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Preparation and Characterization of Catechin Sulfates, Glucuronides, and Methylethers with Metabolic Interest

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Catechins are major polyphenols in many plant foods that have been related to health promotion. In the human organism they are largely metabolized to different conjugates (sulfates, glucuronides, and methylethers), which are further found in plasma and would contribute to the biological effects associated with the intake of the parent compounds. Circulating metabolites are likely to possess biological properties different from those of the original compounds, and therefore, it is important to evaluate their activity, for which sufficient amounts of them are required that cannot be obtained by isolation from biological fluids. This paper describes the preparation of the methyl, sulfate, and glucuronide derivatives of catechins using different chemical syntheses and their characterization by HPLC−DAD−ESI/MS. MS^2 fragmentation of the compounds was also described that allowed the determination of the location of the different substituents on the catechin aglycones. The procedures optimized allowed the preparation of (epi)catechin sulfates, glucuronides, and methylethers conjugated at positions 3′ and 4′, as well as the sulfates at positions 5 and 7 with satisfactory yields for their further isolation by semipreparative-HPLC in view of their use in in vitro/ex vivo assays.

KEYWORDS: catechin metabolites; methylcatechin; sulfates; glucuronides

INTRODUCTION

Flavan-3-ols (catechins and proanthocyanidins) are major polyphenols in many plant foods that have been related to health promotion. Indeed a large number of epidemiological studies demonstrate that the intake of flavan-3-ol-rich products (i.e., tea, red wine, apples, or chocolate) is inversely associated with the risk of coronary heart disease, cancer, and inmunodysfunctions (*1*−*4*). Epidemiological observations are supported by many in vitro and ex vivo studies displaying a remarkable scope of biochemical and pharmacological actions of these compounds, among them antiviral (*5*), anti-inflammatory (*6*), antiallergic (*7*), antioxidant and free radical scavenging properties (*8, 9*).

Because of the potential beneficial role of these compounds in human health, it is essential to understand their bioavailability, i.e., absorption, metabolism, and excretion. It has been shown that catechins are absorbed from the human intestinal tract, largely metabolized and distributed as conjugated derivatives in blood, and that these forms are excreted in urine (*10*). Methyl, sulfate, and glucuronide conjugates have been described as circulating metabolites of catechins. In studies with human volunteers, Donovan et al. (*11*) found that after consumption of wine, catechin was present in plasma as both nonmethylated and 3′-O-methylated (21%) derivatives conjugated with glucuronide and sulfate residues; nonmethylated catechin was mainly found as sulfate and sulfoglucuronide conjugates, whereas 3′-*O*-methylcatechin was present primarily as a glucuronide. Similar results were found in rats by Baba et al. (*12*), which showed that absorbed catechin and epicatechin were mostly present in plasma as glucuronides, sulfates, and sulfoglucuronides, both in the form of nonmethylated or 3′-O-methylated derivatives. Methylation and glucuronidation are already produced in the enterocyte, while additional methylation and sulfation would further take place in the liver (*13*). Perfusion of isolate rat jejunum with flavanols resulted in glucuronidation (∼45%), O-methylation: 3′-*O*-methyl and 4′-*O*-methyl (30%) and *O*-methyl glucuronidation (20%) during transfer across the enterocytes to the serosal side (*14*). Nevertheless, differences may exist in the profile of metabolites between rats and humans. Natsume et al. (*15*) isolated epicatechin 3′-*O*-glucuronide, 4′-*O*-methylepicatechin 3′-*O*-glucuronide, and 4′-*O*-methylepicatechin 5- or 7-*O*-glucuronide from human urine. These same authors purified 3′-*O*-methylepicatechin, epicatechin-7-*O*-glucuronide, and 3′-*O*-methylepicatechin-7-*O*-glucuronide from rat urine. Bhatathi et al. (*16*) indicated that sulfation constitutes the major pathway in epicatechin metabolism in humans, in both liver and intestine, with no glucuronidation occurring. This is not in agreement with the observations made by Natsume et al. (*15*) or Donovan et al. (*11*), who found glucuronide conjugates of (methyl)catechins in either human urine or plasma. These

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latter authors differ, however, in the preferential position of substitution of the methyl residue (i.e., 3′ or 4′) on the catechin.

Circulating metabolites would reach the biological targets, and therefore, they should be the main actors to explain the health effects associated with the intake of catechins and flavonoids in general. Conjugated metabolites are likely to possess different biological properties than do parent compounds, and therefore, in vitro studies should also consider metabolites rather those only commercially available compounds as found in foodstuffs (*17*). Differences should also exist between the distinct type of conjugates (sulfates, glucuronides, and methylated compounds) and the different position at which the conjugation occurs. However, far less is known about the activity and distribution patterns of the metabolites than about their precursors, and relatively few analytical methods have been developed in order to study these metabolites in biological fluids and tissues. Previous work has shown that the positions of the sugar moieties have a large effect on the bioactivity of flavonoids (*18*) and a similar effect was noted for conjugated metabolites (*19*). Thus, the ability to detect, identify, and elucidate the structure of flavonoid metabolites would be extremely useful. In this respect, liquid chromatography−mass spectrometry ($LC-MS^n$) is in many ways an ideal method for analyzing and identifying flavonoid metabolites owing to its high sensitivity, applicability to complex mixtures, and ability to provide structural information.

