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Synthesis and quantitative analysis of plasma-targeted metabolites of catechin and epicatechin

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ABSTRACT: Grape seed polyphenolic extract (GSPE) rich in the flavan-3-ols (+)-catechin and (-) epicatechin beneficially modulates Alzheimer's Disease phenotypes in animal models. The parent molecules in the extract are converted to a series of methylated and glucuronidated derivatives. To fully characterize these metabolites, and establish a robust quantitative assay of their levels in biological fluids, we have implemented a partial synthetic approach utilizing chemical methylation followed by enzymatic glucuronidation. Liquid chromatography/time-of-flight mass spectrometry (LC-TOF-MS) and nuclear magnetic resonance (NMR) spectroscopy were used to assign unequivocal structures to the compounds. An analytical method using solid-phase extraction and LC-MS/MS in selective reaction monitoring mode (SRM) was validated for their quantitation in plasma. These studies provide a basis for improvements in future work on the bioavailability, metabolism and mechanism of action of metabolites derived from dietary flavan-3-ols in a range of interventions.

KEYWORDS: *catechin, glucuronosylation, O-methylation, phase II metabolites, semi-synthesis, structure determination, two-site validation*

■INTRODUCTION

Plant derived polyphenolic compounds possess diverse biological activities, and some have been reported to show anti-tumor, anti-oxidant, anti-inflammatory, or anti-microbial activities (*1-3*). Previous work from our laboratories has shown that polyphenolic compounds from multiple sources, including a specific grape seed polyphenolic extract (GSPE), are capable of improving

cognitive functions and reducing brain neuropathology in animal models of Alzheimer's Disease through multiple mechanisms (*4, 5*). At least a part of this effect may be mediated through interference with aggregation of β-amyloid peptides into neurotoxic, soluble high-molecular weight species (*5*).

GSPE is a complex mixture of proanthocyanidins (PACs, both oligomeric and polymeric) and their monomeric units consisting of the flavan-3-ols (+)-catechin (C) and (-)-epicatechin (EC) (*6*). The flavan-3-ol units are assembled into the various types of oligomers and polymers through C4→C8 or C4→C6 interflavan bonds, and the individual units in the polymer can also be esterified with gallic acid. The monomeric units, rather than the oligomers or polymers, are bioavailable and therefore candidates for being the active components in the GSPE as regards amelioration of Alzheimer's Disease symptoms (*4*). These components, in the form of glucuronidated and/or methylated phase II metabolites, reach the brain at a concentration of 300-400 nM after 10 days of repeated dosing (*4*).

While knowledge of all metabolites found in plasma may be of broader significance for understanding the therapeutic effects of GSPE on a range of disease phenotypes, mechanistic studies require knowledge of the exact structures of metabolites that accumulate in brain or other tissue. To initiate such studies, we have previously implemented a semi-synthetic approach to generate sufficient quantities of flavan-3-ol metabolites for unequivocal identification. We demonstrated that recombinant human glucuronosyltransferases of the UGT1A and UGT2B families can efficiently glucuronidate EC or 3′-*O*-methyl-EC in vitro, suggesting a method for the semi-synthesis of brain-targeted flavan-3-ol metabolites (*7*). Of twelve enzymes tested, UGT1A9 was the most efficient, and this enabled us to generate 3′-*O*-methyl-EC-5-*O*-glucuronide to 50% overall yield (*7*).

In the present study, we have extended the above approach, in combination with chemical methylation of C and EC with iodomethane, to generate authentic standards of the C and EC metabolites found in the plasma and brains of rats treated with GSPE. The structures of the metabolites were rigorously identified, and we present a robust, validated protocol for the measurement of the levels of these compounds in animal fluids.

■MATERIALS AND METHODS

Materials. UDP-glucuronosyl transferase enzymes (recombinant or as mouse microsomes), buffers and UDP-glucuronic acid were purchased from BD Biosciences (San Diego, CA). (-)-Epicatechin, (+)-catechin, DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) and iodomethane were from Sigma Aldrich, St Louis, MO. 3′- and 4′ -*O*-methyl-epicatechin were purchased from Nacalai USA, Inc. (San Diego, CA).

