Food Chemistry 218 (2017) 144–151

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Free radical scavenging potency of quercetin catecholic colonic metabolites: Thermodynamics of $2H^+/2e^-$ processes

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ARTICLE INFO

Article history:
Received 11 March 2016
Received in revised form 11 July 2016
Accepted 5 September 2016
Available online 13 September 2016

Keywords:
Quercetin
Colonic metabolites
Catecholic compounds
Radical scavenging
Double HAT
Double SPLET

ABSTRACT

Reaction energetics of the double ($2H^+/2e^-$), i.e., the first $1H^+/1e^-$ (catechol → phenoxyl radical) and the second $1H^+/1e^-$ (phenoxyl radical → quinone) free radical scavenging mechanisms of quercetin and its six colonic catecholic metabolites (caffeic acid, hydrocaffeic acid, homoprotocatechuic acid, protocatechuic acid, 4-methylcatechol, and catechol) were computationally studied using density functional theory, with the aim to estimate the antiradical potency of these molecules. We found that second hydrogen atom transfer (HAT) and second sequential proton loss electron transfer (SPLET) mechanisms are less energy demanding than the first ones indicating $2H^+/2e^-$ processes as inherent to catechol moiety. The Gibbs free energy change for reactions of inactivation of selected free radicals indicate that catecholic colonic metabolites constitute an efficient group of more potent scavengers than quercetin itself, able to deactivate various free radicals, under different biological conditions. They could be responsible for the health benefits associated with regular intake of flavonoid-rich diet.

1. Introduction

Numerous epidemiological evidences throughout the years have indicated that regular consumption of food and beverages of plant origin is associated with a decreased incidence of cardiovascular diseases, cancer, and neurodiseases, which all in their etiology include oxidative stress (Del Rio et al., 2013). Natural plant products contain many macro- and micronutrients that may be responsible for their health promoting effects (Hollman, 2014). The beneficial effects of fruits, vegetables, grains, olive oil, red wine and tea have been mostly ascribed to polyphenols, ubiquitously present in plant kingdom. In the last decades, one of the most popular explanations of such effects has been ascribed to antioxidant activity of flavonoids, polyphenols present in many fruits and vegetables at concentrations in the low μM to mM range, and which possess strong *in vitro* free radical scavenging activity (Del Rio et al., 2013). This notion has been questioned and almost abandoned by studies of bioavailability, which indicate that *in vivo* flavonoid concentrations in systemic circulation rarely exceed low μM values (Dangles, 2012; Hollman, 2014). Instead, it has been suggested that not parent flavonoid molecules but their metabolites can act as protectors against oxidative stress mediated diseases (Aura, 2008).

Only some 5% of ingested flavonoids are metabolized in small intestine (Clifford, 2004) and over 95% reach colon and undergo microbial metabolism (Duenas, Surco-Laos, Gonzalez-Manzano, Gonzalez-Paramas, & Santos-Buelga, 2011). Gut microbiota can hydrolyse flavonoid glycosides and their conjugates. They also perform degradation reactions of flavonoids such as C-ring cleavage, reduction, decarboxylation, demethylation, and dehydroxylation reactions (Selma, Espin, & Tomas-Barberan, 2009). The resulted metabolites may be potentially more biologically active than the parent compounds. Hydroxylated aromatic compounds can be formed from the A-ring and phenolic acids from the B-ring of flavonoids. Many of the structurally diverse food flavonoids are broken down into the common set of simpler phenolic compounds (e.g., hydroxybenzoic, hydroxyphenylacetic and hydroxyphenylpropionic acids) by action of colonic bacteria (Selma et al., 2009). For example, quercetin glycosides transform, *via* C-ring fission by gut

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http://dx.doi.org/10.1016/j.foodchem.2016.09.018
0308-8146/

microbiota, to simple metabolites including 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxybenzoic acid (Fig. 1) (Rechner et al., 2004).

Some of colonic metabolites can reach high μM concentrations in fecal water (Halliwell, Rafter, & Jenner, 2005) and after efficient absorbance in colon appear in the blood. Since microbial metabolites may be present in systemic circulation in much higher concentration than the intact parent flavonoid, it has been proposed that at least a part of the biological activities ascribed to flavonoids are due to their colonic metabolites (Williamson & Clifford, 2010).

Quercetin (Q) is one of the most abundant dietary flavonoids and can be found in apples, onions, broccoli and red wine. It is the most studied flavonoid and has been reported to exhibit wide range of biological activities including anticancer activity (Duenas et al., 2011). Q possesses the key structural features for effective free radical scavenging: ortho-dihydroxy substitution in B-ring (catechol structure), *enol* moiety in conjugation with a 4-keto group in C-ring and 5-OH group in A-ring forming additional H-bond with 4-keto group (Bors, Heller, Michel, & Saran, 1990). Using artificial large free radicals (e.g., DPPH and ABTS) numerous studies indicate Q as a powerful *in vitro* free radical scavenger (Kim & Lee, 2004). However, quercetin's *in vivo* ability for direct scavenging of common small free radicals such as $HOO^{\cdot}$, $^{\cdot}NO$ and $O_2^{\cdot-}$ (produced by cell metabolic processes as well as endogenous and exogenous impacts) and its biological relevance have yet to be established. If exists, it could be minor due to low plasma concentration of Q. On the contrary, quercetin colonic metabolites could be produced in enough high μM concentrations (e.g., caffeic acid, 670 μM; homoprotocatechuic acid, 277 μM) (Halliwell et al., 2005) that they could be able to exert their beneficial effects as intracellular antioxidants (Del Rio et al., 2013; Galleano, Verstraeten, Oteiza, & Fraga, 2010).

