-54.

The kinetic data (Table 4) show that for all the studied hydrolysis paths the rate determining step is the WA. They also show that this step is very slow in aqueous solution, regardless of the reaction path. Therefore, it can be concluded that the nucleophilic addition of a solvent molecule to **Q** is not viable in aqueous solution. This can be explained based on the chemical nature of the substituent in the catechol moiety (carboxylate), which has not enough electron-withdrawing character. This is in agreement with the findings of Saito et al. [37–40] for alcoholic solutions.

In addition, the fastest paths were found to be Q-33 and Q-44, with rate constants in the order of 10^{-5}–10^{-4} M^{-1} s^{-1}. These paths are just the ones that do not

Scheme 4 Hydrolysis route, equivalent to that proposed by Saito et al. [37–40] for the nucleophilic addition of alcohols to **Q**

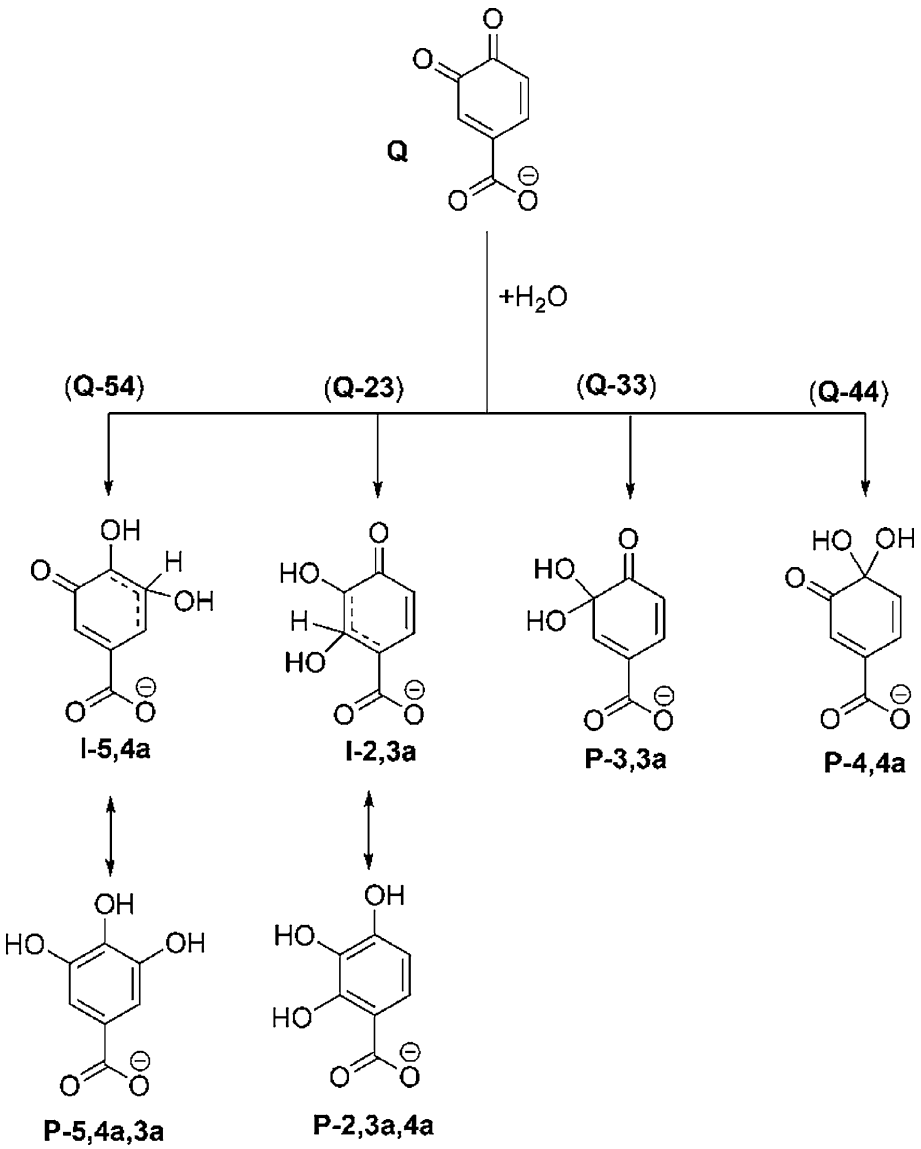
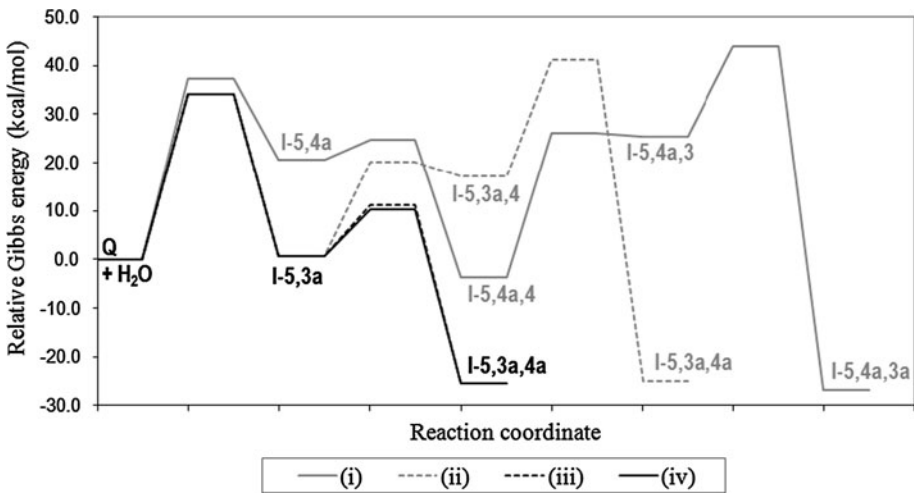
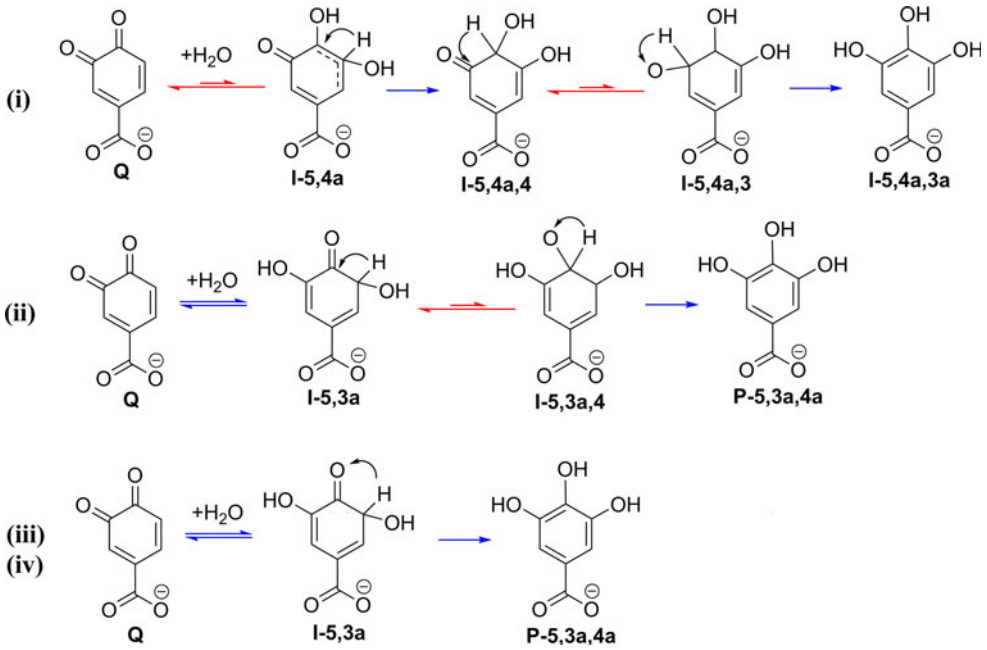


