

Antioxidant Activity of Phenolic Acids and Their Metabolites: Synthesis and Antioxidant Properties of the Sulfate Derivatives of Ferulic and Caffeic Acids and of the Acyl Glucuronide of Ferulic Acid

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ABSTRACT: The main metabolites of caffeic and ferulic acids (ferulic acid-4'-O-sulfate, caffeic acid-4'-O-sulfate, and caffeic acid-3'-O-sulfate), the most representative phenolic acids in fruits and vegetables, and the acyl glucuronide of ferulic acid were synthesized, purified, and tested for their antioxidant activity in comparison with those of their parent compounds and other related phenolics. Both the ferric reducing antioxidant power (FRAP) assay and the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging method were used. Ferulic acid-4'-O-sulfate and ferulic acid-4'-O-glucuronide exhibited very low antioxidant activity, while the monosulfate derivatives of caffeic acid were 4-fold less efficient as the antioxidant than caffeic acid. The acyl glucuronide of ferulic acid showed strong antioxidant action. The antioxidant activity of caffeic acid-3'-O-glucuronide and caffeic acid-4'-O-glucuronide was also studied. Our results demonstrate that some of the products of phenolic acid metabolism still retain strong antioxidant properties. Moreover, we first demonstrate the *ex vivo* synthesis of the acyl glucuronide of ferulic acid by mouse liver microsomes, in addition to the phenyl glucuronide.

KEYWORDS: caffeic acid, ferulic acid, glucuronide, sulfate, ferulic acid acyl glucuronide

INTRODUCTION

Oxidative stress is involved in many human diseases, such as atherosclerosis, cancer, neurodegenerative diseases, diabetes, and aging. Dietary antioxidants have been considered to exert a protective role against the development and progression of these oxidative stress-related pathological conditions.¹ Among dietary antioxidants, polyphenols received particular attention in the past decade due to their antioxidant activity and biological effects. Epidemiological studies have suggested associations between the consumption of polyphenol-rich food and the prevention of many human diseases associated with oxidative stress.^{2–5} On the basis of their daily intake, which greatly exceeds that of other antioxidants (vitamin E, vitamin C, and β -carotene), phenolic compounds may be a major factor in assuring the antioxidant potential of the diet and may contribute to maintaining the endogenous redox balance in humans.

Phenolic acids, a major class of polyphenols, are widely distributed in the human diet, particularly in fruits, vegetables, and beverages (coffee, beer, wine, and fruit juices).^{6,7} Daily intake of phenolic acids has been reported to be in the order of 200 mg/d within a large range depending on nutritional habits and preferences.^{2,6,7} Phenolic acids received special attention because of their relatively high concentrations in food and beverages, strong antioxidant activity, and easy intestinal absorption. Dietary phenolic acids are extensively metabolized in humans.^{8–16} In particular, caffeic acid and ferulic acid, the most representative phenolic acids in the diet, after their absorption from the gastrointestinal tract, circulate in human plasma as conjugated forms, mainly glucuronate and sulfate

derivatives.^{8–10,12,17–24} Glucuronidation and sulfation of polyphenols are generally believed to significantly reduce their antioxidant activity, particularly because both sulfation and glucuronidation occur at the reducing hydroxyl groups of the phenolic structure, which are the functions mainly responsible for the antioxidant properties of polyphenols. However, phenolic acids bear a carboxyl function in addition to the hydroxyl groups in their structure. Consequently, while sulfation (implying an "ether" like bond) can occur only at the hydroxyl group of the phenolic acid, glucuronidation instead might occur both at the hydroxyl group (phenyl-O-glucuronides, in which the hydroxyl group on C-1 of the glucuronide moiety is bound to the hydroxyl group of phenolic acid by an "ether" type bond) or at the carboxylic group (acyl glucuronides, in which the hydroxyl group on C-1 of the glucuronide moiety is bound to the carboxylic group by an "ester" type bond) of the phenolic acid. Acyl glucuronides of many substances (xenobiotics, endogenous compounds, and drugs) bearing a carboxyl group have been recognized in animal and human urine, plasma, and tissues.^{25–27} Noteworthy, acyl glucuronides of phenolic acids, retaining all the reducing hydroxyl functions of the phenolic acid, might possess an antioxidant activity higher than that of their respective phenyl-O-glucuronides, in which at least one reducing hydroxyl group of the phenolic acid binds the glucuronate moiety. In spite of

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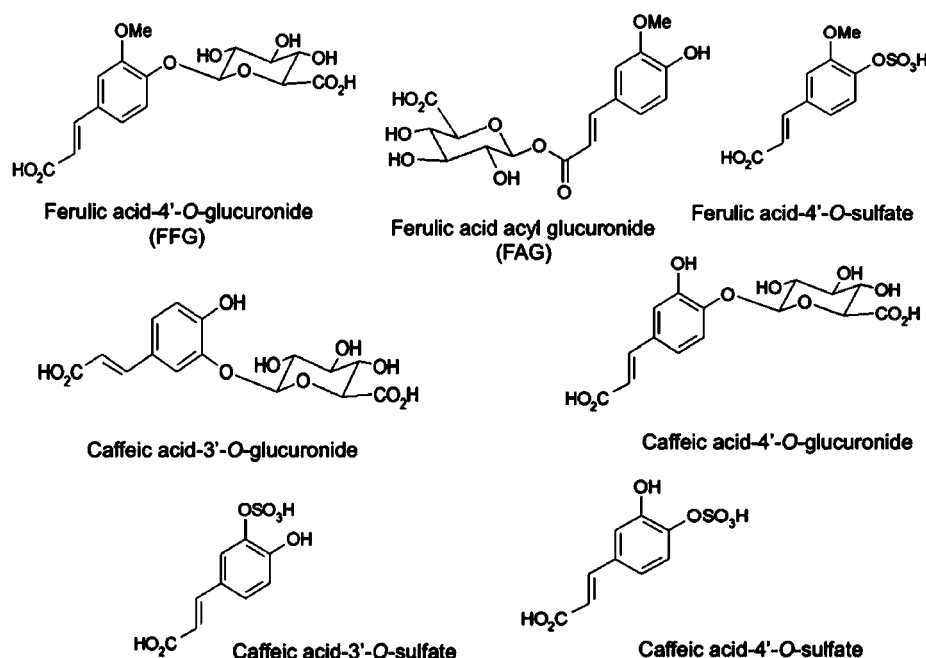


Figure 1. Chemical structure of ferulic and caffeic acids metabolites.

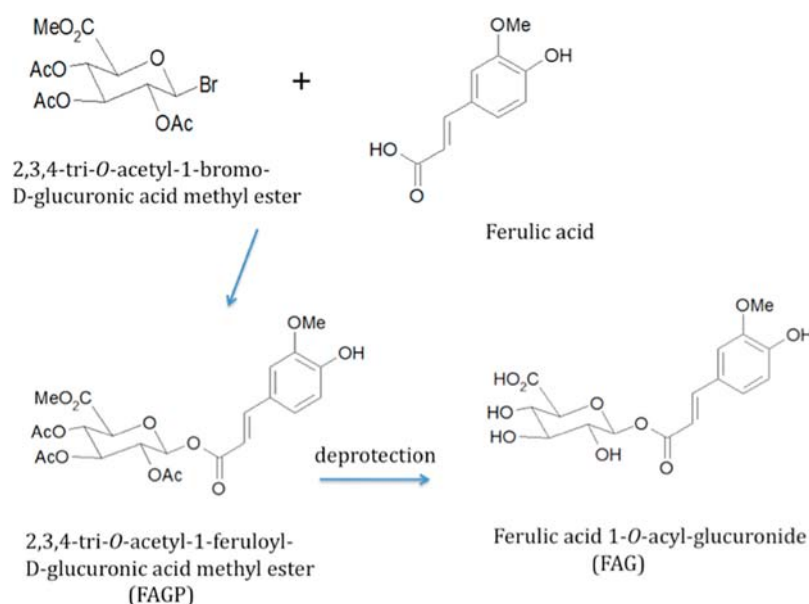


Figure 2. Chemical structures of the precursors (ferulic acid and 2,3,4-tri-O-acetyl-1-bromo-D-glucuronic acid methyl ester) and intermediate (2,3,4-tri-O-acetyl-1-feruloyl-D-glucuronic acid methyl ester, FAGP) in the synthesis of ferulic acid acyl glucuronide (FAG).

the large data concerning the antioxidant activity and biological effects of phenolic acids, very few studies deal with the antioxidant activity and chemical and biological properties of their metabolites^{23,24,28–30} due to the fact that most of these compounds are not commercially available. Moreover, whereas the occurrence in animals and human beings of sulfate derivatives of caffeic and ferulic acid has been well described in the literature,^{8,12,22–25} for glucuronate derivatives all data focused on the phenyl-O-glucuronides, in which the glucuronide moiety is bound to the hydroxyl group of phenolic acid by an “ether” type bond, of caffeic and ferulic acids.^{12,22,25} The first aim of this study was to assess the antioxidant properties of the sulfate and glucuronate metabolites of ferulic and caffeic acids with respect to that of their parent compounds and of

other related phenolic acids. Because of the fact that many of these metabolites are not commercially available, we synthesized and purified some of the metabolites of caffeic and ferulic acids: the sulfate derivative of ferulic acid (ferulic acid 4'-O-sulfate) and the monosulfate derivatives of caffeic acid (caffeic acid 3'-O-sulfate and caffeic acid 4'-O-sulfate). The second aim of our study was to assess the possible occurrence of the acyl glucuronides of phenolic acids, in addition to the well-known phenyl-O-glucuronides. We first synthesized and purified the acyl glucuronide of ferulic acid (FAG) (Figure 1) and compared its antioxidant activity with that of the respective phenyl-O-glucuronide and with the parent ferulic acid.

Finally, we demonstrated for the first time the *ex vivo* synthesis of FAG by mouse liver microsomes.

