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Antioxidant evaluation of *O*-methylated metabolites of catechin, epicatechin and quercetin

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ABSTRACT

Catechins and quercetin are major polyphenols in many plant foods that have been related to health promotion. In the human organism they are largely metabolized to different metabolites, which are further found in plasma and should contribute to the biological effects associated to the intake of the parent compounds. An important step in quercetin and catechins metabolism is the *O*-methylation of the catechol group, which can be expected to have an effect on their antioxidant and scavenging properties. In the present work, the 3′- and 4′-methylethers of catechin and epicatechin have been prepared and characterised and their antioxidant activity evaluated and compared to that of the corresponding quercetin derivatives. The antioxidant activity was assessed using the ferric reducing power (FRAP) assay and two methods based on the ability to scavenge the $ABTS^{\bullet+}$ radical cation at different pH values. In these assays the three flavonoids behave as better radical scavengers and reducing compounds than usually recognised antioxidants like α-tocopherol. The *O*-methylation of the hydroxyls of the catechol B-ring resulted in a decrease of the antioxidant activity with regard to the parent compounds. However, the methylated metabolites still retain significant radical scavenging activity at pH 7.4, suggesting that they could act as potential antioxidants in physiological conditions. Quercetin and its methylated metabolites showed, in general, greater activity than (epi)catechin and their *O*-methyl derivatives, although a relatively high antioxidant activity was found in the case of 3′-*O*-methyl catechin at pH 7.4, comparable to those of its parent compound and the quercetin metabolites. It was confirmed that the antioxidant activity of the flavonoids assayed was strongly dependent on the pH of the medium, showing higher activity at greater pH values. The results obtained are expected to contribute to the understanding of the mechanisms involved in the biological effects attributed to the intake of flavonoid-rich diets.

1. Introduction

Flavonoids are a major class of plant polyphenolics, which comprises several thousand compounds sharing a common phenylchromane skeleton. This basic structure allows a variety of substitution patterns leading to different flavonoid subclasses, such as flavonols, flavones, flavanones, flavanols, anthocyanins, dihydroflavonols, isoflavones and chalcones. Flavonoids are widely distributed in the plant kingdom, being present in a broad range of commonly consumed fruits and vegetables and plant-derived products such as cocoa, tea or wine. The interest in dietary flavonoids has grown in the last fifteen years after the publication of several epidemiological studies showing an inverse correlation between dietary consumption of flavonoid-rich products and reduced incidence and mortality from cardiovascular disease and cancer [1–3]. Indeed a range of pharmacological effects have been demonstrated for different flavonoids in *in vitro*, *ex vivo* and animal assays. In particular, many studies have been carried out with quercetin and (epi)catechin, as they are major flavonoids in the human diet that are easily available as commercial standards. These compounds behave as powerful antioxidants and free radical scavengers [4,5] and are able to interact with several key enzymes [6]. Quercetin has shown to be an effective inhibitor of xanthine oxidase and lipoxygenase, enzymes involved in processes such as inflammation, atherosclerosis, cancer and ageing [7,8]. Catechins are able to reduce platelet aggregation and to inhibit the growth of human cancer cell lines [9], as well as to act as powerful inhibitors of LDL oxidation *in vitro* [10], decrease DNA damage, and delay tumour promotion in mouse [11,12]. However, health effects of these compounds depend on their bioavailability and, therefore, it is important to understand how they are absorbed, metabolized and eliminated from the body, in order to ascertain their *in vivo* actions.

Flavonols like quercetin mostly occur in foodstuffs as glycosides and, in general, the first step in their metabolism is likely to be deglycosylation before absorption in the small intestine

