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Article

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Running title: Antioxidant activity of phenolic acids and their metabolites.

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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 fruit and vegetables, and the acyl glucuronide of ferulic acid were synthesized, purified and tested for their antioxidant activity in comparison with 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 four-fold less efficient as 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 acids metabolism still retain strong antioxidant properties. Moreover, we firstly 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, ageing. 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 last decade due to their antioxidant activity and biological effects. Epidemiological studies have suggested associations between the consumption of polyphenols-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, beta-carotene), phenolic compounds may be a major factor in assuring the antioxidant potential of the diet and may contribute to maintaining the endogeneous redox balance in humans.

Phenolic acids, a major class of polyphenols, are widely distributed in human diet, particularly in fruits, vegetables and beverages (coffee, beer, wine, 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, endogeneous compounds, 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 be supposed to 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 bind the glucuronate moiety. In spite of the large data concerning the antioxidant activity and biological effects of phenolic acids, very few studies deal with the antioxidant activity, 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 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 acid [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 in respect to that of their parent compounds and of other related phenolic acids. Due to 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 mono-sulfate 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 firstly 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 peroxodisulfate, methanol LC/MS grade 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 ml 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, 7% MeOH in twice-distilled water).

Ferulic acid sulfate ester and caffeic acid mono-sulfate esters: synthesis, purification and identification. Ferulic acid 4'-*O*-sulfate and the two mono-sulfate esters of caffeic acid (caffeic acid 4'-*O*-sulfate and caffeic acid 3'-*O*-sulfate) (Figure 1) were synthesized and purified according to Todd [31] et al. Purification of the two mono-sulfate 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 mono-sulfate esters of caffeic acid were identified by ^{1}HNMR spectra (300 MHz, CD_3OD), as already reported [31] and by UPLC-MS analyses, as described in the following [32]. Ferulic acid 4'-*O*-sulfate showed a purity > 95%; for caffeic acid monosulfate purity was > 90%. Yield was 92% for ferulic acid 4'-*O*-sulfate and 47% for caffeic acid monosulfate (as 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 reaction of ferulic acid with 2,3,4-tri-*O*-acetyl-1-bromo-D-glucuronic acid methyl ester following the procedure described by Fialova [33] et al., with minor modification. FAGP was then purified by chromatography on SPE cartridge. Purified FAGP was finally deprotected by 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 ACN and 8.7 ml twice-distilled water, 1.3 ml NaOH 4N were 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 CH_2Cl_2. The organic phase was dried

under reduced pressure at 40°C and about 1 g FAGP was obtained as white powder. For FAGP purification, 0.5 g FAGP dissolved in absolute MeOH were loaded on a 12 ml Supelclean LC18 SPE tube equilibrated with water. The tube was washed in the order with 100 ml twice-distilled water; 100 ml MeOH/H_2O, 75/25; 80 ml MeOH/H_2O 60/40 (v/v). FAGP was eluted with 100 ml 50:50 MeOH/H_2O, the eluated was evaporated under reduced pressure at 40°C and about 200 mg purified FAGP as white crystalline powder were obtained. Purified FAGP was identified by UPLC-MS analyses [32], using the same experimental conditions applied for sulfate derivatives and described in the following. Purity of FAGP was > 90%. Yield was about 25%.

To obtain FAG, purified FAGP was deprotected following the procedure described by Gauthier [34] et al. with minor modifications. 100 mg purified FAGP were added with 47 mg KOH (4.2 equivalents) in 36 ml water. The mixture was stirred at room temperature for 40 min, the pH was brought at about 6.0 with 1N 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 twice-distilled water, centrifuged 5 min at 18 000 x g, then the supernatant was loaded on a Supelclean LC 18 SPE tube (3 ml) equilibrated with water. FAG was eluted with 12 ml twice-distilled water. The eluate was evaporated under reduced pressure at 40°C. The residue was dissolved in 0.5 ml 0.22 M acetic acid and loaded on a Supelclean LC18 SPE tube (3 ml) equilibrated with 0.22 M acetic acid. The tube was washed with 7 ml 0.22 M acetic acid; FAG was eluted with about 10 ml 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. FAG obtained showed a purity > 85%. 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.

183 **UPLC/TOF-MS analyses.** The UPLC/Q/TOF conditions are detailed in Theodoridis [32]
184 et al. In short: UPLC-MS analyses were performed on a Waters Acquity UPLC system
185 coupled with to a Synapt HDMS QTOF-MS (Waters, Manchester, UK) via an electrospray
186 interface (ESI), operating in W-mode and controlled by MassLynx 4.1 and UV-Vis (DAD)
187 detector recording the spectra fro 200 nm to 490 nm. An Aquity UPLC C18 column (2.1 x
188 150 mm, 1.8 μm) (HSS T3, Waters) thermostated at 30°C was used. The column,
189 equilibrated with solvent A (0.1% formic acid in twice-distilled water), was eluted with
190 increasing concentration of solvent B (0.1% formic acid in methanol) at 0.3 ml/min: 0-6
191 min., 100% A; 7-56 min: from 100% A to 100% B; 57-60 min, 100% B. The injection
192 volume was 5 μl. The mass spectrometry data were collected in negative mode (CV 2.5),
193 in centroid mode (mass to charge ratio (m/z) 100-1000). For MS^2 experiments, the
194 collision energy in transfer was settled at 30 V. External calibration with sodium formate
195 and Lock Mass calibration with leucine enkephaline solution (0.5 mg/l, m/z 556.2771 at 0.1
196 ml/min) was applied.
197 In our UPLC conditions, the elution times were: ferulic acid 4'-*O*-sulfate, 19.5 min;
198 caffeic acid 4'-*O*-sulfate, 17.9 min; caffeic acid 3'-*O*-sulfate, 18.6 min; FAGP, 36.2 min.
199 FAG isomers were eluted at 16.6 min, 18.4 min, 18.7 min and 20.0 min.
200 **Orbitrap.** For MS^3 experiments Orbitrap XL mass spectrometer (Thermo Fisher
201 Scientific (Bremen), GmbH, Germany) with a resolution of 100 000 [at m/z 400, full width
202 half maximum (FWHM)] was employed. Capillary temperatures were set at 270° C. ESI
203 capillary voltage of 2.7 kV in negative mode. Scan time was set to 3 microseconds with
204 maximum acquisition time 200 milliseconds. Fourier transform MS (FTMS) full scans from
205 m/z 50 to m/z 1000 were acquired using the Instrument Control Software (LTQ Tune Plus
206 2.5.5 SP1) and Xcalibur 2.1. The Orbitrap was calibrated prior to mass analysis by
207 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 (Coulochem II, ESA, Bedford, MA, USA) or diode array detector (Perkin-Elmer, Norwalk, CT, USA). A cromatography 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 range 200 nA, filter 2 sec. For UV detector, analyses were performed at 320 nm or 230 nm as specified. Chromatographic separations were performed on a Supelcosil LC-18 C_{18} column, 5.0 µm particle size, 250 x 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; 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 min 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; 61-90 min, 98% A, 2% B. Retention times were: 20,9 min, 22.5 min, 25.2 min and 26.0 min for the different positional FAG isomers; 36.9 min for ferulic acid; 61.7 min for FAGP. Prior to HPLC

234 analysis, all samples were filtered using Millex-HV filters (Millipore, Bedford, MA, USA)
235 with 0.45 μm pore size.

