



# Quercetin and related flavonoids conserve their antioxidant properties despite undergoing chemical or enzymatic oxidation



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## ABSTRACT

Oxidation of a phenolic group in quercetin is assumed to compromise its antioxidant properties. To address this assumption, the ROS-scavenging, Folin-Ciocalteu- and Fe-reducing capacities of quercetin and thirteen structurally related flavonoids were assessed and compared with those of mixtures of metabolites resulting from their chemical and enzymatic oxidation. Regardless of the oxidation mode, the metabolites mixtures largely conserved the antioxidant properties of the parent molecules. For quercetin, 95% of its ROS-scavenging and over 77% of its Folin-Ciocalteu- and Fe-reducing capacities were retained. The susceptibility of flavonoids to oxidative disappearance (monitored by HPLC-DAD) and that of the mixtures to retain their antioxidant capacity was favourably influenced by the presence of a catechol (ring-B) and enol (ring C) function. This is the first study to report that mixtures resulting from the oxidation of quercetin and its analogues largely conserve their antioxidant properties.

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## 1. Introduction

Quercetin, the most abundant flavonoid in edible plant foods, has been subject to many studies due to its antioxidant properties (Brown, Khodr, Hider, & Rice-Evans, 1998; Deng, Fang, & Wu, 1997; Rice-Evans, Miller, & Paganga, 1996; Thompson & Williams, 1976; Yoshino & Murakami, 1998). These properties are largely attributed to the capacity of quercetin to reduce the formation of reactive oxygen species (ROS) *via*, for instance, chelation of metals involved in ROS generation (Brown et al., 1998; Thompson & Williams, 1976; Yoshino & Murakami, 1998), inhibition of various ROS-generating enzymes (Chimenti et al., 2006; Leyva-López, Gutierrez-Grijalva, Ambriz-Perez, & Heredia, 2016) and/or down-regulation of expression of relevant enzymes (Leyva-López et al., 2016). The antioxidant actions of quercetin can also arise from its capacity to favour ROS removal *via*, for instance, direct scavenging of such species (Rice-Evans et al., 1996), induction of the synthesis of ROS-removing enzymes (Molina, Sanchez-Reus, Iglesias, & Benedi, 2003; Tang et al., 2012), and/or up-regulation of the expression of genes encoding endogenous antioxidant-synthesizing

enzymes (Lay Saw et al., 2014). In addition, quercetin is intensively studied for its increasingly recognized ROS-scavenging-independent actions (D'Andrea, 2015; Khan et al., 2016; Nabavi, Russo, Daglia, & Nabavi, 2015).

During direct ROS-scavenging action, the quercetin molecule is expected to engage in a series of single-electron transfer and/or hydrogen atom-donation reactions that will initially lead to the oxidation of its catecholic group and subsequently to other oxidative changes that could affect its flavonoid skeleton (Zhou & Sadik, 2008). Such changes have been studied by many investigators; some metabolites generated during the oxidation of quercetin are endowed with clear electrophilic and/or pro-oxidant potential (Boots et al., 2007; Galati, Moridani, Chan, & O'Brien, 2001; Li, Jongberg, Andersen, Davies, & Lund, 2016; Sanda Chedea, Choueiri, Jisaka, & Kefalas, 2012). Chemical, enzymatic, electrochemical and ROS-mediated oxidation studies, among others, have been reported (Zhou & Sadik, 2008). Although many such studies have investigated the chemical identity and possible mechanisms underlying the formation of metabolites (Boots et al., 2007; Galati et al., 2001; Li et al., 2016; Zhou & Sadik, 2008), the actual consequences that the oxidation of quercetin could have on its antioxidant properties have not been evaluated yet. In the present work, we assessed the ROS-scavenging (ORAC), Folin-Ciocalteu (FC)- and Fe-reducing capacities of a mixture of metabolites that resulted from chemical (alkaline dissolution-auto-induced) (Jurasekova, Torreggiani, Tamba, Sanchez-Cortez, & Garcia-Ramos, 2009) and

Abbreviations: ACN, acetonitrile; FRAP, Fe-reducing capacity; ORAC, oxygen radical absorbance capacity; PPO, mushroom tyrosinase; TP, total phenolics.

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enzymatic (mushroom tyrosinase-mediated) oxidation of quercetin (Kubo, Nihei, & Shimizu, 2004). For comparison, and to define possible structural determinants of the susceptibility of quercetin to oxidation, we extended our study to thirteen structurally related flavonoids. The present study reveals that the mixtures of metabolites that result from the oxidation of quercetin or related flavonoids, rather than losing the antioxidant properties of the parent molecules, tend to largely conserve them. Among flavonoids, differences in susceptibility to oxidation and/or retention of their original antioxidant properties were found to primarily depend on structural features and only marginally on the mode of oxidation. Despite undergoing oxidation, quercetin and many other flavonoids conserve a substantial portion of their original antioxidant properties; the potential biological and/or methodological implications are discussed.

## 2. Materials and methods

### 2.1. Materials

Mushroom tyrosinase (2684 units/mg of solid, EC 1.14.18.1) and all flavonoids used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), and their purity was as follows: apigenin ( $\geq 97\%$ ), (+)-catechin ( $\geq 98\%$ ), dideoxyquercetin ( $\geq 98\%$ ), (-)-epicatechin ( $\geq 98\%$ ), ( $\pm$ )-eriodictyol ( $\geq 95\%$ ), fisetin ( $\geq 98\%$ ), galangin ( $\geq 95\%$ ), isorhamnetin ( $\geq 95\%$ ), kaempferol ( $\geq 97\%$ ), luteolin ( $\geq 98\%$ ), morin ( $\geq 97\%$ ), myricetin ( $\geq 96\%$ ), quercetin ( $\geq 95\%$ ) and (+)-taxifolin ( $\geq 90\%$ ). Acetonitrile (ACN) and ethanol were HPLC grade. Formic acid, sodium hydroxide, sodium dihydrogen phosphate and di-sodium hydrogen phosphate were analytical grade and purchased from Merck (Darmstadt, Germany).