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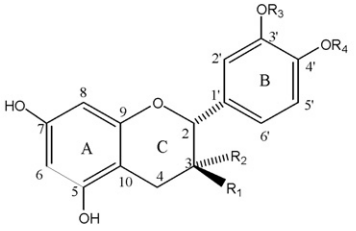
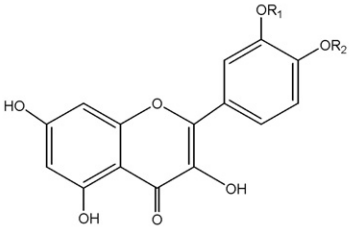
[13,14]. During transfer across the enterocite, and subsequently in the liver, quercetin undergoes *O*-methylation and other conjugation reactions, namely glucuronidation and sulphation. An important step in quercetin metabolism seems to be methylation to isorhamnetin (quercetin 3′-methylether) or tamarixetin (quercetin 4′-methylether). Similarly, flavan-3-ols such as catechin and epicatechin are absorbed from human intestinal tract and are transformed into *O*-methylated derivatives, as well as into glucuronide and sulphate conjugates [15,16]; 3′-O-methyl-(epi)catechin, 4′-*O*-methyl (epi)catechin, epicatechin 3′-*O*-glucuronide, 4′-*O*-methylepicatechin 3′-*O*-glucuronide, and 4′-*O*-methylepicatechin 5- or 7-*O*-glucuronide have been identified as the main circulating forms in human plasma [15,17]. These modifications can be expected to have an effect on their antioxidant and scavenging properties and, therefore, in order to better understand the *in vivo* effects of dietary flavonoids, it is necessary to assess the biological activity of their conjugated metabolites. The assessment of the activity of the methylated derivatives is particularly relevant since glucuronidated and sulphated derivatives are likely to be deconjugated when entering the target cells [18,19].

The *O*-methylation of the catechol group by the enzyme catechol *O*-methyl transferase is an important step in the metabolism of flavonoids. Since the antioxidant properties of flavonoids are assumed to be dependent on the availability of free hydroxyl groups, their methylation is expected to have an influence in this activity. A decrease in the antioxidant activity of quercetin following methylation of the hydroxyl groups was already observed in early studies by Crawford et al. [20]. More recently similar observations have been made regarding methylated derivatives of quercetin [21], catechin [22] and epicatechin [23]. However, as far as we know, no studies have been published comparing the activity of the methylated forms of these three flavonoids and, furthermore, the only data available for epicatechin metabolites referred to a mixture of methylated derivatives [23].

In the present paper, the antioxidant activities of the 3′- and 4′-methylethers of quercetin, catechin and epicatechin (structures shown in Fig. 1) have been compared using three different *in vitro* assays: ferric reducing power (FRAP) and two methods based on the ability to scavenge the $ABTS^{\bullet+}$ radical at different pH values. Previous to those assays the methylethers of catechin and epicatechin were prepared by hemisynthesis, purified and characterised by NMR, due to the lacking of commercial standards.



	R_1	R_2	R_3	R_4
Catechin	OH	H	H	H
Epicatechin	H	OH	H	H
3'-*O*-methyl catechin	OH	H	CH_3	H
4'-*O*-methyl catechin	OH	H	H	CH_3
3'-*O*-methyl epicatechin	H	OH	CH_3	H
4'-*O*-methyl epicatechin	H	OH	H	CH_3



	R_1	R_2
Quercetin	H	H
3'-*O*-methyl quercetin	CH_3	H
4'-*O*-methyl quercetin	H	CH_3

Fig. 1. Structures of catechin, epicatechin, quercetin and their methylated metabolites.

2. Materials and methods

2.1. Standards and reagents

Quercetin, (+)-catechin, (−)-epicatechin, methyl iodide, potassium carbonate, potassium persulphate, horseradish peroxidase type VI-A, hydrogen peroxide 30% (wt, sol. in water), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), and ±(α)-tocopherol were purchased from Sigma–Aldrich (Madrid, Spain). Isorhamnetin and tamarixetin were purchased from Extrasynthèse (Genay, France). HPLC-grade methanol and acetonitrile were purchased from Merck KGaA (Darmstadt, Germany) and CarloErba (Rodano, Italy), respectively. Analytical grade glacial acetic acid, ammonia, glycine and iron trichloride ($FeCl_3{\cdot}6H_2O$) were obtained from Panreac (Barcelona, Spain). ABTS (2,2′-azino-bis-(3-ethylbenzothiazolne-6-sulfonic acid) diammonium salt) and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) were purchased from Fluka (Madrid, Spain).