236 **Microsomes preparation**. Animal studies were performed under conditions approved
237 by the National Health Ministry (Department of Food, Nutrition and Public Animal Health).
238 Balb/c mice (Charles River, Calco (LC), Italy) were anesthetized with pentobarbital
239 injection (10 mg), and the liver excised and placed in cold phosphate-buffered saline
240 (PBS). After weighing, livers were homogenized in 4 volumes of 0.1 M Tris-HCl buffer (pH
241 7.4) containing 1 mM EDTA and 0.1 M KCl with an Ultraturrax homogenizer (IKA, Staufen,
242 Germany) for two 40-s intervals and treated for microsome purification as described by
243 van der Hoeven [35] et al. The purified microsomal fractions were suspended in 0.01 M Tris-
244 acetate buffer pH 7.4 containing 20% glycerol and 0.1 mM EDTA and stored at -80°C until
245 used. Protein concentrations were measured by the method of Lowry [36] using bovine
246 serum albumine as standard.

247 **Biosynthesis of ferulic acid glucuronides in liver microsomes**. The conjugation
248 reaction was carried out according to Jemnitz [37] et al. The incubation mixture contained:
249 0.1 M Tris-HCl buffer pH 6.9; 0.05% Triton X-100; 5 mM UDP-GA; 10 mM $MgCl_2$; 4 mg/ml
250 microsomal protein; 1 mM ferulic acid in a final volume of 1 ml. Microsomes were pre-
251 incubated with Triton X-100 for 30 min at 0°C, and the reaction started with the addition of
252 ferulic acid. Blanks without UDP-GA or ferulic acid were run. Incubation was performed at
253 37 °C for 60 min. The reaction was stopped by addition of 0.05 ml 1 N HCl to the
254 incubation mixture. For phenolic compounds extraction, the pH was brought to 3.0 and
255 300 mg NaCl were added. Samples were then extracted four times for 10 min with 6
256 volume excess ethyl acetate, following by 10 min centrifugation at 2 500 g. The pooled
257 organic supernatant was dried under nitrogen stream and the residue dissolved in 0.5 ml
258 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 [38] et al. 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 0-500 μM, as specified. Sulfate and glucuronide derivatives were dissolved in twice-distilled water. Methanol, used to dissolve most of the phenolic acids, was ≤ 5.0 % in the final text mixture. Aqueous or methanolic (≤ 5.0 % MeOH in the final text mixture) iron sulfate solutions were used in the range 10-100 μM for calibration curves. The working FRAP reagent was prepared daily as follows: 2.5 ml 10 mM TPTZ in 40 mM HCl and 2.5 ml 20 mM $FeCl_3$ added with 25 ml 0.3 M acetate buffer pH 3.6. FRAP reagent (0.9 ml), pre-warmed at 37°C, was mixed with 100 μl 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 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 decolorization assay [39], which is based on the reduction of $ABTS^{+\circ}$ radicals by antioxidants tested. ABTS (7 mM) was dissolved in twice-distilled water, and ABTS radical cation ($ABTS^{+\circ}$) was produced by reacting 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) pre-warmed at 30°C, was mixed with 50 µl 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 µM, as specified. Sulfate and glucuronide derivatives were dissolved in twice-distilled water. Methanol, used to dissolve most of the phenolic acids, was ≤ 0.5% in the final text mixture. Trolox (0-15 nmol), the water-soluble analogue of vitamin E, was used as reference compound. An appropriate solvent blank reading was also taken (A_B). The percentage inhibition of absorbance was calculated using the following formula:

% Inhibition = $[(A_B – A_E)/A_B] \times 100$

where AB= absorbance of the blank sample, and AE= 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 modification. Briefly, FAG was dissolved in twice-distilled water at 1 mg/ml. Aliquots were incubated in 0.7 ml final volume 0.2 M K-phosphate buffer pH 6.8 with 2 000 U β-glucuronidase, dissolved in the same buffer, at 37°C for 2 h. At the end of incubation, samples were centrifuged 10 min at 18 000 g, 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 hydrolysed in the presence of 10 mM EDTA, 1% ascorbic acid, 1.8 N NaOH in 1 ml final volume. After

30 min at 30°C, 4 N HCl was added to bring the pH to 3.0. Samples were filtered and analysed by HPLC with ECD or UV detection, after appropriate dilution.

Statistical analysis. Data presented are means ± 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.

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 ion at m/z 193.0500 and 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 mono-sulfate 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 min and 18.6 min; in panel 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],

peak eluting at 17.9 min min should be attributable to the caffeic acid 4'-*O*-sulfate derivative, while 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 component eluting at 16.6 min, 18.4 min, 18.7 min. In panels B, C, D, 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; 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, m/z 369.0830 respectively, corresponding to the value expected for FAG, and fragment at m/z 175.025 (corresponding to the loss of the glucuronic acid moiety), and m/z 113.024 for all the four peaks. The resulting MS^2 fragmentation patterns show ions at m/z 175.025 and m/z 113.024 for all 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 somewhat differences, suggesting that the conjugates of glucuronic acid with ferulic acid are not identical. A similar behaviour has been described for isomers of the acyl glucuronide of the synthetic flavonoid ipriflavone [37]. These results are in agreement with the isomerisation of the ferulic acid 1-*O*-acyl glucuronide (the C-1 isomer of FAG), due to intramolecular acyl migration from -OH to the adiacent –OH, generating different positional isomers of FAG, with the same molecular ion, in which the ferulic acid moiety is attached to either 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-

363 glucuronidase [25-27,40,41], we further purified the different FAG isomers and treated them
364 with β-glucuronidase as described in method section. Only the fraction containing the more
365 retained and abundant FAG isomer (peak at 20.0 min in the figure 5, panel A) was
366 hydrolyzed by β-glucuronidase, giving 69.0 ± 2.5 % of the calculated ferulic acid,
367 demonstrating that the more retained and abundant FAG isomer, eluting at 20.0 min in
368 UPLC, is the C-1 isomer. Alkaline hydrolysis of purified FAG C1-isomer gave 98.4 ± 1.9 %
369 of the theoretical ferulic acid.
370 To investigate the biological relevance of the acyl glucuronide of ferulic acid (FAG), we
371 studied the biosynthesis of FAG by mouse liver microsomes. Upon incubation of ferulic
372 acid with UDP-GA and microsomal proteins from mouse liver as described in method
373 section, three main components were detected with HPLC-UV detector at 320 nm, as
374 shown in fig. 6, panel B. The first peak at 23.5 min coeluted with standard ferulic acid 4'-*O*-
375 glucuronide (FFG, which is the phenyl glucuronide of ferulic acid) (fig. 6 panel C), the
376 second component at 26.0 min coeluted with FAG (C-1 isomer) (fig. 6 panel D), the third
377 component at 36.9 min was residual ferulic acid. UPLC analyses, with both DAD at 320
378 nm and MS detector, are shown in figure 7, panel A and B respectively. The mass spectra
379 identified the peaks, in the order of elution, as: panel C, ferulic acid 4-*O*- glucuronide
380 (FFG); panel D, ferulic acid 1-*O*-acyl glucuronide (FAG, C-1 isomer); panel E, ferulic acid.
381 Interestingly, the mass spectra of FFG and FAG are almost undistinguishable, with the
382 molecular ion at m/z 369.0818 and m/z 369.0826, respectively, and fragments at m/z
383 193.050, m/z 175.025 and m/z 113.024. The ratio between FFG and FAG is 2.4: 1 on the
384 basis of UPLC-MS analyses (mean of 3 separate experiments). The identity of FAG and
385 FFG was further demonstrated in HPLC-ECD at +600 mV (data not shown). In fact, FAG
386 molecule possesses an unbound, reducing OH-group on the phenolic ring, which is
387 revealed by the electrochemical detector, while FFG was not detected by electrochemical
388 detector, due to the lack of reducing groups (in FFG the OH- group of 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 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 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 compounds. High slope values denote high ferric reducing ability. Caffeic and ferulic acid showed a strong ferric-reducing activity, in the same order of 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% in 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, being 0.0401 the slope for ferulic acid, 0.0383 and 0.0368 the values obtained for FAG (as a mixture of positional isomers) and FAG-1 isomer, respectively. On the contrary, ferulic acid 4'-*O*-glucuronide, which is the