MATERIALS AND METHODS

Chemicals. 2,4,6-Tri-(2-pyridyl)-S-triazine (TPTZ), β -glucuronidase (EC 3.2.1.31 type IX A, from *E. coli*), gallic acid, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, trolox, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), acetobromo- α -D-glucuronic acid methyl ester (2,3,4-tri-O-acetyl-1-bromo-D-glucuronic acid methyl ester), uridine-5'-diphosphoglucuronic acid trisodium salt (UDP-GA), sinapic acid, pyridin, triton X-100, potassium peroxydisulfate, LC/MS grade methanol, and formic acid were from Sigma-Aldrich (St. Louis, MO, USA). 4-Hydroxyphenylacetic acid and *o*-coumaric acid were from Extrasynthese (Genay Cedex, France). Caffeic acid 3'- β -D-O-glucuronide, caffeic acid 4'- β -D-O-glucuronide, and ferulic acid 4'-O- β -D-glucuronide (FFG) were from Toronto Research Chemicals (North York, Ontario, Canada). Supelclean LC18 SPE cartridges (3 or 12 mL tubes, as specified) were from Supelco (Bellefonte, PA, USA). Glacial acetic acid, chlorosulphonic acid, methanol (MeOH), acetonitrile, and diethyl ether were obtained from Carlo Erba (Milano, Italy). For HPLC analysis, ultrapure water from a Milli-Q system (Millipore, Bedford, MA, USA) was used.

Stock solutions of standard phenolic acids were prepared in methanol (MeOH) (1 mg/mL), stored at -80°C , and used within 1 week. Standard solutions of glucuronate and sulfate derivatives of caffeic and ferulic acids were prepared in twice-distilled water (1 mg/mL), stored at -80°C , and used within 1 week. Working standard solutions were prepared daily by dilution in sample buffer (1.25% acetic acid and 7% MeOH in twice-distilled water).

Ferulic Acid Sulfate Ester and Caffeic Acid Monosulfate Esters: Synthesis, Purification, and Identification. Ferulic acid 4'-O-sulfate and the two monosulfate esters of caffeic acid (caffeic acid 4'-O-sulfate and caffeic acid 3'-O-sulfate) (Figure 1) were synthesized and purified according to Todd et al.³¹ Purification of the two monosulfate esters of caffeic acid gave a mixture of the two isomers (4'-O-sulfate and 3'-O-sulfate in the ratio 3:1). Purified ferulic acid sulfate ester and the mixture of the two monosulfate esters of caffeic acid were identified by ^1H NMR spectra (300 MHz, CD_3OD), as already reported³¹ and by UPLC-MS analyses, as described in the following.³² Ferulic acid 4'-O-sulfate showed a purity >95%; for caffeic acid monosulfate, the purity was >90%. The yield was 92% for ferulic acid 4'-O-sulfate and 47% for caffeic acid monosulfate (as a mixture of the two isomers 4'-O-sulfate and 3'-O-sulfate).

Ferulic Acid Acyl-glucuronide: Synthesis, Purification, and Identification. Ferulic acid 1-O-acyl-glucuronide (FAG) was synthesized in two steps (Figure 2). First, the protected form of ferulic acid 1-O-acyl-glucuronide (2,3,4-tri-O-acetyl-1-feruloyl-D-glucuronic acid methyl ester, FAGP) was synthesized by the reaction of ferulic acid with 2,3,4-tri-O-acetyl-1-bromo-D-glucuronic acid methyl ester following the procedure described by Fialova et al.,³³ with minor modifications. FAGP was then purified by chromatography on a SPE cartridge. Purified FAGP was finally deprotected by a hydrolytic procedure to give ferulic acid acyl glucuronide (FAG).

Briefly, for FAGP synthesis, to a solution of ferulic acid (1 g) and 2,3,4-tri-O-acetyl-1-bromo-D-glucuronic acid methyl ester (1 g) in 10 mL of ACN and 8.7 mL of twice-distilled water, 1.3 mL of 4 N NaOH was added. The mixture was stirred for 24 h at room temperature, then the solution was evaporated under reduced pressure at 40°C . To eliminate excess ferulic acid, the residue was extracted twice with 15 mL of CH_2Cl_2 . The organic phase was dried under reduced pressure at 40°C , and about 1 g FAGP was obtained as a white powder. For FAGP purification, 0.5 g of FAGP dissolved in absolute MeOH was loaded onto a 12 mL Supelclean LC18 SPE tube equilibrated with water. The tube was washed, in order, with 100 mL of twice-distilled water; 100 mL of MeOH/ H_2O , 75/25; and 80 mL of MeOH/ H_2O 60/40 (v/v). FAGP was eluted with 100 mL of MeOH/ H_2O (50/50), the eluate was evaporated under reduced pressure at 40°C , and about 200 mg of purified FAGP as a white crystalline powder was obtained. Purified FAGP was identified by UPLC-MS analyses,³² using the same experimental conditions applied for sulfate derivatives and described in the following. The purity of FAGP was >90%. The yield was about 25%.

To obtain FAG, purified FAGP was deprotected following the procedure described by Gauthier et al.³⁴ with minor modifications. One hundred milligrams of purified FAGP was added with 47 mg of KOH (4.2 equivalents) in 36 mL of water. The mixture was stirred at room temperature for 40 min, the pH was brought to about 6.0 with 1 N HCl. The solution was evaporated under reduced pressure at 40°C and the residue purified by solid phase extraction as follows. The residue was dissolved in 0.5 mL of twice-distilled water, centrifuged for 5 min at 18 000g, then the supernatant was loaded onto a Supelclean LC 18 SPE tube (3 mL) equilibrated with water. FAG was eluted with 12 mL of twice-distilled water. The eluate was evaporated under reduced pressure at 40°C . The residue was dissolved in 0.5 mL of 0.22 M acetic acid and loaded onto a Supelclean LC18 SPE tube (3 mL) equilibrated with 0.22 M acetic acid. The tube was washed with 7 mL of 0.22 M acetic acid; FAG was eluted with about 10 mL of 0.22 M acetic acid/MeOH 90/10 (v/v). The eluate was evaporated under reduced pressure at 40°C . The purified FAG obtained was identified by UPLC-MS analyses,³² as described in the following. The FAG obtained showed a purity >85%. The yield was about 20%. FAG was obtained as a mixture of different positional isomers (see below). To purify and separate the different FAG isomers, the last purification step was repeated, the eluate fractionated, and each fraction analyzed on HPLC-ECD.

UPLC/TOF-MS Analyses. The UPLC/Q/TOF conditions are detailed in Theodoridis et al.³² In short, UPLC-MS analyses were performed on a Waters Acquity UPLC system coupled with to a Synapt HDMS QTOF-MS (Waters, Manchester, UK) via an electrospray interface (ESI), operating in W-mode and controlled by MassLynx 4.1 and a UV-vis (DAD) detector recording the spectra from 200 to 490 nm. An Aquity UPLC C18 column (2.1×150 mm, $1.8 \mu\text{m}$) (HSS T3, Waters) thermostated at 30°C was used. The column, equilibrated with solvent A (0.1% formic acid in twice-distilled water), was eluted with increasing concentration of solvent B (0.1% formic acid in methanol) at 0.3 mL/min, 0–6 min, 100% A; 7–56 min, from 100% A to 100% B; 57–60 min, 100% B. The injection volume was 5 μL . The mass spectrometry data were collected in negative mode (CV 2.5), in centroid mode (mass to charge ratio (m/z) 100–1000). For MS² experiments, the collision energy in transfer was settled at 30 V. External calibration with sodium formate and Lock Mass calibration with leucine enkephaline solution (0.5 mg/L, m/z 556.2771 at 0.1 mL/min) was applied.

In our UPLC conditions, the elution times were ferulic acid 4'-O-sulfate, 19.5 min; caffeic acid 4'-O-sulfate, 17.9 min; caffeic acid 3'-O-sulfate, 18.6 min; and FAGP, 36.2 min. FAG isomers were eluted at 16.6, 18.4, 18.7, and 20.0 min.

Orbitrap. For MS³ experiments, an Orbitrap XL mass spectrometer (Thermo Fisher Scientific (Bremen), GmbH, Germany) with a resolution of 100 000 [at m/z 400, full width half-maximum (fwhm)] was employed. Capillary temperatures were set at 270°C . An ESI capillary voltage of 2.7 kV in negative mode was used. Scan time was set to 3 μs with maximum acquisition time 200 ms. Fourier transform MS (FTMS) full scans from m/z 50 to m/z 1000 were acquired using Instrument Control Software (LTQ Tune Plus 2.5.5 SP1) and Xcalibur 2.1. Orbitrap was calibrated prior to mass analysis by external calibration using standard peptide mixtures (ProteoMass MALDI calibration kit for LTQ XL and LTQ hybrids, Sigma-Aldrich) for the normal mass range. The samples were diluted with methanol–water 1:1 and infused into the instrument by glass syringe.

HPLC Instrumentation. The synthesis and purification of the derivatives of caffeic acid and ferulic acid were followed by HPLC with both electrochemical detection (ECD) and UV detection. The HPLC consists of a Perkin-Elmer Liquid Chromatograph (Perkin-Elmer, Norwalk, CT, USA) with gradient pump, column thermoregulator, autosampling injector equipped with electrochemical coulometric detector (Coulchem II, ESA, Bedford, MA, USA), or diode array detector (Perkin-Elmer, Norwalk, CT, USA). A chromatography workstation with Totalchrom software (Perkin-Elmer, Norwalk, CT, USA) was used for data processing. Operating conditions were as follows: column temperature, 30°C ; flow rate, 1 mL/min; injection volume, 100 μL ; electrochemical detection at +600 mV, sensitivity

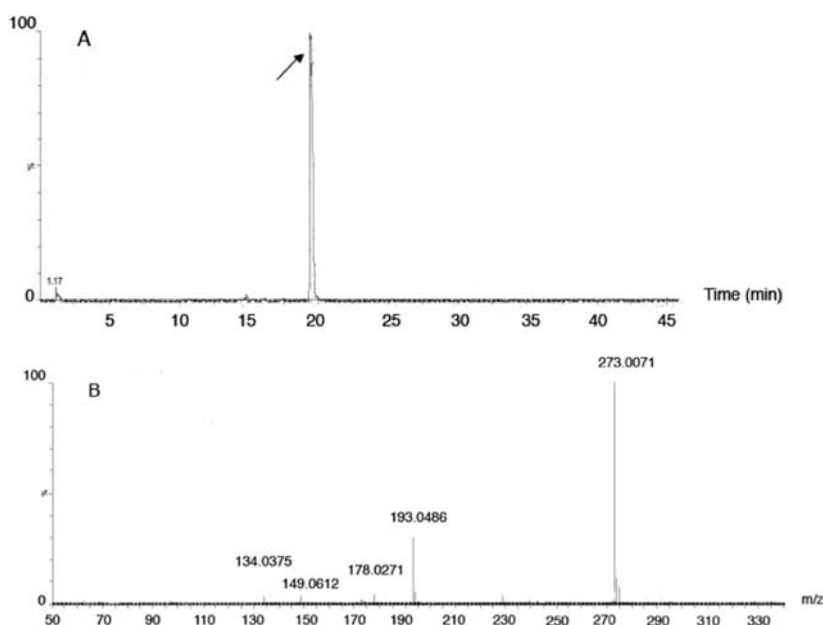


Figure 3. UPLC-MS analysis of purified ferulic acid-4'-O-sulfate. Panel A: UPLC-MS chromatogram, with ferulic acid-4'-O-sulfate eluting at 19.5 min; Y-axis, relative abundance (%); X-axis, time (min). Panel B: MS spectrum of ferulic acid-4'-O-sulfate; Y-axis, relative abundance (%); X-axis, m/z ratio.

