JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Characterization of Sulfated Quercetin and Epicatechin Metabolites

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ABSTRACT: Different monosulfates of quercetin and epicatechin with metabolic interest were obtained by hemisynthesis and characterized regarding their chromatographic behavior and absorption and mass spectra. Three of these compounds were further isolated, and their structures were elucidated by mass spectrometry and ^{1}H and ^{13}C nuclear magnetic resonance using one- and two-dimensional techniques (heteronuclear single-quantum coherence and heteronuclear multiple-bond correlation). The calculation of the proton and carbon shifts caused by sulfation allowed for the assignment of the position of the sulfate group in the flavonoids, so that the compounds were identified as quercetin-3′-*O*-sulfate, quercetin 4′-*O*-sulfate, and epicatechin 4′-*O*-sulfate. It was found that sulfation at position 3′ induced a large upfield shift in the carbon bearing the sulfate group and downfield displacements of the adjacent carbons, whereas no significant upfield or downfield shifts were observed with respect to the parent flavonoid when sulfation was produced at position 4′.

KEYWORDS: *Quercetin, epicatechin, metabolites, sulfates, NMR*

INTRODUCTION

Flavonoids are plant secondary metabolites of phenolic nature that are widely distributed in the human diet, being present in a broad range of commonly consumed fruits and vegetables and derived products, such as cocoa, tea, or wine. In recent years, there has been a growing interest in the potential health benefits of dietary flavonoids, because they have been related to a reduced risk of different chronic diseases and especially cardiovascular disease.[1−3] Flavonols and flavan-3-ols (i.e., catechins and proanthocyanidins) are major classes of flavonoids in the human diet, and among them, quercetin and catechins have been particularly used in studies of biological activity. These compounds are generally known to possess antioxidant and radical-scavenging properties,[4,5] as well as the ability to modulate the activity of several metabolically relevant enzymes and exert a wide range of biological effects.[6] Quercetin acts as an effective inhibitor of xanthine oxidase and lipoxygenase that play a key role in processes such as inflammation, atherosclerosis, cancer, or aging,[7] shows vasodilatation and anti-aggregant effects *in vitro*, and reduces blood pressure, oxidative status, and end-organ damage in animal models of hypertension.[8,9] Catechins are able to reduce platelet aggregation and inhibit the growth of human cancer cell lines,[10] as well as to act as powerful inhibitors of low-density lipoprotein (LDL) oxidation *in vitro*,[11] decrease DNA damage, and delay tumor promotion in mice.[12,13]

Health effects of flavonoids depend upon their bioavailability. Flavonols, such as 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.[14,15] During transfer across the enterocyte and, subsequently, in the liver, quercetin undergoes O-methylation and other conjugation reactions, namely, glucuronidation and sulfation. Catechins are not usually glycosylated and can be partially absorbed as such from the human intestinal tract to be further transformed into O-methylated derivatives and glucuronide and sulfate conjugates.[16,17] The biological activity of these metabolites is expected to be different of that of the parent flavonoids, as affected by the nature and position of the conjugating moieties. Therefore, to better understand the *in vivo* effects of dietary flavonoids, it is important to take into account the distribution, activity, and metabolic fate of the metabolites, as well as to be in the disposition of analyzing and identifying them in biological fluids and organs, for which adequate standards are required. However, most flavonoid metabolites are not commercially available and must, therefore, be prepared in the laboratory. Some works have been published dealing with the preparation of conjugated metabolites of quercetin (glucuronides and sulfates), using enzymatic or chemical synthesis,[18−24] but fewer studies exist regarding conjugated catechins.[18,25] The lack of suitable procedures is especially important in the case of sulfates that are more chemically labile than other metabolites, such as glucuronides or methylethers. In a previous paper of our group,[25] the hemisynthesis of catechin sulfates using an adaptation of the method described by Jones et al.[20] for quercetin sulfates was reported. In that paper, the identification of the synthesized compounds was based on their mass spectra fragmentation patterns that only allowed for establishing their nature as sulfates and the flavonoid ring on which the sulfation occurred but not the precise substituted hydroxyl. In view to confirm their actual structure, in the present work, different monosulfates of quercetin and epicatechin (Figure 1) were prepared and characterized by nuclear magnetic resonance (NMR).

MATERIALS AND METHODS

Standards and Reagents. High-performance liquid chromatography (HPLC)-grade methanol was purchased from Carlo Erba (Rodano, Italy). Pyridine and HPLC-grade acetonitrile were obtained

Received: April 15, 2011
Revised: March 8, 2012
Accepted: March 16, 2012
Published: March 16, 2012

dx.doi.org/10.1021/jf2050203 | *J. Agric. Food Chem.* 2012, 60, 3592−3598

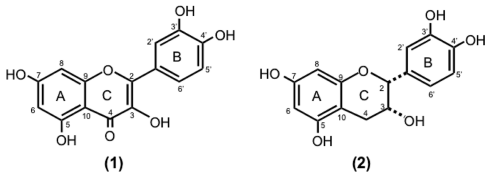

Figure 1. Structures of quercetin (**1**) and epicatechin (**2**). In the case of the different sulfated metabolites, the corresponding −OH is substituted by a $-O-SO_3H$ group.

from Merck KGaA (Darmstadt, Germany). Trifluoroacetic acid (TFA) was from Riedel-de-Haën (Seeize, Germany), and analytical-grade glacial acetic acid was from Panreac (Barcelona, Spain). Quercetin, epicatechin, sulfur trioxide−*N*-triethylamine complex, Sephadex LH-20, and dioxane were purchased from Sigma-Aldrich (Madrid, Spain).