phenyl-*O*-glucuronide of ferulic acid, showed a ferric-reducing ability very low (slope 0.0006) in respect not only to that of the parent ferulic acid, but also in respect to FAG. From our data, the activity of ferulic acid 4'-*O*-glucuronide was about seventy fold lower in respect to that of ferulic acid and about sixty fold lower in 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 in respect to that of the parent caffeic acid. On the contrary, the ferric-reducing ability of caffeic acid 4'-*O*-glucuronide was about ten fold lower in 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 the 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 opposite the mono-substituted 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 in respect to that of 4-OH-phenyl acetic acid, and the higher antioxidant activity of ferulic acid in 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, being the antioxidant activity of syringic acid a little bit higher than that of sinapic acid. Therefore, beside the presence of the 4'-hydroxyl group, also the number (degree) of substitutions (hydroxyl or methoxyl groups) on the aromatic ring is determinant for the ferric-reducing antioxidant activity [44,45].

Antioxidant activity determined by 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 reference compound. Again, high slope values denote high antioxidant

activity, therefore slope values allow for a comparison of the antioxidant efficiencies of the tested compounds.

Caffeic and ferulic acid showed a strong antioxidant activity. The antioxidant activity of caffeic acid is about a half in 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 2.75: 1) is four fold lower in respect to that of the parent caffeic acid, while the antioxidant activity of ferulic acid 4'-*O*-sulfate is about eleven fold lower in respect to that of the parent ferulic acid. Among glucuronides, the antioxidant activity of FAG is about a half of that of the parent ferulic acid and quite close or slightly lower to 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 thirty-forty fold lower in respect to the antioxidant activity of FAG, the acyl glucuronide of ferulic acid and about eighty fold lower in 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 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 substitutions in 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 in respect to that of 4-OH-phenyl acetic acid,

and the higher antioxidant activity of ferulic acid in 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, being the antioxidant activity of syringic acid quite similar to that of sinapic acid.

DISCUSSION

Dietary phenolic acids, abundant in most common fruits, vegetables and beverages (coffee, beer, wine, fruit juices) are absorbed and extensively metabolized in humans, and circulate in plasma mainly as sulfate and glucuronate derivatives [8-16]. Very few data are 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 in 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 beta-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

493 the parent compound, while in the respective phenyl-*O*-glucuronide at least one hydroxyl
494 group of the phenolic acid is bound to the glucuronate moiety. In this study, the synthesis
495 of the sulfate derivatives of ferulic and caffeic acids and of the acyl glucuronide of ferulic
496 acid (FAG) allowed for the first time the comparison of their antioxidant activity with that of
497 their parent compounds and of other common phenolic acids. The antioxidant activity of
498 commercially available *O*-phenyl glucuronides of ferulic and caffeic acids was also
499 measured. FRAP and ABTS assays were used to evaluate the antioxidant activity. Both
500 assays take advantage of electron-transfer reactions, in which the probe itself is an oxidant
501 that abstracts an electron from the antioxidant. ABTS assay is carried out at neutral pH,
502 while FRAP assay under acidic conditions. The pH values have an important effect on the
503 reducing capacity of antioxidants. In particular, at acidic pH the reducing capacity may be
504 suppressed due to protonation of antioxidants, whereas at basic pH proton dissociation of
505 phenolic compounds would enhance their reducing [47].

506 With regard to glucuronide derivatives, our results show that FAG, the acyl glucuronide
507 of ferulic acid, retain a very good antioxidant activity, similar to that of the parent ferulic
508 acid with FRAP assay, while with ABTS assay it was about a half in respect to ferulic acid.
509 On the contrary, the antioxidant activity of ferulic acid 4'-*O*-glucuronide, the phenyl-*O*-
510 glucuronide of ferulic acid, is much lower in respect to that of both FAG (about sixty fold
511 lower with the FRAP assay and thirty-forty fold lower with the ABTS assay) and ferulic acid
512 (about seventy fold lower with the FRAP assay and eighty fold lower with the ABTS
513 assay). Caffeic acid 3'-*O*-glucuronide retains a strong antioxidant activity, comparable to
514 that of the parent caffeic acid with ABTS assay, while with FRAP assay it was a half in
515 respect to that of caffeic acid. Caffeic acid 4'-*O*-glucuronide displayed a good antioxidant
516 activity with ABTS assays, which is about a third in respect to that of caffeic acid, while
517 with the FRAP assay it was about twenty-fold lower than that of caffeic acid. Concerning
518 the sulfate derivatives of ferulic acid and caffeic acid, the antioxidant activity seems to be

519 low compared with that of the parent compounds. Ferulic acid 4'-*O*-sulfate shows an
520 antioxidant activity about eleven-fold lower than that of ferulic acid in the ABTS assay, and
521 about eighteen-fold lower in the FRAP assay. Caffeic acid monosulfate (a mixture of
522 caffeic acid 4'-*O*-sulfate and caffeic acid 3'-*O*-sulfate in the ratio 2.75: 1) displayed an
523 antioxidant efficiency about four-fold lower in the ABTS assay and approximately
524 seventeen-fold lower than that of the parent caffeic acid in the FRAP assay.
525 Overall, these results underline the role of the reducing hydroxyl functions in the
526 antioxidant activity of phenolic acids and their metabolites.
527 Among all the metabolites tested, the acyl glucuronide of ferulic acid and the phenyl 3'-
528 *O*-glucuronide of caffeic acid retained a strong antioxidant activity comparable or about a
529 half in respect to that of their parent compounds. This could be explained by the fact that
530 all these derivatives retained the 4'-hydroxyl function on the aromatic ring, which is
531 determinant for the antioxidant activity [44,45]. Noteworthy, for most of the phenolic acids
532 tested, the lowest effective concentrations used in this study to evaluate their antioxidant
533 activity are quite close to the physiological reachable concentrations [8,10,12,28]. The order of
534 reactivity for gallic acid, caffeic acid, ferulic acid and trolox (the water-soluble analogue of
535 vitamin E) obtained with both FRAP and ABTS assays is in agreement with the results of
536 previous studies [45,48,49], however the concentrations used in these studies were very high.
537 Summarizing, our results demonstrate that, although ferulic and caffeic acids are
538 extensively metabolized upon absorption, some of these metabolites retain a strong
539 antioxidant activity and might still exert a significant antioxidant action *in vivo.* These
540 results are particularly remarkable for human health, due to the fact that both ferulic and
541 caffeic acids are the most representative phenolic acids in human diet and, upon
542 absorption, they circulate in human plasma almost exclusively as conjugated forms
543 (glucuronates and sulfates), while only traces, if any, of the free, unbound forms were
544 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 acids-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, 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 on 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 firstly reported evidences 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 conjugates [40] 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 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.

ABBREVIATIONS USED: FAG: ferulic acid acyl glucuronode; 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.

SUPPORTING INFORMATION AVAILABLE:

Figure A Nardini: chemical structures of the most representative phenolic acids.

This material is available free of charge via the Internet at http://pubs.acs.org.