range, 200 nA; and filter, 2 s. For the UV detector, analyses were performed at 320 or 230 nm as specified. Chromatographic separations were performed on a Supelcosil LC-18 C_{18} column, 5.0 μ m particle size, and 250 \times 4.6 mm ID (Supelco, Bellefonte, PA, USA) including a guard column. For gradient elution, mobile phases A and B were employed. Solution A was 1.25% glacial acetic acid in twice-distilled water; solution B was absolute methanol. For the sulfate derivatives of caffeic acid and ferulic acid, the following gradient was used: 0–30 min, from 98% A, 2% B to 88% A, 12% B, linear gradient; 31–60 min, from 88% A, 12% B to 80% A, 20% B, linear gradient; 61–90 min, from 80% A, 20% B to 74% A, 26% B, linear gradient; and 91–98 min, 55% A, 45% B; 99–129 min, 98% A, 2% B. Retention times were 25.3 min for ferulic acid 4'-O-sulfate; 15.6 and 16.5 min for caffeic acid 4'-O-sulfate and caffeic acid 3'-O-sulfate, respectively. For the synthesis, purification, and characterization of FAGP and FAG, the following gradient was used: 0–50 min, from 98% A, 2% B to 50% A, 50% B, linear gradient; 51–60 min, 50% A, 50% B; and 61–90 min, 98% A, 2% B. Retention times were 20.9, 22.5, 25.2, and 26.0 min for the different positional FAG isomers; 36.9 min for ferulic acid; and 61.7 min for FAGP. Prior to HPLC analysis, all samples were filtered using Millex-HV filters (Millipore, Bedford, MA, USA) with 0.45 μ m pore size.

Microsome Preparation. Animal studies were performed under conditions approved by the National Health Ministry (Department of Food, Nutrition and Public Animal Health).

Balb/c mice (Charles River, Calco (LC), Italy) were anesthetized with pentobarbital injection (10 mg) and the liver excised and placed in cold phosphate-buffered saline (PBS). After weighing, livers were homogenized in 4 volumes of 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 0.1 M KCl with an Ultraturrax homogenizer (IKA, Staufen, Germany) for two 40-s intervals and treated for microsome purification as described by van der Hoeven et al.³⁵ The purified microsomal fractions were suspended in 0.01 M Tris-acetate buffer at pH 7.4 containing 20% glycerol and 0.1 mM EDTA and stored at -80°C until used. Protein concentrations were measured by the method of Lowry³⁶ using bovine serum albumine as the standard.

Biosynthesis of Ferulic Acid Glucuronides in Liver Microsomes. The conjugation reaction was carried out according to Jemnitz et al.³⁷ The incubation mixture contained 0.1 M Tris-HCl buffer at pH 6.9; 0.05% Triton X-100; 5 mM UDP-GA; 10 mM MgCl_2 ; 4 mg/mL

microsomal protein; and 1 mM ferulic acid in a final volume of 1 mL. Microsomes were preincubated with Triton X-100 for 30 min at 0°C and the reaction started with the addition of ferulic acid. Blanks without UDP-GA or ferulic acid were run. Incubation was performed at 37°C for 60 min. The reaction was stopped by the addition of 0.05 mL of 1 N HCl to the incubation mixture. For phenolic compound extraction, the pH was brought to 3.0, and 300 mg of NaCl was added. Samples were then extracted four times for 10 min with 6 volume excess of ethyl acetate, following by 10 min of centrifugation at 2500g. The pooled organic supernatant was dried under nitrogen stream and the residue dissolved in 0.5 mL of 1.25% glacial acetic acid containing 7% (v/v) MeOH and analyzed by HPLC with ECD or UV detection after appropriate dilution. The extraction procedure allows the quantitative recovery of ferulic acid, ferulic acid 4'-O-glucuronide, and FAG (range 87.6%–99.4%).

Antioxidant Activity. The antioxidant activity on phenolic acids and their metabolites was measured by both the ferric reducing antioxidant power (FRAP) and the ABTS radical scavenging assay.

FRAP Assay. Ferric-reducing antioxidant power was measured as described by Benzie et al.³⁸ The FRAP assay is a simple method of determining the reduction of a ferric-tripyridyltriazine complex to its ferrous, colored form in the presence of antioxidants. Phenolic compounds were tested at various concentrations in the range of 0–500 μ M, as specified. Sulfate and glucuronide derivatives were dissolved in twice-distilled water. Methanol, used to dissolve most of the phenolic acids, was $\leq 5.0\%$ in the final test mixture. Aqueous or methanolic ($\leq 5.0\%$ MeOH in the final test mixture) iron sulfate solutions were used in the range 10–100 μ M for calibration curves. The working FRAP reagent was prepared daily as follows: 2.5 mL of 10 mM TPTZ in 40 mM HCl and 2.5 mL of 20 mM FeCl_3 added with 25 mL of 0.3 M acetate buffer at pH 3.6. FRAP reagent (0.9 mL), prewarmed at 37°C , was mixed with 100 μ L of test sample. The reaction was followed for up to 4 min at 593 nm at 37°C . Blanks containing FRAP reagent and water or methanol instead of phenolic compounds were also run; the difference between sample absorbance and blank absorbance at 593 nm after 4 min of reaction at 37°C was used for calculation. Each sample was analyzed in triplicate. Simple regression analysis was performed to calculate the dose–response relationship of each compound tested.

ABTS Assay. The free radical scavenging capacity of phenolic acids and their metabolites was also studied using the ABTS radical cation

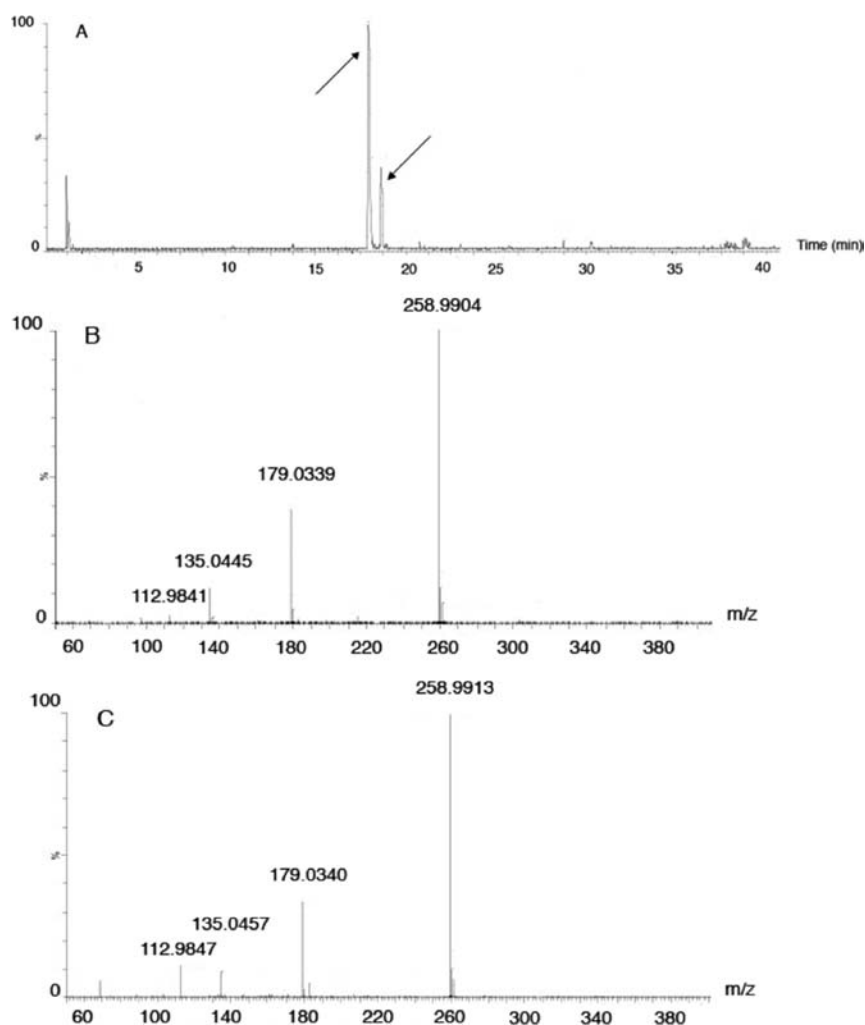


Figure 4. UPLC-MS analyses of purified caffeic acid-4'-O-sulfate and caffeic acid-3'-O-sulfate. Panel A: UPLC-MS chromatogram, with caffeic acid-4'-O-sulfate eluting at 17.9 min and caffeic acid-3'-O-sulfate eluting at 18.6 min; Y-axis, relative abundance (%); X-axis, time (min). Panel B: mass spectrum of caffeic acid-4'-O-sulfate; Y-axis, relative abundance (%); X-axis, m/z ratio. Panel C: mass spectrum of caffeic acid-3'-O-sulfate; Y-axis, relative abundance (%); X-axis, m/z ratio.

decolorization assay,³⁹ which is based on the reduction of ABTS^{•+} radicals by antioxidants tested. ABTS (7 mM) was dissolved in twice-distilled water, and the ABTS radical cation (ABTS^{•+}) was produced by reacting the ABTS solution with 2.45 mM potassium persulfate (final concentration) at room temperature in the dark for 16 h before use. A working solution was diluted to absorbance values between 1.2 and 1.3 AU at 734 nm with twice-distilled water and equilibrated at 30 °C. ABTS reagent (0.95 mL) prewarmed at 30 °C was mixed with 50 μ L of test sample. The absorbance reading was taken at 734 nm, 30 °C, at 20 s intervals up to 6 min after initial mixing (A_E). Phenolic compounds were tested at various concentrations in the range 0–20 μ M, as specified. Sulfate and glucuronide derivatives were dissolved in twice-distilled water. Methanol, used to dissolve most of the phenolic acids, was $\leq 0.5\%$ in the final test mixture. Trolox (0–15 nmol), the water-soluble analogue of vitamin E, was used as the reference compound. An appropriate solvent blank reading was also taken (A_B). The percentage inhibition of absorbance was calculated using the following formula:

$$\% \text{Inhibition} = [(A_B - A_E)/A_B] \times 100$$

where A_B = absorbance of the blank sample, and A_E = absorbance of the sample tested (at 6 min reaction) and plotted as a function of the concentration of the compound tested. All solutions were prepared daily. All measurements were made in triplicate. Simple regression

analysis was performed to calculate the dose–response relationship of each phenolic compound tested.