### 2.2. Methods

#### 2.2.1. Alkali-mediated oxidative disappearance of flavonoids

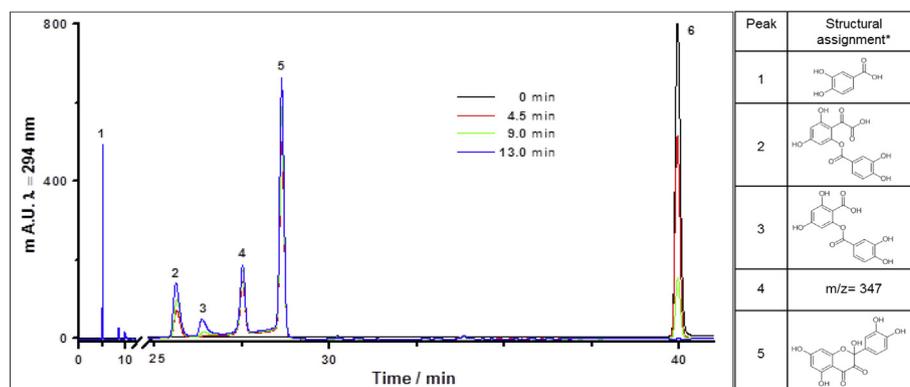
Each flavonoid was dissolved in 1 M NaOH to a final concentration of 0.1 M (pH = 12) and immediately incubated at 22 °C for a maximum of 300 min. Prior to analysis of the remnant concentration, samples of the incubated solutions were neutralized by the addition of sodium phosphate buffer (75 mM, pH 7.0) and diluted immediately with the mobile phase (see below). Control oxidation experiments were performed in parallel by incubating flavonoids dissolved in pure ethanol instead of alkali. After sampling, the ethanolic solutions were treated identically to the alkali samples.

#### 2.2.2. Enzyme-mediated oxidative disappearance of flavonoids

Stock solutions of each flavonoid were prepared fresh in ethanol to 20 mM; prior to their incubation these were diluted to a final concentration of 100  $\mu$ M with phosphate buffer (75 mM, pH 6.6). Oxidation was induced by incubating samples of the latter solutions with 42.5 U/mL of mushroom tyrosinase (PPO) at 30 °C for a maximum of 300 min. Prior to the analysis of the remnant concentration of the flavonoids, incubated samples were diluted with an equal volume of a 40:60 v/v mixture of ACN and water acidified with formic acid (at 0.1% v/v) to stop the oxidation reaction. As control oxidation experiments, solutions of each flavonoid (containing 0.5% v/v ethanol) were incubated in parallel in the absence of PPO.

#### 2.2.3. HPLC analysis of the flavonoids

HPLC analysis was performed using an Agilent 1200 series bomb, equipped with an autosampler and a photodiode array detector (Santa Clara, CA, USA). The HPLC system was controlled by Agilent ChemStation (Agilent Technologies 2010). The concentration of each flavonoid was assessed in incubated samples and estimated from the area under the curve of its chromatographic peak using the standard curves prepared for each flavonoid on the day of the experiment. The standard deviation of each determination was always less than 5%. Detection wavelengths were 294 nm for (+)-catechin, epicatechin, eriodictyol and taxifolin; 334 nm for apigenin; 370 nm for dideoxyquercetin, fisetin, galangin, isorhamnetin, kaempferol, luteolin, morin, myricetin and quercetin. Other chromatographic conditions were as follows: flow rate of 0.8 mL/min, column (250 x 4.6 mm i.d., 5  $\mu$ m, RP-18e Puro-spher® Star, Merck, Darmstadt, Germany), oven column at 25 °C. All flavonoids were analysed under isocratic conditions using a mobile phase mixture of ACN (A) and water/formic acid (0.1%) (B), whose composition (v/v) varied according to the analysed flavonoid, as follows: catechin and epicatechin, 20% A and 80% B ( $t_R$  = 6.4 and 7.6 min, respectively); taxifolin, 25% A and 75% B ( $t_R$  = 10.7 min); fisetin and myricetin, 30% A and 70% B ( $t_R$  = 8.8 and 9.5 min, respectively); quercetin, eriodictyol and luteolin, 35% A and 65% B ( $t_R$  = 11.4, 10.9 and 10.6 min, respectively); apigenin, dideoxyquercetin, isorhamnetin, kaempferol and morin, 40% A and 60% B ( $t_R$  = 11.1, 9.7, 13.1, 10.1, 6.7 min, respectively); galangin, 55% A and 45% B ( $t_R$  = 12.6 min). For quercetin, in addition to assessing the changes in the area under the curve of the characteristic peak, the appearance of new peaks with progressive oxidative disappearance was investigated (shown in Fig. 1). The disappearance of quercetin and appearance of its metabolites was monitored at 294 nm employing the following HPLC gradient



**Fig. 1.** HPLC chromatogram of the time-dependent disappearance of quercetin, and the appearance of major oxidation products during the exposure of quercetin to alkaline conditions. Under the chromatographic conditions employed in this study, quercetin corresponds to peak 6, with a retention time of 39.9 min. The  $t_R$  of peaks 1–5, associated with quercetin oxidation products, were 5.0, 25.6, 26.4, 27.5 and 28.6 min, respectively. Based on ESI-MS/MS analysis, the figure includes the chemical structure assigned to compounds in peaks 1–5.

program: 10% A and 90% B (0–15 min), linear increase to 60% A over 35 min, and a return to the starting conditions within 5 min. Under these chromatographic conditions, quercetin exhibits a  $t_R = 39.9$  min. The  $t_R$  for the emerging quercetin oxidation-related peaks are described in Fig. 1.