Some studies have been published regarding the activity of conjugated metabolites of quercetin (*20*, *21*), but hardly any data exist concerning catechin metabolites. In order to evaluate the activity of catechin metabolites in in vitro and cell model assays, as well as to optimize methods for their analysis, sufficient amounts of pure compounds are required that cannot be obtained by isolation from biological fluids. This work deals with the synthesis and characterization of catechin metabolites, i.e., sulfate, glucuronide, and methylated derivatives. The knowledge of their chromatographic behavior, mass spectra, and fragmentation patterns, as provided by HPLC−DAD−ESI/MS, is expected contribute to their correct identification in biological samples and to the understanding of their metabolism in vivo.

MATERIALS AND METHODS

Standards and Reagents. Catechin, acetobrom-α-D-glucuronic acid methyl ester, sodium methylate, methyl iodide, and potassium carbonate were purchased from Sigma-Aldrich (Milwaukee, WI). Pyridine, dioxane, and sulfur trioxide−*N*-triethylamine were from Sigma (Poole, U.K.). HPLC grade methanol and ethanol were purchased from Carlo Erba (Milan, Italy). Acetone, glacial acetic acid, formic acid, and hydrochloric acid were of analytical grade and obtained from Panreac (Barcelona, Spain). Trifluoroacetic acid (TFA) was purchased from Riedel-de Haën (Seelze, Germany).

Preparation of (Epi)Catechin Methylethers. The methylethers of (epi)catechin were synthesized on the basis of the protocol described by Donovan et al. (*22*). 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 solvent was filtered and concentrated in rotary evaporator to dryness. The methylethers of (epi)catechin synthesized were characterized by HPLC−DAD−MS. The major products of the reaction (3′- and 4′-methylethers of catechin and epicatechin) were further purified by semipreparative HPLC.

Preparation of Catechin Glucuronides. Synthesis of the catechin glucuronides was based on the Koenigs−Knorr previously employed by Tsushida et al. (*23*) for the preparation of quercetin glucosides. Catechin (500 mg) and acetobrom-α-D-glucuronic acid methyl ester (2.5 g) were dissolved in 25 mL of acetone. After addition of potassium carbonate (500 mg), the mixture was stirred for 2 h at room temperature. The resulting mixture was added to 150 g of cooled water and then adjusted to acidic condition by adding a few drops of formic acid. The acetone was removed in a Büchi R-124 rotary evaporator (Büchi Labortechnik AG, Switzerland) at 30 °C to dryness. The residue obtained was redissolved in ultrapure water (Direct-Q, Millipore, Molsheim, France) and submitted to semipreparative HPLC to remove the remaining reagents and side products of the reaction. A fraction containing intermediate compounds was collected, evaporated to dryness under vacuum, and redissolved in 50 mL of methanol. This solution was analyzed HPLC−DAD−MS, showing that it contained a mixture of five compounds with a pseudomolecular ion $[M - H]^-$ at *m*/*z* 605 corresponding to the different catechin triacetylglucuronic methyl esters. Then 750 μL of sodium methylate (28:72, v/v sodium methylate/methanol) was added to the solution and kept at 4 °C for 30 min to remove acetyl moieties bound to the glucuronide residue. Further addition of 7.5 mL of ultrapure water was made and allowed to react 30 min at room temperature in order to hydrolyze the methyl esters. The solution was neutralized (1.85 mL of 2 N hydrochloric acid), adjusted to acidic condition by adding a few drops of formic acid, and then evaporated to dryness and redissolved in water. The catechin glucuronides obtained were characterized by HPLC−DAD−MS. For their isolation a fractionation was first made in a Sephadex LH-20 column (350 mm × 30 mm) eluted with 10% aqueous ethanol (500 mL) and 20% aqueous ethanol (500 mL), and further purification of the compounds was performed by semipreparative HPLC.

Preparation of (Epi)Catechin Sulfates. These metabolites were synthesized by a modification of the method described by Jones et al. (*24*) for the preparation of quercetin sulfates. First, water associated with (epi)catechin (500 mg) was removed by adding dry pyridine until it dissolved. Pyridine was rotary-evaporated, and the dry compound was dissolved in dioxane (50 mL) and allowed to react with a 10-fold molar excess of sulfur trioxide−*N*-triethylamine complex under argon to avoid contact with air. This reaction took place in a water bath (40 °C) for 90 min, after which products of sulfation precipitated out and stuck to the glass. Then dioxane was decanted and the product (a mixture of different mono- and disulfates of (epi)catechin, as well as the aglycone) redissolved in ethanol and water (10:90). The compounds were fractionated on a Sephadex LH-20 column (350 mm × 30 mm) eluted with 10% aqueous ethanol (500 mL) and 20% aqueous ethanol (500 mL) to separate (epi)catechin monosulfates, disulfates, and aglycone. The fraction containing the monosulfates was collected, concentrated to dryness under vacuum, redissolved in ultrapure water, and analyzed by HPLC−DAD−MS. Different (epi)catechin sulfates were then isolated from this fraction by semipreparative HPLC.

HPLC−DAD−ESI/MS Analyses. They 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 (revision A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C8, 3 μm (4.6 mm × 150 mm) column thermostated at 30 °C was used. Solvents employed for the analysis of catechin methylethers and glucuronides were (A) 2.5% acetic acid, (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 $mL \cdot min^{-1}$. For the analysis of catechin monosulfates solvents were (A) 0.1% TFA in water and (B) acetonitrile; the elution gradient was 0% A to 10% B over 5 min, 0−15% A in B over 20 min, and 15−20% C in B over 5 min, using a flow rate of 0.5 $mL \cdot min^{-1}$.