2.2. Preparation of (epi)catechin methylethers

The methylethers of catechin and epicatechin were prepared by hemisynthesis based on the protocol described by Donovan et al. [15] further modified by González-Manzano et al. [24]. Briefly, a mixture of catechin or epicatechin (250 mg), potassium carbonate (500 mg) and methyl iodide (1 mL) was prepared in acetone (20 mL) and irradiated in an ultrasonic bath. The progress of the reaction was monitored by HPLC. After a reaction time of 3.5 h the mixture was filtered and concentrated in a rotary evaporator to dryness. The methylethers of (epi)catechin synthesized were characterised by HPLC-DAD-MS. The major products of the reaction (3′- and 4′-methylethers of catechin and epicatechin) were further separated and purified by semi-preparative HPLC.

2.3. Isolation of methylated catechin and epicatechin

A Waters 600 chromatograph coupled to a UV–vis model 486 detector and a Ultracarb C18 ODS20 5 μm (10 mm × 250 mm, i.d.) column from Phenomenex (Supelco Ascentis, Bellefonte, PA, USA) were used. The separation conditions were as follows: 3 mL/min flow rate; room temperature; solvent A, water/acetic acid (95:5, v/v); solvent B, methanol; elution gradient from 0 to 20% B over 15 min, from 20 to 30% B over 25 min and from 30 to 40% over 5 min, followed by washing and re-equilibration of the column. Detection was carried out at 280 nm, and the peaks were collected in a fraction collector. Fractions containing the individual compounds were concentrated to dryness under vacuum, redissolved in water and freeze dried. The purity and identity of the compounds were checked by HPLC-DAD-MS.

2.4. HPLC-DAD-MS

Analyses were carried out in a Hewlett-Packard 1100 chromatograph (Agilent Technologies, Waldbronn, Germany) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C8, 3 μm (4.6 mm × 150 mm) column thermostatted at 30 °C was used. The solvents used were: (A) 2.5% acetic acid in water, (B) acetic acid/acetonitrile (10:90, v/v) and (C) acetonitrile. The elution gradient established was 100% A to 100% B over 5 min, 0–15% C in B over 35 min, and 15–40% C in B over 10 min, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm (catechins) and 370 nm (quercetin) as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in a Finnigan LCQ detector (Thermoquest, San Jose, CA, USA) equipped with an ESI source and an ion trap mass analyzer, which were controlled by the LCQ Xcalibur software. Both the auxiliary and the sheath gases were nitrogen at flow rates of 20 and 80 L/min, respectively. The source voltage was 4.5 kV, the capillary voltage was 11 V, and the capillary temperature was 220 °C. Spectra were recorded in negative ion mode between *m/z* 150 and 2000. The MS detector was programmed to perform a series of three consecutive scans: a full mass scan, an MS/MS scan of the most abundant ion in the first scan and an MS^3 of the most abundant ion in the MS^2 using normalized collision energy of 45%.

2.5. NMR analysis of catechin methylethers

^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were measured in CD_3OD on a Bruker Avance DRX-400 spectrometer at 298 K. The resonances at 3.30 ppm of the residual methanol in the ^1H and at 39.50 ppm for CD_3OD in the ^{13}C spectra were used as internal references. ^1H chemical shifts were assigned using 1D and 2D ^1H NMR (COSY), while ^{13}C resonances were assigned using 2D NMR (HMBC and HMQC).

2.6. In vitro evaluation of the antioxidant activity

2.6.1. ABTS/peroxidase assay

The assay was carried out according to Cano et al. [25] with minor modifications performed by Villaño et al. [26]. Free radicals were generated by an enzymatic system consisting of horseradish peroxidase enzyme, its oxidant substrate (hydrogen peroxide) and the ABTS chromophore. The radical was generated by a reaction between 1.5 mM ABTS, 15 μM hydrogen peroxide and 0.25 μM peroxidase in 50 mM glycine–HCl buffer (pH 4.5). The final volume was 60 mL, yielding a final concentration of 30 μM of the $ABTS^{\bullet+}$ radical cation. The blank reference cuvette contained glycine–HCl buffer. Once the radical was formed, the sample was added and the decrease in absorbance was monitored. The assay was carried out at room temperature. The reaction started by adding 100 μL of test sample to 2 mL of $ABTS^{\bullet+}$ solution, the samples were vortexed for 10 s, and the absorbance at 414 nm was measured after 2 min of reaction using a Hewlett Packard UV–Visible HP 8453 spectrophotometer (Palo Alto, CA, USA). Two independent experiments in triplicate were performed for each of the assayed compounds. In each case, six different dilutions were prepared in 50% aqueous methanol and submitted to the reaction. Results were expressed as Trolox equivalent antioxidant capacity (TEAC), defined as the millimolar concentration of a Trolox solution whose antioxidant capacity is equivalent to a 1.0 mM solution of the substance under study [27]. TEAC values were obtained by interpolating the decrease in absorbance on the calibration curve obtained using Trolox solutions from 15 to 500 μM.