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Table 1. Antioxidant activity, measured with FRAP assay, of phenolic acids and their metabolites in comparison with trolox

Compounds	Concentration range (μmol/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 [a]	2.5-25	0.0383	+ 0.0115	0.9997
FAG-1 [b]	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
p-Coumaric acid	5-50	0.0079	+ 0.0176	0.9977
o-Coumaric acid	2.5-25	0.0050	+ 0.0094	0.9992
Caffeic acid monosulfate [c]	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

FRAP assay was performed as reported in 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 *t*-test.

[a] Mixture of positional FAG isomers; [b] FAG, C1 isomer; [c] mixture of caffeic acid-4'-*O*-sulfate and caffeic acid-3'-*O*-sulfate in the ratio 2.75:1.

Table 2- Antioxidant activity, measured with ABTS assay, of phenolic acids and their metabolites in comparison with trolox

Compounds	Concentration range (μmol/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
p-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
o-Coumaric acid	3-20	2.2009	+ 0.7565	0.9971
Vanillic acid	0.5-5	2.1909	+ 0.6495	0.9924
FAG [a]	1.5-20	2.1382	+ 1.3584	0.9941
Chlorogenic acid	2-25	1.8891	- 0.2395	0.9970
FAG-1 [b]	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 [c]	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

ABTS assay was performed as described in 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 *t*-test.

[a] Mixture of positional FAG isomers; [b] FAG, C1 isomer; [c] mixture of caffeic acid-4'-*O*-sulfate and caffeic acid-3'-*O*-sulfate, in the ratio 2.75 :1

LEGEND TO FIGURES

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

Figure 2. Chemical structure of 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).

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.

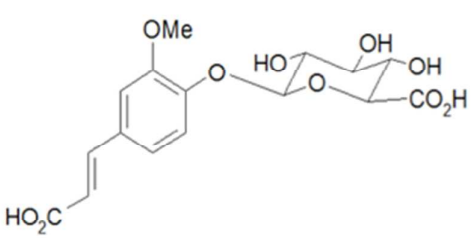
Figure 4. UPLC-MS analyses of purified caffeic acid-4'-*O*-sulfate and caffeic acid-3'-*O*-sulfate. Panel A: UPLC-MS chromatogram, with caffeic acid-4'-*O*-sulfate eluting at 17.9 min and caffeic acid-3'-*O*-sulfate eluting at 18.6 min; Y-axis, relative abundance (%); X-axis, time (min). Panel B: mass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

Figure 5. UPLC-MS analysis of purified FAG. Panel A: UPLC-MS chromatogram, with FAG isomers eluting at 16.6 min, 18.4 min, 18.7 min 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): 19.9 min (E); Y-axis, relative abundance (%); X-axis, m/z ratio.

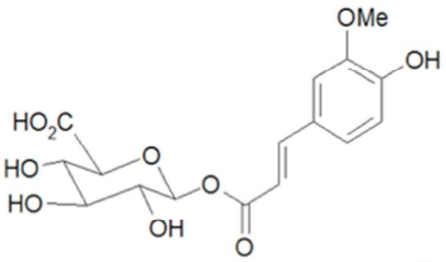
Figure 6. HPLC-UV analysis of microsomal preparation for FAG biosynthesis; Y-axis, detector response (mV) at 320 nm; X-axis, time (min). Panel A: HPLC-UV chromatogram of standard ferulic acid-4'-*O*-glucuronide (FFG) eluting at 23.5 min; standard ferulic acid 1-*O*-acyl glucuronide (FAG) eluting at 26.0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

Figure 7. UPLC-DAD-MS analyses of microsomal preparation for FAG biosynthesis. Panel A: UPLC-DAD chromatogram at 320 nm of a typical microsomal sample after 1 h incubation with ferulic acid and UDP-GA; Y-axis, detector response (AU) at 320 nm; X-axis, time (min); 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.

Figure 1



Ferulic acid-4'-*O*-glucuronide
(FFG)



Ferulic acid acyl glucuronide
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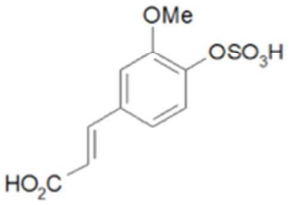
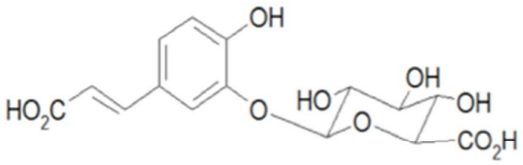
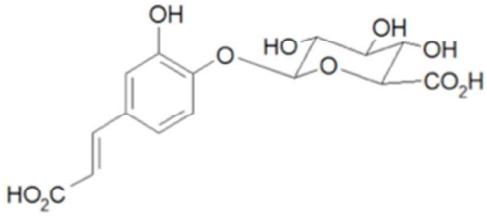