Fig. 3 Reaction profiles for route Q-54. *i* Without any other solvent molecule assisting, *ii* with one water molecule assisting the nuchelophilic addition, *iii* with one solvent molecule assisting all the reaction steps, *iv* with one solvent molecule assisting the water addition and two water molecules assisting the proton migration



Scheme 5 Reaction routes for the hydrolysis of the quinone. **i** Without any other solvent molecule assisting, **ii** with one water molecule assisting the nucleophilic addition, **iii** with one solvent molecule assisting all the reaction steps, **iv** with one solvent molecule assisting the water addition and two water molecules assisting the proton migration



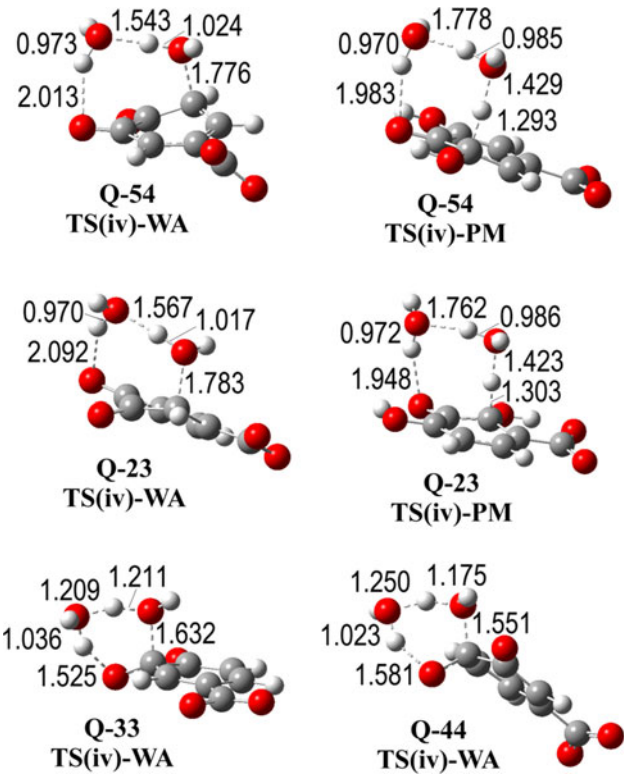


Fig. 4 Fully optimized structures of the **Q** hydrolysis routes within approach (iv), the distances are reported in Å. *WA* water addition step, *PM* proton migration step

lead to regeneration of the catechol moiety. It can be hypothesized that for other catechol compounds, with more electron-withdrawing substituents, these would also be the more favored hydrolysis paths. If that is the case the hydrolysis of **Q** would only have minor contributions to the regeneration of the catechol moiety. However, it can be also hypothesized that **Q**, **P-3**, **3a**, and **P-3**, **4a** might act as free radical scavengers themselves. These points remain as open questions that escape the purpose of the present work. However, further investigations to test both hypotheses are highly encouraged.

Table 4 Standard Gibbs energies of reaction (ΔG^0, kcal/mol), Gibbs energies of activation ($\Delta G^{0\neq}$, kcal/mol), and rate constants (k, M^{-1} s^{-1}), at 298.15 K, for path the hydrolysis of Q, using approach (iv)

Path	ΔG^0	$\Delta G^{0\neq}$	k
Water addition			
Q-54	0.72	34.11	6.09×10^{-13}
Q-23	−1.93	28.86	4.39×10^{-9}
Q-33	−0.83	22.44	2.30×10^{-4}
Q-44	−0.01	22.98	8.55×10^{-5}
Proton migration			
Q-54	−26.08	9.58	1.04×10^{6}
Q-23	−29.59	9.67	9.75×10^{5}

With one solvent molecule assisting the water addition (WA) and two water molecules assisting the proton migration (PM)

4 Conclusions

The free radical scavenging activity of protocatechuic acid has been studied in aqueous and lipid solutions. It was found that in non-polar environments (lipid) its protective

effect is moderate. On the other hand, in aqueous solution it is predicted to be an excellent peroxyl radical scavenger.

In such media, the pH of the environments is predicted to have an important role in the free radical scavenging activity of protocatechuic acid. At physiological pH, it was found to react very fast with the first radical via SET from the di-anion, which can also be labeled as SPET from the mono-anion, which is the dominant form of protocatechuic acid under such conditions.

After the first peroxyl radical has been scavenged, and in the presence of a good electron-donor species, such as the superoxide radical anion, the latter is consumed and H_2Prc^- is regenerated (route **B**). Therefore, unless a reaction with other species in the surroundings consume H_2Prc^- or some of the intermediates this scavenger would be "recycled" as it traps two free radicals per cycle. This means that protocatechuic acid has the ability of scavenging more than 4 radical equivalents in aqueous solution, at least at pH around 7.4.

The hydrolysis of the quinone, formed during the oxidation of protocatechuic acid, has also been tested, since it was previously reported to be important for protocatechuates in alcoholic solution. It was found that this process is assisted by other solvent molecules. It was also found that it is so slow that it should not be experimentally observed.

According to our results, protocatechuic acid can scavenge several free radicals in aqueous solution because it is regenerated through route B, which is competing with the quinone formation in the second stage of the whole oxidation process.

An equivalent cyclic process seems to be possible for other scavengers with the catechol moiety. If this hypothesis is confirmed the role of compounds with a catechol moiety as free radical scavengers might be even more important that what has been assumed so far.

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