Different reaction mechanisms involved in free radical scavenging by polyphenols have been proposed (Dangles, 2012; Leopoldini, Russo, & Toscano, 2011; Vaganek, Rimarčik, Dropkova, Lengyel, & Klein, 2014). They can generally be grouped into two types of processes: H-atom abstraction and radical adduct formation (RAF). H-atom abstraction processes may occur *via* at least three different mechanisms: hydrogen atom transfer (HAT), electron transfer followed by proton transfer (ET-PT) and sequential proton loss electron transfer (SPLET). These and some other possible mechanisms (such as proton-coupled electron transfer (PCET) and sequential proton-loss hydrogen-atom transfer (SPLHAT)) have been very recently reviewed by Galano et al. (2016). Already published studies dealing with free radical scavenging mechanisms of natural polyphenols have mostly been based on single, $1H^+/1e^-$ processes (Galano et al., 2016; Leopoldini et al., 2011). However, depending on a number and position of OH groups they may proceed as multiple sequential $1H^+/1e^-$ mechanisms (Dangles, 2012). Catecholic (*o*-diphenolic) compounds are able to scavenge two free radicals *via* semiquinone formation, in two successive $1H^+/1e^-$ processes which results in *o*-quinone formation (Amić et al., 2014; Iuga, Alvarez-Idaboy, & Vivier-Bunge, 2011). In this study, we have computationally investigated energetics of $2H^+/2e^-$ (two sequential $1H^+/1e^-$ processes) mechanisms of homolytic and heterolytic O—H bond cleavage in catechol moiety of Q and its catecholic metabolites.

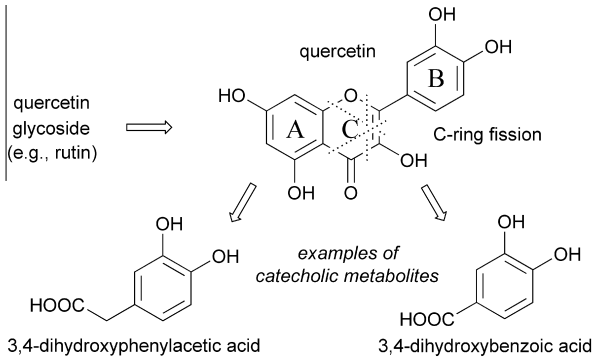

Fig. 1. Proposed segment of the colonic degradation of quercetin (Rechner et al., 2004).

Scavenging of free radicals is a complex process influenced by many factors. Among them, characteristics of scavenged free radicals should not be neglected because they highly influence scavenging mechanism (Galano et al., 2016). To explore free radical scavenging potential of Q and its catecholic metabolites, a set of ten free radicals has been chosen. It embraces oxygen-centered radicals with different reactivities: $^{\cdot}OH$ (hydroxyl radical), $^{\cdot}OOH$ (hydroperoxyl radical), $^{\cdot}OCH_3$ (methoxyl radical), $^{\cdot}OC(CH_3)_3$ (t-butoxyl radical), $PhO^{\cdot}$ (phenoxyl radical), $O_2^{\cdot-}$ (superoxide radical anion), $CH_3—OO^{\cdot}$ (methyl peroxyl radical), $CH_2=CH—OO^{\cdot}$ (vinyl peroxyl radical), $CH_2=CH—CH_2—OO^{\cdot}$ (allyl peroxyl radical), and $Cl_3C—OO^{\cdot}$ (trichloromethyl peroxyl radical). The $^{\cdot}OH$ radical is the main source of biological damage in living organisms because it is so electrophilic that it may strip an electron or H-atom from almost any compound with which it comes in contact (Galano et al., 2016). Radicals $^{\cdot}OCH_3$ and $^{\cdot}OC(CH_3)_3$ are examples of alkoxyl radicals. The smallest alkoxyl radical $^{\cdot}OCH_3$ is abundant damaging free radical in the human body. In comparison, peroxyl radicals $^{\cdot}OOH$, $CH_2=CH—OO^{\cdot}$ and $CH_2=CH—CH_2—OO^{\cdot}$ are less reactive. They may mimic lipid peroxyl radicals ($LOO^{\cdot}$) which are abundantly formed in biological systems. $Cl_3C—OO^{\cdot}$ is very electronegative, highly reactive peroxyl radical. $O_2^{\cdot-}$ is an important radical with relatively low reactivity that occurs during *in vivo* metabolism. Phenoxyl type radicals ($PhO^{\cdot}$) are involved in biological redox processes and in the biosynthesis of natural products.

Quercetin colonic metabolites with catecholic structure are of particular interest because they retain basic structural motif for superior antioxidant activity. In this report we theoretically investigated free radical scavenging potency of selected six quercetin colonic catecholic metabolites, i.e., caffeic acid (CA), hydrocaffeic acid (HCA), homoprotocatechuic acid (HPA), protocatechuic acid (PCA), 4-methylcatechol (MC) and catechol (C), and compared to that of parent quercetin molecule. All these selected catecholic compounds are naturally occurring in plant kingdom in free or esterified form (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). Their *in vitro* free radical scavenging activities have been assayed experimentally (Duenas et al., 2011; Kim & Lee, 2004) and investigated theoretically (Leopoldini et al., 2011). Mechanisms of free radical inactivation by CA, widespread plant polyphenol and one of the most abundant colon metabolite of polyphenols (Halliwell et al., 2005), which may exert a variety of biological functions, have been recently computationally studied (Leon-Carmona, Alvarez-Idaboy, & Galano, 2012). HCA has been indicated as the one that potentially may act as scavenger of intracellular reactive oxygen species (Huang, de Paulis, & May, 2004). Theoretical investigation of antiradical activity of HCA has also been performed (Leon-Carmona et al., 2012). HPA, phenolic acid colon metabolite of diverse flavonoids, has been shown to may act as a potent free radical scavenger even at very low μM concentrations (Merfort, Heilmann, Weiss, Pietta, & Gardana, 1996). Both HCA and HPA have a cancer-preventive potential. PCA can be found in many edible and medicinal plants and is the major human colon metabolite of cyanidin glycosides, and has been shown to have numerous health effects. Among others, PCA has been investigated for its free radical scavenging (Galano & Perez-Gonzalez, 2012) and anticancer activity (Masella et al., 2012). MC (also known as 3,4-dihydroxytoluene), a colonic metabolite of Q, has high antioxidant activity and inhibits growth of melanoma cells *in vitro* (Payton, Bose, Alworth, Kumar, & Ghosh, 2011).