2.6.2. ABTS/persulphate assay [28]

In this assay, the $ABTS^{\bullet+}$ radical was produced by the oxidation of 7 mM ABTS with potassium persulphate (2.45 mM final concentration) in water. The mixture was allowed to stand in the dark at room temperature for 12–16 h before use, and then the $ABTS^{\bullet+}$ solution was diluted with phosphate buffered saline (PBS) at pH 7.4 and equilibrated at 30 °C to give an absorbance of 0.7 ± 0.02 at 734 nm. A 50 μL 50% aqueous methanol of the test compound was mixed with 2 mL of the $ABTS^{\bullet+}$ preparation, vortexed for 10 s, and the absorbance measured at 734 nm after 4 min of reaction at 30 °C. Different dilutions of each of the test compounds were assayed and the results were obtained by interpolating the absorbance on a calibration curve obtained with Trolox (30–1000 μM). The results were expressed as TEAC values. Two independent experiments in triplicate were performed for each of the assayed compounds.

2.6.3. FRAP assay

Ferric reducing ability was evaluated according to Benzie and Strain [29] with minor modifications. The FRAP reagent contained 10 mM of TPTZ solution in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$, and acetate buffer (300 mM, pH 3.6) (1:1:10, v/v/v). A 100 μL 50% aqueous methanol of the test compounds was added to 3 mL of the FRAP reagent, and the absorbance was measured at 593 nm after incubation at room temperature for 6 min, using the FRAP reagent as a blank. Different dilutions of each of the test compounds were assayed and the results were obtained by interpolating the absorbance on a calibration curve obtained with Trolox (30–1000 μM). The FRAP value was defined as the milliequivalents of Trolox having the antioxidant power equivalent to a 1.0 mM solution of the substance under study. Two independent experiments in triplicate were performed for each of the assayed compounds.

2.7. Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) using the PC software package, SPSS (version 13.0; SPSS Inc., Chicago). Significant differences were assessed with an LSD test ($p < 0.05$).

3. Results and discussion

3.1. Preparation and characterisation of (epi)catechin methylethers

The 3′- and 4′-methylethers of catechin and epicatechin were obtained by chemical hemisynthesis as described in a previous work [24]; further isolation was carried out by semi-preparative HPLC and the purity and identity of the compounds was established by HPLC-DAD-ESI/MS, as also reported by González-Manzano et al. [24]. Pseudomolecular ions ($[M-H]^-$) of the compounds at *m/z* 303 confirmed their nature as (epi)catechin methylethers and MS^2 fragmentation patterns demonstrated the location of the *O*-methyl group on the B-ring. However, MS analyses did not make it possible to assign the position of methylation unequivocally (*i.e.*, C3′ or C4′), which was initially tentatively assigned based on the elution behaviour, assuming that the 3′-*O*-methyl derivative eluted earlier than the 4′-*O*-methyl derivative in reversed-phase HPLC [15]. Thus, in order to unequivocally identify the structure of the catechin methylethers NMR-1D and 2D experiments were also done. Table 1 shows the results obtained in the NMR analyses. ^1H NMR data are similar to those reported by Donovan et al. [15] for the 3′ and 4′-*O*-methylated derivatives of catechin. Those authors obtained only ^1H NMR spectra and assigned the position of the methoxyl groups based on the NOE difference spectra. In our case, the ^{13}C NMR was also obtained and the assignment of the location of the methoxyl groups was based on the HMQC and HMBC experiments.

Table 1
^{1}H, ^{13}C NMR data and HMBC correlations obtained for 3′- and 4′-*O*-methyl-catechin determined in CD_3OD.