Chemical Methylation of C/EC. (Epi)catechin (1 g) was dissolved in 240 mL dimethylformamide in a 1 L bottle, and the bottle saturated with nitrogen gas for 5 min. DBU (14 mL) and iodomethane (300 mL) were then added, mixed well, and the solution re-saturated with nitrogen gas for 30 s. The bottle was capped and placed at 40 ^{o}C in the dark for 3 h. The reaction was then quenched by the addition of 400 mL methanol with mixing. The solution was dried under nitrogen and re-suspended in HPLC grade methanol for HPLC analysis and purification.

Large Scale Purification of Methylated C/EC. Methylated (epi)catechins were purified on a large scale using a Biotage Isolera flash chromatography system equipped with a 10 g SNAP C18 cartridge at a flow rate of 18 mL/min, monitoring at 280 nm with a UV/Vis detector. The sample in methanol was added to the column via a preloading concentrator disc.

Solvent A was milli-Q water, and solvent B was HPLC grade acetonitrile. The column was equilibrated at 5% B, and the gradient for elution was as follows: isocratic at 5% B for 12 column volumes (CV) or 180 mL; from 5% B to 13% B in 48 CVs or 720 mL; from 13% B to 100% B in 4 CVs or 60 mL; then washed in 100% B for 20 CVs or 300 mL. Fractions were collected in 16 CV or 240 mL fractions.

Glucuronidation of EC and Methyl-C/EC and Purification of the Products.

The glucuronidation reaction was performed at room temperature, and the mixture consisted of 210 μL purified water, 40 μL UGT Reaction Mix Solution A (containing UDP-glucuronic acid, BD Biosciences),100 μL UGT Reaction Mix Solution B (containing $MgCl_2$, Tris-HCl buffer and alamethicin, BD Biosciences), 10 μL of 20 mM (epi)catechin derivative, and 120 μL human UGT1A9 (for EC and methyl-EC) or pooled male mouse liver microsomes (5 mg protein per mL) (for C and methyl-C). After 6 h, an additional 40 μL of UGT Reaction Mix Solution A, 40 μL of Reaction Mix Solution B, 120 μL UGT1A9 or mouse microsomes, and 10 μL of 20 mM (epi)catechin derivative were added. Then, at 12 h, a further 40 μL UGT Reaction Mix Solution A and 120 μL UGT1A9 or mouse microsomes were added. The reactions were stopped after 24 h by the addition of 400 μL of methanol containing 3% of 85% phosphoric acid. The solvent was removed under nitrogen gas, and the aqueous fractions were desalted using a Waters Sep-Pak Plus C18 cartridge. The cartridge was first washed with 10 mL methanol, followed by 20 mL water. The sample (5 mL) was loaded onto the cartridge, then washed twice with 5 mL water, and finally eluted twice with 5 mL methanol. The sample was dried under nitrogen and then re-suspended in a small volume of methanol for HPLC purification. Product peaks were partially purified on the analytical HPLC and the solvent removed under nitrogen gas. The aqueous fractions were then acidified to 1% phosphoric acid final concentration, and desalted using a

Waters Sep-Pak Plus C18 cartridge. The methanol was dried under nitrogen and the fractions analyzed for comparison to products found in the plasma of mice/rats fed the monomer enriched GSPE fraction. All samples were dissolved in 100 μL acidified water and acetonitrile (4:1, v/v), and further analyzed by HPLC coupled to LC-TOF-MS (see below).

HPLC for Analysis and Purification of C/EC Metabolites. HPLC analysis and purification of (epi)catechin derivatives were performed on an Agilent HP1200 HPLC, monitoring at 280 nm. The HPLC with ChemStation software version B.02.01.SRI was equipped with a G1322A degasser, a G1311A quaternary pump, a G1367B autosampler, a G1316A thermostatic column compartment, and a G1315C diode array detector. A Varian Metasil 5 Basic C18 250 x 4.6 mm column was used for analytical and micro-preparative HPLC on the Agilent HPLC system. Analytical and preparative HPLC were performed using the following gradient: isocratic at 5% B for 5 min; 5% B to 10% B in 5 min; 10% B to 17% B in 15 min; 17% B to 23% B in 5 min; then 23% B to 50% B in 35 min. Alternatively, a shorter method was used which retained the same gradient except that the last step gradient was from 23% B to 28% B in 5 min, reducing the run time by 30 min. Solvent A was 1% phosphoric acid in MilliQ water, and solvent B was HPLC grade acetonitrile. Flow rate was 1 mL/min.