#### 2.2.4. ESI-MS/MS analysis of peaks arising from DAD-HPLC analysis of the alkali-induced quercetin oxidation solution

To establish the possible chemical structure of the compounds present in the major peaks described in Fig. 1, each peak was collected from an Agilent 1260 Infinity automated fraction collector (coupled to the HPLC system), dried under  $N_2$  at 40 °C and subjected to ESI-MS/MS analysis. ESI-MS/MS spectra were acquired using an AB Sciex Triple Quad™ 4500 triple–quadrupole tandem mass spectrometer equipped with a turbo spray ionization source. The mass spectrometer was operated in negative ionization mode, and data were acquired in multiple reaction monitoring (MRM) mode. The source temperature was 650 °C, and the ESI voltage (IS) and entrance potential (EP) were set at  $-4.5$  kV and  $-10$  V, respectively. The gas flows were as follows: ion spray gas (GS1), 50 psi; heater gas (GS2), 40 psi; curtain gas, 20 psi; and collision gas, 7 psi. The declustering potentials (DPs), collision energies (CEs), and collision exit potentials (CXPs) for each compound were adjusted at each tandem MS stage to determine the optimum parameters at which a maximum amount of signal information

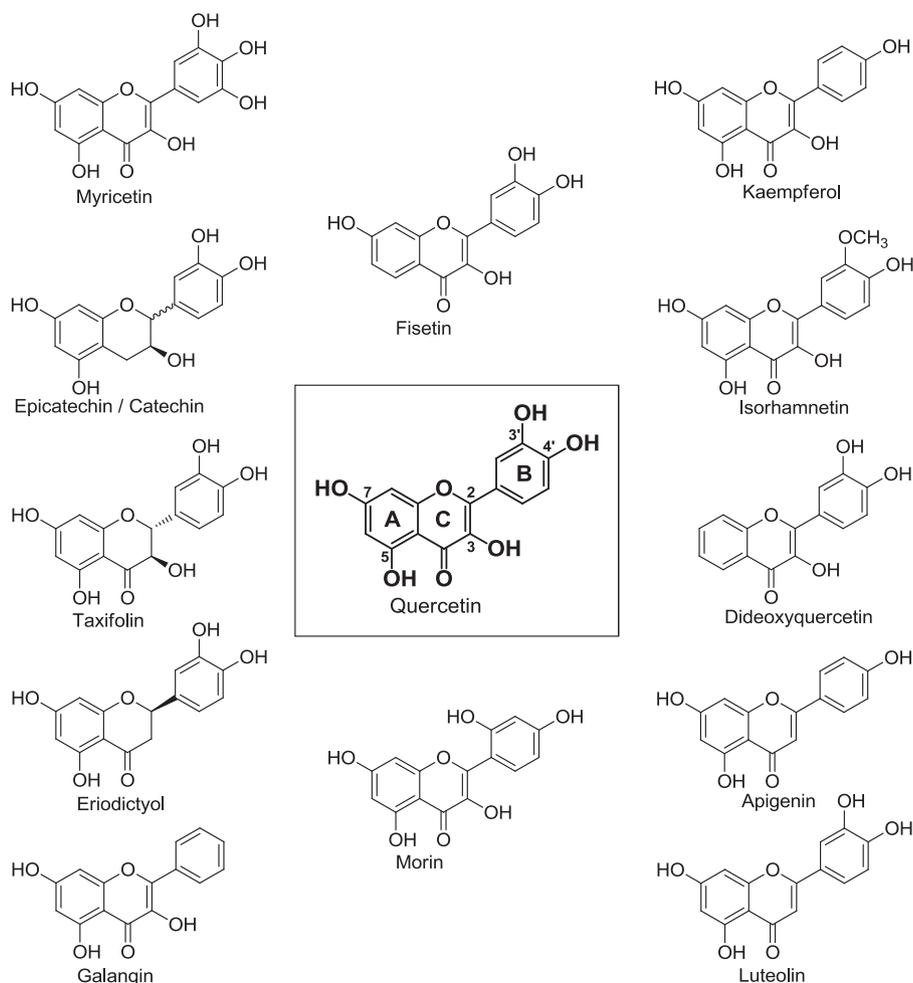
could be obtained, and a scanning rate of 150 ms was used. The ESI-MS/MS system was controlled by Analyst 1.6.2 software.