Ring	Position	δ ^{1}H (ppm); m; *J* (Hz)	δ ^{13}C (ppm)	HMBC
4′-*O*-methyl-catechin				
C	2	4.59; d, *J* = 7.4	82.6	H-5′, H-2′, H-4
	3	3.97; m	68.8	H-2, H-4
	4eq	2.50; dd; *J* = 8.1, 16.1	28.5	H-2
	4ax	2.69; dd; *J* = 5.4, 16.1		
	9	–	156.8	H-4
	10	–	100.8	H-8, H-6, H-4
A	5	–	157.6	H-6, H-4
	6	5.85; d; *J* = 2.3	95.5	H-8
	7	–	157.8	H-8, H-6
	8	5.92; d; *J* = 2.2	96.3	H-6
B	1′	–	133.6	H-2, H-5′
	2′	6.85; bs	112.5	–
	3′	–	147.5	H-5′, H-2′
	4′	–	148.8	H-6′, H-2′,OCH_3
	5′	6.90; d; *J* = 8.2	115.1	H-6′, H-2
	6′	6.82; dd; *J* = 8.2, 1.6	119.8	H-2′, H-2
	OCH_3	3.84; s	56.9	
3′-O-methyl-catechin				
C	2	4.58; d, *J* = 7.8	83.0	H-2′, H-6′
	3	3.99; m	68.9	H-2, H-4
	4eq	2.50; dd; *J* = 8.4, 16.0	28.9	H-2
	4ax	2.88; dd; *J* = 5.5, 16.0		
	9	–	156.9	H-8, H-4
	10	–	100.9	H8, H-6, H-4
A	5	–	157.5	H-6
	6	5.85; d; *J* = 2.2	95.5	H-8
	7	–	157.9	H-8, H-6
	8	5.92; d; *J* = 2.2	96.4	H-6
B	1′	–	132.1	H-5′, H-2
	2′	6.96; bs	112.0	H-6′, H-2
	3′	–	148.9	H-2′, H-5′, OCH_3
	4′	–	147.5	H-2′, H-6′
	5′	6.78; d; *J* = 8.0	115.9	–
	6′	6.84; dd; *J* = 8.2, 1.6	121.3	H-2′
	OCH_3	3.83; s	56.4	

s: singlet; d: doublet; dd: double doublets; bs: broad singlet; m: multiplet.

Protons and carbons were easily assigned according to their ^{1}H and ^{13}C NMR chemical shifts by comparison with data published for catechin [30] and included in the database NAPROC-13 [31]. The relative *trans* configuration of catechin between C2 and C3 was deduced from the coupling constants value between H-2 and H-3 (*J* = 7.4 Hz) in the ^{1}H NMR spectrum. The assignment of the methoxyl group at the 3′ position was based on the HMBC correlation between the carbon C3′ (148.9 ppm) with the protons H-2′ (6.96 ppm) and H-5′ (6.78 ppm) as well with the methoxyl protons (3.83 ppm). Similarly, 4′-*O*-methyl catechin was characterised based on the correlation between the carbon C4′ (148.8 ppm) and protons H-2′ (6.85 ppm) and H-6′ (6.82 ppm) and methoxyl protons (3.84 ppm). In addition, ^{1}H and ^{13}C NMR analyses of a standard of (+)-catechin were also made and the results compared with those obtained for the methylated derivatives, which allowed the confirmation of the position of the methoxyl group based on the shifts in the carbon signals located on the B-ring.

The results obtained in the NMR analyses allowed the unequivocal identification of 3′-*O*-methyl and 4′-*O*-methyl catechin and permitted to confirm the earlier elution in RP-HPLC of the 3′-*O*-methyl derivative with regard to the 4′-*O*-methyl derivative.

3.2. In vitro antioxidant evaluation

The antioxidant activity of quercetin, catechin, epicatechin and their methylated conjugates was assessed using FRAP and ABTS scavenging assays. The FRAP assay evaluates the ability of a substance to reduce Fe^{3+} to Fe^{2+}, which is measured by the formation of a coloured complex with TPTZ that can be read spectrophotometrically at 593 nm. Since the antioxidant activity of a substance is usually correlated to its reducing capacity, this assay provides a reliable method to evaluate the antioxidant activity [29]. ABTS assays measure the ability of an antioxidant to scavenge the $ABTS^{\bullet+}$ radical cation. In the original method developed by Miller et al. [32], metmyoglobin and H_2O_2 were used to generate ferrylmyoglobin, which then reacted with ABTS to form the $ABTS^{\bullet+}$ radical. Different strategies have been further implemented for $ABTS^{\bullet+}$ generation, using either chemical or enzyme reactions; chemical generation usually requires longer times, whereas enzyme generation is faster and the reaction conditions are milder. In this study two different assays have been employed differing in the way of generation of the $ABTS^{\bullet+}$ radical and the pH value used: an enzymatic protocol using horseradish peroxidase and pH 4.5 [25], and a chemical assay using persulphate and a pH value of 7.4, close to physiological conditions [28].