Hydrolytic Treatments. *β -Glucuronidase Treatments.* Enzymatic hydrolysis of FAG was performed as already described,⁸ with minor modifications. Briefly, FAG was dissolved in twice-distilled water at 1 mg/mL. Aliquots were incubated in 0.7 mL of final volume 0.2 M K-phosphate buffer at pH 6.8 with 2000 U of β -glucuronidase, dissolved in the same buffer, at 37 °C for 2 h. At the end of incubation, samples were centrifuged for 10 min at 18000g, filtered, and analyzed by HPLC with ECD or UV detection, after appropriate dilution.

Alkaline Hydrolysis. Alkaline hydrolysis of FAG was performed by the alkaline hydrolytic treatment previously described.¹⁰ FAG dissolved in twice-distilled water was hydrolyzed in the presence of 10 mM EDTA, 1% ascorbic acid, and 1.8 N NaOH in 1 mL of final volume. After 30 min at 30 °C, 4 N HCl was added to bring the pH to 3.0. Samples were filtered and analyzed by HPLC with ECD or UV detection, after appropriate dilution.

Statistical Analysis. Data presented are the means \pm standard error. Statistical analysis was performed using a statistical package running on a PC (KaleidaGraph 4.0, Synergy Software, Reading, PA, USA). Student's *t* test was used for regression analyses. The probability of $p < 0.05$ was considered statistically significant.

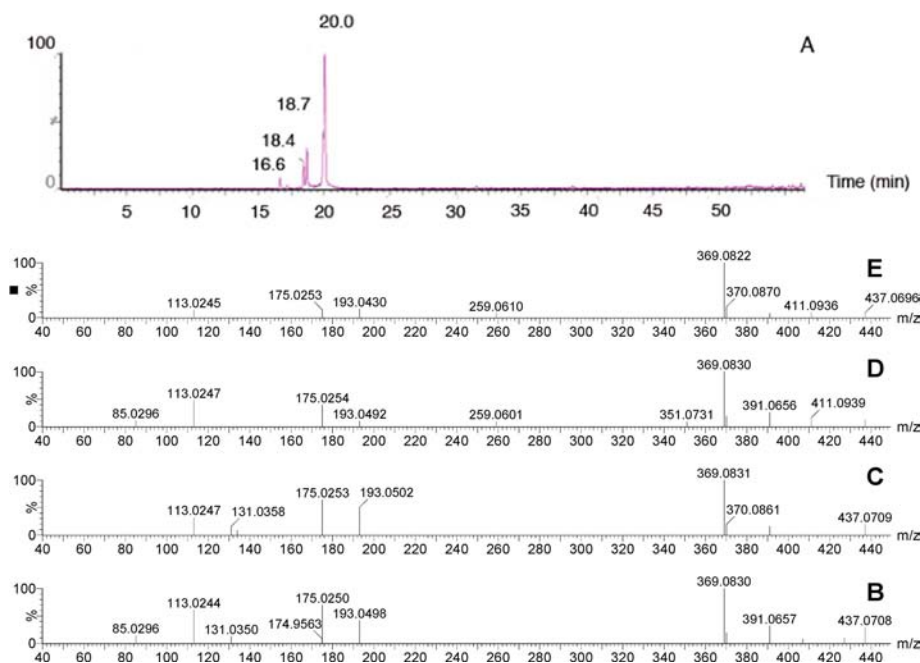


Figure 5. UPLC-MS analysis of purified FAG. Panel A: UPLC-MS chromatogram, with FAG isomers eluting at 16.6, 18.4, 18.7, and 20.0 min; Y-axis, relative abundance (%); X-axis, time (min). Panels B–E: mass spectra of FAG isomers eluting at 16.6 min (B); 18.4 min (C); 18.7 min (D); and 19.9 min (E); Y-axis, relative abundance (%); X-axis, m/z ratio.

RESULTS

Characterization of Sulfate Derivatives of Ferulic and Caffeic Acids. Figure 3 shows the UPLC-MS profile of ferulic acid 4'-O-sulfate: in panel A, the UPLC-MS elution chromatogram shows a major component eluting at 19.5 min; in panel B, the mass spectrum of the peak eluting at 19.5 min shows the presence of the negatively charged molecular ion $[M - H]^-$ at m/z 273.0071 corresponding to the value expected for ferulic acid 4'-O-sulfate and a major fragment at 193.0486 corresponding to the ferulic acid moiety. Further confirmation of the compound was obtained by Orbitrap mass spectrometry, which gave the resulting MS^2 fragmentation pattern with an ion at m/z 193.0500 and an MS^3 fragmentation pattern with ions at m/z 134.0369, 149.0603, and 178.0265 (data not shown). These data are in agreement with the literature.¹²

Figure 4 shows the UPLC-MS profile of caffeic acid monosulfate derivatives (caffeic acid 4'-O-sulfate and caffeic acid 3'-O-sulfate): in panel A, the UPLC-MS elution chromatogram shows two major peaks eluting at 17.9 and 18.6 min; in panels B and C, the mass spectra of the two peaks eluting at 17.9 min (panel B) and 18.6 min (panel C) show the presence of the negatively charged molecular ion $[M - H]^-$ at m/z 258.9904 and 258.9913, respectively, corresponding to the value expected for both caffeic acid 4'-O-sulfate and caffeic acid 3'-O-sulfate, with a major fragment at 179.0340 for both peaks, corresponding to the caffeic acid moiety. The resulting MS^2 fragmentation pattern gave ions at m/z 135.0449 and 179.0341 for both peaks (data not shown). According to the literature,¹² the peak eluting at 17.9 min should be attributable to the caffeic acid 4'-O-sulfate derivative, while the peak eluting at 18.6 min should be ascribed to caffeic acid 3'-O-sulfate. The ratio of caffeic acid 4'-O-sulfate and caffeic acid 3'-O-sulfate is about 2.75: 1.

Characterization of Ferulic Acid Acyl Glucuronide and Microsomal Biosynthesis. In the present study, we synthesize for the first time the acyl glucuronide of ferulic

acid (FAG). Figure 5 shows the UPLC-MS analyses of purified FAG: in panel A, the UPLC-MS elution chromatogram exhibits a main component eluting at 20.0 min and three minor components eluting at 16.6 min, 18.4 min, and 18.7 min. In panels B, C, D, and E, the mass spectra of the four peaks in order of elution (16.6 min, panel B; 18.4 min, panel C; 18.7 min, panel D; and 20.0 min, panel E) show the presence of the negatively charged molecular ion $[M - H]^-$ at m/z 369.0822, m/z 369.0830, m/z 369.0831, and m/z 369.0830, respectively, corresponding to the value expected for FAG, and fragments at m/z 175.025 (corresponding to the loss of the glucuronic acid moiety) and m/z 113.024 for all of the four peaks. The resulting MS^2 fragmentation patterns show ions at m/z 175.025 and m/z 113.024 for all of the four peaks (data not shown). Fragments at 175 and 113 have been already described for dihydroferulic acid-4'-O-glucuronide.¹² In spite of the same molecular ion, the ratio of fragments in the mass spectra of the four peaks shows some differences, suggesting that the conjugates of glucuronic acid with ferulic acid are not identical. A similar behavior has been described for isomers of the acyl glucuronide of the synthetic flavonoid ipriflavone.³⁷ These results are in agreement with the isomerization of the ferulic acid 1-O-acyl glucuronide (the C-1 isomer of FAG), due to intramolecular acyl migration from -OH to the adjacent -OH, generating different positional isomers of FAG, with the same molecular ion, in which the ferulic acid moiety is attached to the C-2, -3, or -4 position of the glucuronic acid ring. Intramolecular acyl migration of substituted sugars is a known process occurring under slightly alkaline conditions, and it has been already described for several acyl glucuronides.^{25–27,40,41} Since only C1-isomers are substrates for β -glucuronidase,^{25–27,40,41} we further purified the different FAG isomers and treated them with β -glucuronidase as described in the Materials and Methods section. Only the fraction containing the more retained and abundant FAG isomer (peak at 20.0 min in Figure 5, panel A) was hydrolyzed by β -glucuronidase, giving