As poorly bioavailable, powerful *in vitro* antioxidant Q could not exert significant *in vivo* antiradical activity. Hence, estimation of antiradical potency of highly bioavailable Q colonic metabolites is of particular interest. The aim of this study was to theoretically evaluate free radical scavenging potency of Q and its six catecholic colon-derived metabolites. This was achieved by calculation of reaction enthalpies and free energies involved in individual steps of double ($2H^+/2e^-$) HAT and SPLET mechanisms. The data obtained enable the estimation of thermodynamically preferred mechanism of radical inactivation, as well as ranking of Q and its metabolites as scavengers of a set of the chosen free radicals of different characteristics.

2. Computational details

Catechol moiety ($Ph(OH)_2$) of polyphenolic molecule is able to undergo double ($2H^+/2e^-$) free radical scavenging mechanisms, i.e., to scavenge two free radicals ($RO^\cdot$), with corresponding net reactions given by Eqs. (1) and (2):

$$Ph(OH)_2 + RO^\cdot \rightarrow Ph(OH)O^\cdot + ROH \quad (1)$$

$$Ph(OH)O^\cdot + RO^\cdot \rightarrow Ph(=O)_2 + ROH \quad (2)$$

where $Ph(OH)O^\cdot$ and $Ph(=O)_2$ are phenoxyl radical and *o*-quinone, respectively. We considered two double ($2H^+/2e^-$) mechanisms: double HAT and double SPLET. In double HAT mechanism, the first $1H^+/1e^-$ process is related to H-atom abstraction from one of the catecholic O—H groups resulting in formation of phenoxyl radical, and the second $1H^+/1e^-$ process is related to H-atom abstraction from another O—H group and formation of *o*-quinone (Fig. 2a).

The first bond dissociation enthalpy (BDE1) is calculated according to Eq. (3):

$$BDE1 = H(Ph(OH)O^\cdot) + H(H^\cdot) - H(Ph(OH)_2) \quad (3)$$

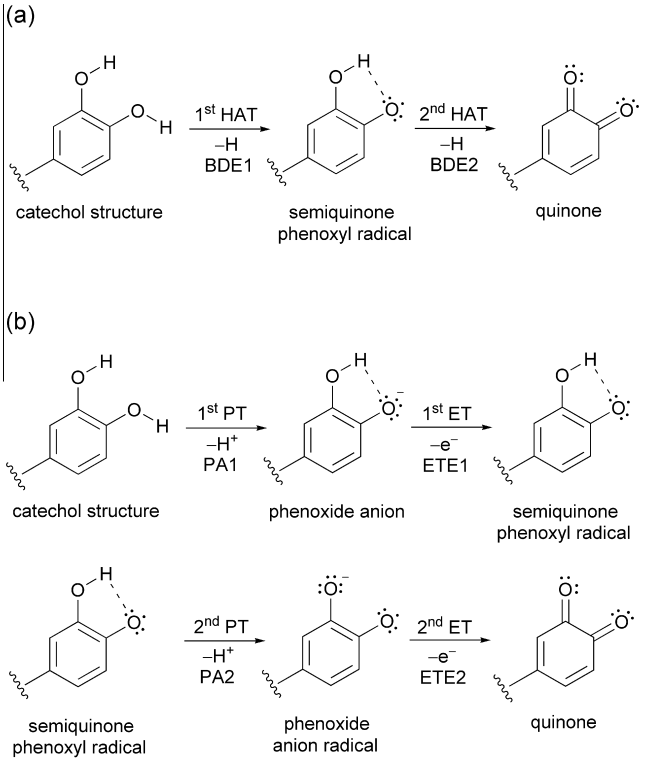

Fig. 2. (a) Double HAT mechanism in pentyl ethanoate and (b) double SPLET mechanism in water.

where $H(Ph(OH)_2)$, $H(Ph(OH)O^\cdot)$ and $H(H^\cdot)$ are enthalpies of catecholic molecule, its phenoxyl radical, and H-atom, respectively. The second bond dissociation enthalpy (BDE2) is calculated using Eq. (4):

$$BDE2 = H(Ph(=O)_2) + H(H^\cdot) - H(Ph(OH)O^\cdot) \quad (4)$$

where $H(Ph(=O)_2)$ is enthalpy of *o*-quinone. A lower BDE value, usually attributed to a greater ability to donate a hydrogen atom released by the homolytic cleavage of the O—H bond, results in an easier free radical scavenging reaction. In general, HAT is preferred in non-polar solvents because it does not involve charge separation.

The double SPLET mechanism (Fig. 2b) starts with deprotonation of catecholic structure, i.e., by heterolytic cleavage of one of its O—H bonds, producing phenoxide anion $Ph(OH)O^-$ (Eq. (5)):

$$Ph(OH)_2 \rightarrow Ph(OH)O^- + H^+ \quad (5)$$

Phenoxide anion then gives an electron to a free radical, Eq. (6):

$$Ph(OH)O^- \rightarrow Ph(OH)O^\cdot + e^- \quad (6)$$

Related first proton affinity (PA1) and electron transfer enthalpy (ETE1) are calculated using Eqs. (7) and (8), respectively:

$$PA1 = H(Ph(OH)O^-) + H(H^+) - H(Ph(OH)_2) \quad (7)$$

$$ETE1 = H(Ph(OH)O^\cdot) + H(e^-) - H(Ph(OH)O^-) \quad (8)$$

where $H(Ph(OH)O^-)$, $H(H^+)$ and $H(e^-)$ are enthalpies of phenoxide anion, proton and electron, respectively. Phenoxyl radical generated by the first $1H^+/1e^-$ process then undergoes the second $1H^+/1e^-$ process *via* phenoxide anion radical ($PhO^\cdot O^-$) according to Eqs. (9) and (10), respectively:

$$Ph(OH)O^\cdot \rightarrow PhO^\cdot O^- + H^+ \quad (9)$$

$$PhO^\cdot O^- \rightarrow Ph(=O)_2 + e^- \quad (10)$$

Associated the second proton affinity (PA2) and the second electron transfer enthalpy (ETE2) can be calculated by Eqs. (11) and (12), respectively:

$$PA2 = H(PhO^\cdot O^-) + H(H^+) - H(Ph(OH)O^\cdot) \quad (11)$$

$$ETE2 = H(Ph(=O)_2) + H(e^-) - H(PhO^\cdot O^-) \quad (12)$$

where $H(PhO^\cdot O^-)$ is enthalpy of the radical anion. The net result of both mechanisms is the same, that is, the production of *o*-quinone. In general, SPLET mechanism is favoured in polar media, since it involves charge separation process.