#### 2.2.5. Antioxidant activity determinations

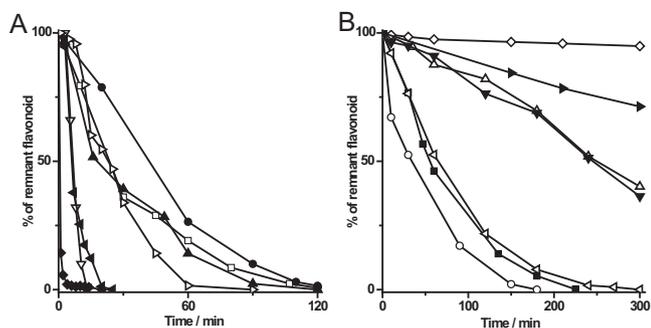
The antioxidant capacity of the fourteen flavonoids and the mixture of metabolites that result from their chemical (alkaline) or enzymatic (mushroom tyrosinase) oxidation was simultaneously assessed using the three following assays: (1) oxygen radical absorbance capacity, (2) Folin-Ciocalteu-reducing capacity (total phenolics or TP), and (3) Fe-reducing capacity (FRAP). The ORAC and Folin-Ciocalteu-reducing assays were performed as described by Wu et al. (2004). In the case of FRAP, the assay was performed as described by Benzie and Strain (1996), except that 30 min of incubation was employed as the reading time (Henríquez, López-Alarcón, Gómez, Lutz, & Speisky, 2011). All antioxidant assays were conducted using 96-well plates, and the results were read in a multi-mode reader Synergy 2, Biotek (Winooski, VT, USA).

#### 2.2.6. Statistics

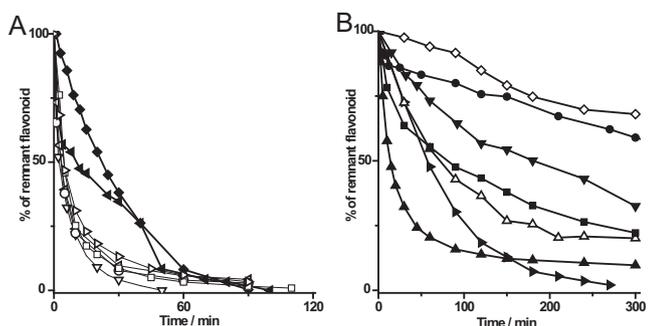
Data points plotted in Figs. 3 and 4, and results expressed in Tables 1 and 2, represent the means of at least three independent experiments, each conducted in quadruplicate. For the sake of simplicity—since the standard deviation values represented less than 5% of the means—the standard deviations were omitted from Fig. 3 and 4. In the case of Tables 1 and 2, however, since some means exhibited standard deviations greater than 5%, all were



**Fig. 2.** Chemical structure of quercetin and thirteen related flavonoids. The structure of quercetin is depicted in the centre of the figure, with labelled A, B and C rings, numbering the double bond situated in carbons 2 and 3 and hydroxyl groups bound to carbons 3', 4', 3, 5 and 7.



**Fig. 3.** Time-dependent disappearance of quercetin and related flavonoids following chemical oxidation. After the initial dissolution of each flavonoid in alkali, samples incubated for various durations (0–300 min, at 22 °C) were neutralized and immediately assayed by HPLC, as described in the Materials and methods Section 2.2.3. For each flavonoid, data are presented as the remnant percentages of initial concentration. Part A of the figure shows decay profiles of flavonoids that underwent total disappearance in the first 120 min of exposure to alkaline conditions. Part B depicts decay profiles of flavonoids whose disappearance was either total or partial between 120 and 300 min. Flavonoids presented in Part A are myricetin (◆), quercetin (▽), fisetin (◄), dideoxyquercetin (▷), taxifolin (▲), eriodictyol (□) and isorhamnetin (●). In part B symbols are epicatechin (○), luteolin (■), catechin (◁), kaempferol (▼), galangin (△), morin (►) and apigenin (◇).



**Fig. 4.** Time-dependent disappearance of quercetin and related flavonoids following enzymatic oxidation. After initial dissolution of each flavonoid in phosphate buffer (pH 6.6), aliquots were incubated in the presence or absence of polyphenol oxidase with varying duration (0–300 min, at 30 °C). Immediately after incubation, samples were collected and subjected to HPLC analysis (further details in Materials and methods Section 2.2.3). Data are presented as the remnant percentage of the initial concentration of each flavonoid. Decay profiles of flavonoids that underwent total disappearance in the first 120 min of exposure to enzyme are shown in part A; part B shows profiles of those whose oxidative disappearance was total or partial between 120 and 300 min. Flavonoids in Part A are, as follows: quercetin (▽), epicatechin (○), catechin (◁), dideoxyquercetin (▷), myricetin (◆), fisetin (◄) and eriodictyol (□). In part B, symbols are taxifolin (▲), morin (►), galangin (△), luteolin (■), kaempferol (▼), isorhamnetin (●) and apigenin (◇).

included. The statistical significance of the difference between curves (as in Figs. 3 and 4) was assessed with paired *t*-tests or analysis of variance (ANOVA), as appropriate. Differences at *p* below 0.05 were considered significant. Disappearance profiles and data treatment (linear regression analysis) were performed employing Origin 6.0 software. In all tests, *P* < 0.05 was considered statistically significant.

### 3. Results

To study the effect of dissolving quercetin in an alkaline medium (pH 12) on the stability of the flavonoid (i.e., changes in concentration), samples were taken at various time points (from 0–13 min), brought immediately to pH 7.0 (by addition of phosphate

**Table 1**

ORAC, total phenolics and FRAP of quercetin and related flavonoids following exposure to alkaline-oxidation.