Table 2 shows the values of antioxidant activity obtained for quercetin, catechin, epicatechin, their methylated derivatives and α-tocopherol used as standard in the three different assays. The three studied flavonoids behaved in all the assays as significantly better antioxidants than α-tocopherol, being quercetin the most potent compound, with an activity three to sevenfold greater than that of the vitamin. The three flavonoids presented higher TEAC values in the ABTS/persulphate assay carried out a 7.4. This result agrees with the observations of Lemanska et al. [33] and Muzolf et

Table 2
Values of antioxidant activity of quercetin, catechin, epicatechin and their methylated derivatives in the different *in vitro* antioxidant assays, expressed as Trolox equivalents.

Compound	ABTS/peroxidase	ABTS/persulphate	FRAP
Quercetin	$3.08 \pm 0.05^{a}{}_{j}$	$6.76 \pm 0.30^{b}{}_{i}$	$3.07 \pm 0.01^{a}{}_{i}$
Catechin	$1.34 \pm 0.02^{a}{}_{g}$	$3.84 \pm 0.01^{b}{}_{gh}$	$1.53 \pm 0.22^{a}{}_{fgh}$
Epicatechin	$2.07 \pm 0.09^{b}{}_{i}$	$4.26 \pm 0.25^{c}{}_{h}$	$1.34 \pm 0.01^{a}{}_{efg}$
3′-*O*-Methylquercetin (isorhamnetin)	$1.71 \pm 0.06^{a}{}_{h}$	$2.13 \pm 0.04^{b}{}_{ef}$	$1.82 \pm 0.11^{a}{}_{h}$
4′-*O*-Methylquercetin (tamarixetin)	$1.85 \pm 0.06^{a}{}_{h}$	$3.39 \pm 0.03^{b}{}_{g}$	$1.65 \pm 0.06^{a}{}_{gh}$
3′-*O*-Methyl-catechin	$0.25 \pm 0.01^{a}{}_{de}$	$3.59 \pm 0.37^{c}{}_{g}$	$1.29 \pm 0.03^{b}{}_{ef}$
4′-*O*-Methyl-catechin	$0.21 \pm 0.00^{a}{}_{d}$	$2.24 \pm 0.09^{c}{}_{ef}$	$0.86 \pm 0.03^{b}{}_{d}$
3′-*O*-Methyl-epicatechin	$0.39 \pm 0.01^{a}{}_{e}$	$1.88 \pm 0.06^{c}{}_{e}$	$0.92 \pm 0.01^{b}{}_{d}$
4′-*O*-Methyl-epicatechin	$0.34 \pm 0.01^{a}{}_{de}$	$2.54 \pm 0.02^{c}{}_{f}$	$1.12 \pm 0.08^{b}{}_{de}$
α-Tocopherol	$1.01 \pm 0.02^{a}{}_{f}$	$0.98 \pm 0.03^{a}{}_{d}$	$1.18 \pm 0.01^{b}{}_{de}$

[a,b,c]Mean values in the same row with different letters are significantly different: LSD ($p < 0.05$). [d,e,f,g,h,i,j]Mean values in the same column with different letters are significantly different: LSD ($p < 0.05$).

al. [34] that found that the radical scavenging capacity of polyhydroxylflavones and catechins, respectively, increased with the pH of the medium, due to an increase in the electron donating ability upon deprotonation.

Nonetheless, the two ABTS assays also differed in their reaction time (4 min in the persulphate assay and 2 min in the peroxidase assay). Thus, in order to check if the difference in the TEAC values obtained could be due to the different reaction time rather than to the pH value, kinetic studies were carried out for all the compounds using increasing reaction times from 2 to 20 min. In all cases, the TEAC values obtained in the persulphate assay (pH 7.4) were higher than in the peroxidase assay (pH 4.5), indicating that the different results obtained in each assay were actually related to the pH value irrespective of the time of assay. The fact that greater activity as radical scavengers was obtained at pH 7.4 suggests that similar ability can be expected at physiological pH values.