To take into account characteristics of selected free radicals on reaction with the catecholic molecules, calculations of Gibbs free energy of reactants and products involved in the studied double mechanisms have been performed. In general, reaction free energy represents the criterion of the thermodynamically preferred process. The reaction between antioxidant and particular free radical is thermodynamically favourable if it is exergonic, Eq. (13):

$$\Delta_r G = [G(\text{products}) - G(\text{reactants})] < 0 \quad (13)$$

$\Delta_r G_{BDE1}$ and $\Delta_r G_{BDE2}$ represent the free energy changes of the first and the second HAT mechanism (Fig. 2a), respectively, and are calculated by Eqs. (14) and (15), respectively:

$$\Delta_r G_{BDE1} = [G(Ph(OH)O^\cdot) + G(ROH)] - [G(Ph(OH)_2) + G(RO^\cdot)] \quad (14)$$

$$\Delta_r G_{BDE2} = [G(Ph(=O)_2) + G(ROH)] - [G(Ph(OH)O^\cdot) + G(RO^\cdot)] \quad (15)$$

Lower $\Delta_r G_{BDE}$ value is assumed to correspond to a greater reactivity of catecholic molecule with considered free radical.

In case of double SPLET, $\Delta_r G_{PA1}$ and $\Delta_r G_{ETE1}$ are related to the first SPLET mechanism (Eqs. (7) and (8), Fig. 2b), and are calculated by Eqs. (16) and (17), respectively:

$$\Delta_r G_{PA1} = [G(Ph(OH)O^-) + G(ROH)] - [G(Ph(OH)_2) + G(RO^-)] \quad (16)$$

$$\Delta_r G_{ETE1} = [G(Ph(OH)O^\cdot) + G(RO^-)] - [G(Ph(OH)O^-) + G(RO^\cdot)] \quad (17)$$

$\Delta_r G_{PA2}$ and $\Delta_r G_{ETE2}$ are related to the second SPLET mechanism (Eqs. (11) and (12), Fig. 2b), and are calculated by Eqs. (18) and (19), respectively:

$$\Delta_r G_{PA2} = [G(PhO^\cdot O^-) + G(ROH)] - [G(Ph(OH)O^\cdot) + G(RO^-)] \quad (18)$$

$$\Delta_r G_{ETE2} = [G(Ph(=O)_2) + G(RO^-)] - [G(PhO^\cdot O^-) + G(RO^\cdot)] \quad (19)$$

All calculations were performed using Gaussian 09 program package (Frisch et al., 2013). Density functional theory (DFT) represents a powerful tool for studying free radical scavenging mechanisms of natural compounds (Galano et al., 2016; Leopoldini et al., 2011). Geometry optimizations and frequency calculations for Q and six catecholic metabolites, corresponding radicals, anions, radical anions, and *o*-quinones, as well as for ten selected free radicals and their species involved in the considered radical scavenging pathways were carried out using the M05-2X functional (Zhao, Schultz, & Truhlar, 2006) in conjunction with the 6-311++G(d,p) basis set. The M05-2X functional has been chosen because it is designed for main-group chemistry and recommended for kinetic and thermodynamic calculations by its developers (Zhao et al., 2006). Among the suite of density functionals, M05-2X is particularly suitable for systems where thermochemistry and kinetics are important. It is among the best performing functionals for calculations of reaction energies involving free radicals and its reliability has been independently confirmed by other authors (Galano & Perez-Gonzalez, 2012). In addition, all calculations were also performed using the B3LYP-D3 functional. The B3LYP-D3 is a dispersion-corrected hybrid GGA (Generalized Gradient Approximation) functional which contains an additional term in the formula for the total energy, whose purpose is to model short- and medium-range interatomic interactions (Grimme, Antony, Ehrlich, & Kreig, 2010). One can expect that B3LYP-D3 will describe the parent molecules and all species issued from them more reliably and accurately than traditional density functional methods. The influence of water and pentyl ethanoate as solvents was calculated with an implicit continuum solvation model, SMD, which takes into account the full solute electron density in estimation of free energy of solvation (Marenich, Cramer, & Truhlar, 2009). SMD is a universal solvation model applicable to any charged or uncharged solute in any solvent or liquid medium for which a few key descriptors are known (in particular, dielectric constant, refractive index, bulk surface tension, acidity and basicity parameters). In conjunction with the M05-2X density functional, SMD model has been successfully used for study of thermodynamics and kinetics of free radical scavenging mechanisms (Leon-Carmona et al., 2012; Perez-Gonzalez, Alvarez-Idaboy, & Galano, 2015). Spin unrestricted calculations were used for open-shell systems. Relative enthalpies and free energies were calculated at 298.15 K. Published values of the gas-phase enthalpy of proton and electron as well as of the solvation enthalpy of hydrogen atom, proton and electron were used (Tables S1a and S1b in Supplementary data).

3. Results and discussion

3.1. Evaluation of free radical scavenging potency of Q and its catecholic metabolites

The most stable structures obtained by the conformational analysis of Q and its six selected colonic metabolites are presented in Fig. 3. Obtained results in non-polar medium and related discussions presented in this paper are based on these neutral conformations. In aqueous medium the results are related to the carboxylate anions of CA, HCA, HPA and PCA which predominate at physiological pH of 7.4.