Synthesis of Quercetin and Epicatechin Sulfates. These metabolites were synthesized by an adaptation of the method described by Jones et al.[20] First water associated with quercetin or epicatechin (500 mg each) was removed by adding dry pyridine until dissolved. Pyridine was rotary evaporated, and the dry compound was dissolved in dioxane (50 mL) and allowed to react in a water bath (40 °C) for 90 min with a 10-fold molar excess of sulfur trioxide−*N*-triethylamine complex under nitrogen to avoid contact with air. Products of sulfation precipitated out and stuck to the glass. Dioxane was decanted, and the synthesized mixture redissolved in 10% methanol in water. The mixtures of quercetin and epicatechin sulfates were fractioned on a Sephadex LH-20 column (350 × 30 mm), successively eluted with 10% aqueous ethanol (500 mL) and 20% aqueous ethanol (500 mL). The fractions containing monosulfates were collected, concentrated to dryness under vacuum, redissolved in ultrapure water, and analyzed by high-performance liquid chromatography−diode array detector−mass spectrometry (HPLC−DAD−MS). Individual quercetin and epicatechin sulfates were further isolated by semi-preparative HPLC.

Semi-preparative HPLC. A Waters 600 chromatograph coupled to an ultraviolet−visible (UV−vis) model 486 detector and an Ultracarb C18ODS20 5 μm (10 × 250 mm inner diameter) column from Phenomenex (Supelco Ascentis, Bellefonte, PA) were used. Solvents and the gradient employed for the separation of quercetin sulfates were as follows: solvent A, water; solvent B, methanol; gradient, from 0 to 15% B in 5 min, from 15 to 25% B in 30 min, from 25 to 50% B in 20 min, and isocratic 50% B for 15 min. For the separation of epicatechin sulfates, the solvents were (A) 5% acetic acid and (B) methanol. The gradient used was from 0 to 10% B in 40 min and from 10 to 12% B in 20 min. The flow rate was 3 mL min^{-1} in both cases. Detection was carried out at 370 nm (quercetin) and 280 nm (epicatechin). Peaks were automatically collected in a fraction collector, concentrated under vacuum, and freeze-dried. The identity of the compounds was checked by HPLC−DAD−electrospray ionization (ESI)/MS and NMR.

HPLC−DAD−MS Analyses. Analyses were carried out in a Hewlett-Packard 1100 chromatograph (Agilent Technologies, Waldbronn, Germany) with a quaternary pump and a DAD coupled to a HP Chem Station (revision A.05.04) data-processing station.

Separation was achieved on an AQUA (Phenomenex, Torrance, CA) reversed-phase C18 column (5 μm, 150 × 4.6 mm inner diameter) thermostatted at 35 °C. For the analysis of quercetin sulfates, solvents used were (A) 0.1% TFA in water and (B) acetonitrile and the elution gradient was from 10 to 15% B over 5 min, from 15 to 25% B over 5 min, from 25 to 35% B over 10 min, from 35 to 50% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, at a flow rate of 0.5 mL/min. For the analysis of epicatechin sulfates, a Water Spherisorb S3OD-2 C18 (3 μm, 150 × 4.6 mm inner diameter) column was used. The solvents were (A) 0.1% TFA in water and (B) acetonitrile, and the elution gradient was from 0 to 10% B over 5 min, from 10 to 15% B over 20 min, and from 20 to 40% C in B over 5 min, using a flow rate of 0.5 mL min^{-1}. Double online detection was carried out in the DAD using 280 nm (epicatechin) and 370 nm (quercetin) as preferred wavelengths and in a mass spectrometer connected to the HPLC system via the DAD cell outlet.

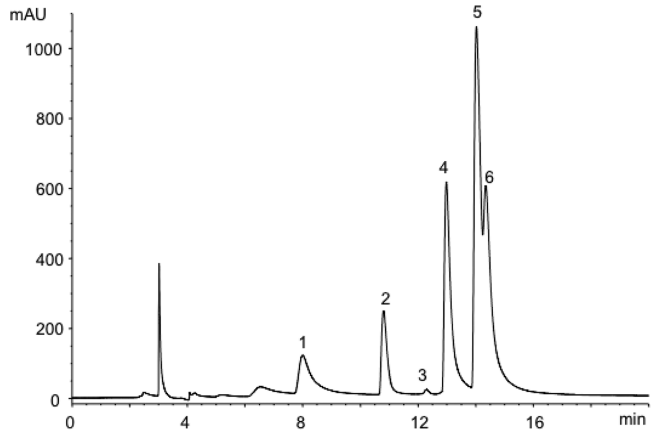

Figure 2. Chromatogram recorded at 370 nm showing the profile of quercetin monosulfates obtained by hemisynthesis and further separation on a Sephadex LH-20 column. See Table 1 for peak identification.