Flavonoid	ORAC <sub>NaOH</sub> / ORAC <sub>EtOH</sub>	TP <sub>NaOH</sub> / TP <sub>EtOH</sub>	FRAP <sub>NaOH</sub> / FRAP <sub>EtOH</sub>
Quercetin	0.95 ± 0.11	0.77 ± 0.02	0.77 ± 0.01
Epicatechin	0.49 ± 0.01	0.63 ± 0.02	0.44 ± 0.06
Catechin	0.50 ± 0.03	0.57 ± 0.09	0.42 ± 0.06
Morin	0.84 ± 0.05	0.95 ± 0.04	0.67 ± 0.02
Kaempferol	0.68 ± 0.08	0.60 ± 0.02	0.21 ± 0.03
Galangin	0.72 ± 0.22	0.89 ± 0.01	0.53 ± 0.09
Eriodictyol	0.48 ± 0.07	0.39 ± 0.02	0.35 ± 0.10
Taxifolin	0.71 ± 0.01	0.51 ± 0.07	0.37 ± 0.05
Fisetin	1.01 ± 0.14	0.95 ± 0.03	0.68 ± 0.01
Isorhamnetin	1.27 ± 0.28	0.68 ± 0.05	0.35 ± 0.04
Myricetin	0.82 ± 0.21	0.55 ± 0.04	0.30 ± 0.02
Luteolin	0.69 ± 0.09	0.62 ± 0.06	0.12 ± 0.01
Apigenin	1.23 ± 0.09	1.19 ± 0.06	0.98 ± 0.02
Dideoxyquercetin	0.99 ± 0.07	0.56 ± 0.11	0.50 ± 0.12

Each flavonoid was exposed to NaOH until either total or partial disappearance of the flavonoid, as shown in Fig. 3. For each flavonoid, data are expressed as the ratios of corresponding assay run in NaOH- versus ethanol-dissolved (control) samples.

**Table 2**

ORAC, total phenolics and FRAP of quercetin and its related flavonoids following exposure to enzymatic oxidation.

Flavonoid	ORAC <sub>PPPO</sub> / ORAC <sub>control</sub>	TP <sub>PPPO</sub> / TP <sub>control</sub>	FRAP <sub>PPPO</sub> / FRAP <sub>control</sub>
Quercetin	1.08 ± 0.07	0.91 ± 0.01	0.83 ± 0.02
Epicatechin	0.55 ± 0.01	0.61 ± 0.01	0.27 ± 0.03
Catechin	0.45 ± 0.05	0.62 ± 0.01	0.28 ± 0.07
Morin	0.87 ± 0.03	0.68 ± 0.02	0.34 ± 0.03
Kaempferol	0.87 ± 0.01	1.08 ± 0.01	0.72 ± 0.01
Galangin	1.26 ± 0.07	1.40 ± 0.20	0.73 ± 0.06
Eriodictyol	0.46 ± 0.02	0.45 ± 0.02	0.16 ± 0.10
Taxifolin	0.61 ± 0.01	0.66 ± 0.03	0.27 ± 0.03
Fisetin	1.01 ± 0.02	0.91 ± 0.08	0.56 ± 0.01
Isorhamnetin	1.96 ± 0.76	0.97 ± 0.02	0.96 ± 0.10
Myricetin	0.91 ± 0.01	0.92 ± 0.04	0.31 ± 0.02
Luteolin	0.55 ± 0.04	0.77 ± 0.06	0.49 ± 0.09
Apigenin	1.06 ± 0.06	0.87 ± 0.04	0.99 ± 0.01
Dideoxyquercetin	0.91 ± 0.04	0.93 ± 0.01	0.46 ± 0.04

After dissolving each flavonoid in phosphate buffer (pH 6.6), aliquots were incubated in the presence or absence of polyphenol oxidase until either total or partial disappearance of the flavonoid, as shown in Fig. 4. For each flavonoid, data are expressed as ratios of corresponding assay run in the presence versus absence of PPO.

buffer) and subjected to HPLC analysis. Fig. 1 depicts the DAD chromatogram (run at 294 nm) of the time-dependent disappearance of quercetin. In the mixture of metabolites, quercetin was identified as peak 6 by running its standard under identical HPLC-DAD conditions. As shown, the concentration of quercetin decreased by 40% and 82% after 4.5 and 9.0 min of incubation, respectively. Its total disappearance was observed after 13 min. These changes were accompanied by the emergence of five new peaks (numbered 1 to 5). To establish the chemical identity of these peaks, DAD-HPLC fractions were collected and subjected to ESI-MS/MS analysis. The molecular ion [M–H]<sup>−</sup> and characteristic fragments of peaks 1–5 were, according to their *m/z*, as follows: 153 and 109/108 for peak 1; 333 and 179/197 for peak 2; 305 and 169/151 for peak 3; 347 and 275/203 for peak 4; and 317 and 191/163 for peak 5. On the basis of the similarity between the *m/z* data and that reported in quercetin oxidation studies conducted by Zhou and Sadik (2008), the following chemical structures were tentatively assigned: protocatechuic acid (peak 1); 2-(2-((3,4-dihydroxybenzoyl)oxy)-4,6-dihydroxyphenyl)-2-oxoacetic acid (peak 2); 2-((3,4-dihydroxybenzoyl)oxy)-4,6-dihydroxybenzoic acid

(peak 3); unknown (peak 4); and 2,5,7,3',4'-pentahydroxy-3,4-flavan-dione (peak 5), (Fig. 1).