The analysis of free radical scavenging mechanisms under physiological conditions is the most relevant one. In the polar environment, polyphenolic compounds have a tendency to deprotonate and are at least partly present as phenoxide anions. It is well known that pH influences the antioxidant activity. The free radical scavenging activity increases with increasing pH, because phenoxide anions are better radical scavengers than corresponding neutral molecules (Musialik, Kuzmicz, Pawlowski, & Litwinienko, 2009). Obviously, in the aqueous environment at pH = 7.4, Q is to some extent deprotonated and exist partly in anionic form (Alvarez-Diduk, Ramirez-Silva, Galano, & Merkoci, 2013; Musialik et al., 2009). The most acidic phenolic group of Q and its corresponding pK_a value have still not been unequivocally identified. Some authors indicate 7-OH group as the first site of deprotonation (pK_{a1} = 8.45) (Musialik et al., 2009), while others consider 4′-OH group as the most acidic (pK_{a1} = 6.41) (Alvarez-Diduk et al., 2013). Our calculated proton affinities (PA, kcal/mol) indicate 4′-OH group of Q slightly more acidic than 7-OH group, as illustrated in Fig. S1a (in Supplementary data). Carboxylate group (—COOH) of hydroxycarboxylic acids is more acidic than their phenolic (—OH) groups. The pK_{a1} of CA, HCA, HPA and PCA are 4.37, 4.45, 4.18 and 3.86 (Table S1c), respectively, indicating that these compounds primarily exist as carboxylate anions at physiological pH of 7.4. So, to provide a detailed insight into the radical scavenging potency of these acids in aqueous environment at pH = 7.4, the carboxylate anions have been considered. The acidity dissociation constants for catechol are both above pH 7: pK_{a1} = 9.25 and pK_{a2} = 13.0 (Schweigert, Zehnder, & Eggen, 2001). However, it has been assumed that the catechol radical is primarily in dissociated form at physiological pH (Schweigert et al., 2001). This could also

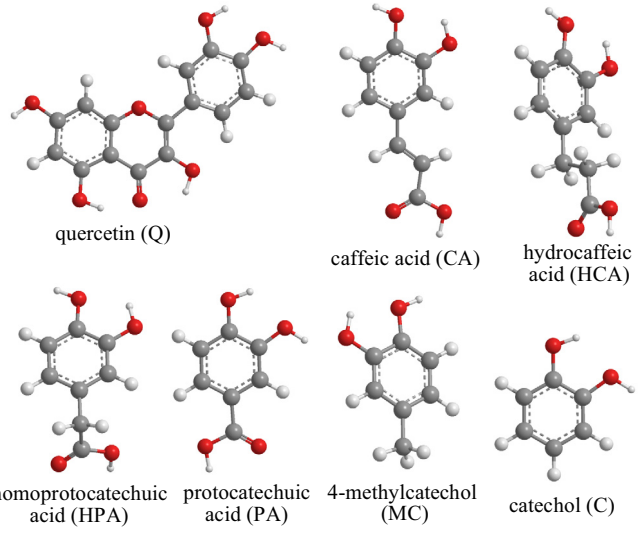

Fig. 3. Optimised structures of quercetin and its catecholic colonic metabolites calculated by SMD/M05-2X/6-311++G(d,p) approach.

be predicted for MC. In non-polar lipid environment (membrane bilayer), which does not support charge separation, Q and its catecholic metabolites exist in the non-dissociated form.

Reaction enthalpies related to double HAT and double SPLET mechanisms for Q and its six colonic catecholic metabolites calculated at the M05-2X/6-311++G(d,p) SMD level of theory are listed in Table 1. Calculations were performed in pentyl ethanoate for HAT and water for SPLET, representing membrane lipids and biological liquids, respectively, i.e., the two kinds of natural environments of radical inactivation. All studied compounds (Fig. 3) possess catecholic structure which enables scavenging of two free radicals: semiquinone phenoxyl radical formed after inactivation of the first free radical may further scavenge the second free radical resulting in *o*-quinone formation (Fig. 2). Usually, only the first HAT and the first SPLET have been theoretically examined. Recently, it has been demonstrated that the possibility of the second HAT and SPLET mechanisms significantly contribute to overall free radical scavenging potency of polyphenolic compounds (Amić et al., 2014).

Data presented in Table 1 indicate that in non-polar medium (pentyl ethanoate) catecholic metabolites and parent quercetin molecule possess nearly equal potency for scavenging the first free radical *via* HAT mechanism because corresponding BDE1 values are nearly identical: 80.59 kcal/mol (average value) for six metabolites and 80.55 kcal/mol for Q, respectively. Due to catecholic moiety quercetin metabolites not only retain high free radical scavenging ability of parent molecule, but even possess increased potency for the second free radical inactivation because BDE2 is on average by 6 kcal/mol less energy demanding than BDE1 (74.36 *vs* 80.59 kcal/mol). Corresponding difference for Q is nearly 4 kcal/mol (76.36 *vs* 80.55 kcal/mol), indicating catecholic metabolites as potentially more effective free radical scavengers.

Similar results were obtained in polar (aqueous) environment where SPLET mechanism is thermodynamically preferred. Because at physiological pH of 7.4 catecholic molecules exist at least partly in anionic (phenoxide) form, significant enthalpy (driving force) related to SPLET mechanism is ETE (Amić et al., 2014; Lespade & Bericon, 2012). On average, ETE1 and ETE2 of metabolites are by 5 kcal/mol less than corresponding values for Q (Table 1), indicating metabolites as better free radical scavengers *via* SPLET mechanism. As in the case of HAT, the second SPLET mechanism is less energy demanding than the first one (90.73 *vs* 98.71 kcal/mol).

Regardless of the reaction medium, the products of $2H^+/2e^-$ processes are *o*-quinones. It should be noted that *o*-quinones are redox active electrophilic species, which may exert pro-oxidant activities by binding with proteins and nucleic acids, resulting in cell death and also oncogenic mutations. On the contrary, *o*-quinones can react with the parent polyphenols to form dimers (Dangles, 2012) and/or are rapidly hydrated to unreactive species and hence are unlikely to damage biological macromolecules (Tu, Giblin, & Gross, 2011).