Solid Phase Extraction (SPE) of C and EC Metabolites from Rodent Plasma. Rodent plasma samples were obtained from a previous pharmacokinetic study using GSPE (*4*) that received approval from the Animal Care and Use Committee at Purdue University. Plasma aliquots from control animals (no GSPE) and treated animals (with GSPE) that were collected at sacrifice were thawed from -80 ^{o}C storage and quickly pooled separately at Purdue University. These control and GSPE plasma samples were then re-aliquoted into individual vials and re-frozen at -80 °C until analysis. For target assessment purposes, typical

plasma was diluted fivefold with blank plasma to produce low dose plasma. Blank, low, and normal plasma samples were then used for analytical validation at Purdue University and a parallel set of samples was sent to the University of North Texas for cross-lab validation.

Samples of frozen plasma were thawed at room temperature, and 300 µL of acidified saline (9g NaCl in 1 L 0.1% v/v formic acid/H_2O) were added to 200 µL of plasma with vortex mixing. A SPE cartridge (30 mg Oasis HLB column, Waters Co, Milford, MA), was activated by addition of 1 mL MeOH followed by 1 mL DI H_2O, after which the plasma/saline mixture was loaded. The loaded SPE column was first washed with 1 mL 1.5 M formic acid, followed by 1 mL 95:5 v/v water: MeOH, and the eluate discarded. Metabolites were then eluted with 2 mL 0.1% v/v formic acid/MeOH.

Extraction recovery was assessed in a pilot experiment by spiking 10 µL of ethyl gallate stock solution (10 µM) into freshly thawed plasma samples prior to extraction and analysis. Extraction recovery was found to be 96.0 ± 3.1%. Following validation of recovery, ethyl gallate was used as a daily internal standard (10 µL of stock 10 µM solution) added to the eluates from solid phase extraction prior to samples being dried under vacuum (<50 mm Hg) at 37 °C. The residues were re-suspended in 160 µL 0.1% v/v formic acid/H_2O + 40 µL 0.1% v/v formic acid/acetonitrile, sonicated for 10s, vortex mixed for 10s, then transferred to centrifuge tubes which were centrifuged at 18,000 *g* for 5 min to remove any particulates. The clear supernatants were carefully transferred to 300 µL HPLC vials for analysis.

The ability of this extraction method to account for potential matrix effects was assessed by comparing the response of individual metabolite standards in the presence or absence of blank plasma extracted matrix. Briefly, standards of C and its major metabolites in GSPE rodent plasma at 50, 100, and 500 nM were resuspended either in pure solvent (160 µL 0.1% v/v formic

acid/H_2O + 40 µL 0.1% v/v formic acid/acetonitrile -Standard) or in resolubilized blank plasma extracted by SPE (Standard+Matrix). By comparison of response between standards injected with or without plasma matrix an estimate of matrix effects was established by using the following equation: % matrix effect (% ME) = (peak area of standard with matrix – peak area of pure standard)/(peak area of pure standard) x 100 (*8*).

NMR Analysis

NMR spectroscopy was performed using a Bruker Avance DRX 500 MHz spectrometer equipped with a cryoprobe. The metabolites were dissolved in CD_3OD (99.96% atom D; Sigma, St. Louis, MO) and then transferred to a 5 mm Shigemi tube (Wilmad Glass, Vineland, NJ).

HPLC/MS Analysis of C/EC Metabolites from Rodent Plasma.