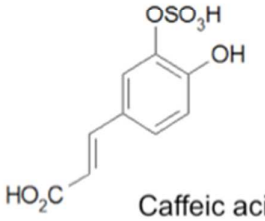
Ferulic acid-4'-*O*-sulfate



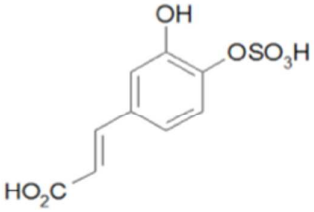
Caffeic acid-3'-*O*-glucuronide



Caffeic acid-4'-*O*-glucuronide

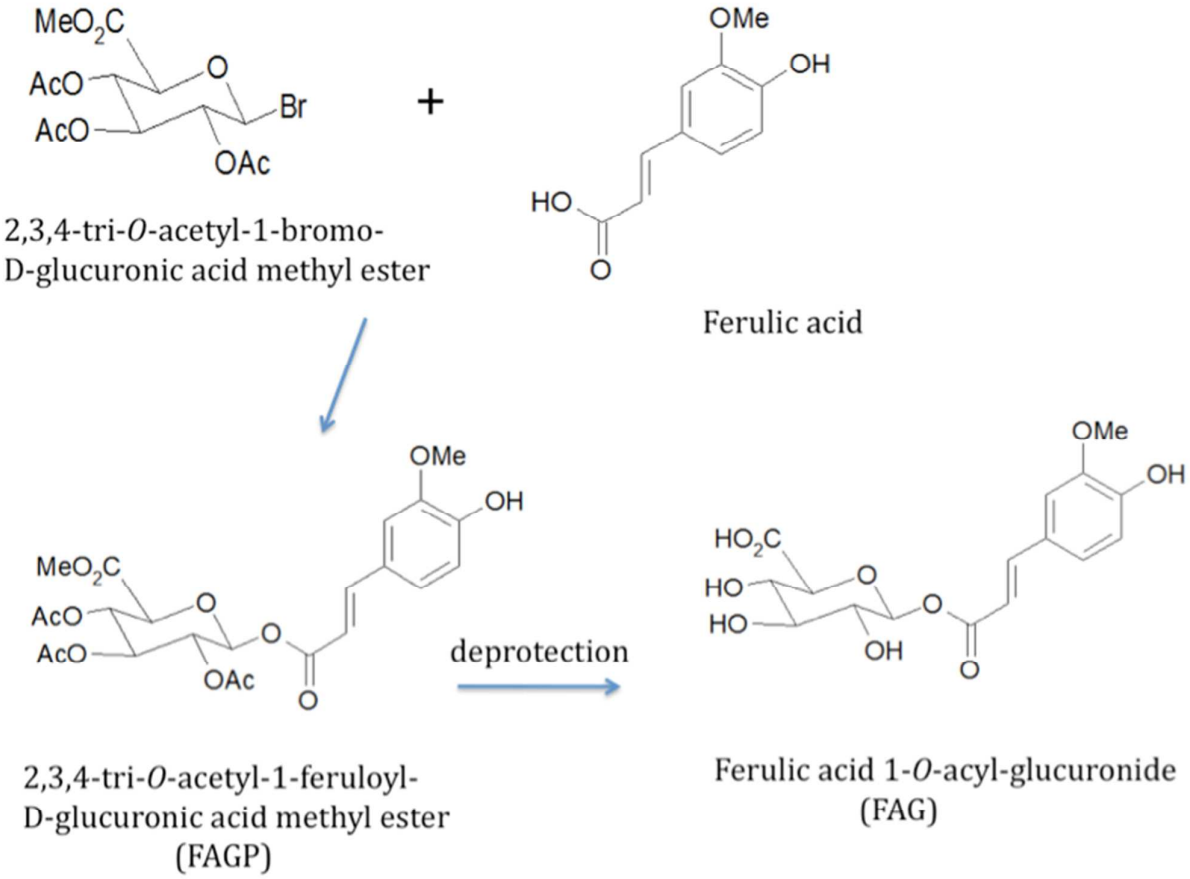


Caffeic acid-3'-*O*-sulfate



Caffeic acid-4'-*O*-sulfate

Figure 2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AcO
AcO
O
Br
OAc
+
OMe
OH
HO
O
2,3,4-tri-O-acetyl-1-bromo-D-glucuronic acid methyl ester
Ferulic acid
OMe
OH
MeO2C
AcO
AcO
O
OAc
O
deprotection
HO2C
HO
HO
OH
O
2,3,4-tri-O-acetyl-1-feruloyl-D-glucuronic acid methyl ester (FAGP)
Ferulic acid 1-O-acyl-glucuronide (FAG)

Figure 3

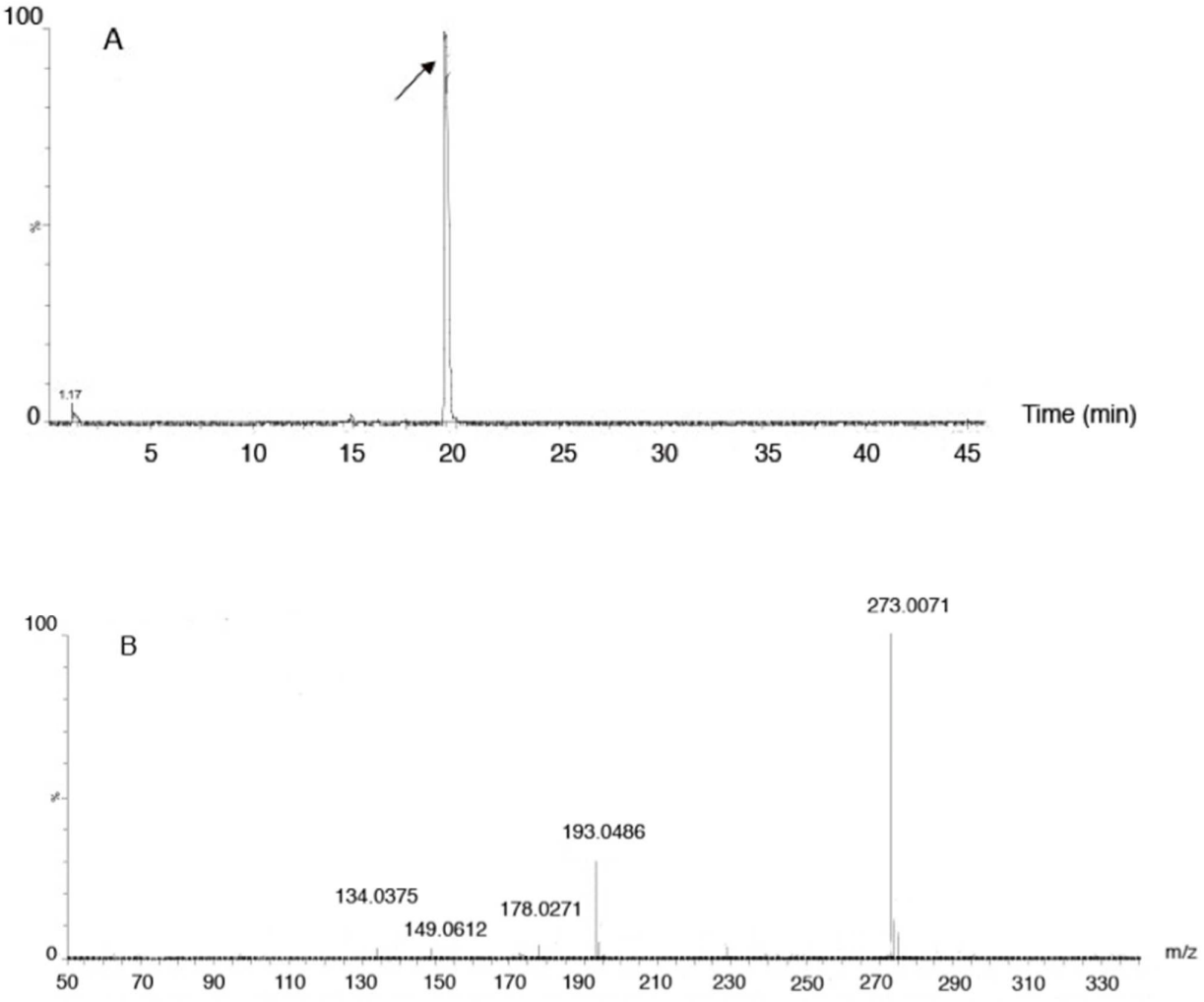
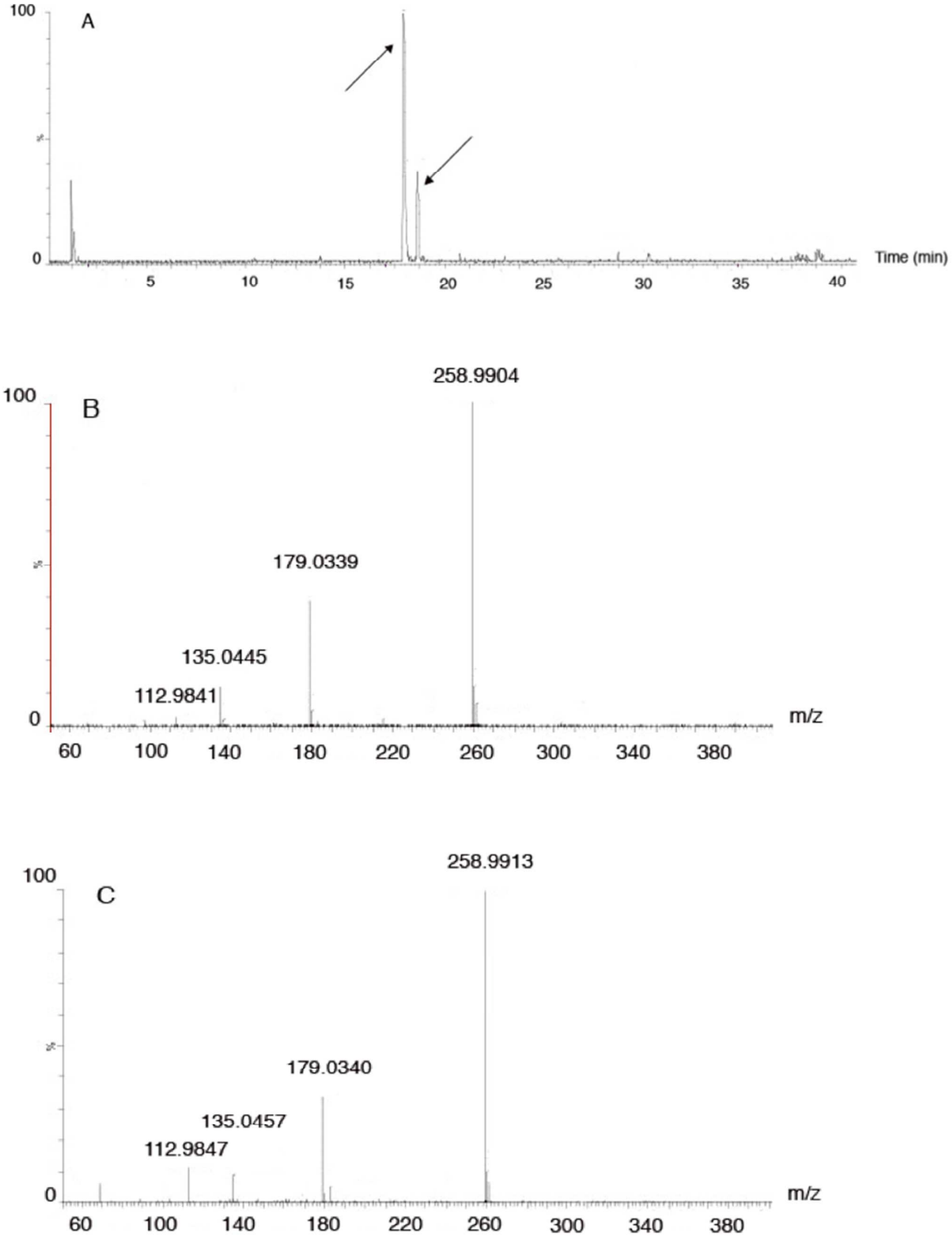