3.2. Estimation of the reliability of calculated results

Because there are still no experimental values of reaction enthalpies for investigated molecules and mechanisms, obtained results can be only compared with other available theoretical results. Obviously, theoretical results primarily represent relative trend between calculated quantities. Currently, computationally obtained data related to O—H bond reaction enthalpies of single ($1H^+/1e^-$) processes (BDE1, PA1, ETE1, etc.) are available (Galano et al., 2016; Vaganek et al., 2014). However, application of diverse computational methods (different levels of theory, different functionals and basis sets, calculations in gas-phase or in solution) and methodologies (for calculations in solutions including or not solvation enthalpies) do not allow correct comparison. To the best of our knowledge there is no published DFT data related to thermodynamics of second step of double ($2H^+/2e^-$) processes (BDE2, PA2, ETE2, etc.) by which obtained M05-2X/6-311++G(d,p) results may be compared. This was the reason why we have calculated reaction enthalpies of investigated double processes by using another approach, i.e., B3LYP-D3/6-311++G(d,p) level of theory. As can be seen from Tables S2 and S3, basic results obtained by these methods offer analogous trends of calculated enthalpies and free energies with shift exerted by B3LYP-D3 method to proportionally more negative values. Accordingly, both methods show analogous trends between estimated reaction enthalpies involved in studied double ($2H^+/2e^-$) free radical scavenging mechanisms (compare Table 1 *vs* Table S4).

To assess the reliability of applied calculating strategies we firstly compared our B3LYP-D3 results for Q reaction enthalpies related to single ($1H^+/1e^-$) processes with theoretical results obtained by Klein's group (Vaganek et al., 2014). The reason for this is that we used analogous methodology of reaction enthalpies calculation utilized by Klein's group (Rimarčik, Lukeš, Klein, & Ilčin, 2010). BDE1 value of 74.91 kcal/mol (Table S4), calculated by using SMD/B3LYP-D3/6-311++G(d,p) method in non-polar solvent pentyl ethanoate, is in excellent agreement with Vaganek et al. (2014) value of 313 kJ/mol (74.81 kcal/mol), obtained in non-polar benzene by using IEF-PCM/B3LYP/6-311++G** method. ETE1 value of 76.92 kcal/mol, calculated by us in water as a solvent, is in fair accordance with ETE1 value of 344 kJ/mol (82.22 kcal/mol) obtained by Vaganek et al. (2014). However, PA1 value (19.27 kcal/mol) is not in good accordance with Vaganek et al. (2014) result of 159 kJ/mol (38.00 kcal/mol).

Additionally, we compared our results with those obtained by using somewhat different methodology. For example, by using PCM/B3LYP/6-31+G* level of theory, Fiorucci, Golebiowski,

Table 1
The SMD/M05-2X/6-311++G(d,p) reaction enthalpies (in kcal/mol) for double HAT in pentyl ethanoate and double SPLET in water of catecholic metabolites and parent quercetin molecule.

	Catecholic metabolite	1st HAT	2nd HAT	1st SPLET		2nd SPLET	
		BDE1	BDE2	PA1	ETE1	PA2	ETE2
1	Caffeic acid (CA)	80.34	76.80	19.95	77.96	15.90	77.35
2	Hydrocaffeic acid (HCA)	79.61	72.96	23.02	74.86	15.85	73.87
3	Homoprotocatechuic acid (HPA)	80.56	73.51	23.09	74.76	15.96	73.49
4	Protocatechuic acid (PCA)	83.87	76.55	20.96	80.00	13.79	77.59
5	4-Methylcatechol (MC)	78.31	72.63	22.46	75.01	15.45	74.19
6	Catechol (C)	80.85	73.70	21.66	78.48	13.92	77.00
Average value (1–6)		*80.59*	*74.36*	*21.86*	*76.85*	*15.15*	*75.58*
Total enthalpy requirement for SPLET				*98.71*		*90.73*	
	Quercetin (Q)	80.55	76.36	18.02	82.59	12.81	80.63
Total enthalpy requirement for SPLET				*100.61*		*93.44*	

Table 2
The Gibbs free energy changes (Δ_rG, in kcal/mol) of scavenging HOO· radical *via* double HAT mechanism in pentyl ethanoate and double SPLET mechanism in water. The results were obtained using the M05-2X functional in combination with the 6-311++G(d,p) basis set and SMD solvation model.

HOO· radical	HAT		SPLET			
	1st HAT	2nd HAT	1st SPLET		2nd SPLET	
	Δ_rG_{BDE1}	Δ_rG_{BDE2}	Δ_rG_{PA1}	Δ_rG_{ETE1}	Δ_rG_{PA2}	Δ_rG_{ETE2}
Quercetin (Q)	−5.64	−9.65	−11.49	6.49	−17.74	5.05
Caffeic acid (CA)	−5.74	−9.90	−9.52	1.38	−14.70	1.97
Hydrocaffeic acid (HCA)	−6.43	−13.27	−6.51	−1.57	−13.63	−1.98
Homoprotocatechuic acid (HPA)	−5.31	−12.27	−6.45	−1.46	−13.82	−2.57
Protocatechuic acid (PCA)	−1.94	−10.10	−8.47	3.39	−16.10	1.22
4-Methylcatechol (MC)	−7.15	−13.53	−7.28	−1.49	−14.26	−1.42
Catechol (C)	−5.04	−12.66	−7.83	2.07	−16.13	0.74

Cabrol-Bass, and Antonczak (2007) obtained PA1 value of 286.3 kcal/mol for Q in water as a solvent. This PA1 was calculated without the inclusion of the enthalpy of proton and hydration enthalpy of proton. By inclusion proton enthalpies, recalculated value amounts 23.28 kcal/mol, which is in accord with our result of 19.27 kcal/mol. Lespade and Bericon (2012) used B3LYP/6-31+G(d)//B3LYP/6-311+G(2d,2p) approach with SCRF-PCM method for the calculation of ETE1 for Q in water solution. They obtained ETE1 value of 102.8 kcal/mol. ETE1 was calculated without the inclusion of the enthalpy of electron and hydration enthalpy of electron. By inclusion electron enthalpies, recalculated value amounts 72.65 kcal/mol, which is in good agreement with our result of 76.92 kcal/mol (Table S4).