To understand the possible structural determinants of the susceptibility of quercetin to undergo alkaline decomposition, the disappearance of thirteen structurally related flavonoids was also assessed. The oxidative, incubation and pre-analysis treatment conditions were identical to those applied to quercetin, with the sole exception that the incubation time was, for some flavonoids, extended up to 300 min. As shown in Fig. 2, the structures of the thirteen selected flavonoids differ from that of quercetin in terms of the presence or absence, change in position or derivatization of the hydroxyl groups in rings A (i.e., fisetin and dideoxyquercetin), B (i.e., myricetin, kaempferol, isorhamnetin, apigenin, morin and galangin) and C (i.e., eriodictyol, luteolin and apigenin); the presence or absence of a double bond in C2–C3 of ring C (i.e., catechins, eriodictyol and taxifolin); or a carbonyl group in C4 of ring C (i.e., catechins).

Fig. 3 (parts A and B) depicts the time-course disappearance profiles of quercetin and its structural analogues. As shown in part A, myricetin, followed by quercetin and fisetin, were the three fastest decomposing flavonoids, as they had fully disappeared after 4, 13 and 25 min, respectively. Dideoxyquercetin, taxifolin, eriodictyol and isorhamnetin underwent total decomposition between 60 and 120 min. Finally, as depicted in part B, epicatechin, luteolin and catechin underwent a comparatively slower but still complete disappearance between 180 and 300 min. By the end of this period, kaempferol, galangin and morin exhibited only partial (and apigenin, negligible (6%)) disappearance.

In light of the above-described results, we investigated the extent to which the disappearance of quercetin and its related flavonoids affected the ROS-scavenging (ORAC assay) and redox properties (Folin-Ciocalteu and FRAP assays) of the solutions resulting from exposure to alkaline conditions. The assays were applied to samples at the exact time that each flavonoid fully disappeared (as in Fig. 3A and B). For flavonoids that did not fully disappear, samples were taken at minute 300. For comparison, each flavonoid was also evaluated in samples obtained after direct dissolution in ethanol (instead of alkali). Table 1 expresses the results as ratios of the ORAC, TP or FRAP values obtained after analysis of alkali- and ethanol-dissolved flavonoids (NaOH and EtOH as subscripts for each assay). Such ratios represent the portion of the ORAC, TP and FRAP values from non-oxidized flavonoid when the same flavonoid underwent alkali treatment. In the case of ORAC, the alkali-treated flavonoids isorhamnetin, apigenin, fisetin, dideoxyquercetin, quercetin, morin and myricetin were found to conserve at least 82% of the activity displayed by their non-oxidized forms. Galangin, followed by taxifolin, luteolin and kaempferol, conserved 68%–72% of their original activity. The flavonoids that showed the lowest relative ORAC (yet all above 48%) upon alkali-treatment were catechin, epicatechin and eriodictyol. In the case of the Folin-Ciocalteu assay, apigenin, fisetin, morin and galangin conserved over 88% of the original reducing activity, followed by quercetin, isorhamnetin, epicatechin, luteolin, kaempferol, catechin, dideoxyquercetin, myricetin and taxifolin, which displayed over 50% of the activity shown by the non-oxidized forms. For the ferric-reducing antioxidant potential, exposure of flavonoids to alkali conditions led quercetin, fisetin and morin to conserve over 67% of their control values; over 42% of the original ferric-reducing potential was conserved by galangin, dideoxyquercetin, epicatechin and catechin. Finally, taxifolin, eriodictyol, isorhamnetin and myricetin conserved less than 30%.

Prompted by the observation that despite undergoing alkali-mediated oxidation, quercetin and many of its structurally related flavonoids could largely conserve their ROS-scavenging and/or redox properties, we extended our studies to the use of polyphenol oxidase to induce the oxidative disappearance of such flavonoids.

Fig. 4 (parts A and B) depicts the profiles of the PPO-induced disappearance of quercetin and its analogues. As shown in Fig. 4A, while PPO induced the total disappearance of quercetin after 50 min, the disappearance of epicatechin, catechin, dideoxyquercetin, myricetin, fisetin and eriodictyol reached over 90% after a 60 min incubation. The latter flavonoids underwent total disappearance after a 110 min incubation. As shown in Fig. 4B, morin fully disappeared after 270 min, and after a 300 min incubation, over 75% of the initial concentration of taxifolin, galangin and luteolin had disappeared. Among the three latter flavonoids, taxifolin was unique; despite only partial disappearance, it showed a very fast initial consumption, decreasing in concentration by nearly 80% after 60 min. Taxifolin, kaempferol, isorhamnetin and apigenin also failed to undergo total disappearance; after 300 min; their concentrations decreased by 68%, 41% and 32%, respectively.

Table 2 summarizes the extent to which the PPO-induced disappearance of quercetin and its related flavonoids affects their ROS-scavenging and redox properties. The ORAC, Folin-Ciocalteu and FRAP assays were applied to samples taken at the exact time each flavonoid fully disappeared (data from Fig. 4, part A and B). For flavonoids that did not fully disappear, samples were taken at minute 300. For comparison, each flavonoid was also evaluated in samples obtained following incubation in the absence of PPO. Table 2 expresses the results as ratios of the ORAC, TP and FRAP values obtained after analysis of flavonoids incubated in the presence versus absence of PPO (PPO and Control subscripts for each assay). In the case of ORAC, the PPO-treated flavonoids isorhamnetin, galangin, quercetin, apigenin and fisetin were found to conserve at least 100% of the activity displayed by their non-oxidized forms. Between 86% and 91% of the original ORAC values were conserved after PPO-treatment of myricetin, dideoxyquercetin, morin and kaempferol. After exposure to PPO, taxifolin, luteolin, epicatechin, eriodictyol and catechin showed 45% to 61% of their control ORAC values. In the case of the TP assay, galangin and kaempferol fully conserved their original Folin-Ciocalteu reducing capacity, followed by isorhamnetin, dideoxyquercetin, myricetin, fisetin, quercetin and apigenin, which conserved over 87% of the activity displayed by their non-oxidized forms. Finally, luteolin, morin, taxifolin and catechin displayed over 62% of the control values, and eriodictyol and epicatechin showed over 38% of the original activity. With regard to the FRAP assay, while apigenin, isorhamnetin, quercetin, galangin and kaempferol were conserved by at least 72%, fisetin, luteolin, dideoxyquercetin, morin and myricetin showed over 31% of the control activity. The largest PPO-induced loss of FRAP was seen in the case of catechin, epicatechin, taxifolin and eriodictyol exposed to PPO (16% to 28% of original values).