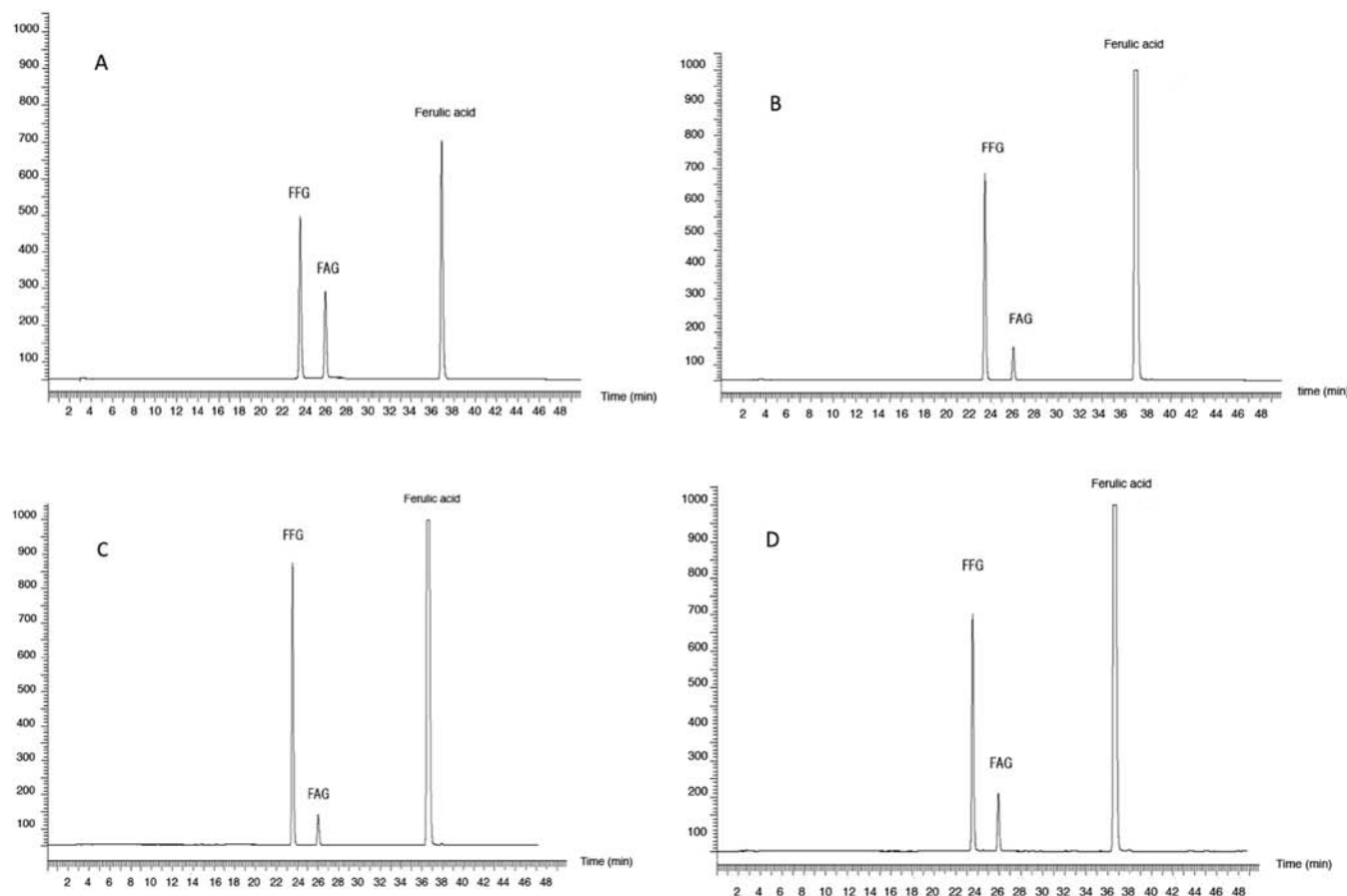


Figure 6. HPLC-UV analysis of the microsomal preparation for FAG biosynthesis; Y-axis, detector response (mV) at 320 nm; X-axis, time (min). Panel A: HPLC-UV chromatogram of standard ferulic acid-4'-O-glucuronide (FFG) eluting at 23.5 min; standard ferulic acid 1-O-acyl glucuronide (FAG) eluting at 26.0 min; standard ferulic acid, eluting at 36.9 min. Panel B: chromatographic profile of a typical microsomal sample after 1 h of incubation with ferulic acid and UDP-GA, as described in the Materials and Methods section. Panel C: the same sample as in B, added with standard FFG. Panel D: the same sample as in B, added with the standard FAG C1-isomer. Analyses were performed at 320 nm.

69.0 \pm 2.5% of the calculated ferulic acid, demonstrating that the more retained and abundant FAG isomer, eluting at 20.0 min in UPLC, is the C-1 isomer. Alkaline hydrolysis of the purified FAG C1-isomer gave 98.4 \pm 1.9% of the theoretical ferulic acid.

To investigate the biological relevance of the acyl glucuronide of ferulic acid (FAG), we studied the biosynthesis of FAG by mouse liver microsomes. Upon incubation of ferulic acid with UDP-GA and microsomal proteins from mouse liver as described in the Materials and Methods section, three main components were detected with a HPLC-UV detector at 320 nm, as shown in Figure 6, panel B. The first peak at 23.5 min coeluted with standard ferulic acid 4'-O-glucuronide (FFG, which is the phenyl glucuronide of ferulic acid) (Figure 6, panel C), the second component at 26.0 min coeluted with FAG (C-1 isomer) (Figure 6, panel D), and the third component at 36.9 min was residual ferulic acid. UPLC analyses, with both DAD at 320 nm and an MS detector, are shown in Figure 7, panels A and B, respectively. The mass spectra identified the peaks, in the order of elution, as follows: panel C, ferulic acid 4-O-glucuronide (FFG); panel D, ferulic acid 1-O-acyl glucuronide (FAG, C-1 isomer); panel E, ferulic acid. Interestingly, the mass spectra of FFG and FAG are almost undistinguishable, with the molecular ion at m/z 369.0818 and m/z 369.0826, respectively, and fragments at m/z 193.050, m/z 175.025, and m/z 113.024. The ratio between FFG and FAG is 2.4: 1 on the basis of

UPLC-MS analyses (mean of 3 separate experiments). The identity of FAG and FFG was further demonstrated in HPLC-ECD at +600 mV (data not shown). In fact, the FAG molecule possesses an unbound, reducing OH-group on the phenolic ring, which is revealed by the electrochemical detector, while FFG was not detected by an electrochemical detector due to the lack of reducing groups (in FFG, the OH group of the ferulic acid moiety is esterified with glucuronic acid). Overall, these results demonstrate for the first time the synthesis of the acyl glucuronide of ferulic acid by liver microsomes in ex vivo experiments. Noteworthy, microsomal proteins from mouse liver seem to synthesize only the C-1 isomer of FAG.

Antioxidant Activity Determined by the FRAP Assay.

The antioxidant activity of metabolites of phenolic acids, and more generally of polyphenols, has been poorly investigated,^{29,30,42} mainly due to the lack of commercially available standards.

In Table 1, we compared the ferric reducing ability (FRAP) of the caffeic acid and ferulic acid metabolites synthesized in this study with that of their respective parent compounds and with the activity of some commercially available caffeic acid and ferulic acid metabolites and of other related phenolic acids, as a measure of their antioxidant power. Iron sulfate was used as the reference compound. The slope of the curve reflects the antioxidant's reducing capacity; therefore, slope values allow for a comparison of the antioxidant efficiencies of the tested

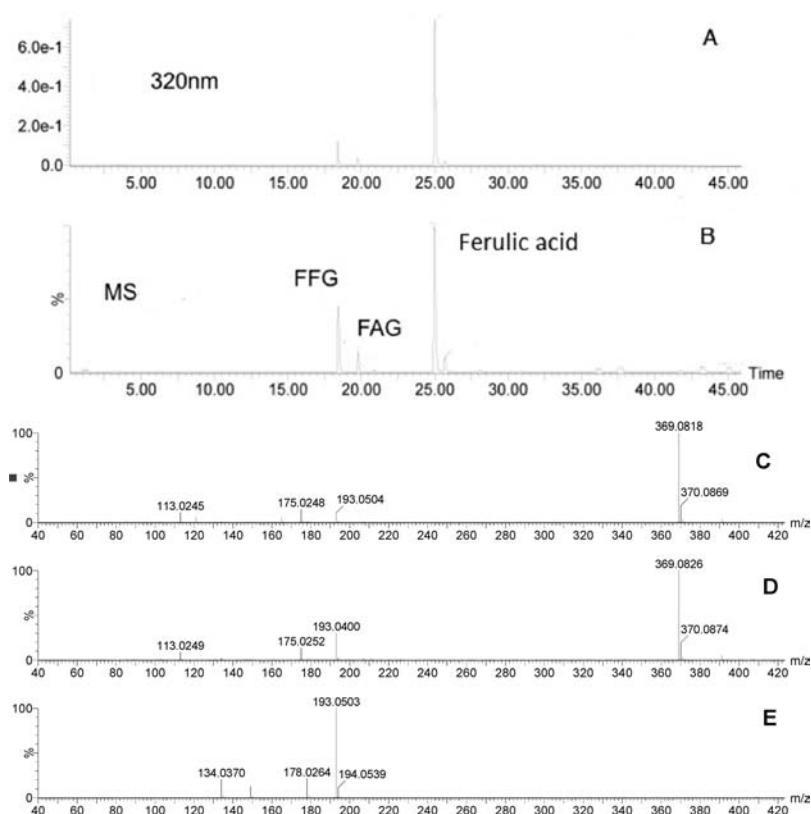


Figure 7. UPLC-DAD-MS analyses of the microsomal preparation for FAG biosynthesis. Panel A: UPLC-DAD chromatogram at 320 nm of a typical microsomal sample after 1 h of incubation with ferulic acid and UDP-GA; Y-axis, detector response (AU) at 320 nm; X-axis, time (min). Panel B: UPLC-MS chromatogram of the same sample; Y-axis, relative abundance (%); X-axis, time (min). Panels C–E: Y-axis, relative abundance (%); X-axis, m/z ratio. Mass spectra of C, peak eluting at 18.5 min, identified as ferulic acid-4'-O-glucuronide (FFG); D, peak eluting at 20.0 min, identified as ferulic acid 1-O-acyl glucuronide (FAG); E, peak eluting at 25.0 min, identified as ferulic acid.

compounds. High slope values denote high ferric reducing ability. Caffeic and ferulic acid showed a strong ferric-reducing activity, in the same order as that of trolox, the water-soluble analogue of vitamin E.

The ferric-reducing activity of the sulfate derivatives, caffeic acid monosulfate (a mixture of caffeic acid 4'-O-sulfate and caffeic acid 3'-O-sulfate in the ratio 2.75: 1), and ferulic acid 4'-O-sulfate was very low, compared to that of the respective parent compounds. Infact, the antioxidant activity of caffeic acid monosulfate was about 6.0% with respect to that of the parent caffeic acid, while the antioxidant activity of the ferulic acid 4'-O-sulfate was 5.5% of that of the parent ferulic acid.

Among glucuronides, FAG, the acyl glucuronide of ferulic acid, exhibited an antioxidant activity comparable to that of the parent ferulic acid, the slope being 0.0401 for ferulic acid, and 0.0383 and 0.0368 the values obtained for FAG (as a mixture of positional isomers) and the FAG-1 isomer, respectively. On the contrary, ferulic acid 4'-O-glucuronide, which is the phenyl-O-glucuronide of ferulic acid, showed a very low ferric-reducing ability (slope 0.0006) not only with respect to that of the parent ferulic acid but also with respect to that of FAG. From our data, the activity of ferulic acid 4'-O-glucuronide was about 70-fold lower with respect to that of ferulic acid and about 60-fold lower with respect to that of the acyl glucuronide of ferulic acid (FAG). The ferric-reducing activity of caffeic acid 3'-O-glucuronide was high, in the same order of that of iron sulfate, and it was about a half with respect to that of the parent caffeic acid. On the contrary, the ferric-reducing ability of caffeic acid 4'-O-glucuronide was about 10-fold lower with respect to that

of caffeic acid 3'-O-glucuronide. Overall, these results suggest that the 4'-hydroxyl group on the aromatic ring is crucial for ferric-reducing activity.