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. The mass spectrometer was connected to the HPLC system via the DAD cell outlet. Both the auxiliary and the sheath gases were nitrogen at flow rates of 20 and 80 L min^{-1}, 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 positive (catechin methylethers) and in negative (catechin sulfates and glucuronides) ion modes between *m*/*z* 150 and 2000. The MS detector was programmed to perform a series of three consecutive scans: a full mass scan, an

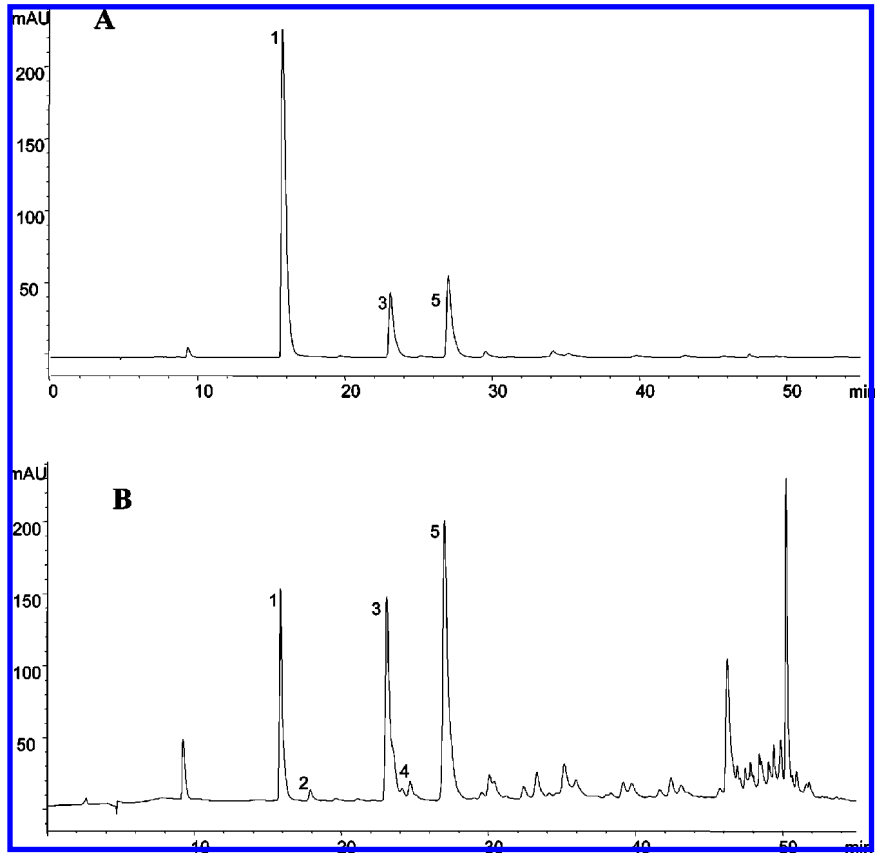

Figure 1. Chromatograms recorded at 280 nm showing the result obtained in the hemisynthesis of catechin methylethers after (A) 2 h and (B) 3.5 h of reaction. Peaks are as follows: (1) catechin; (2) 5-*O*-methylcatechin; (3) 3′-*O*-methylcatechin; (4) 7-*O*-methylcatechin; (5) 4′-*O*-methylcatechin.

MS/ MS scan of the most abundant ion the first scan, and an MS^3 of the most abundant ion in the MS^2 using normalized collision energy of 45%.

Semipreparative HPLC. A Waters 600 chromatograph coupled to a UV−vis model 486 detector and an Ultracarb C18ODS20 5 μm (10 mm × 250 mm, i.d.) column from Phenomenex (Supelco Ascentis, Bellefonte, PA) were used. The solvents were (A) 5% acetic acid and (B) methanol. The flow rate was 3 mL·min^{-1}. The gradient used was different for the different catechin derivatives. For the intermediate products in the synthesis of catechin glucuronides (i.e., catechin triacetylglucuronic mehylesters), the gradient was 10−20% B in 15 min, 20−40% B in 15 min, 40−55% B in 10 min. For the separation of catechin glucuronides, the gradient was 0−10% B in 5 min and 10−30% B in 20 min. For the separation of the catechin sulfates, the gradient was 0−10% B in 40 min and 10−12% B in 20 min, and for the catechin methylethers, it was 0−20% B in 15 min, 20−30% B in 25 min, and 30−40% B in 5 min. Detection was carried out at 280 nm, and the peaks were collected in a fraction collector. Fractions obtained were concentrated under vacuum at low temperature and freeze-dried. The identity of the compounds was checked by HPLC−DAD−MS.

RESULTS AND DISCUSSION

Preparation of Catechin Conjugates. The method for the preparation of catechin methylethers described by Donovan et al. (*22*) was applied with small modifications regarding the time of reaction. In the original method a time of 2 h was used; however, in our conditions low amounts of conjugates were accumulated at that time and the reaction was kept 3.5 h, at which the peaks of the methylethers reached their maximum area. The majority formation of two products was observed (**Figure 1**), and they were assigned to the 3′- and 4′-methylethers of catechin, as previously shown by Donovan et al. (*22*). These same authors (*11*) indicated that the catechin was methylated preferentially in the 3′ position, although in our case higher yield in the formation of the 4′-methylether than of the 3′ derivative was observed. Minor formation of the methylated derivatives at 5- and 7-positions was also found (**Figure 1**), whereas no production of the 3-methylether could be observed, indicating that the B-ring is the preferential site for methylation in the reaction conditions used. No mention to the formation of none of these latter products was made by Donovan et al. (*22*). Application of the same procedure using epicatechin instead catechin gave similar results, with formation of the corresponding 3′- and 4′-methylethers.