#### 4. Discussion

The present study investigated the susceptibility of quercetin and thirteen other structurally related flavonoids to alkali- and PPO-mediated oxidation and addressed the consequences of such oxidation on the original ROS-scavenging, Folin-Ciocalteu- and Fe-Triazine-reducing properties. As discussed below, the results reveal that the mixtures of metabolites that result from such oxidations, rather than decreasing initial antioxidant properties, tend to largely conserve them. At least in the case of quercetin, retention of the antioxidant capacity can be partly attributed to the quercetin oxidation product 2,5,7,3',4'-pentahydroxy-3,4-flavan-dione (peak 5 in Fig. 1), which has been observed to conserve the DPPH-scavenging capacity of quercetin (Ramos et al., 2006). Differences in the susceptibility of each flavonoid to oxidation were found to primarily depend on certain structural features and only marginally on how the oxidation was induced. In the case of alkali-induced oxidation, the fastest disappearing flavonoids (i.e.,

myricetin, quercetin, fisetin and dideoxyquercetin) were found to share the following structural features: hydroxyl groups in the 3' and 4' carbons of ring B, a double bond in C2–C3, a hydroxyl group in C3 and a carbonyl in C4 in ring C. The molecules that do not exhibit such catechols (i.e., kaempferol, galangin, morin and apigenin) underwent a substantially slower and incomplete disappearance. Morin exhibits hydroxyl groups at positions 2' and 4' of ring B, supporting the notion that the catechol configuration prompts oxidation, rather than the presence of these two moieties. Revealing the particular importance of the hydroxyl moiety in the 3' position, its O-methylation was associated with 10-fold longer times to total disappearance (isorhamnetin versus quercetin). Finally, the presence of an additional hydroxyl group at position 5' of ring B (as in myricetin) was found to translate into substantially faster (up to threefold) total disappearance.

The first step in the oxidation of a catecholic flavonoid involves the initial removal of either an electron or a hydrogen atom from one of its hydroxyl groups to generate an o-semiquinone intermediate (Jovanovic, Steeden, Tosic, Marjanovic, & Simicg, 1994). The stability of the latter species depends on the probability of hydrogen bridge formation with the oxygen of a vicinal hydroxyl group (which may explain the extremely fast disappearance of myricetin) and/or the delocalization of an unpaired electron through resonance along the C ring and, eventually, the A ring (Van Acker et al., 1996). To take place, the latter mechanism requires the existence of a double bond in the C2–C3 position of ring C (Van Acker et al., 1996). In fact, for the flavonoids taxifolin, eriodictyol, epicatechin and catechin, which lack such a bond, total alkali-induced oxidation was between 9- and 23-fold slower than that of quercetin. Most likely, the substantially lower susceptibility of these molecules to total oxidation reflects the limited possibility of the phenoxyl radicals that formed in the B ring to undergo stabilization (Awad et al., 2001). When the double bond concurs with a hydroxyl group in the C3 position, the planarity of the molecule is favoured (Van Acker et al., 1996). The latter seems to favour oxidation, as it would allow the stabilization of the resonant phenoxyl radical through the formation of a hydrogen bond with the vicinal carbonyl group, as well as the formation of tautomers of the o-quinone generated after the two-electron oxidation of the catechol (Kubo et al., 2000). In fact, in the case of luteolin, the time needed to undergo total disappearance was nearly 17-fold longer than that of quercetin. Furthermore, apigenin, which lacks the catechol and C3 hydroxyl, showed almost negligible disappearance after 300 min of exposure to alkali.

When fourteen flavonoids were exposed to mushroom tyrosinase-mediated oxidation, all were found to undergo some degree of disappearance. Unlike studies in which quercetin and other flavonoids were assessed for their potential to act as PPO inhibitors (Gasowska-Bajger & Wojtasek, 2016; Kubo et al., 2000), the present study addressed the disappearance of flavonoids in the absence of additional PPO substrates. As observed previously by Kubo et al. (2004) and our oxidation in-alkali results, the susceptibility of most flavonoids to early and total disappearance through enzymatic oxidation is also favoured by the presence of a 3' and 4' catechol configuration. In the absence of such hydroxyl groups (e.g., kaempferol or isorhamnetin) or in the absence of both (e.g., galangin), substantially slower and incomplete disappearance was observed. An exception was luteolin, whose unexpectedly slow disappearance might be explained in terms of the difficulty of its highly reactive o-quinone intermediate to undergo subsequent stabilization (Awad et al., 2001; Balyan, Kudugunti, Hamad, Yousef, & Moridani, 2015). However, the relatively early PPO-mediated disappearance of the flavonoids catechin, epicatechin, eriodictyol and taxifolin, which lack a C2–C3 double bond, could be explained by the following hypothesis: the oxidation of such molecules, which is confined to the B ring (Mochizuki,

Yamazaki, Kano, & Ikeda, 2002), would lead to the formation of highly reactive o-quinone species that are capable of spontaneously polymerizing with their non-oxidized forms (Awad et al., 2001; Jiménez-Atiéndzar, Escribano, Cabanes, Gandía-Herrero, & García-Carmona, 2005; Osman, 2011; Sang et al., 2003).