100
A
%
0
1.17
5
10
15
20
25
30
35
40
45
Time (min)
100
B
%
0
273.0071
193.0486
134.0375
178.0271
149.0612
50
70
90
110
130
150
170
190
210
230
250
270
290
310
330
m/z

Figure 4


A
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%
0
5
10
15
20
25
30
35
40
Time (min)
B
100
%
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179.0339
135.0445
112.9841
60
100
140
180
220
260
300
340
380
m/z
C
100
%
0
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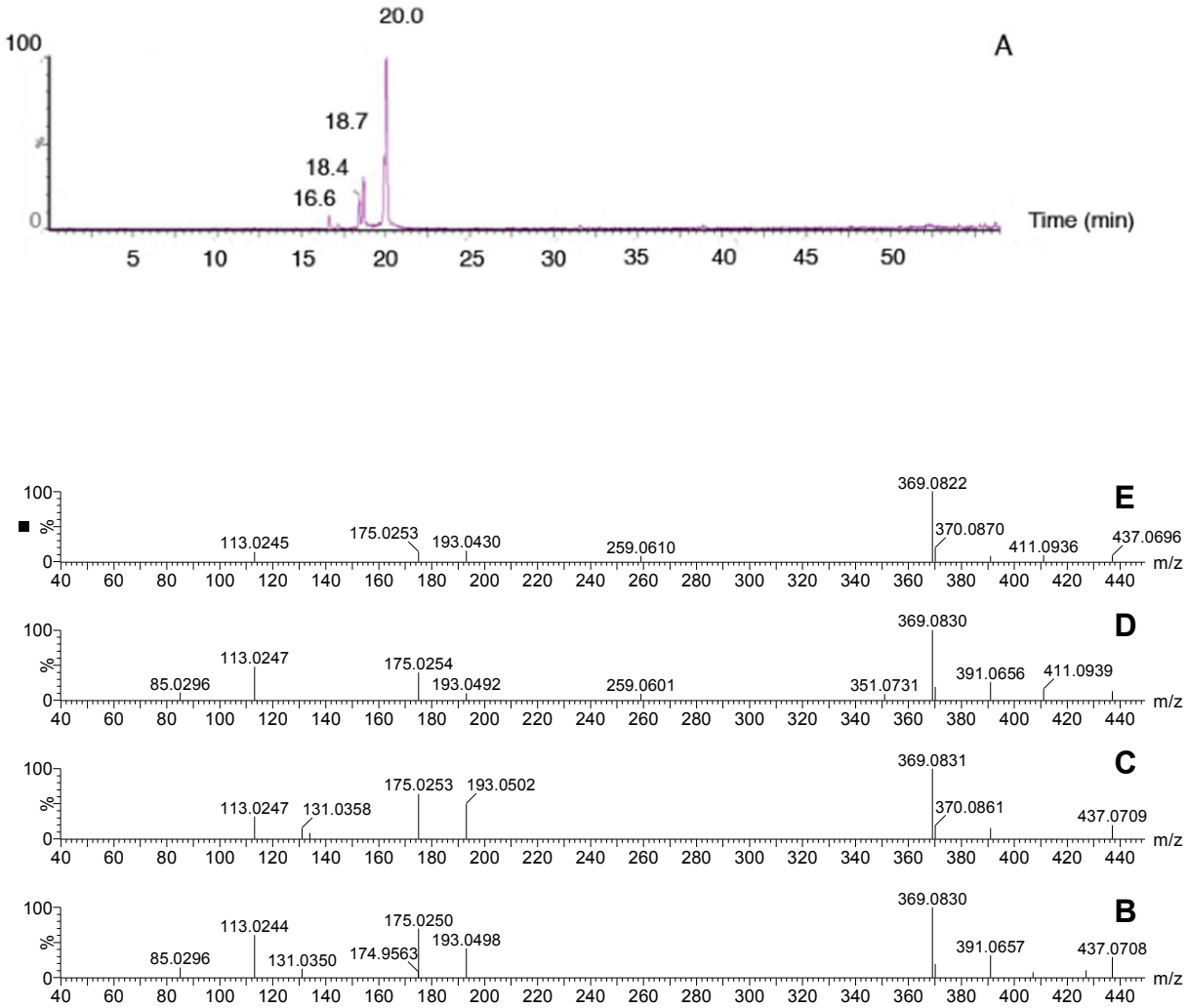
135.0457
112.9847
60
100
140
180
220
260
300
340
380
m/z

Figure 5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18.4
16.6
100
0
Time (min)
5 10 15 20 25 30 35 40 45 50
E
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370.0870
113.0245
175.0253
193.0430
259.0610
411.0936
437.0696
D
369.0830
113.0247
85.0296
175.0254
193.0492
259.0601
351.0731
391.0656
411.0939
C
369.0831
175.0253
193.0502
113.0247
131.0358
370.0861
437.0709
B
369.0830
113.0244
175.0250
193.0498
85.0296
131.0350
174.9563
391.0657
437.0708
m/z
40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440