Table 1. Retention Time and UV and Mass Spectral Data Obtained for Quercetin and Sulfated Metabolites Observed in the Chromatogram of Figure 2

compounds	peak number	retention time (min)	λ_{max} (nm)	molecular ion $[M + H]^+$ (m/z)	MS^2 (m/z)
quercetin disulfate	1	8.0	368	461	381, 301
quercetin 3-*O*-sulfate	2	10.8	351	381	301
quercetin 5-*O*-sulfate	3	12.2	364	381	301
quercetin 7-*O*-sulfate	4	12.9	371	381	301
quercetin 4′-*O*-sulfate	5	14.0	366	381	301
quercetin 3′-*O*-sulfate	6	14.3	368	381	301
quercetin		22.1	372	301	273, 257, 229, 179, 151

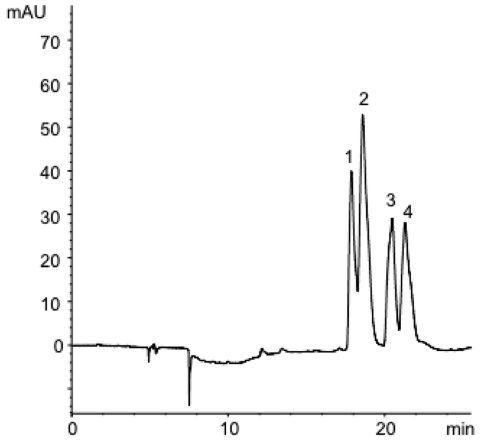

Figure 3. Chromatogram recorded at 280 nm showing the profile of epicatechin monosulfates obtained. See Table 4 for peak identification.

dx.doi.org/10.1021/jf2050203 | *J. Agric. Food Chem.* 2012, 60, 3592−3598

Table 2. ^{1}H and ^{13}C NMR Data and HMBC Correlations Obtained for Quercetin, Quercetin 3′-*O*-Sulfate, and Quercetin 4′-*O*-Sulfate Determined in CD_3OD

ring	position	δ ^{1}H (ppm); m; J (Hz)[a]	δ ^{13}C (ppm)	HMBC
		Quercetin		
C	2		147.9	H-6′, H-2′
	3		137.2	
	4eq		177.3	
	4ax			
	9		158.2	H-8
	10		104.5	H-8, H-6
A	5		162.4	H-6
	6	6.16; d; J = 2.0	99.2	H-8
	7		165.5	H-8, H-6
	8	6.36; d; J = 2.0	94.3	H-6
B	1′		124.1	H-5′
	2′	7.72; d; J = 2.1	115.9	H-6′
	3′		146.2	H-5′, H-2′
	4′		148.7	H-6′, H-2′, H-5′
	5′	6.86; d; J = 8.5	116.2	H-6′
	6′	7.61; dd; J = 8.4, 2.2	121.6	H-2′
		Quercetin 4′-*O*-Sulfate		
C	2		147.9	H-2′, H-6′
	3		137.2	
	4eq		177.3	
	4ax			
	9		158.2	H-8
	10		104.5	H-8, H-6
A	5		162.5	H-6
	6	6.16; d; J = 2.0	99.2	H-8
	7		165.5	H-8, H-6
	8	6.37; d; J = 2.0	94.3	H-6
B	1′		124.1	H-5′
	2′	7.72; d; J = 2.1	115.9	H-6′
	3′		146.2	H-2′, H-5′
	4′		148.7	H-6′, H-2′, H-5′
	5′	6.87; d; J = 8.4	116.2	H-6′
	6′	7.62; dd; J = 8.4, 2.2	121.6	H-2′
		Quercetin 3′-*O*-Sulfate		
C	2		147.0	H-6′, H-2′
	3		137.6	
	4eq		177.4	
	4ax			
	9		158.2	H-8
	10		104.5	H-8, H-6
A	5		162.5	H-6
	6	6.16; d; J = 2.0	99.3	H-8
	7		165.7	H-8, H-6
	8	6.41; d; J = 2.0	94.4	H-6
B	1′		124.3	H-5′
	2′	8.19; d; J = 2.1	123.8	H-6′
	3′		141.3	H-5′, H-2′
	4′		152.6	H-6′, H-2′, H-5′
	5′	7.00; d; J = 8.6	118.2	unresolved
	6′	8.00; dd; J = 8.6, 2.1	127.4	H-2′

[a]s, singlet; d, doublet; dd, double doublets; bs, broad singlet; m, multiplet.

MS detection was performed in a Finnigan LCQ detector (Thermoquest, San Jose, CA) equipped with an ESI source and an ion-trap mass analyzer, which were controlled by the LCQ Xcalibur software. Both the auxiliary and 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 two consecutive scans: a full scan and a MS−MS scan of the most abundant ion in the first scan, using normalized collision energy of 45%.

NMR Analysis of Quercetin and Epicatechin Sulfates. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra of the isolated metabolites 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 ^{1}H spectra and at 39.50 ppm for CD_3OD in the ^{13}C spectra were used as internal references. ^{1}H chemical shifts were assigned using one-dimensional (1D) and two-dimensional (2D) ^{1}H NMR [correlation spectroscopy (COSY)], while ^{13}C resonances were assigned using 2D NMR [heteronuclear multiple-bond correlation (HMBC) and heteronuclear multiple-quantum coherence (HMQC)].