Further, we compared our results obtained using M05-2X functional (SMD/M05-2X/6-311++G(d,p) method) with the results published by Galano et al. (2016) computed at the very similar method SMD/M05-2X/6-311+G(d,p). As expected, BDE1 value for quercetin (80.55 kcal/mol) and BDE1 value for caffeic acid (80.34 kcal/mol) calculated in pentyl ethanoate (Table 1) are in agreement with BDE1 values of 79.4 and 78.8 kcal/mol, respectively, obtained in benzene by Galano et al. (2016).

It should be noted that the experimentally determined BDE values can be found for simple phenolics, such as phenol molecule. Using benzene as a solvent value of 88.3 kcal/mol was experimentally determined by Lucarini, Pedrielli, Pedulli, Cabiddu, and Fattuoni (1996). By using SMD/M05-2X/6-311++G(d,p) level of theory, we obtained value of 88.62 kcal/mol in benzene (this value embraces the H-atom solvation enthalpy of 1.53 kcal/mol (Rimarčik et al., 2010)) and 88.25 kcal/mol in pentyl ethanoate. Since the agreement with the experimentally obtained value is excellent it could indicate the employed approach as appropriate.

As mentioned in *Computational details* section the M05-2X functional is considered as one of the best performing functionals for calculating reaction energies involving free radicals (Galano et al., 2016; Zhao et al., 2006). Our results related to phenol molecule are in line with this statement because we obtained the better agreement with the experimentally determined BDE value by using M05-2X than by using B3LYP-D3 functional. Namely, latter gives values of 84.20 and 83.74 kcal/mol in benzene and pentyl ethanoate, respectively, i.e. deviation of cca. 4 kcal/mol with respect to the experimental data appears. All numerical data of individual reactions enthalpies presented and discussed in this section are summarised in Table S5.

3.3. Influence of nature of free radicals on scavenging potency of Q and its catecholic metabolites

Because the scavenging processes are highly influenced by the properties of the scavenged free radical species, we also calculated (using SMD/M05-2X/6-311++G(d,p) level of theory) the free energy of reactions (Δ_rG) of Q and its six metabolites with each of ten selected free radicals (·OH, ·OOH, ·OCH_3, ·$OC(CH_3)_3$, PhO·, $O_2^{\cdot-}$, CH_3—OO·, CH_2=CH—OO·, CH_2=CH—CH_2—OO· and Cl_3C—OO·) for double HAT and double SPLET mechanisms.

Scavenging free radicals by Q and its catecholic metabolites can be illustrated by inactivating of ·OOH radical (Table 2 and Fig. 4). The second and third columns in Table 2 are related to free energy change of reaction of inactivation of ·OOH radical *via* the 1st HAT (Δ_rG_{BDE1}) and 2nd HAT (Δ_rG_{BDE2}) mechanisms, respectively. The fourth (Δ_rG_{PA1}) and fifth (Δ_rG_{ETE1}), and the sixth (Δ_rG_{PA2}) and seventh (Δ_rG_{ETE2}) columns are related to free energy changes of reaction of scavenging of ·OOH radical *via* the 1st SPLET and the 2nd SPLET mechanisms, respectively. Δ_rG values can be helpful for assignment of the specific free radical scavenging potency because more negative values indicate thermodynamically more preferred processes. Results indicate that in non-polar medium potency of metabolites to scavenge first ·OOH radical *via* 1st HAT mechanism is at least equal to potency of quercetin parent molecule. The only exception is PCA which appears to be less effective scavenger. Scavenging of second ·OOH radical *via* 2nd HAT mechanism is on average by 6 kcal/mol less energy demanding. Similar trend appears in aqueous environment where double SPLET mechanism occurs (Table 2 and Fig. 4). Since exergonicity of Δ_rG_{PA} overwhelms endergonicity of Δ_rG_{ETE} (in the case of Q, CA, PA and C), and by taking into account Hess's law the 1st and 2nd SPLET mechanisms are, in summary, exergonic (thermodynamically unfeasible reaction can be driven by a thermodynamically feasible reaction that is coupled to it). In polar medium, all catecholic metabolites (due to lower $\Delta_rG_{PA} + \Delta_rG_{ETE}$ values) possess higher potency for inactivating ·OOH radical *via* both the 1st and 2nd SPLET

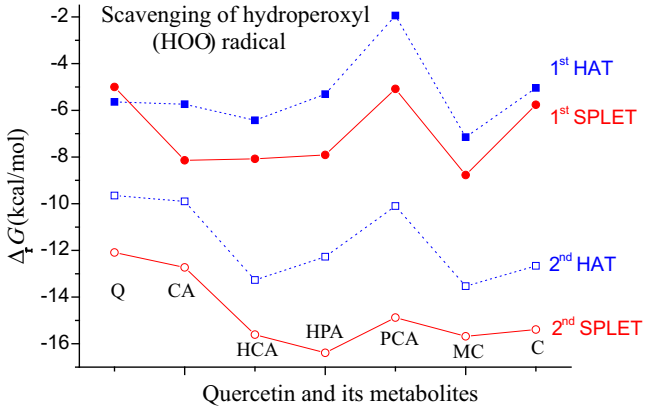

Fig. 4. The Gibbs free energy change for double HAT (hydrogen atom transfer) and double SPLET (sequential proton loss electron transfer) mechanisms of HOO· radical scavenging by quercetin (Q) and its metabolites: caffeic acid (CA), hydrocaffeic acid (HCA), homoprotocatechuic acid (HPA), protocatechuic acid (PCA), 4-methylcatechol (MC) and catechol (C).

mechanisms, in comparison to the parent Q molecule. This is more obvious in the case of the 2nd SPLET mechanisms (Fig. 4). Total energy requirement for the 2nd SPLET mechanism (Δ_rG_{PA2} + Δ_rG_{ETE2}) is in average by 7 kcal/mol less demanding than the corresponding value for the 1st SPLET mechanism (Δ_rG_{PA1} + Δ_rG_{ETE1}). Catecholic metabolites possess higher potency for inactivating the second free radical than Q itself. Moreover, data presented in Fig. 4 clearly show that catecholic structures (Q and its metabolites) possess higher potency for scavenging of ·OOH radical in polar environment (*via* SPLET mechanisms) than in non-polar environment (*via* HAT mechanisms). This is in accordance with the published results regarding free radical scavenging potency of PCA (Galano & Perez-Gonzalez, 2012).