Methods for the preparation of catechin glucuronides and sulfates had to be optimized on the basis of methodologies previously described for the hemisynthesis of similar quercetin derivatives. It is noticed that relevant differences exist between both types of flavonoids regarding the acidity of their various hydroxyl groups, which provokes differences in the regioselectivity of the syntheses. Thus, distinct types and/or distribution of the reaction products should be expected in each case. Flavonols have A- and B-rings conjugated to the 4-carbonyl, and relevant differences exist between the distinct hydroxyls. In the case of quercetin, the 5-hydroxyl is by far the least acidic because of its hydrogen bonding with the 4-carbonyl. By contrast, 7- and 4′-hydroxyls are the most acidic, which is explained by their para positions regarding the conjugated chain. Compared to them, 3- and 3′-hydroxyls show reduced acidity, explained by the meta orientation of the 3′-hydroxyl in relation to the conjugated chain, and the possibility of weak hydrogen bonding with the 4-carbonyl in the case of the 3-hydroxyl; in addition this position is more hindered than the others. The more acidic hydroxyls of flavonols, made more nucleophilic by deprotonation under the mildly basic conditions commonly

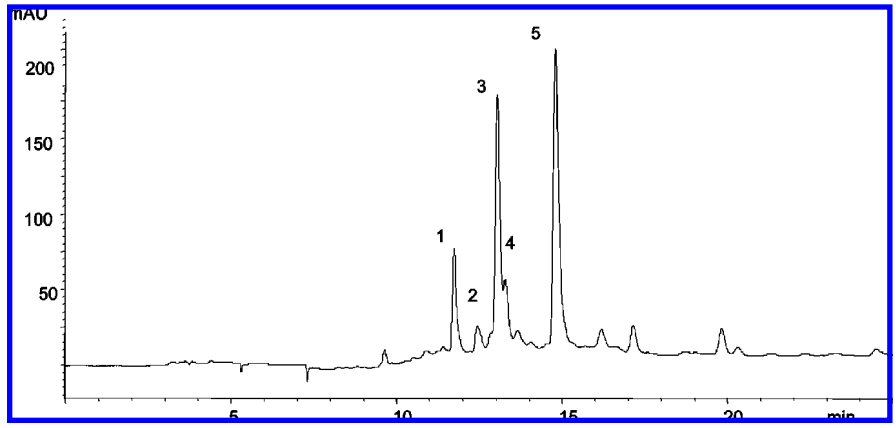

Figure 2. Chromatogram recorded at 280 nm showing the profile of catechin glucuronides obtained by hemisynthesis. Peaks are as follows: (1) catechin-5-*O*-glucuronide; (2) catechin-3-*O*-glucuronide; (3) catechin-3′-*O*-glucuronide; (4) catechin-7-*O*-glucuronide; (5) catechin-4′-*O*- glucuronide.

employed for conjugation, are often more reactive, and therefore, preferential formation of those conjugates should occur. The case of catechins is quite different. The lack of the 4-carbonyl and the 2,3-double bond results in similar acidities for all the phenolic hydroxyls, and therefore, no regioselective conjugation is expected (*25*). Thus, modifications had to be introduced in the protocols described for the preparation of the quercetin conjugates to adapt them to the synthesis of the catechin derivatives and also particular attention had to be paid to the steps of purification and characterization of the metabolites because of their similar characteristics.

Catechin glucuronides were chemically synthesized using the Koenigs−Knorr reaction following the protocol described in the section of experimental. Major products obtained were catechin-4′-*O*-glucuronide (40%) and catechin-3′-*O*-glucuronide (30%), followed by catechin-5-*O*-glucuronide (11%), catechin-7-*O*-glucuronide (9%), and catechin-3-*O*-glucuronide (3%) (**Figure 2**), indicating that in the reaction conditions phenolic hydroxyls in the B-ring are more reactive than those in the A-ring and that the nonphenolic hydroxyl at position 3 is the less reactive. This is in agreement with the acidities calculated for the four catechin hydroxyl groups by Cren-Olivé et al. (*26*), according to which hydroxyls at 5 and 7 positions possess slightly higher pK_a values (9.48 and 9.58) than those at 3′ and 4′ (9.02 and 9.12).

Figure 3 shows the HPLC chromatogram of the crude extract resulting from the synthesis of catechin sulfates obtained in the same conditions used for the separation of catechin methylethers and glucuronides, in which mono- and disulfates appear as two unresolved peaks. Both types of products could be easily separated in different fractions in a Sephadex LH-20 column. Monosulfates could be resolved by changing the analytical HPLC gradient conditions using trifluoroacetic acid instead of acetic acid as elution solvent (**Figure 4**). Four compounds were detected corresponding to the monosulfates at positions 5, 3′, 7, and 4′ of the catechin, respectively. Their elution order was established based on their chromatographic behavior compared with other catechin conjugates and MS^n fragmentation pattern (see below). No formation of the sulfate at position 3 was observed. Higher yield was obtained in the formation of catechin-3′-*O*-sulfate (38% of the monosulfates prepared) than for the other three monosulfates (around 20% each). Similar results were found when the hemisynthesis was carried out with epicatechin. No good separation and characterization of the complex mixture of catechin bisulfates obtained in the hemisynthesis procedure could be obtained.