Table 3. ^{13}C NMR Shifts Induced by 3′- or 4′-Sulfation in Quercetin[a]

carbon	quercetin 3′-*O*-sulfate	quercetin 4′-*O*-sulfate
2	+0.9	0.0
3	−0.4	0.0
4	−0.1	0.0
10	0.0	0.0
1′	−0.2	0.0
2′	−7.9	0.0
3′	+4.9	0.0
4′	−3.9	0.0
5′	−2.0	0.0
6′	−5.8	0.0

[a]Refer to $\delta_{aglycone} - \delta_{ester}$.

Table 4. Retention Time and UV and Mass Spectral Data Obtained for Epicatechin and Their Monosulfated Metabolites Observed in the Chromatogram of Figure 3

compounds	peak number	retention time (min)	λ_{max} (nm)	molecular ion $[M - H]^-$ (m/z)	MS^2 (m/z)
epicatechin 5-*O*-sulfate	1	17.9	278	369	217, 245, 247, 289
epicatechin 3′-*O*-sulfate	2	18.5	277	369	201, 231, 245, 289
epicatechin 7-*O*-sulfate	3	20.4	275	369	217, 245, 247, 289
epicatechin 4′-*O*-sulfate	4	21.1	274	369	201, 231, 245, 289
epicatechin		27.8	279	289	245, 203, 179, 151, 137

RESULTS AND DISCUSSION

Preparation of Quercetin and Epicatechin Sulfates. Chemical hemisynthesis of quercetin and epicatechin sulfates was performed on the basis of the procedure described by Jones et al.[20] for the preparation of sulfates of quercetin, with some modifications, as previously reported by our group.[25] For both flavonoids, complex mixtures presenting diversely sulfated compounds and the aglycones were obtained that were fractionated on Sephadex LH-20 to separate respective fractions containing mostly monosulfates (Figures 2 and 3).

Identification of Quercetin Sulfates. Table 1 shows the retention and absorption and mass spectral data of the peaks corresponding to quercetin sulfates observed in the

dx.doi.org/10.1021/jf2050203 | J. Agric. Food Chem. 2012, 60, 3592−3598

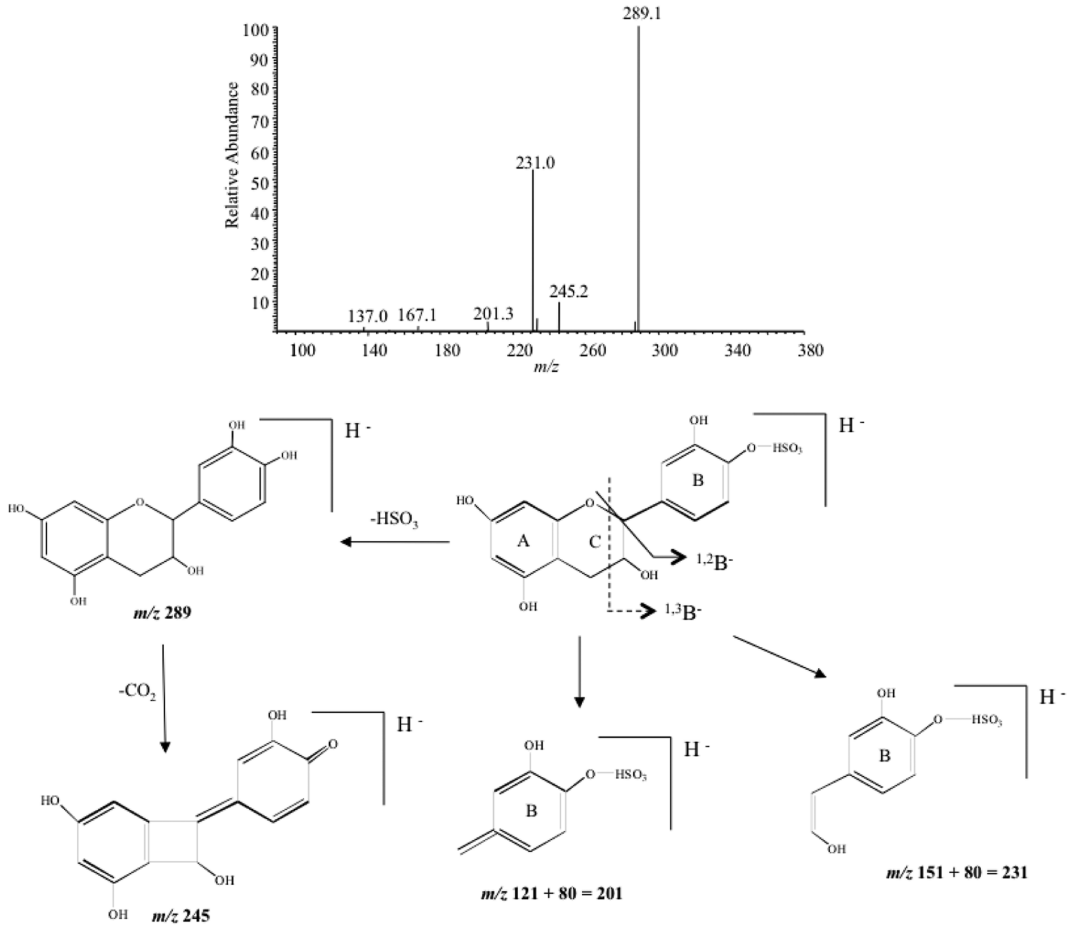

Figure 4. MS^2 spectrum of epicatechin 4′-*O*-sulfate in negative-ion mode.

chromatogram of Figure 2, as well as those of a standard of quercetin obtained in the same analytical conditions.