Figure 6 panel A

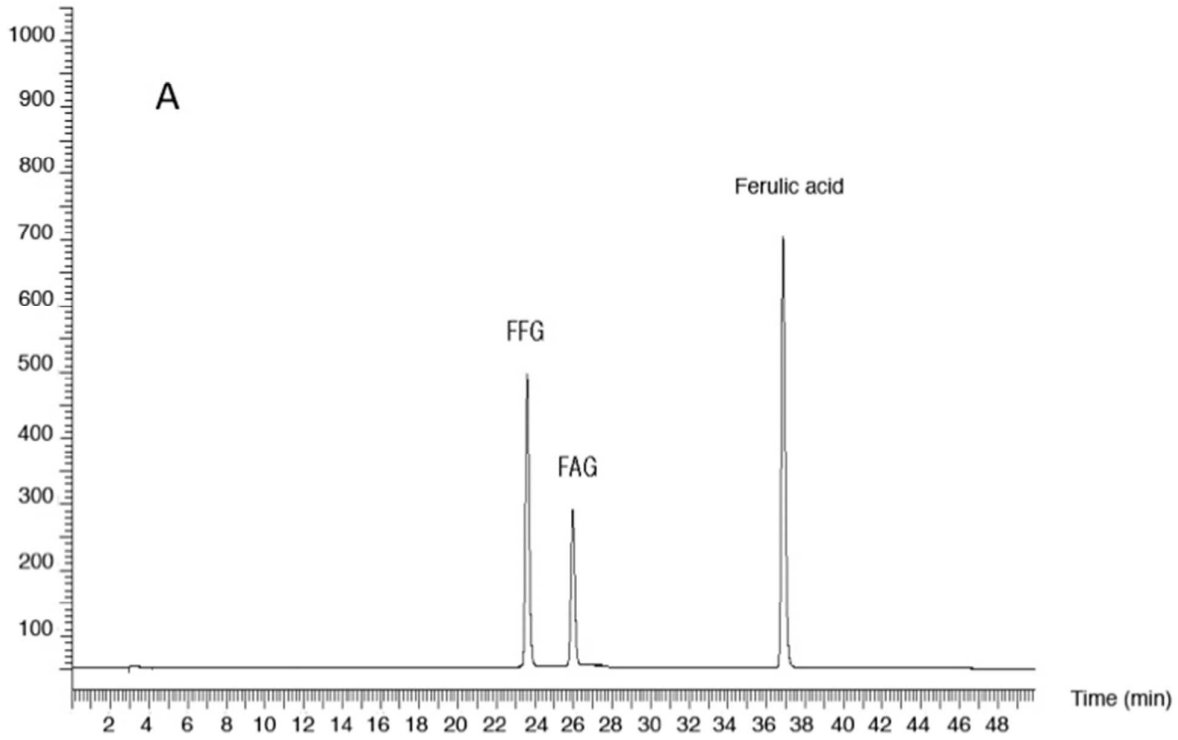


Figure 6 panel B

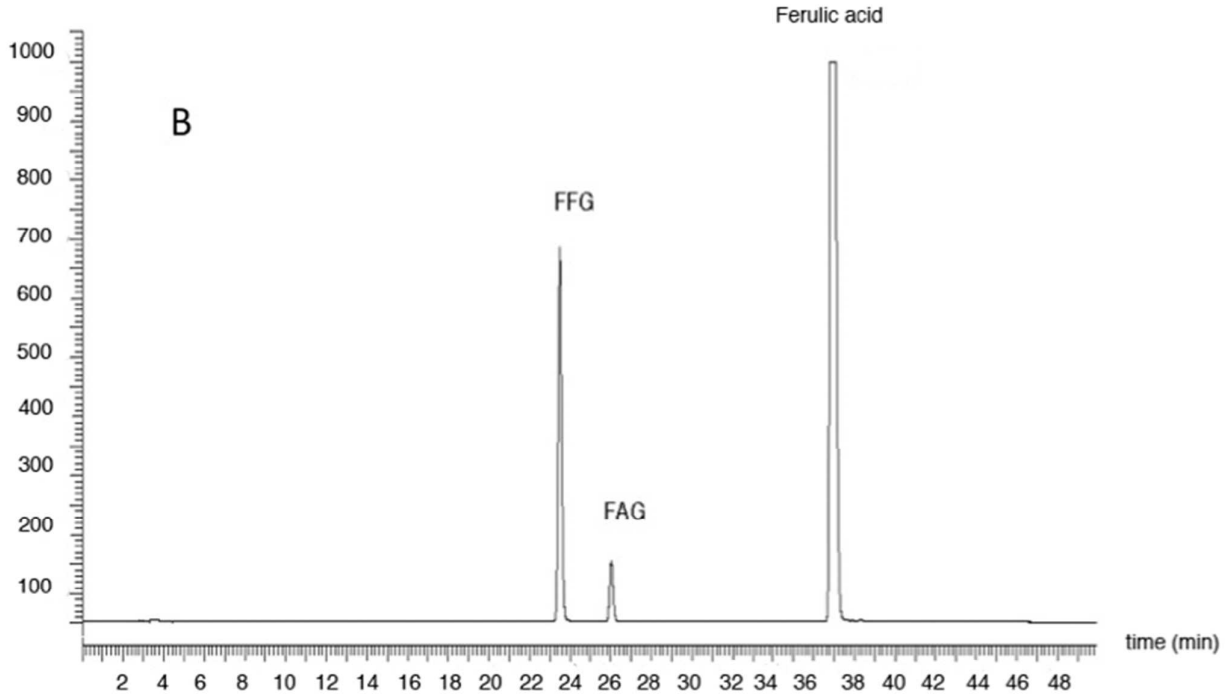


Figure 6 panel C

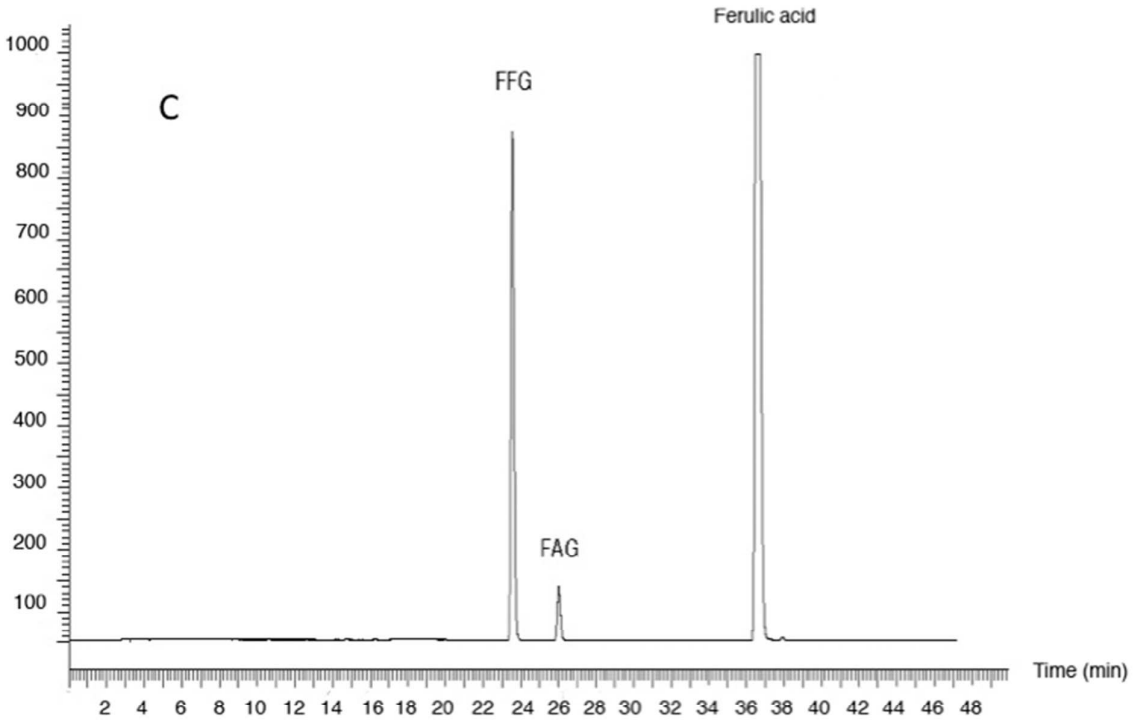


Figure 6 panel D