The procedures above-described allowed the preparation with satisfactory yields for their further isolation by semipreparative

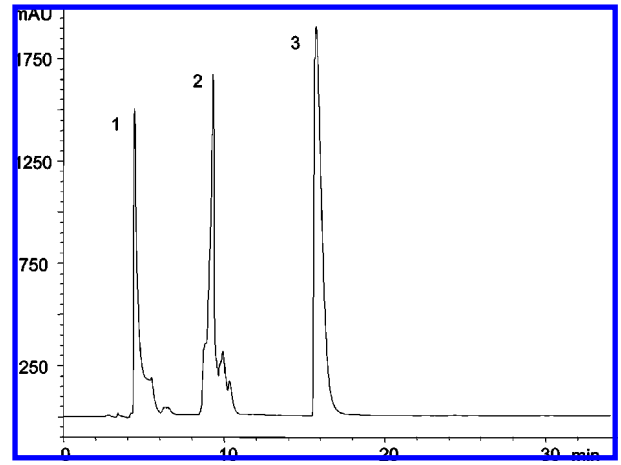

Figure 3. Chromatogram recorded at 280 nm obtained in the same HPLC conditions as the chromatograms of **Figures 1** and **2** showing the result of the hemisynthesis of catechin sulfates. Peaks are as follows: (1) mixture of catechin disulfates; (2) mixture of catechin monosulfates; (3) catechin.

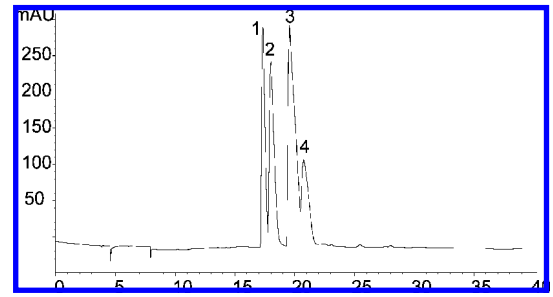

Figure 4. Chromatogram recorded at 280 nm showing the profile of catechin monosulfates obtained. HPLC conditions are different from those employed for the chromatograms shown in the **Figures 1**−**3**. Peaks are as follows: (1) catechin-5-*O*-sulfate; (2) catechin-3′-*O*-sulfate; (3) catechin-7-*O*-sulfate; (4) catechin-4′-*O*- sulfate.

HPLC of the following metabolites: 3′-methylether and 4′-methylether of (epi)catechin, 3′-*O*-glucuronide and 4′-*O*-glucuronide of (epi)catechin-, and 3′-*O*-sulfate, 4′-*O*-sulfate, 5-*O*-sulfate, and 7-*O*-sulfate of (epi)catechin. In the conditions described these compounds were obtained at the level of tens of milligrams, sufficient for their use in in vitro/ex vivo activity assays, as well as to optimize their LC−MS characterization, useful for their analysis in biological fluids.

Characterization of Catechin Conjugates by HPLC−DAD−ESI/MS. The same HPLC conditions were used for the