Tentative assignment of the monosulfates in the chromatograms was first made assuming the same elution order in reversed phase reported by Jones et al.,[20] i.e., quercetin 3-*O*-sulfate, quercetin 7-*O*-sulfate, quercetin 3′-*O*-sulfate, and quercetin 4′-*O*-sulfate. It should be indicated that no formation of quercetin 5-*O*-sulfate was reported by those authors. Indeed, the hydroxyl group at position 5 of quercetin is by far the least acidic,[18] which makes it less likely to be substituted under the mildly basic conditions employed for the sulfation reaction. Therefore, the sulfate derivative at position 5 would be expected to be produced to a lower extent than the other quercetin monosulfates, so that it could be associated to peak 3 in our chromatogram.

It has been reported that conjugation of the hydroxyl group at position 3 of quercetin and other flavonols causes a band I hypsochromic shift between 13 and 30 nm,[22,26−29] whereas smaller or no shifts in this band occurred when the conjugation takes place in other positions.[22,28] In our case, a large hypsochromic shift of λ_{max} in band I (−21 nm) in relation to quercetin was observed for peak 2, whereas small displacements of 4−8 nm were found for peaks 3, 5, and 6 and no displacement for peak 4. This observation supports the proposed identity of peak 2 as quercetin 3-*O*-sulfate.

Separation of the sulfate mixture by semi-preparative HPLC allowed us to obtain compounds **5** (24 mg, representing a final yield of 4.8%) and **6** (10 mg, representing a final yield of 2%) in the pure state to be submitted to NMR analysis. The other peaks observed in the chromatogram of Figure 2 could not be isolated in a sufficient amount and/or with a sufficient degree of purity for their identification by NMR.

The results obtained in the NMR analysis of peaks 5 and 6 are shown in Table 2 compared to a standard of quercetin. Data obtained for peak 6 agree with those previously published for quercetin 3′-*O*-sulfate by other authors.[20,21,30] The assignment of the location of the sulfate group at position 3′ was based on the characteristic up- and downfield displacements of protons and carbons with respect to quercetin (Table 3). In the ^{1}H NMR spectrum, representative downfield shifts of H-2′ (8.19 ppm) and H-6′ (8.00 ppm), which are in positions *ortho* and *para* with respect to the sulfate group, compared to quercetin (H-2′ and H-6′ at 7.72 and 7.61 ppm, respectively) are observed. Further confirmation of the presence of the sulfate group at position 3′ was obtained from the carbon shifts observed in the ^{13}C NMR spectrum. A large upfield shift of 4.9 ppm was produced in C-3′ in relation to quercetin, while C-2′/C-4′ and C-6′ (in *ortho* and *para* positions with regard to C-3′, respectively) underwent significant downfield shifts (−7.9, −3.9, and −5.8 ppm, respectively). These observations are also in agreement with the expected upfield displacement for carbons carrying sulfate groups and downfield shifts for the α carbons in relation to the carbon bearing the sulfate.[31]

Interestingly, in the case of peak 5, tentatively assigned as quercetin 4′-*O*-sulfate based on its elution characteristics, neither up- nor downfield displacements for protons and carbons with respect to quercetin were observed in the NMR spectra (Table 3). The possibility that this result could be due

dx.doi.org/10.1021/jf2050203 | J. Agric. Food Chem. 2012, 60, 3592−3598

to a loss of the sulfate group during compound isolation or preparation for NMR analysis was discarded, because further analysis of the sample by HPLC−DAD−ESI/MS after being submitted to NMR analysis confirmed that the peak of the compound appeared at the expected retention time (14 min) and showed the same absorption spectrum and pseudomolecular ion ($[M - H]^-$ at m/z 381) as the original peak. Therefore, it was concluded that the presence of the sulfate at 4′ position does not modify the magnetic field of the molecule with respect to the parent quercetin. As far as we know, no previous NMR data have been reported for quercetin 4′-*O*-sulfate, although Barron and Ibrahim[30] performed NMR analysis of quercetin-3,4′-*O*-disulfate, suggesting some effect on the shift pattern for the 4′ position. However, those authors obtained the spectra in dimethyl sulfoxide (DMSO), whereas our analysis was performed in CD_3OD, which may have an influence on the chemical shifts. On the other hand, the NMR behavior of the 4′ position of a quercetin disulfate should not be necessarily reflected in the corresponding monosulfate. Furthermore, the apparently unexpected behavior observed in our study for quercetin 4′-*O*-sulfate could not be totally unforeseen, because it was in agreement with data predicted by the "Predict ^{1}H NMR" option of ChemBioDraw Ultra software based on the tables of chemical shifts for structural determination of organic compounds.[32] Finally, the same behavior was also observed in the case of epicatechin 4′-*O*-sulfate, as discussed below.