1. LC-TOF-MS for unknown metabolite characterization. Separation was achieved on an Agilent 1100 HPLC system using a Waters XBridge BEH shield RP-C18 XP (2.1 x 100 mm 2.5 µm) column following a 2 µm frit filter. The column and autosampler were maintained at 40 °C and 10 °C, respectively. Mobile phase A was 0.1% v/v formic acid/water and B was 0.1% v/v formic acid/acetonitrile. A linear gradient for elution was used at a constant flow rate of 0.26 mL/min. Initial conditions 5% B; 0-12 min: 5-15% B; 12-18 min: hold at 15% B; 18-24 min: 15-40% B; 24-30 min: 40-50% B; 30-31 min: 50-5% B; 31-36 min: hold at 5% B to reset gradient. Injection volume was set to 10 µL. Mass spectra of flavan-3-ol metabolites were obtained using an Agilent 6200 time-of-flight (TOF) mass spectrometer, with negative mode electrospay ionization, using a mass range of 100-1000 m/z and acquisition rate of 1.4 spectra/s. Source parameters were as follows: gas temperature = 350 °C, drying gas = 9 L/min, nebulizer pressure = 35 psi, capillary potential = 3500 V, fragmentor voltage = 145 V, skimmer voltage = 60 V, and OCT 1 RF Vpp = 250 V.

184 *2. LC-MS/MS for metabolite quantification.* Separation was achieved on an Agilent 1200
185 HPLC system as described in the previous section, except for the gradient conditions as follows:
186 initial conditions 10% B; 0-9 min: 10-35% B; 9-10 min: 35-65% B; 10-12 min: 65-10% B; 12-16
187 min: hold at 10% B to reset gradient. Analytes were quantified using tandem mass spectrometry
188 (MS/MS) with an Agilent 6460 triple quadrupole with electrospray ionization (ESI) in negative
189 mode using selected reaction monitoring (SRM) mass transitions. Collision energy was 17 eV.
190 Acquisition MRMs were: C/E, 289 → 245 m/z; C/E glucuronide, 465 → 289 m/z; methyl C/E
191 glucuronide, 479 → 303 m/z; ethyl gallate, 197 → 169 m/z. All mass transitions used a dwell
192 time of 200 ms and fragmentor voltage of 135 V. Source parameters were as follows: gas
193 temperature = 350 °C, gas flow = 11 L/min, nebulizer pressure = 30 psi, sheath gas temperature
194 = 350 °C, sheath gas flow = 11 L/min, capillary potential = 3500 V, and nozzle voltage = 1000
195 V. Intraday repeatability was assessed by extracting and analyzing pooled plasma $n = 5$ times.
196 Interday reproducibility was determined across $n = 5$ days using aliquot pooled plasma.
197 Repeatability and reproducibility were determined as relative coefficient of variation (% CV) as
198 calculated by standard deviation/mean x 100. Limit of detection (LOD) was defined as S/N = 3
199 and limit of quantification (LOQ) as S/N = 5. Both values were obtained by producing serial
200 dilutions of parent non-metabolized compounds and metabolites. Data are presented as mean ±
201 standard deviation. Signal to noise (S/N) ratio was calculated using MassHunter software using
202 the peak-to-peak method.
203 *3. Second site validation.* Samples were analyzed using a Waters Xevo-TQ mass
204 spectrometer interfaced with a Waters Acquity UPLC system. Sample preparation and LC
205 parameters were according to the original protocol above. Mass spectrometry source parameters
206 were as follows: ESI, negative mode; capillary voltage, 2 kV; source temperature, 150 ^{o}C; sheath

gas flow, 800 L/h; desolvation temperature, 400 ^{o}C; cone voltage, 28 V. Acqusition MRMs were: C/EC glucuronide, 456 → 289 m/z, collision energy 22; C/EC methyl glucuronide, 479 → 303 m/z, collision energy 22; ethyl gallate, 197 → 169 m/z, collision energy 16. Dwell time was 200 ms.

■RESULTS AND DISCUSSION

Metabolites of Flavan-3-ols in Rat Plasma and Strategy for Generation of Standards. Multiple metabolites derived from the flavan-3-ols C (2,3-*trans*) and EC (2,3-*cis*) are found in mouse or rat plasma following the feeding of monomer enriched GSPE. LC-MS analysis has indicated that these compounds are primarily glucuronides of C/EC or methyl-C/EC (*4*) (Figure 1A,B). One of the most abundant metabolites in plasma was previously identified by micro NMR as 3′-*O*-methyl-EC-5-*O*-β-D-glucuronide (Figure 1C) (*7*), and we have developed a semi-synthetic route to this compound (*7*). Because of the low levels of the metabolites, and the need to develop a robust method for their quantitation, we decided to develop semi-synthetic routes to the remaining compounds.