Among the other phenolic compounds tested, gallic acid with three hydroxyl groups on the aromatic ring is the strongest antioxidant; on the contrary, the monosubstituted phenolic acids (*p*-coumaric acid, *o*-coumaric acid, and 4-OH-phenyl acetic acid) showed very low activity. In general, the presence of the propenoid chain seems to have a positive effect on the reducing properties of the hydroxyl group, as demonstrated by the higher antioxidant activity of *p*-coumaric acid with respect to that of 4-OH-phenyl acetic acid and the higher antioxidant activity of ferulic acid with respect to that of vanillic acid, due to a stabilizing resonance effect on the phenoxyl radical.⁴³ Syringic acid and sinapic acid represent an exception, the antioxidant activity of syringic acid being a little bit higher than that of sinapic acid. Therefore, besides the presence of the 4'-hydroxyl group, also the number (degree) of substitutions (hydroxyl or methoxyl groups) on the aromatic ring is a determinant of the ferric-reducing antioxidant activity.^{44,45}

Antioxidant Activity Determined by the ABTS Assay.

In Table 2, we examined the ability of the same above-reported compounds to reduce the ABTS radical cation, using the ABTS radical cation decolorization assay. Trolox, the water-soluble analogue of vitamin E, was used as a reference compound. Again, high slope values denote high antioxidant activity; therefore, the slope values allow for a comparison of the antioxidant efficiencies of the tested compounds.

Table 1. Antioxidant Activity, Measured with the FRAP Assay, of Phenolic Acids and Their Metabolites in Comparison with Trolox^a

compsds	concentration range ($\mu\text{mol/L}$)	slope	intercept	correlation (r)
gallic acid	2.5–25	0.1083	+0.0334	0.9923
syringic acid	2.5–25	0.0668	−0.0109	0.9998
sinapic acid	2.5–25	0.0564	+0.0272	0.9978
caffeic acid	2.5–25	0.0485	+0.0066	0.9995
trolox	5–50	0.0443	+0.0299	0.9987
ferulic acid	2.5–25	0.0401	+0.0841	0.9985
FAG ^b	2.5–25	0.0383	+0.0115	0.9997
FAG-1 ^c	2.5–25	0.0368	+0.0750	0.9992
chlorogenic acid	5–50	0.0349	+0.0018	0.9992
caffeic acid 3'-O-glucuronide	2.5–25	0.0231	+0.0167	0.9991
FeSO ₄	10–100	0.0222	−0.0128	0.9988
vanillic acid	5–50	0.0178	+0.0035	0.9981
<i>p</i> -coumaric acid	5–50	0.0079	+0.0176	0.9977
<i>o</i> -coumaric acid	2.5–25	0.0050	+0.0094	0.9992
caffeic acid monosulfate ^d	2.5–50	0.0029	+0.0022	0.9972
caffeic acid 4'-O-glucuronide	2.5–25	0.0025	+0.0025	0.9974
ferulic acid-4'-O-sulfate	2.5–50	0.0022	+0.0528	0.9937
4-OH-phenyl acetic acid	50–500	0.0006	−0.0520	0.9987
ferulic acid 4'-O-glucuronide	25–250	0.0006	−0.0370	0.9883

^aThe FRAP assay was performed as reported in the Materials and Methods section. Equations were calculated with at least six different concentrations assayed in triplicate ($P < 0.01$). All equations followed a linear regression model. Regression analysis was performed by Student's *t*-test. ^bMixture of positional FAG isomers. ^cFAG, C1 isomer. ^dMixture of caffeic acid-4'-O-sulfate and caffeic acid-3'-O-sulfate at a ratio of 2.75:1.

Caffeic and ferulic acids showed a strong antioxidant activity. The antioxidant activity of caffeic acid is about a half with respect to that of ferulic acid and quite similar to the antioxidant activity of trolox. The antioxidant activity of caffeic acid monosulfate (a mixture of caffeic acid 4'-O-sulfate and caffeic acid 3'-O-sulfate, in the ratio of 2.75: 1) is 4-fold lower with respect to that of the parent caffeic acid, while the antioxidant activity of ferulic acid 4'-O-sulfate is about 11-fold lower with respect to that of the parent ferulic acid. Among glucuronides, the antioxidant activity of FAG is about half of that of the parent ferulic acid and quite close to or slightly lower than the antioxidant activity of trolox and caffeic acid. Noteworthy, the antioxidant activity of ferulic acid 4'-O-glucuronide, the phenyl-O-glucuronide of ferulic acid, is about 30–40-fold lower with respect to the antioxidant activity of FAG, the acyl glucuronide of ferulic acid, and about 80-fold lower with respect to that of ferulic acid. Interestingly, the antioxidant activity of caffeic acid 3'-O-glucuronide is quite similar to that of the parent caffeic acid, while the antioxidant ability of caffeic acid 4'-O-glucuronide is about one-third of that of caffeic acid and caffeic acid 3'-O-glucuronide. Again, the presence of a 4'-hydroxyl group on the aromatic ring seems to be crucial for the antioxidant activity, as observed also with the FRAP assay. Among the other phenolic compounds tested, gallic acid with three hydroxyl groups on the aromatic ring is the strongest antioxidant. The presence of one or two methoxy

Table 2. Antioxidant Activity, Measured with the ABTS Assay, of Phenolic Acids and Their Metabolites in Comparison with Trolox^a

compsds	concentration range ($\mu\text{mol/L}$)	slope	intercept	correlation (r)
gallic acid	0.5–5	7.354	+1.3745	0.9915
ferulic acid	1–20	4.3967	+2.5438	0.9988
syringic acid	1–10	4.1088	+0.0467	0.9978
<i>p</i> -coumaric acid	0.5–5	3.9137	+2.4677	0.9859
sinapic acid	1–10	3.7791	+0.6338	0.9971
caffeic acid	3–20	2.4284	−0.4172	0.9994
trolox	2.5–15	2.3314	−0.2180	0.9977
caffeic acid 3'-O-glucuronide	1.5–20	2.2477	+0.3472	0.9995
<i>o</i> -coumaric acid	3–20	2.2009	+0.7565	0.9971
vanillic acid	0.5–5	2.1909	+0.6495	0.9924
FAG ^b	1.5–20	2.1382	+1.3584	0.9941
chlorogenic acid	2–25	1.8891	−0.2395	0.9970
FAG-1 ^c	1.5–20	1.7351	+1.0054	0.9936
4-OH-phenylacetic acid	0.5–5	1.2017	+0.8557	0.9797
caffeic acid 4'-O-glucuronide	1.5–20	0.7499	+0.9698	0.9902
caffeic acid monosulfate ^d	2.5–20	0.6159	+0.1875	0.9966
ferulic acid 4'-sulfate	2.5–20	0.3805	+0.2422	0.9918
ferulic acid 4'-O-glucuronide	2.5–20	0.0543	+0.0058	0.9273

^aThe ABTS assay was performed as described in the Materials and Methods section. Equations were calculated with at least six different concentrations assayed in triplicate ($P < 0.01$). All equations followed a linear regression model. Regression analysis was performed by Student's *t*-test. ^bMixture of positional FAG isomers. ^cFAG, C1 isomer. ^dMixture of caffeic acid-4'-O-sulfate and caffeic acid-3'-O-sulfate, at a ratio of 2.75:1.

substitutions in the ortho position to the 4'-hydroxyl group increased the antioxidant efficiency: substitution of the 3'-hydroxyl group of caffeic acid by a methoxy group (ferulic acid) considerably enhances the antioxidant activity, and syringic acid is more active than vanillic acid.⁴³ Again, as already observed with the FRAP assay, the presence of the propenoid chain seems to have a positive effect on the reducing properties of the hydroxyl group, as demonstrated by the higher antioxidant activity of *p*-coumaric acid with respect to that of 4-OH-phenyl acetic acid, and the higher antioxidant activity of ferulic acid with respect to that of vanillic acid, due to a stabilizing resonance effect on the phenoxyl radical.⁴³ Syringic acid and sinapic acid represent an exception, the antioxidant activity of syringic acid being quite similar to that of sinapic acid.

DISCUSSION

Dietary phenolic acids, abundant in most common fruits, vegetables, and beverages (coffee, beer, wine, and fruit juices) are absorbed and extensively metabolized in humans, and circulate in plasma mainly as sulfate and glucuronate derivatives.^{8–16} Very little data is present in the literature concerning the antioxidant activity of polyphenols metabolites. Quercetin glucuronides and quercetin 3-O-sulfate have been reported to retain antioxidant properties, although to a minor extent with respect to quercetin.²⁹ Ferulic acid β -glucuronide, prepared from the plasma of feruloyl arabinose fed rats, has been reported to possess an antioxidant activity stronger than

that of ferulic acid in the LDL oxidation system;³⁰ however, in this study ferulic acid β -glucuronide was isolated from plasma, and its concentration was measured by the release of ferulic acid by β -glucuronidase hydrolysis, which is known not to be completely exhaustive.⁴⁶ The glucuronidation and sulfation processes modify the hydrophobicity and the possibilities of electron delocalization so that the antioxidant activity of conjugates might be different from that of the parent compounds. Glucuronidation and sulfation of polyphenols are generally believed to significantly reduce their antioxidant activity due to the fact that both sulfation and glucuronidation occur at the reducing hydroxyl groups of the phenolic structure, which are the functions mainly responsible for the antioxidant properties of polyphenols. For phenolic acids, bearing a carboxyl function in addition to the hydroxyl groups, glucuronidation can occur, other than at the reducing hydroxyl group (phenyl-*O*-glucuronides), also at the carboxylic group (acyl glucuronides) of the phenolic acid. Therefore, acyl-glucuronides retain all the free, unbound, reducing hydroxyl functions of the parent compound, while in the respective phenyl-*O*-glucuronide, at least one hydroxyl group of the phenolic acid is bound to the glucuronate moiety. In this study, the synthesis of the sulfate derivatives of ferulic and caffeic acids and of the acyl glucuronide of ferulic acid (FAG) allowed for the first time the comparison of their antioxidant activity with that of their parent compounds and of other common phenolic acids. The antioxidant activity of commercially available *O*-phenyl glucuronides of ferulic and caffeic acids was also measured. FRAP and ABTS assays were used to evaluate the antioxidant activity. Both assays take advantage of electron-transfer reactions, in which the probe itself is an oxidant that abstracts an electron from the antioxidant. The ABTS assay is carried out at neutral pH, while the FRAP assay under acidic conditions. The pH values have an important effect on the reducing capacity of antioxidants. In particular, at acidic pH the reducing capacity may be suppressed due to protonation of antioxidants, whereas at basic pH proton dissociation of phenolic compounds would enhance their reducing.⁴⁷