Analogous results as for ·OOH radical were obtained for free radical inactivation of other studied free radicals (Tables S6–S14, Figs. S6–S14). Furthermore, these results are also in accordance with results obtained by using B3LYP-D3/6-311++G(d,p) computations (Tables S15–S24 and Figs. S15–S24). On the basis of exergonicity of the calculated reaction free energies, the reactivity of Q and its metabolites toward 10 studied free radicals was predicted to decrease as follows: $^{\cdot}OH > {}^{\cdot}OC(CH_3)_3 \approx {}^{\cdot}OCH_3 \gg Cl_3C{-}OO^{\cdot} > PhO^{\cdot} > {}^{\cdot}OOH \approx CH_2{=}1CH{-}OO^{\cdot} \approx CH_3{-}OO^{\cdot} \approx CH_2{=}CH{-}CH_2{-}OO^{\cdot} \gg O_2^{\cdot-}$.

Presented results indicate that quercetin catecholic metabolites constitute an efficient group of more potent scavengers than Q itself, able to deactivate various free radicals, under different biological conditions. Second HAT and SPLET mechanisms of free radical scavenging are less energy demanding than the first ones indicating double $2H^+/2e^-$ processes as inherent to catecholic moiety. This could be one of the possible explanations of the major role of catecholic moiety in direct scavenging of free radicals which has been noted more than two decades ago (Bors et al., 1990). In the case of HPA, which usually has been described as a main metabolite from the colonic degradation of Q, our theoretical predictions are in line with published experimental results. For example, Kim and Lee (2004) found that HPA possesses higher VCEAC (vitamin C equivalent antioxidant capacity) than Q: 316.7 mg/L *vs* 229.4 mg/L, respectively. The antioxidant capacity of vitamin C was designated at a value of 100 mg/L. Consequently, HPA is more than three-fold active than vitamin C (and more active than Q) in deactivating of ABTS radicals. HPA is also more active in another *in vitro* antioxidant assay, i.e., the FRAP (ferric reducing antioxidant power) assay. In this assay results are expressed as TEAC (Trolox-equivalent antioxidant capacity) values. Values obtained for HPA and Q amount 4.03 mM and 3.07 mM, respectively, indicating HPA as 4.03 times active than Trolox (synthetic analogue of vitamin E) in reducing of Fe(III) to Fe(II) (Duenas et al., 2011). Recently, the high activity of catecholic group (in the case of PCA) in scavenging of hydroperoxyl radicals HOO·, the protonated form of superoxide radical anion $O_2^{\cdot-}$, has been explained by regeneration of catecholic moiety in aqueous solution what may results in scavenging of several radical equivalents, two per cycle (Galano & Perez-Gonzalez, 2012).

Thermodynamic parameters here presented may be important factors governing the free radical scavenging reactions of Q and its catecholic metabolites, while a more complete understanding would require kinetic analysis. For example, on the basis of our results (Table S13 and Fig. S13) it appears that in polar environment Q and CA are not able to scavenge $O_2^{\cdot-}$ radical by the first SPLET mechanism because of endergonicity of the involved electron transfer process (for Q: Δ_rG_{PA1} = −32.94 kcal/mol, Δ_rG_{ETE1} = 44.08 kcal/mol, Δ_rG_{SPLET1} = 11.14 kcal/mol; for CA: Δ_rG_{PA1} = −30.97 kcal/mol, Δ_rG_{ETE1} = 38.97 kcal/mol, Δ_rG_{SPLET1} = 8.00 kcal/mol). However, it should be noted that small positive values of Δ_rG (<10 kcal/mol) do not necessarily mean that the corresponding free radical scavenging reaction should be neglected. Such process may represent notable reaction pathway if it takes place at a significant rate (Perez-Gonzalez et al., 2015). Despite the fact that experimentally determined rate constants of the inactivation of the $O_2^{\cdot-}$ radical by Q and CA are low, $k = 4.7 \times 10^4$ M^{-1} s^{-1} (Jovanovic & Simic, 2000) ($k = 1.0 \times 10^6$ M^{-1} s^{-1} (Taubert et al., 2003)) and $k = 5.0 \times 10^5$ M^{-1} s^{-1} (Taubert et al., 2003), respectively, in comparison with typical radical reactions ($k \sim 10^9$ M^{-1} s^{-1}), this could be enough for efficient *in vivo* scavenging of the $O_2^{\cdot-}$ radical (Jovanovic & Simic, 2000).

4. Conclusions

Increasing evidence suggests that intestinal metabolites of polyphenols significantly contribute to human health. Colonic metabolites of flavonoids unequivocally have a potential to promote human health *via* several possible *in vivo* mechanisms, associated or not with antioxidant actions. Our results indicate that quercetin catecholic colonic metabolites possess potential for inactivating various free radicals by direct scavenging *via* double SPLET and double HAT mechanisms depending upon polarity of environment. As revealed by other authors some of the catecholic colonic metabolites may be produced in concentrations sufficient for this activity *in vivo*. Probability of their direct radical scavenging activity could be more relevant *in situ* because after entering systemic circulation one of the vicinal phenolic OH groups could be blocked by metabolic biotransformations such as methylation, glucuronidation and sulfation. Certainly, much more work is necessary to identify whether direct free radical inactivation by colonic metabolites is of biological significance.

Acknowledgements

This work was supported by the Ministry of Science, Education and Sports of the Republic of Croatia, and by the Ministry of Science of the Republic of Serbia (Projects No. 172015 and 174028). We also gratefully acknowledge the anonymous reviewers for their constructive comments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.09.018.

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