The antioxidant properties of the polyphenols have been related to their ability to scavenge free radicals and thus act as chain-breaking compounds. Whereas the resorcinol A-ring acts mainly through H-atom transfer reaction, the catechol B-ring is specifically involved in electronic transfer from the phenolate. This latter has been indicated to be the main mechanism to explain the scavenging activity of the flavonoids [22,33]. In our results (Table 2), lower antioxidant activity was found for the three studied flavonoids in the assays performed at acidic pH values (*i.e.*, ABTS/peroxidase and FRAP assays), at which neutral forms of the molecules predominate and hydrogen atom donation would contribute to the antioxidant effect. This result would confirm that electron transfer from the phenolate ion is a more important mechanism to explain the antioxidant activity of the assayed compounds. In the assays performed at acidic pH quercetin also behaves as better antioxidant than catechins, suggesting that it is not only a better electron donor but also more efficient hydrogen-donating compound.

The observation that catechins showed lower antioxidant activity than quercetin was already made by other authors [4,35–37]. Although catechins have the same number and location of the hydroxyl substituents that quercetin (Fig. 1), differences exist between both types of flavonoids regarding the acidities of their phenolic hydroxyls and the ability to stabilise the phenoxyl radical upon formation by electron delocalisation. All the hydroxyl phenolic moieties in (epi)catechin have similar pK_a values, ranging 9.02–9.58 [38], whereas in the case of quercetin the pK_a values are dependent on the position of the hydroxyl, having those at C7 and C4′ the easiest deprotonation with pK_a situated in the range 7.0–8.3 [21,37]. Therefore, for a same pH value greater proportion of phenolate ions and higher electron donating activity should exist in the case of quercetin than in that of (epi)catechin. In addition, quercetin structure has greater ability for electron delocalisation and stabilisation of the phenoxyl radicals due to the conjugation of rings A and B as provided by the 4-oxo group, hydroxyl at C3 and 2,3-unsaturation in the C-ring [4], leading to more stable oxidation products than in the case of catechins [35].

Comparison of the antioxidant activities of the two studied catechins shows that epicatechin presents higher TEAC values than catechin in the ABTS scavenging assays, although the differences were only significant in the peroxidase assay (Table 2). No differences were found between them in the FRAP assay. Greater ability of epicatechin than catechin to scavenge the ABTS radical was also observed by Muzolf et al. [34]. Since both compounds present similar structure and no relevant differences exist between them regarding the acidity of their phenolic hydroxyl groups nor their ability to act as hydrogen and electron donors [39], a possible explanation of their distinct behaviour might be found in the different stereochemistry of the hydroxyl group at C3 and the larger charge area of epicatechin compared to catechin [40], which could provide a more effective electron delocalisation.

The *O*-methylation of the hydroxyls at C3′ or C4′ of the studied flavonoids resulted in a decrease of their TEAC values in the three antioxidant assays (Table 2), which is in agreement with previous observations by other authors [20–23]. Nevertheless, despite the decrease produced, the methylated derivatives still behaved as better antioxidants than α-tocopherol in the radical scavenging assays carried out at pH 7.4, indicating that they still retain significant antioxidant activity at physiological pH. In the case of quercetin greater antioxidant activity than the vitamin is also retained in the assays performed in acidic conditions.

In our study no significant differences between the antioxidant activities of the two methylated metabolites of quercetin were observed in the assays performed in acidic media, but significantly higher radical scavenging activity was demonstrated by the 4′-*O*-methyl quercetin in the assay carried out at pH 7.4 (Table 2). An activation of the 3′-OH when a methoxyl group exists at 4′ position was observed by van Acker et al. [41] when comparing the relative antioxidant activities of different flavonoids, which might contribute to explain this result. However, this result does not agree with those obtained by other authors [21,23] that found greater radical scavenging activity for 3′-*O*-methyl quercetin than for the corresponding 4′ derivative, even though the differences were little and might be not significant (significance was not reported by the authors). The decrease in the radical scavenging activity of quercetin upon methylation of the catechol moiety has been explained by an increase in the pK_a of the molecule that results in lower levels of deprotonation at physiological pH; furthermore, the *O*-methylation would affect the electronic properties (especially of the deprotonated forms) decreasing the ability for electron and hydrogen atom donation [21]. According to Lemanska et al. [21], the *O*-methylation could increase the pK_a of quercetin by up to 1 pH unit, being the extent of that increase somewhat larger in the case of the 4′-*O*-methyl derivative, which would explain its lower radical scavenging ability compared to 3′-*O*-methyl quercetin. No