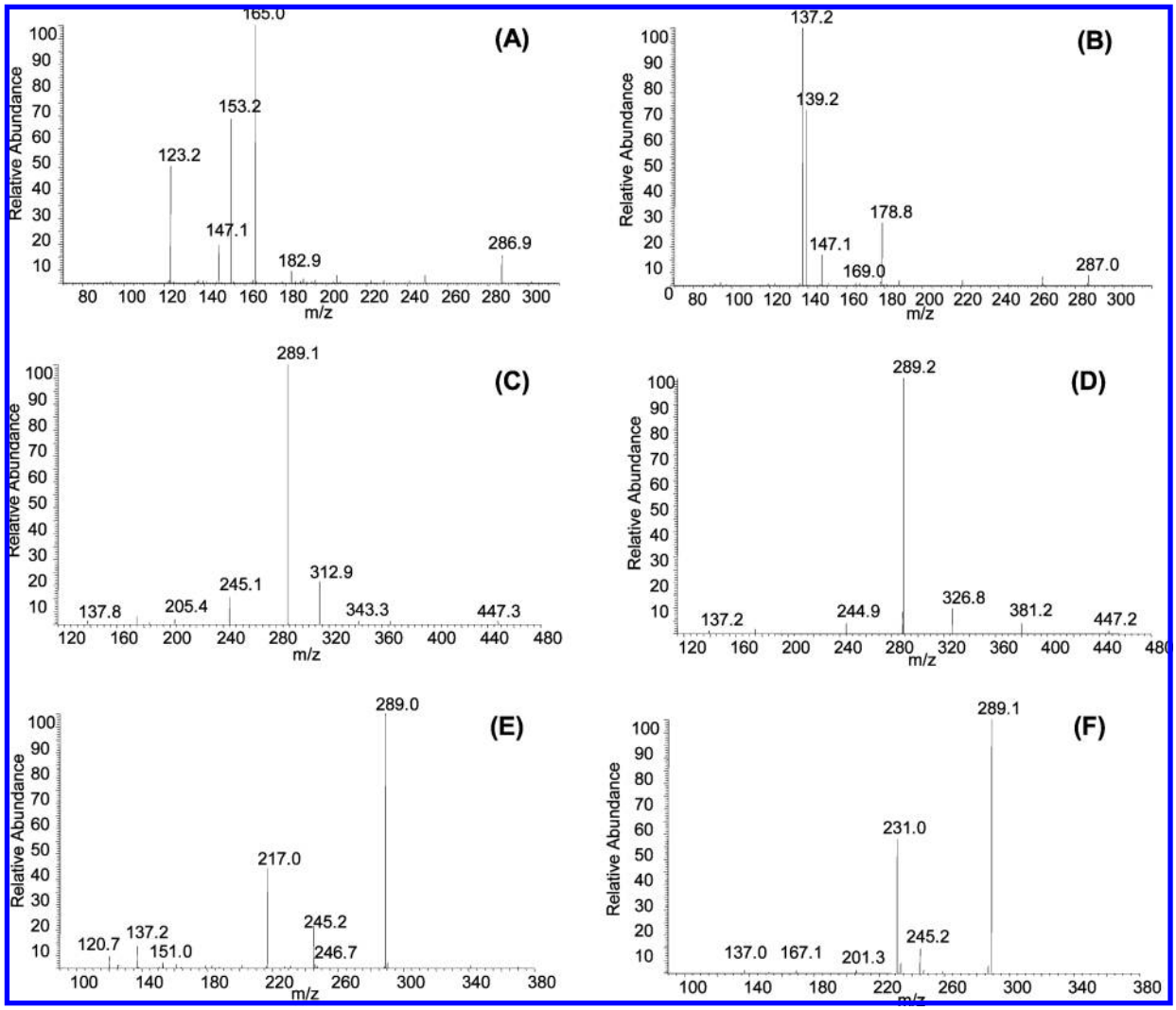

Figure 5. MS^2 spectra of different catechin metabolites: catechin methylethers (pseudomolecular ion $[M + H]^+$ at *m*/*z* 305) conjugated on the A-ring (**A**) and B-ring (**B**); catechin glucuronides (pseudomolecular ion $[M - H]^-$ at *m*/*z* 465) conjugated on the A-ring (**C**) and B-ring (**D**); catechin sulfates (pseudomolecular ion $[M - H]^-$ at *m*/*z* 369) conjugated on the A-ring (**E**) and B-ring (**F**). Spectra of the catechin methylethers were obtained in positive ion mode and spectra of catechin glucuronides and sulfates in negative ion mode.

separation of (epi)catechin methylethers and glucuronides, whereas different conditions were optimized for the separation of the monosulfates using TFA instead of acetic acid as HPLC solvent. ESI/MS identification of the methylether derivatives was performed using positive ionization mode so that comparison could be made with the results previously reported by Donovan et al. (*22*) and Cren-Olivé et al. (*28*). ESI/MS characterization of catechin glucuronides and sulfates was made using negative ionization mode. The fragmentation ion nomenclature recently proposed for flavones and flavonols (*27*) has been adopted to denote the product ions. The labels ^{ij}A and ^{ij}B refer to the fragment containing A and B ring, respectively, and the superscripts *i* and *j* indicate the C-ring bonds that have been broken.

The chromatogram in **Figure 1** shows the result obtained in the synthesis of the catechin methylethers. Four peaks with a pseudomolecular $[M - H]^+$ ion at *m*/*z* 305 were detected, which were associated with different products of the methylation on each of the phenolic hydroxyl groups of the catechin. Similar synthesis carried out by Donovan et al. (*22*) revealed the preferential formation of the 3′- and 4′-*O*-methyl derivatives of catechin, as identified by NMR, the first one eluting earlier in reversed-phase HPLC. Studies carried out by Cren-Olivé et al. (*28*) by LC−MS also showed that 3′-*O*-methylcatechin eluted before the 4′-*O*-methyl derivative and that 5-*O*-methylcatechin eluted before the corresponding 7-*O*-methyl analogue.

No relevant differences were found in the MS^2 spectra of compounds **2** and **4** (**Figure 5A**), indicating that they possessed the same fragmentation pattern (**Figure 6**). The ion at *m*/*z* 153 results from the retro Diels−Alder (RDA) fission and gives rise to a $^{1,3}A^+$ ion with the methyl group (139 + 14=153). The ion at *m*/*z* 165 would correspond to the $^{1,4}B^+$ ion from the 1,4 fission of the C-ring. Further dehydration of this product yields the ion at *m*/*z* 147. The ion at *m*/*z* 123 results from the 1,2 fission of the B ring that gives rise to a $^{1,2}B^+$ ion. The ion at *m*/*z* 183 corresponds to the 1,2 fission of the C ring that gives rise to a $^{1,2}A^+$ ion with the methyl group (169 + 14=183), and the ion at *m*/*z* 287 is attributed to the loss of the hydroxyl in C3 position, which was suggested to be the most labile and easily lost (*28*). These results clearly showed that the methyl residue in peaks 2 and 4 was located on the A-ring, that is, at either positions 5 or 7.

Peaks 3 and 5 also showed the same MS^2 spectra (**Figure 5B**) and fragmentation pattern. This is in agreement with Donovan et al. (*22*) who also found that the fragmentation pattern did not differ significantly for methylation at the 3′ or 4′ position. In these compounds the observation of the product

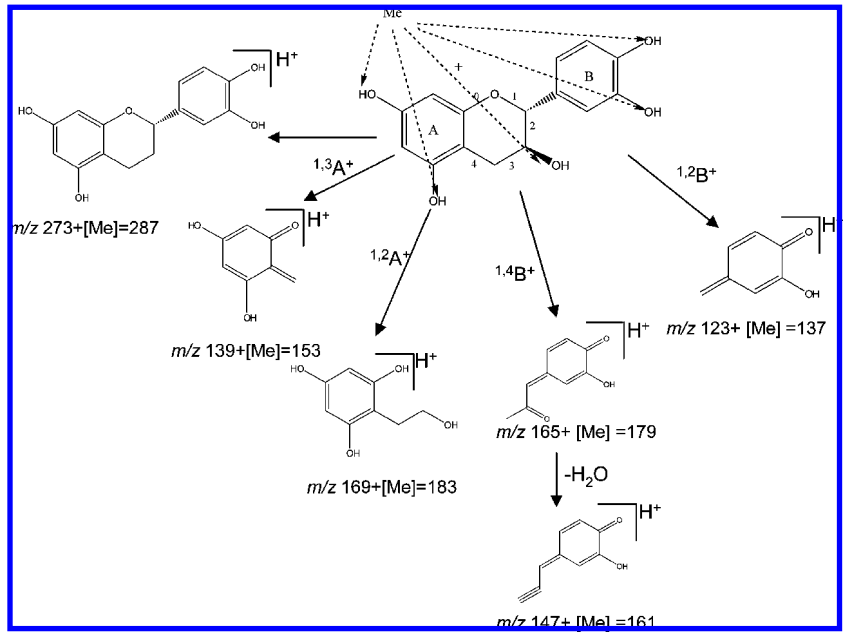

Figure 6. MS^2 fragmentation patterns of catechin methylethers (pseudomolecular ion at *m/z* 305) in positive ion mode.