With regard to glucuronide derivatives, our results show that FAG, the acyl glucuronide of ferulic acid, retain a very good antioxidant activity, similar to that of the parent ferulic acid with FRAP assay, while with the ABTS assay it was about a half with respect to ferulic acid. On the contrary, the antioxidant activity of ferulic acid 4'-*O*-glucuronide, the phenyl-*O*-glucuronide of ferulic acid, is much lower with respect to that of both FAG (about 60-fold lower with the FRAP assay and 30–40-fold lower with the ABTS assay) and ferulic acid (about 70-fold lower with the FRAP assay and 80-fold lower with the ABTS assay). Caffeic acid 3'-*O*-glucuronide retains a strong antioxidant activity, comparable to that of the parent caffeic acid with the ABTS assay, while with the FRAP assay, it was a half with respect to that of caffeic acid. Caffeic acid 4'-*O*-glucuronide displayed a good antioxidant activity with ABTS assays, which is about a third with respect to that of caffeic acid, while with the FRAP assay, it was about 20-fold lower than that of caffeic acid. Concerning the sulfate derivatives of ferulic acid and caffeic acid, the antioxidant activity seems to be low compared with that of the parent compounds. Ferulic acid 4'-*O*-sulfate shows an antioxidant activity about 11-fold lower than that of ferulic acid in the ABTS assay and about 18-fold lower in the FRAP assay. Caffeic acid monosulfate (a mixture of caffeic acid 4'-*O*-sulfate and caffeic acid 3'-*O*-sulfate in the ratio 2.75:1) displayed an antioxidant efficiency about 4-fold lower in

the ABTS assay and approximately 17-fold lower than that of the parent caffeic acid in the FRAP assay.

Overall, these results underline the role of the reducing hydroxyl functions in the antioxidant activity of phenolic acids and their metabolites. Among all the metabolites tested, the acyl glucuronide of ferulic acid and the phenyl 3'-*O*-glucuronide of caffeic acid retained a strong antioxidant activity comparable to or about a half with respect to that of their parent compounds. This could be explained by the fact that all these derivatives retained the 4'-hydroxyl function on the aromatic ring, which is the determinant of the antioxidant activity.^{44,45} Noteworthy, for most of the phenolic acids tested, the lowest effective concentrations used in this study to evaluate their antioxidant activity are quite close to the physiological reachable concentrations.^{8,10,12,28} The order of reactivity for gallic acid, caffeic acid, ferulic acid, and trolox (the water-soluble analogue of vitamin E) obtained with both FRAP and ABTS assays is in agreement with the results of previous studies;^{45,48,49} however, the concentrations used in these studies were very high.

In summary, our results demonstrate that, although ferulic and caffeic acids are extensively metabolized upon absorption, some of these metabolites retain a strong antioxidant activity and might still exert a significant antioxidant action *in vivo*. These results are particularly remarkable for human health, due to the fact that both ferulic and caffeic acids are the most representative phenolic acids in the human diet, and upon absorption, they circulate in human plasma almost exclusively as conjugated forms (glucuronates and sulfates), while only traces, if any, of the free, unbound forms were detected.^{8–10,12,17–24} Therefore, the strong antioxidant activity exhibited by some of these metabolites might account for the increase in plasma antioxidant potential measured upon ingestion of phenolic acid-rich beverages and food, reported by many authors.^{1,50–53}

Moreover, it should be taken into consideration that the simultaneous presence of different metabolites might produce additional synergistic effects *in vivo*. Further, an accumulation of phenolic acids and their metabolites might occur in many tissues (lung, liver, and heart) as described for 3-palmitoylcatechin, epigallocatechin gallate, and resveratrol.^{54–56}

Whereas the occurrence of phenyl glucuronides and sulfate derivatives of caffeic and ferulic acids in human plasma and urine has been widely reported,^{8–10,12,17–24} little or no attention has been paid to acyl glucuronides of phenolic compounds. The acyl glucuronide of ipriflavone, an isoflavon derived from genistein, has been identified in dog, rat, and human liver microsomes.³⁷ In this study, in addition to the chemical synthesis of the acyl glucuronide of ferulic acid (FAG), we first reported evidence demonstrating the biosynthesis of FAG by mouse liver microsomes, in addition to the ferulic acid phenyl 4'-*O*-glucuronide. The ability of liver tissue to synthesize FAG and the strong antioxidant activity exhibited by FAG, much higher than that of the respective ferulic acid phenyl 4'-*O*-glucuronide, might suggest a role of this metabolite in contributing to the plasma antioxidant potential. Although acyl glucuronides had been known as mammalian metabolites for half a century,^{57,58} with the development of LC-MS it became evident that many carboxylic acid-containing compounds were metabolized extensively to their acyl glucuronides.²⁵ Acyl glucuronides react with sulfhydryl and hydroxyl groups and can be hydrolyzed back to the aglycone under physiological conditions.²⁵ They undergo covalent binding to

plasma proteins,⁵⁹ can react with glutathione, and transacylate cellular macromolecules.²⁷ Further, acyl glucuronides undergo rearrangement due to intramolecular acyl migration from –OH to the adjacent –OH, generating different positional isomers. The potential rearrangement of glucuronides from the biosynthetic C-1 isomers to other positional isomers is important since only 1-O-substituted acyl glucuronides are substrates of β -glucuronidase, which is commonly used to identify such conjugates⁴⁰ and must be taken into account when measuring the concentrations of phenolic acids present in biological fluids as glucuronic acid esters.²⁷ Acyl group migration can occur in plasma, bile, and urine;²⁷ therefore, the concentrations of glucuronic acid conjugates of carboxylic acid might be underestimated if β -glucuronidase hydrolysis is used to liberate the aglycones in the sample containing the rearranged isomers, and this will be particularly important in bioavailability studies.²⁷

Our results demonstrate the biological relevance of the acyl glucuronide of ferulic acid and its strong antioxidant activity. Additional studies will be necessary to evaluate the in vivo role of acyl glucuronides of phenolic acids.

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Notes

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ABBREVIATIONS USED

FAG, ferulic acid acyl glucuronide; FAGP, protected form of ferulic acid acyl glucuronide; FFG, ferulic acid 4'-O- β -D-glucuronide; UDP-GA, uridine-5'-diphosphoglucuronic acid trisodium salt; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; TPTZ, 2,4,6-tri-(2-pyridyl)-S-triazine; EDTA, ethylenediamine-tetraacetic acid; FRAP, ferric reducing antioxidant activity

REFERENCES

- (1) Pulido, R.; Hernandez-Garcia, M.; Saura-Calixto, F. Contribution of beverages to the intake of lipophilic and hydrophilic antioxidants in the Spanish diet. *Eur. J. Clin. Nutr.* **2003**, *57*, 1275–1282.
- (2) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S–2085S.
- (3) Block, G.; Patterson, B.; Subar, A. Fruit, vegetables and cancer prevention: a review of the epidemiological evidence. *Nutr. Cancer* **1992**, *18*, 1–30.
- (4) Fuhrman, B.; Lavy, M.; Aviram, M. Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. *Am. J. Clin. Nutr.* **1995**, *61*, 549–554.
- (5) Renaud, S.; de Lorgeril, M. Wine, alcohol, platelets and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523–1526.
- (6) Clifford, M. N. Chlorogenic acids and other cinnamates: nature, occurrence and dietary burden. *J. Sci. Food Agric.* **1999**, *79*, 362–372.
- (7) Herrmann, K. Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. *Crit. Rev. Food Sci. Nutr.* **1989**, *28*, 315–347.
- (8) Nardini, M.; Natella, F.; Scaccini, C.; Ghiselli, A. Phenolic acids from beer are absorbed and extensively metabolized in humans. *J. Nutr. Biochem.* **2006**, *17*, 14–22.
- (9) Nardini, M.; Forte, M.; Vrhovsek, U.; Mattivi, F.; Viola, R.; Scaccini, C. White wine phenolics are absorbed and extensively metabolized in humans. *J. Agric. Food Chem.* **2009**, *57*, 2711–2718.
- (10) Nardini, M.; Cirillo, E.; Natella, F.; Scaccini, C. Absorption of phenolic acids in humans after coffee consumption. *J. Agric. Food Chem.* **2002**, *50*, 5735–5741.
- (11) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S–2085S.
- (12) Stalmach, A.; Mullen, W.; Barron, D.; Uchida, K.; Yokota, T.; Cavin, C.; Steiling, H.; Williamson, G.; Crozier, A. Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: identification of biomarkers of coffee consumption. *Drug Metab. Dispos.* **2009**, *37*, 1749–1758.
- (13) Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747.
- (14) Rechner, A. R.; Kuhnle, G.; Bremner, P.; Hubbard, G. P.; Moore, K. P.; Rice-Evans, C. A. The metabolic fate of dietary polyphenols in humans. *Free Radical Biol. Med.* **2002**, *33*, 220–235.
- (15) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230S–242S.
- (16) Jimenez, J. P.; Hubert, J.; Hooper, L.; Cassidy, A.; Manach, C.; Williamson, G.; Scalbert, A. Urinary metabolites as biomarkers of polyphenol intake in humans: a systemic review. *Am. J. Clin. Nutr.* **2010**, *92*, 801–809.
- (17) Rondini, L.; Peyrat-Maillard, M.-N.; Marsset-Baglieri, A.; Berset, C. Sulfated ferulic acid is the main in vivo metabolite found after short-term ingestion of free ferulic acid in rats. *J. Agric. Food Chem.* **2002**, *50*, 3037–3041.
- (18) Azuma, K.; Ippoushi, K.; Nakayama, M.; Ito, H.; Higashio, H.; Terao, J. Absorption of chlorogenic acid and caffeic acid in rats after oral administration. *J. Agric. Food Chem.* **2000**, *48*, 5496–5500.
- (19) Wittemer, S. M.; Ploch, M.; Windeck, T.; Muller, S. C.; Drewelow, B.; Derendorf, H.; Veit, M. Bioavailability and pharmacokinetics of caffeoylquinic acids and flavonoids after oral administration of artichoke leaf extracts in humans. *Phytomedicine* **2005**, *12*, 28–38.
- (20) Baba, S.; Osakabe, N.; Natsume, M.; Terao, J. Orally administered rosmarinic acid is present as the conjugated and/or methylated forms in plasma, and is degraded and metabolized to conjugated forms of caffeic acid, ferulic acid, and m-coumaric acid. *Life Sci.* **2004**, *75*, 165–178.
- (21) Choudhury, R.; Srail, S. K.; Debnam, E.; Rice-Evans, C. A. Urinary excretion of hydroxycinnamates and flavonoids after oral and intravenous administration. *Free Radical Biol. Chem.* **1999**, *27*, 278–286.
- (22) Omar, M. H.; Mullen, W.; Stalmach, A.; Auger, C.; Rouanet, J. M.; Teissedre, P. L.; Caldwell, S. T.; Hartley, R. C.; Crozier, A. Absorption, disposition, metabolism and excretion of [3–14C]caffeic acid in rats. *J. Agric. Food Chem.* **2012**, *60*, 5205–5214.
- (23) Fumeaux, R.; Menozzi-Smarrito, C.; Stalmach, A.; Munari, C.; Kraehenbuehl, K.; Steiling, H.; Crozier, A.; Williamson, G.; Barron, D. First synthesis, characterization, and evidence for the presence of hydroxycinnamic acid sulfate and glucuronide conjugates in human biological fluids as results of coffee consumption. *Org. Biomol. Chem.* **2010**, *8*, 5199–5211.
- (24) Rondini, L.; Peyrat-Maillard, M. N.; Marsset-Baglieri, A.; Berset, C. Sulfated ferulic acid is the main in vivo metabolite found after short-term ingestion of free ferulic acid in rats. *J. Agric. Food Chem.* **2002**, *50*, 3037–41.
- (25) Spahn-Langguth, H.; Benet, L. Z. Acyl glucuronides revisited: is the glucuronidation process a toxification as well as detoxification mechanism? *Drug Metab. Rev.* **1992**, *24*, 5–48.