Identification of Epicatechin Sulfates. Figure 3 shows the profile of monosulfates collected after fractionation of the crude reaction mixture in the Sephadex LH-20 column. Four compounds were obtained that were tentatively identified as epicatechin 5-*O*-sulfate, epicatechin 3′-*O*-sulfate, epicatechin 7-*O*-sulfate, and epicatechin 4′-*O*-sulfate based on their mass spectra fragmentation patterns and retention characteristics, as discussed in a previous paper by our group.[25] In catechins, there are no large differences in the acidities of the four phenolic hydroxyls, which possess pK_a values between 9.02 and 9.58[33] and are more reactive in the synthesis conditions used than the non-phenolic hydroxyl at position 3, which explains that no relevant formation of the corresponding sulfate was observed.

Table 4 shows the chromatographic and UV and mass spectral data of the four prepared monosulfates and epicatechin. In this case, modifications in the absorption spectrum are not much useful for identification purposes, because for the compounds considered, only a small hypsochromic shift (less than 4 nm) in the maximum wavelength (band II) was produced in relation to epicatechin. Obviously, all monosulfates showed the same pseudomolecular $[M - H]^-$ ion at m/z 369, although their fragmentation patterns differed depending upon the ring of substitution on either A or B, as previously reported.[25] Thus, characteristic signals in the MS^2 spectrum are observed at m/z 217 (137 + 80; $^{1,3}A^-$ fragment ion bearing a sulfate moiety) and 247 (167 + 80; $^{1,2}A^-$ ion + HSO_3) when the sulfate moiety was located on the A ring, whereas signals at m/z 231 (151 + 80; $^{1,3}B^-$ ion + HSO_3) and 201 (121 + 80; $^{1,2}B^-$ ion + HSO_3) appeared when the sulfate moiety was located on the B ring, as shown in the example of Figure 4.

Further fractionation of the monosulfate mixture by semipreparative HPLC allowed for the separation of peak 4 (20 mg, representing a final yield of 4%) in the pure state for its analysis by ^{1}H and ^{13}C NMR. The rest of the compounds could not be obtained with the sufficient degree of purity for NMR analysis.

Table 5. ^{1}H and ^{13}C NMR Data and HMBC Correlations Obtained for Epicatechin and Epicatechin 4′-*O*-Sulfate Determined in CD_3OD

ring	position	δ ^{1}H (ppm); m; J (Hz)[a]	δ ^{13}C (ppm)	HMBC
		Epicatechin		
C	2	4.88; s	79.8	H-6′, H-2′, H-4
	3	4.16; m	67.4	H-2, H-4
	4eq	2.72; dd; J = 2.8, 16.8	29.2	H-2
	4ax	2.85; dd; J = 4.5, 16.7		
	9		157.3	H-4
	10		100.8	H-8, H-6, H-4
A	5		157.6	H-6, H-4
	6	5.90; d; J = 2.3	95.8	H-8
	7		158.0	H-8, H-6
	8	5.93; d; J = 2.3	96.3	H-6
B	1′		132.3	H-2, H-5′
	2′	6.96; d; J = 1.9	115.3	H-2, H-6′
	3′		145.9	H-5′,H-2′
	4′		145.7	H-6′, H-2′
	5′	6.74; d; J = 8.1	115.8	H-6′
	6′	6.79; dd; J = 8.1, 1.8	119.3	H-2′, H-2
		Epicatechin 4′-*O*-Sulfate		
C	2	4.80; s	79.7	H-6′, H-2′, H-4
	3	4.16; m	67.3	H-4
	4eq	2.72; dd; J = 2.9, 16.8	29.1	unresolved
	4ax	2.85; dd; J = 4.4, 16.8		
	9		156.9	unresolved
	10		100.0	H-8, H-6, H-4
A	5		157.6	H-6
	6	5.90; d; J = 2.2	95.7	H-8
	7		157.9	H-8, H-6
	8	5.92; d; J = 2.2	96.2	unresolved
B	1′		132.2	H-5′, H-2
	2′	6.99; d; J = 1.9	115.2	H-6′, H-2
	3′		145.8	H-5′, H-2′
	4′		145.6	H-6′, H-2′
	5′	6.69; d; J = 8.1	115.7	H-6′
	6′	6.80; dd; J = 8.2, 1.9	119.2	H-2′, H-2

[a] s, singlet; d, doublet; dd, double doublets; bs, broad singlet; m, multiplet.

The results obtained are shown in Table 5 compared to epicatechin. All carbons and aromatic protons could be assigned on the basis of their chemical shifts and HMBC correlations. As for quercetin 4′-*O*-sulfate, no significant displacements were found in protons or carbons with regard to epicatechin, and further analysis by HPLC−DAD−ESI/MS of the solution employed in the NMR analysis also confirmed that the compound was not altered and no loss of the sulfate residue was produced during NMR processing. Overall, this result supports that the presence of a unique sulfate residue at position 4′ does not produce upfield displacement in the carbons bearing the sulfate group nor downfield shifts of the carbons in positions *ortho* and *para*. In our knowledge, this is the first time that ^{1}H and ^{13}C NMR data of an epicatechin sulfate have been published.