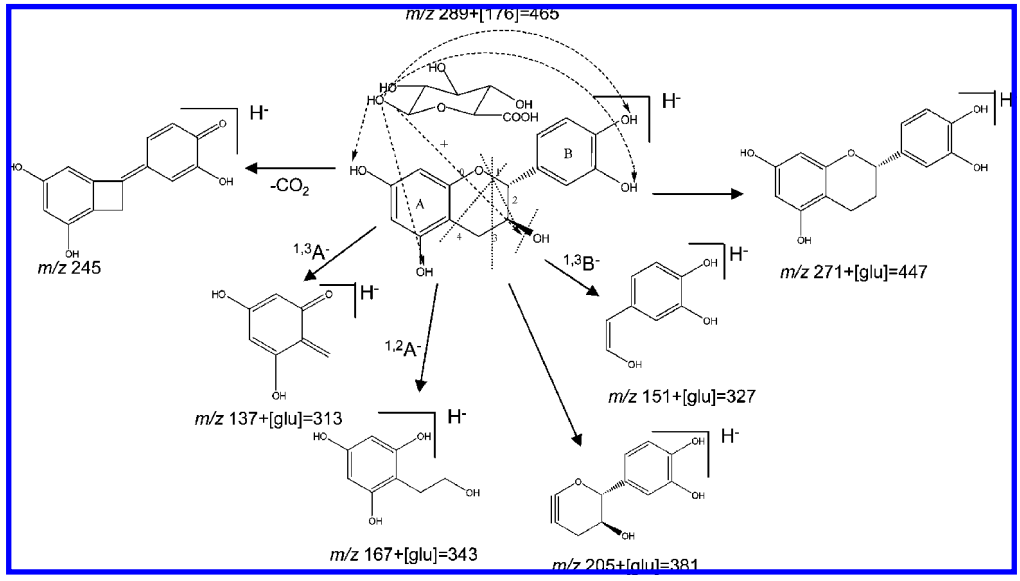

Figure 7. MS^2 fragmentation patterns of catechin glucuronides (pseudomolecular ion at *m/z* 465) in negative ion mode.

ions $^{1,2}B^+$ at *m/z* 137 (123 + 14 = 137) and $^{1,3}A^+$ at *m/z* 179 (165 + 14 = 179) demonstrated the location of the methyl residue on the B-ring (i.e., positions 3′ or 4′). On the basis of these results and the previous observations made by Donovan et al. (*22*) and Cren-Olivé et al. (*28*) regarding elution of the catechin methylethers in RP-HPLC, peaks 2−5 in the chromatogram of the **Figure 1** were assigned to 5-*O*-methylcatechin, 3′-*O*-methylcatechin, 7-*O*-methylcatechin, and 4′-*O*-methylcatechin, respectively.

Chemical synthesis of catechin glucuronides gave rise to a mixture of five intermediate compounds showing a pseudomolecular ion $[M - H]^-$ at *m/z* 605, corresponding to the different triacetylglucuronic methylesters at each of the five hydroxyl groups of the catechin. Further removal of the acetyl and methyl residues produced a mixture of five catechin monoglucuronides (**Figure 2**). All of them possessed a pseudomolecular ion $[M - H]^-$ at *m/z* 465 that released a main MS^2 fragment at *m/z* 289 (catechin; loss of the glucuronide moiety $[(M - H) - 176]^-$). Secondary product ions were useful to establish the location of the glucuronide moiety on the catechin and thus to identify the different metabolites. As it happened for the catechin methylethers, similar MS^2 spectra were observed for compounds **1** and **4** (**Figure 5C**) and compounds **3** and **5** (**Figure 5D**), indicating that the glucuronide moiety was located in either the A- or B-ring in each case.

The fragmentation patterns of these compounds are shown in **Figure 7**. Four secondary ions were observed for peaks 1 and 4 at *m/z* 313 due to the RDA fission that gives rise to a $^{1,3}A^-$ ion with the glucuronide moiety (137 + 176 = 313), at *m/z* 343 that corresponds to the 1,2 fission of the C releasing a $^{1,2}A^-$ ion with the glucuronide moiety (167 + 176 = 343), at *m/z* 447 that results from the loss of the OH at C3, and at *m/z* 245 from the loss of CO_2 $[(M - H) - 44]^-$ from the ion at *m/z* 289; this loss of CO_2 seems to be characteristic of the fragmentation of flavonoids in negative ion mode (*29*), and the resulting ion (*m/z* at 245) was observed as the main product ion in the MS^3 spectra. These results demonstrated that in these compounds the glucuronide residue was located on the catechin A-ring.