actual explanation for the differences regarding the behaviour of the two methylated quercetin derivatives between our study and those performed by other authors can be offered, but for the different experimental approaches used in each case.

Different *in vitro* studies reported genotoxic effects for quercetin associated to the pro-oxidant activity of reactive electrophilic quinones resulting from its oxidation [42,43], although *in vivo* carcinogenicity has failed to be demonstrated for quercetin. It has been recently shown that methylation of a catechol hydroxyl group considerably attenuates the cellular implications of the pro-oxidant activity of quercetin [44]. Since the extent of the pro-oxidant characteristics of quercetin are dependent on its electron donating ability, the decrease in this capacity associated to the methylation may also provide an explanation for the attenuation in the genotoxicity of quercetin [44]. Thus, the *O*-methylation would contribute to reduce the *in vivo* toxicity of these compounds keeping a part of their antioxidant activity.

The decrease in the antioxidant activity of catechins upon methylation was already observed by Cren-Olivé et al. [22] and Pollard et al. [23] using different methods. The first authors evaluated the ability of distinct methylated catechin derivatives to inhibit LDL oxidation, while Pollard et al. studied the capacity of a mixture of 3′- and 4′-*O*-methyl epicatechin to inhibit peroxynitrite-induced tyrosine nitration and to scavenge the ABTS radical. However, as far as we know, no previous studies have been published regarding the antioxidant activity of the individual 3′- and 4′-*O*-methyl epicatechin neither the antioxidant activity of the methyl derivatives of catechin and epicatechin has been compared.

In catechins similar reactivity exists for the hydroxyls at C3′ and C4′ [38] and, therefore, similar modifications in the pK_a values could be expected upon methylation at any of these positions, as well as a similar decrease in the antioxidant activities with regard to the parent compound. However, our results revealed significant differences in the behaviour of the 3′- and 4′-*O*-methyl derivatives of the two catechins. Thus, 3′-*O*-methylcatechin showed to be a more efficient scavenger than 4′-*O*-methylcatechin at pH 7.4, whereas the opposite was observed in the case of the epicatechin metabolites (Table 2). Since the only relevant difference between both types of catechins is the stereochemistry of the hydroxyl at C3 position, it can be speculated that this might account for the different behaviour observed for these compounds, although it is unclear how this influence could be exerted. Particularly noteworthy is the scavenging activity demonstrated by 3′-*O*-methyl catechin, which showed not significant differences with that of the parent compound and behaved as the best antioxidant methylated metabolite among those studied in the ABTS/persulphate assay at pH 7.4 (Table 2). This observation is relevant since 3′-*O*-methyl catechin is a major metabolite of catechin in humans [15,16].

4. Conclusions

The results obtained in this study confirm that quercetin, catechin and epicatechin are better radical scavengers and reducing compounds than usually recognised antioxidants like α-tocopherol. The *O*-methylation of the hydroxyls of the catechol B-ring, a main metabolic pathway for these flavonoids, resulted in a decrease of the antioxidant activity with regard to the parent compounds. However, the methylated metabolites still retain significant radical scavenging activity at pH 7.4, suggesting that they could act as potential antioxidants in physiological conditions. Quercetin and its methylated metabolites showed, in general, greater activity than (epi)catechin and their *O*-methyl derivatives, although a relatively high antioxidant activity was found in the case of 3′-*O*-methyl catechin at pH 7.4 comparable to those of catechin and the methylated forms of quercetin. It was confirmed that the antioxidant activity of these flavonoids is strongly dependent on the pH of the medium, higher activity being shown at greater pH values. Whereas most antioxidant assays are usually performed at acidic pH's, this observation stresses the importance of measuring the antioxidant ability at pH values close to the neutrality in order to better assess what occurs in physiological conditions.

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