- (26) Stachulski, A. V.; Harding, J. R.; Lindon, J. C.; Maggs, J. L.; Park, B. K.; Wilson, J. D. Acyl glucuronides: biological activity, chemical reactivity and chemical synthesis. *J. Med. Chem.* **2006**, *49*, 6931–6945.
- (27) Faed, E. M. Properties of acyl glucuronides: implications for studies of the pharmacokinetics and metabolism of acidic drugs. *Drug Metab. Rev.* **1984**, *15*, 1213–1249.
- (28) Zhao, Z.; Egashira, Y.; Sanada, H. Ferulic acid sugars are recovered in rat plasma and urine mainly as the sulfoglucuronide of ferulic acid. *J. Nutr.* **2003**, *133*, 1355–1361.
- (29) Manach, C.; Morand, C.; Crespy, V.; Demigné, C.; Texier, O.; Régérat, F.; Rémésy, C. Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant activity. *FEBS Lett.* **1998**, *426*, 331–336.
- (30) Ohta, T.; Nakano, T.; Egashira, Y.; Sanada, H. Antioxidant activity of ferulic acid beta-glucuronide in the LDL oxidation system. *Biosci. Biotechnol. Biochem.* **1997**, *61*, 1942–1943.
- (31) Todd, J. S.; Zimmerman, R. C.; Crews, P.; Alberte, R. S. The antifouling activity of natural and synthetic phenolic acid sulphate esters. *Phytochemistry* **1993**, *34*, 401–404.
- (32) Theodoridis, G.; Gika, H.; Franceschi, P.; Caputi, L.; Arapitsas, P.; Scholz, M.; Masuero, D.; Wehrens, R.; Vrhovsek, U.; Mattivi, F. LC-MS based global metabolite profiling of grapes: solvent extraction protocol optimization. *Metabolomics* **2012**, *8*, 175–185.
- (33) Fialova, P.; Weignerova, L.; Rauvolfova, J.; Prikrylova, V.; Pisevcova, A.; Ettrich, R.; Kuzma, M.; Sedmera, P.; Kren, V. Hydrolytic and transglycosylation reactions of N-acyl modified substrates catalysed by β -N-acetylhexosaminidases. *Tetrahedron* **2010**, *60*, 693–701.
- (34) Gauthier, C.; Legault, J.; Rondeau, S.; Pichette, A. Synthesis of betulinic acid acyl glucuronide for application in anticancer prodrug monotherapy. *Tetrahedron Lett.* **2009**, *50*, 988–991.
- (35) van der Hoeven, T.; Coon, M. J. Preparation and properties of partially purified cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase from rabbit liver microsomes. *J. Biol. Chem.* **1974**, *249*, 6302–6310.
- (36) Lowry, O. H.; Rosebrough, N. J.; Fare, A. L.; Randall, R. J. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–271.
- (37) Jemnitz, K.; Levai, F.; Monostory, K.; Szatmari, I.; Vereczkey, L. The *in vitro* biosynthesis and stability measurement with acyl-glucuronide isoforms of the main metabolite ipriflavone. *Eur. J. Drug Metab. Pharmacokinet.* **2000**, *25*, 153–160.
- (38) Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76.
- (39) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231–1237.
- (40) Sinclair, K. A.; Cardwell, J. The formation of β -glucuronidase resistant glucuronides by the intramolecular rearrangement of glucuronide acid conjugates at mild alkaline pH. *Biochem. Pharmacol.* **1982**, *31*, 953–957.
- (41) Skordi, E.; Wilson, I. D.; Lindon, J. C.; Nicholson, J. K. Kinetic studies on the intramolecular acyl migration of β -1-O-acyl glucuronides: application to the glucuronides of (R)- and (S)- ketoprofen, (R)- and (S)- hydroxy-ketoprofen metabolites, and tolmetin by ^1H -NMR spectroscopy. *Xenobiotica* **2005**, *35*, 715–725.
- (42) Cano, A.; Arnao, M. B.; Williamson, G.; Garcia-Conesa, M. T. Superoxide scavenging by polyphenols: effect of conjugation and dimerization. *Redox Rep.* **2002**, *7*, 379–383.
- (43) Rice-Evans, C.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933–956.
- (44) Hodnick, W. F.; Milosevjevic, E. B.; Nelson, J. H.; Pardini, R. S. Electrochemistry of flavonoids. *Biochem. Pharmacol.* **1988**, *37*, 2606–2611.
- (45) Pulido, R.; Bravo, L.; Saura-Calixto, F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *J. Agric. Food Chem.* **2000**, *48*, 3396–3402.
- (46) Guy, F.; Renouf, M.; Barron, D.; Cavin, C.; Dionisi, F.; Kochhar, S.; Rezzi, S.; Williamson, G.; Steiling, H. Quantitative analysis of plasma caffeic and ferulic acid equivalents by liquid chromatography tandem mass spectrometry. *J. Chromatogr., B* **2009**, *877*, 3965–3974.
- (47) Huang, D.; Ou, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841–1856.
- (48) Nenadis, N.; Lazaridou, O.; Tsimidou, M. Z. Use of reference compounds in antioxidant activity assessment. *J. Agric. Food Chem.* **2007**, *55*, 5452–5460.
- (49) Villano, D.; Fernandez-Pachon, M. S.; Troncoso, A. M.; Garcia-Parrilla, M. C. Comparison of antioxidant activity of wine phenolic compounds and metabolites *in vitro*. *Anal. Chim. Acta* **2005**, *538*, 391–398.
- (50) Ghiselli, A.; Natella, F.; Guidi, A.; Montanari, L.; Fantozzi, P.; Scaccini, C. Beer increases plasma antioxidant capacity in humans. *J. Nutr. Biochem.* **2000**, *11*, 76–80.
- (51) Natella, F.; Nardini, M.; Giannetti, I.; Dattilo, C.; Scaccini, C. Coffee drinking influences plasma antioxidant capacity in humans. *J. Agric. Food Chem.* **2002**, *50*, 6211–6216.
- (52) Serafini, M.; Bugianesi, R.; Salucci, M.; Azzini, E.; Raguzzini, A.; Maiani, G. Effect of acute ingestion of fresh and stored lettuce (*Lactuca sativa*) on plasma total antioxidant capacity and antioxidant levels in human subjects. *Br. J. Nutr.* **2002**, *88*, 615–623.
- (53) Simonetti, P.; Gardana, C.; Pietta, P. Plasma levels of caffeic acid and antioxidant status after red wine intake. *J. Agric. Food Chem.* **2001**, *49*, 5964–5968.
- (54) Das, N. P.; Griffiths, L. A. Studies of flavonoid metabolism. Metabolism of (+)-[^{14}C] catechin in rat and guinea pig. *Biochem. J.* **1969**, *115*, 831–836.
- (55) Suganuma, M.; Okabe, S.; Oniyama, M.; Tada, Y.; Ito, H.; Fujiki, H. Wide distribution of [^3H](–)epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. *Carcinogenesis* **1998**, *19*, 1771–1776.
- (56) Bertelli, A. A.; Giovannini, L.; Stradi, R.; Bertelli, A.; Tillement, J. P. Plasma, urine and tissue levels of trans- and cis-resveratrol (3,4',5-trihydrostilbene) after short-term or prolonged administration of red wine to rats. *Int. J. Tissue React.* **1996**, *18*, 67–71.
- (57) Goebel, W. F. Derivatives of glucuronic acid. VIII. The structure of benzoylglucuronic acid. *J. Biol. Chem.* **1938**, *122*, 649–653.
- (58) Stachulski, A. V.; Harding, J. R.; Lindon, J. C.; Maggs, J. L.; Park, K. B.; Wilson, I. D. Acyl glucuronides: biological activity, chemical reactivity and chemical synthesis. *J. Med. Chem.* **2006**, *49*, 6931–6945.
- (59) Zhou, S. F.; Paxton, J. W.; Tingle, M. D.; Kestell, P.; Jameson, M. B.; Thompson, P. I.; Baguley, B. C. Identification and reactivity of the major metabolite (beta-1-glucuronide) of the antitumour agent 5,6-dimethylxanthene-4-acetic acid (DMXAA) in humans. *Xenobiotica* **2001**, *31*, 277–293.