In summary, monosulfate derivatives of quercetin and epicatechin were prepared by hemisynthesis and characterized according to their chromatographic behavior, absorption, and mass spectra. Three compounds could be further isolated and identified by ^{1}H and ^{13}C NMR as quercetin 3′-*O*-sulfate, quercetin 4′-*O*-sulfate, and epicatechin 4′-*O*-sulfate. It was observed that,

while sulfation at position 3′ induced a large upfield shift in the carbon bearing the sulfate group and downfield displacements of the adjacent carbons, no significant up- or downfield shifts were observed with respect to parent flavonoid when sulfation was produced at position 4′. All of the observations made allowed us to assign the peaks corresponding to the different monosulfate metabolites of quercetin and epicatechin in the HPLC chromatograms. The obtained results are of interest because they contribute patterns for the identification of these metabolites in biological samples and for their preparation in view to their use as standards with analytical purposes and their employment in *in vitro* studies.

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Funding

GIP/USAL is financially supported by the Spanish Ministerio de Ciencia e Innovación through the projects AGL2007-66108-C04-02 and AGL2009-12001 and the Consolider-Ingenio 2010 Programme (FUN-*c*-FOOD, CSD2007-00063). Montserrat Dueñas thanks the Spanish "Ramón y Cajal" Programme for a contract.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Anna Lithgow for her help in NMR analysis and interpretation.

REFERENCES

(1) Hertog, M. G. L.; Feskens, E. J. M.; Kromhout, D.; Hertog, M. G.; Hollman, P. C. H.; Katan, M. B. Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen elderly study. *Lancet* **1993**, *342*, 1007−1011.

(2) Knekt, P.; Kumpulainen, J.; Jarvinen, R.; Rissanen, H.; Heliovaara, M.; Reunanen, A.; Hakulinen, T.; Aromaa, A. Flavonoid intake and risk of chronic diseases. *Am. J. Clin. Nutr.* **2002**, *76*, 560−568.

(3) Garcia-Lafuente, A.; Guillamon, E.; Villares, A.; Rostagno, M. A.; Martinez, J. A. Flavonoids as anti-inflammatory agents: Implications in cancer and cardiovascular disease. *Inflammation Res.* **2009**, *58*, 537−552.

(4) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure−antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.

(5) Mira, L.; Silva, M.; Rocha, R.; Manso, C. F. Measurement of relative antioxidant activity of compounds: A methodological note. *Redox Rep.* **1999**, *4*, 69−74.

(6) Di Carlo, G.; Mascolo, N.; Izzo, A. A.; Capasso, F. Flavonoids: Old and new aspects of a class of natural therapeutic drugs. *Life Sci.* **1999**, *35*, 941−952.

(7) Boots, A. W.; Haenen, G. R. M. M.; Bast, A. Health effects of quercetin: From antioxidant and nutraceutical. *Eur. J. Pharmacol.* **2008**, *585*, 325−337.

(8) Gryglewski, R. J.; Korbut, R.; Robak, J.; Swies, J. On the mechanism of antithrombotic action of flavonoids. *Biochem. Pharmacol.* **1987**, *36*, 317−322.

(9) Perez-Vizcaino, F.; Duarte, J. Flavonols and cardiovascular disease. *Mol. Aspects Med.* **2010**, *31*, 478−494.

(10) Cooper, K. A.; Donovan, J. L.; Waterhouse, A. L.; Williamson, G. Cocoa and health: A decade of research. *Br. J. Nutr.* **2008**, *99*, 1−11.

(11) Steinberg, F. M.; Bearden, M. M.; Keen, C. L. Cocoa and chocolate flavonoids: Implication for cardiovascular health. *J. Am. Diet. Assoc.* **2003**, *103*, 215−223.

(12) Agarwal, C.; Veluri, R.; Kaur, M.; Chou, S. C.; Thompson, J. A.; Agarwal, R. Fractionation of high molecular weight tannins in grape seed extract and identification of procyanidin B2-3,3′-di-*O*-gallate as a major active constituent causing growth inhibition and apoptotic death of DU145 human prostate carcinoma cells. *Carcinogenesis* **2007**, *28*, 1478−1484.

(13) Fraga, C. G.; Oteiza, P. I. Dietary flavonoids: Role of (−)-epicatechin and related procyanidins in cell signaling. *Free Radical Biol. Med.* **2011**, *51*, 813−823.

(14) Day, A. J.; Du Pont, M. S.; Ridley, S.; Rhodes, M.; Rhodes, M. J. C.; Morgan, M. R. A.; Williamson, G. Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver *β*-glucosidase activity. *FEBS Lett.* **1998**, *436*, 71−75.

(15) Nemeth, K.; Plumb, G. W.; Berrin, J. G.; Juge, N.; Jacob, R.; Naim, H. Y.; Williamson, G.; Swallow, D. M.; Kroon, P. A. Deglycosylation by small intestinal epithelial cell *β*-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. *Eur. J. Nutr.* **2003**, *42*, 29−42.