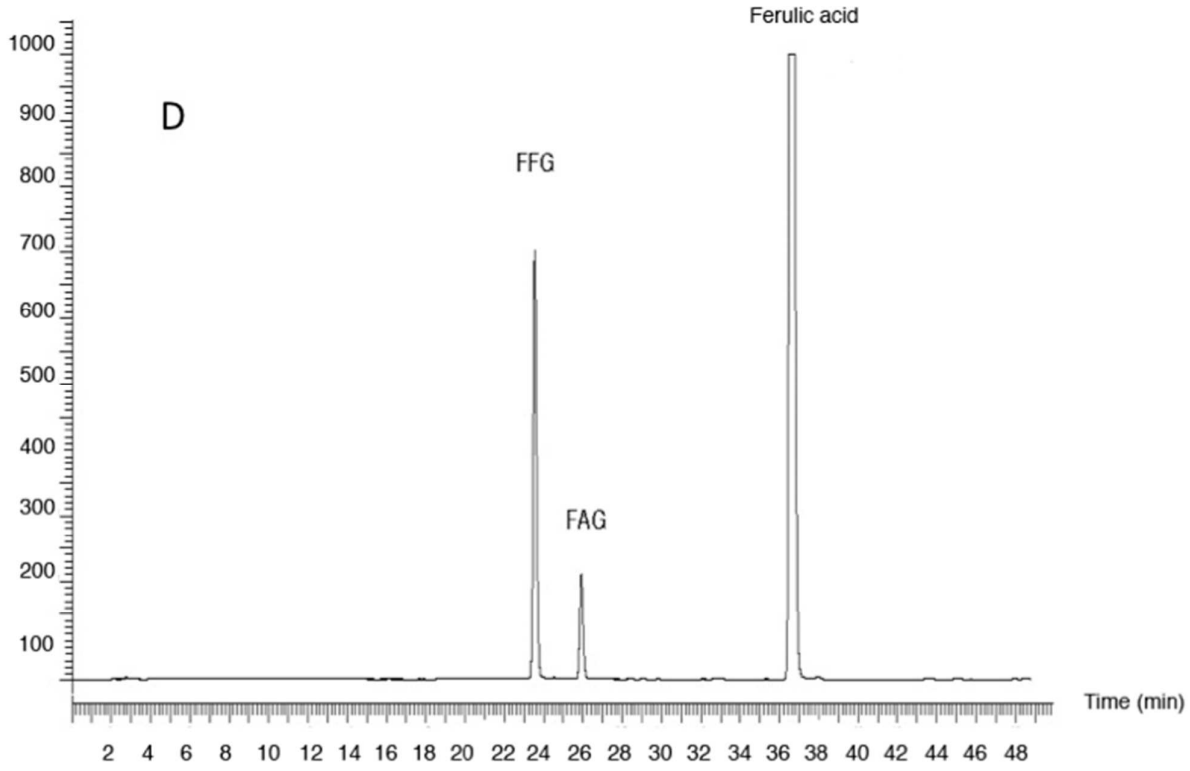


Figure 7 Panels A,B

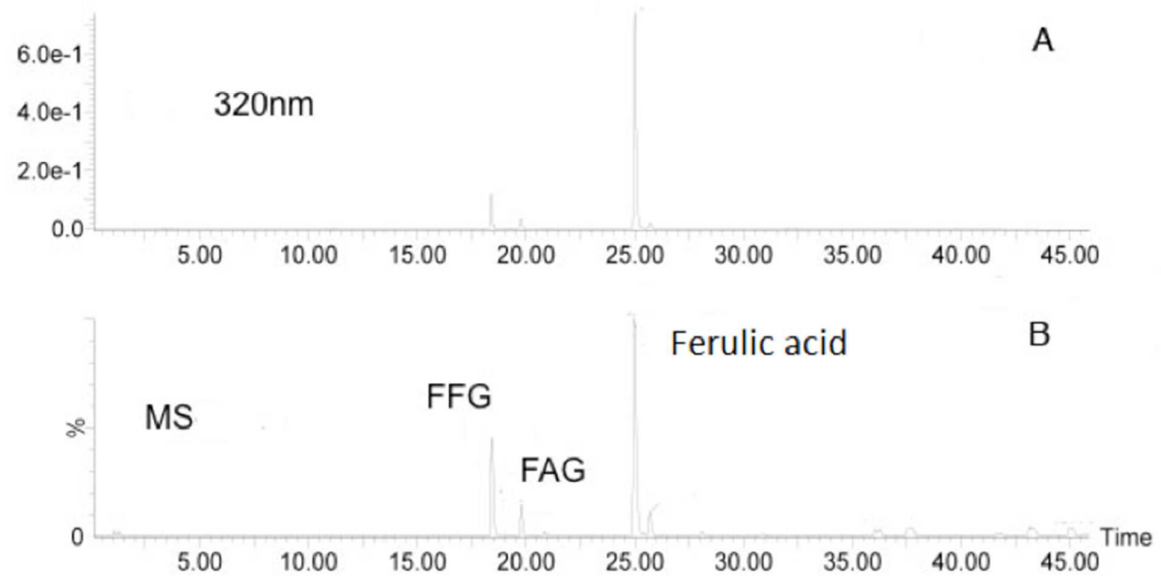
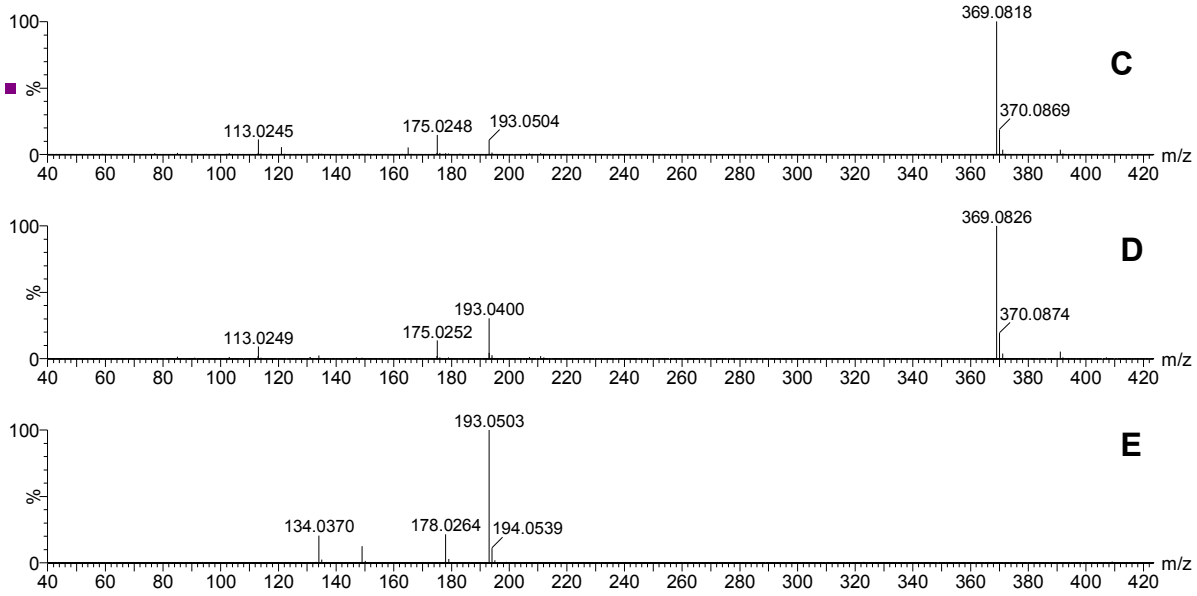


Figure 7 panels CDE



TOC graphic

Ferulic acid acyl glucuronide
(FAG)