A major, and completely novel, finding of the present study is that despite undergoing oxidation, each of the tested flavonoids generated a mixture of metabolites that conserved the original ROS-scavenging, Folin-Ciocalteu- and Fe-Triazine-reducing activities to varying extents. The latter effects were seen regardless of whether the oxidation was chemical or enzymatic. Nine of the 14 oxidized flavonoids were found to exhibit ROS-scavenging remnant activities greater than 70%, as inferred from alkali-to-ethanol and PPO-to-control ratios greater than 0.7 (data from Tables 1 and 2). Thus, regardless of the mode of oxidation used, the oxidized mixtures of most flavonoids largely appear to conserve their hydrogen-transferring capacity. However, over 50% of the original Folin-Ciocalteu-reducing properties were conserved in 13 of 14 alkali-oxidized flavonoids; nine conserved over 62%. In the case of PPO-treated flavonoids, 12 of 14 molecules conserved at least 62% of such activity. In the case of the iron-reducing properties, 10 alkali-exposed and 8 PPO-treated flavonoids maintained over 35% of their original activity. Although the effects of both modes of oxidation were more stringent for iron-reducing properties, for some flavonoids, the remnant FRAP activity was significantly higher, for example, quercetin was conserved at 77% and 83% after alkali and PPO treatment, respectively. Thus, the results for ROS-scavenging, Folin-Ciocalteu- and Fe-Triazine-reducing properties reveal that, for most flavonoids, oxidation-arising mixtures conserve a substantial portion of their original antioxidant properties.

Under time-controlled oxidation conditions (alkali- and PPO-induced), eight of the 14 tested flavonoids (i.e., quercetin, epicatechin, catechin, eriodictyol, taxifolin, fisetin, myricetin and dideoxyquercetin) underwent nearly 100% disappearance. Interestingly, when the alkali and PPO mixtures obtained from the oxidation of each of these flavonoids were compared in terms of their remnant ROS-scavenging activities, the values were highly correlated ( $r = 0.95$ ), describing a linear regression equation whose slope is 1.0 (data not shown). The two following implications are suggested. First, the eight flavonoids discussed above differ in the magnitude of the change to their original ROS-scavenging capacity. Second, for each flavonoid, alkali and PPO treatment appear to have an almost identical effect on the original ROS-scavenging property. Establishing the chemical identity of the metabolites in each oxidation mixture is beyond the scope of the present work. However, it is tempting to speculate that the oxidation process would not grossly alter the structural moieties that are primarily responsible for ROS-scavenging and/or redox-reducing properties of the flavonoids. Presumably, such moieties would comprise phenolic groups that are capable of both stabilizing ROS and reducing the Folin-Ciocalteu and Ferric-Triazine reagents. In addition, other structural features that favourably stabilize the resulting phenoxyl radical(s) are also likely to be present in the structure of the putative metabolites (i.e., electron-delocalizing and resonance-permitting moieties). Interestingly, among the eight flavonoids that underwent total disappearance in alkali and PPO, the three that showed greater retention of their original ORAC, Folin-Ciocalteu- and Ferric-Triazine-reducing activities were the catecholic flavonols. This was particularly true for flavonoids whose structure simultaneously includes a catechol in ring B and an enol (C2–C3 double bond with a C3-hydroxyl) moiety in ring C (i.e., quercetin, dideoxyquercetin and fisetin). However, flavonoids that have a catechol in ring B but lack a double bond in the C2–C3 position of ring C (flavanols and flavanones) exhibited the lowest degree of antioxidant retention (i.e., catechin, epicatechin, eriodictyol and taxifolin).

In addition to its antioxidant implications, the ability of oxidized flavonoids to scavenge ROS and/or reduce the Folin-Ciocalteu and Fe-Triazine reagents may have methodological implications (Prior, Wu, & Schaich, 2005). That is, whenever a flavonoid is assayed using these methods, a mixture of compounds is likely to contribute to the observed results. This mixture would comprise the reduced flavonoid plus several redox-active metabolites generated during the assay of the flavonoid, which could be particularly important when the sum of the activities of such metabolites is comparable to that of the flavonoid from which they originate. In such cases, the antioxidant activity believed to arise strictly from the reduced flavonoid is likely to be overestimated, eventually limiting the interpretation of studies of the structure-antioxidant activity relationship. On the other hand, at this point, it is not possible to demonstrate that for a given flavonoid, the three former assays will generate the same set of oxidized metabolites. The putative contribution of the latter to the overall results of these assays must still be assessed and may not necessarily be the same. Nonetheless, for the various methods employed to induce flavonoid oxidation, it is worth mentioning that at least in the case of quercetin (employed here as a reference), a similar set of metabolites has been reported to form from free radical-, enzymatically, and/or electrochemically induced oxidation (Zhou & Sadik, 2008). However, the present results cannot be taken as an indication that, in complex food matrices, the oxidation of flavonoids that better retain their original antioxidant properties will favourably affect the net antioxidant capacity of such foods. Finally, there is an additional potential biological implication of the discussed results: following their cellular uptake, quercetin or its structural analogues undergo some form of oxidation reactions that can also generate metabolites with biologically relevant ROS-scavenging and/or redox activities.

### Conflict of interest

The authors have no conflict of interest to disclose.

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