(16) Donovan, J. L.; Luthria, D. L.; Stremple, P.; Waterhouse, A. L. Analysis of (+)-catechin, (−)-epicatechin and their 3′ and 4′-O-methylated analogs: A comparison of sensitive methods. *J. Chromatogr., B: Biomed. Sci. Appl.* **1999**, *726*, 277−283.

(17) Baba, S.; Osakabe, N.; Natsume, M.; Terao, J. Absorption and urinary excretion of procyanidin B2 [epicatechin-(4*β*-8)-epicatechin] in rats. *Free Radical Biol. Med.* **2002**, *33*, 142−148.

(18) Barron, D.; Cren-Olivé, C.; Needs, P. W. Chemical synthesis of flavonoid conjugates. In *Methods in Polyphenols Analysis*; Santos-Buelga, C., Williamson, G., Eds.; Royal Society of Chemistry: Cambridge, U.K., 2003; pp 187−213.

(19) Barron, D. Recent advances in the chemical synthesis and biological activity of phenolic metabolites. In *Recent Advances in Polyphenol Research*; Daayf, F., Lattanzio, V., Eds.; Blackwell Publishing: Oxford, U.K., 2008; Vol. *1*, pp 317−358.

(20) Jones, D. J. L.; Jukes-Jones, R.; Verschoyle, R. D.; Farmer, P. B.; Gescher, A. A synthetic approach to the generation of quercetin sulfates and the detection of quercetin 3′-*O*-sulfate as a urinary metabolite in the rat. *Bioorg. Med. Chem.* **2005**, *13*, 6727−6731.

(21) Needs, P. W.; Kroon, P. A. Convenient syntheses of metabolically important quercetin glucuronides and sulfates. *Tetrahedron* **2006**, *62*, 6862−6868.

(22) Day, A. J.; Bao, Y.; Morgan, M. R. A.; Williamson, G. Conjugation position of quercetin glucuronides and effect on biological activity. *Free Radical Biol. Med.* **2000**, *29*, 1234−1243.

(23) Plumb, G. W.; O'Leary, K.; Day, A. J.; Williamson, G. Enzymatic synthesis of quercetin glucosides and glucuronides. In *Methods in Polyphenols Analysis*; Santos-Buelga, C., Williamson, G., Eds.; Royal Society of Chemistry: Cambridge, U.K., 2003; pp 177−185.

(24) Dueñas, M.; Mingo-Chornet, H.; Pérez-Alonso, J. J.; Di Paola-Naranjo, R.; González-Paramás, A. M.; Santos-Buelga, C. Preparation of quercetin glucuronides and characterization by HPLC−DAD−ESI/MS. *Eur. Food Res. Technol.* **2008**, *227*, 1069−1076.

(25) González-Manzano, S.; González-Paramás, A.; Santos-Buelga, C.; Dueñas, M. Praparation and characterization of catechin sulfates, glucuronides, and methylethers with metabolic interest. *J. Agric. Food Chem.* **2009**, *57*, 1231−1238.

(26) Barron, D.; Ibrahim, R. K. Hydrocloric acid and aryl-sulphatase as reagents for UV-spectral detection of 3- and 4′-sulphated flavonoids. *Phytochemistry* **1988**, *27*, 2335−2338.

(27) Barron, D.; Ibrahim, R. K. Synthesis of flavonoid sulfates 2. The use of aryl sulfatase in the synthesis of flavonol-3-sulfates. *Z. Naturforsch., C: J. Biosci.* **1988**, *43*, 625−630.

(28) Day, A. J.; Mellon, F.; Barron, D.; Sarrazin, G.; Michael, R. A. M.; Williamson, G. Human metabolism of dietary flavonoids: Identification of plasma metabolites of quercetin. *Free Radical Res.* **2001**, *35*, 941−952.

(29) Singh, R.; Wu, B.; Tang, L.; Liu, Z.; Hu, M. Identification of the position of mono-*O*-glucuronide of flavones and flavonols by analyzing shift in online UV spectrum (λ_{max}) generated from an online diode array detector. *J. Agric. Food Chem.* **2010**, *58*, 9384−9395.

(30) Barron, D.; Ibrahim, R. K. Quercetin and patuletin 3,3′-disulphates from *Flaveria chloraefolia*. *Phytochemistry* **1987**, *26*, 1181−1184.
(31) Ragan, M. A. Phenol sulfate esters: Ultraviolet, infrared, ^{1}H and ^{13}C nuclear magnetic resonance spectroscopic investigation. *Can. J. Chem.* **1978**, *56*, 2681−2685.
(32) Pretsch, E.; Bühlmann, P.; Affolter, C. Protón de compuestos heteroaromáticos. In *Determinación Estructural de Compuestos Orgánicos*; Herrera, A., Martínez, R., Eds.; Springer-Verlag Ibérica: Barcelona, Spain, 2001; pp 186−197.
(33) Cren-Olivè, C.; Rolando, C. Polyphenols: Measurement and calculation of their physical chemistry properties. In *Methods in Polyphenols Analysis*; Santos-Buelga, C., Williamson, G., Eds.; Royal Society of Chemistry: Cambridge, U.K., 2003; pp 157−175.