Product ions observed for peaks 3 and 5 were at *m/z* 327, corresponding to the RDA fission yielding the $^{1,3}B^-$ fragment

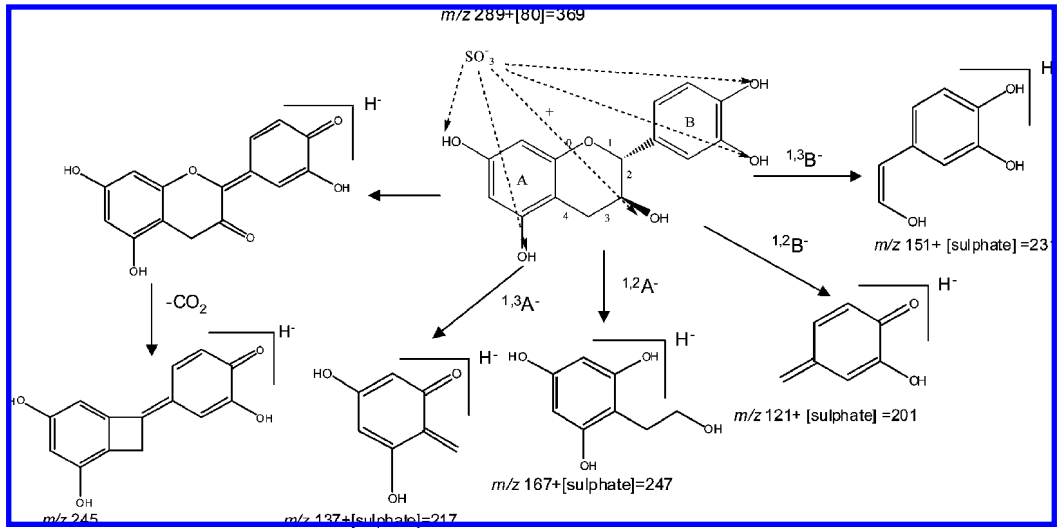

Figure 8. MS^2 fragmentation patterns of catechin monosulfates (pseudomolecular ion at *m/z* 369) in negative ion mode.

with the glucuronide moiety (151 + 176 = 327), at *m/z* 447 (loss of the OH in C3 position), at *m/z* 245 (loss of CO_2 from catechin), and at *m/z* 381 attributed to the loss of the A-ring (205 + 176=381). This last product ion is unusual in catechins, although it was reported by Cren-Olive et al. (*28*) for methylcatechins. This fragmentation pattern revealed that in peaks 3 and 5 the glucuronide moiety was attached to catechin B-ring. The MS^2 spectrum of peak 2 showed only a relevant signal at *m/z* 289 (catechin).

Taking into account these observations and the comments already made regarding the elution profile of the methylcatechins, peaks 1−5 obtained in the synthesis of catechin glucuronides were assigned to catechin-5-*O*-glucuronide, catechin-3-*O*-glucuronide, catechin-3′-*O*-glucuronide, catechin-7-*O*-glucuronide, and catechin-4′-*O*-glucuronide, respectively.

The chromatogram of the **Figure 3** shows the result obtained in the synthesis of catechin sulfates. The three peaks observed possessed negative ions at *m/z* 449, *m/z* 369, and *m/z* 289, which correspond to catechin disulfates, monosulfates, and aglycone, respectively. Further fractionation on Sephadex LH-20 allowed the separation of the disulfates (first compounds to elute) from the monosulfates and catechin (last one to elute). Monosulfates were collected and analyzed by HPLC−DAD−MS using TFA as acid modifier for the elution as above commented. Four compounds were detected (**Figure 4**) that showed a pseudomolecular ion $[M - H]^-$ at *m/z* 369 that released a main MS^2 fragment at *m/z* 289 (catechin). Various secondary product ions were also produced that were useful to establish the location of the sulfate residue on the catechin. Compounds **1** and **3** showed similar MS^2 spectra (**Figure 5E**) and fragmentation pattern (**Figure 8**). The ion at *m/z* 217 corresponds to $^{1,3}A^-$ ion with a sulfate moiety (137 + 80) produced from RDA fission of the parent metabolite, and the small signal at *m/z* 247 is associated with the $^{1,2}A^-$ ion with the sulfate moiety (167 + 80). These fragments clearly demonstrated that the sulfate moiety in compounds **1** and **3** was linked to the catechin A-ring.

Peaks 2 and 4 also showed similar MS^2 spectra (**Figure 5F**) with product ions that revealed that their sulfate residues were linked to the catechin B-ring. In particular, ions at *m/z* 231 (151 + 80) and 201 (121 + 80) correspond respectively to the $^{1,3}B^-$ and $^{1,2}B^-$ fragments with the sulfate moiety. The ion at *m/z* 245 produced in all peaks is attributed to the loss of CO_2 $[(M - H) - 44]^-$ from the aglycone; this fragment was observed as the main product ion in the MS^3 spectra. These fragmentation patterns and the expected HPLC elution behavior by analogy with that of the methylcatechins allowed the identification of peaks 1, 2, 3, and 4 in the chromatogram of **Figure 3** as catechin-5-*O*-sulfate, catechin-3′-*O*- sulfate, catechin-7-*O*- sulfate, and catechin-4′-*O*-sulfate, respectively.

Conclusions. Procedures for the hemisynthesis of (epi)catechin sulfates, glucuronides, and methylethers of metabolic interest have been optimized. In the conditions used, phenolic hydroxyl groups at the 3′ and 4′ positions in the catechin B-ring were shown as the most reactive for conjugation, followed by phenolic hydroxyls at positions 5 and 7 on the A-ring, while hardly any derivatives at position 3 (C-ring) were produced. Five catechin monoglucuronides, four catechin monosulfates, and four catechin methylethers could be obtained and characterized by HPLC−DAD−MS. Identification of the compounds was made on the basis of their MS^2 fragmentation and elution characteristics in RP-HPLC, thus establishing patterns for their further analysis in biological fluids. The methodologies used allowed the preparation of (epi)catechin sulfates, glucuronides, and methylethers conjugated at positions 3′ and 4′, as well as the sulfates at positions 5 and 7 with satisfactory yields for their further isolation by semipreparative-HPLC in view of their use in in vitro/ex vivo assays for their biological activity.

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