Our strategy for generation of standards of the other C/EC metabolites found in rat plasma was to: 1) synthesize methyl derivatives of C and EC; 2) subject the (methylated) C and EC to enzymatic glucuronidation; 3) compare LC-MS mobility and fragmentation pattern of the synthetic compound to those of the metabolites in plasma; 4) scale up the synthesis of compounds that match those in plasma and confirm their structures by NMR.

Methylation of C and EC. Although 3′-and 4′-*O*-methyl-EC are commercially available, they are expensive (synthetic 4′-*O*-methyl-EC currently costs over 1,000 Euros per mg; tebu-bio, Le Perray-en-Yvelines, France). We have previously explored the methylation of

EC using catechol-*O*-methyltransferase from porcine liver (*7*); this leads predominantly to the 3′-*O*-methyl derivatives. Although this method was effective (and is a useful approach if it is desired to incorporate radiolabel in the methyl group through incubation with labeled methyl donor S-adenosyl-L-methionine), it is also relatively expensive, and we therefore investigated the cheaper alternative of chemical methylation of the core flavan-3-ols with iodomethane, using previously established procedures (*9*). This approach leads to generation of multiple methylated products from both C and EC (Figure 2), with the more complex multi-methylated products constituting a larger proportion of the reaction products with longer incubation times. However, because the different products and isomers can easily be separated by preparative HPLC, and because of the low cost of the starting materials, this method proved effective for generation of 3′- and 4′-*O*-mono-methyl derivatives of both C and EC, suitable for subsequent glucuronidation. The methylated EC derivatives were authenticated by comparison to the commercial standards. Standards are not available for the methyl C derivatives; identity was first inferred based on the retention times of the corresponding EC derivatives, and subsequently confirmed (for the 3 ′-*O*-methyl derivative) by NMR of the resulting glucuronidated products (see below). Optimization of reaction conditions, particularly reaction time, resulted in maximum yields of 3′-*O*-methyl-C/EC of around 20%. The methylated compounds were purified by chromatography using a Biotage Isolera system.

Glucuronidation of (Methyl)C/EC, and Matching of Synthetic Compounds to Metabolites in Plasma. Human recombinant glucuronosyltransferase UGT1A9, in the presence of UDP-glucuronic acid, converts EC to a number of products, the major one being the 3′-*O*-glucuronide (a compound not found in the plasma of rats or mice fed GSPE), with minor amounts of the 5-*O*-glucuronide (a minor metabolite in the plasma) (*7*). 3′-*O*-Methyl-EC is

efficiently converted by UGT1A9 to 3′-*O*-methyl-EC-5-*O*-glucuronide (a major plasma metabolite). The other compounds in plasma appear to be derivatives of C. We therefore investigated conditions (reaction times, enzyme and substrate concentrations and supplementation of reaction mixtures with additional enzyme/substrate) for the optimal glucuronidation of methylated and non-methylated C by mammalian glucuronosyltransferases and UDP-glucuronic acid, as previously described for EC (*7*). In contrast to EC, we found that pooled male mouse microsomes were more efficient at glucuronidation of C and its derivatives than was recombinant UGT1A9, resulting in two major products initially named CG14 and CG18 based on their approximate retention times by HPLC (Supplemental Figure 1A). CG14 and CG18 were further purified by preparative HPLC (Supplemental Figure 1B). Both compounds possessed a molecular ion of 465, and matched peaks in the plasma of rats fed GSPE: CG14 was a minor component (Supplemental Figure 1C), whereas CG18 corresponded to the second most abundant metabolite (Supplemental Figure 1D).

In a similar manner, glucuronidation of (presumed) 3′-*O*-methyl-C by mouse microsomes in the presence of UDP-glucuronic acid led to the formation of multiple products of which two, of retention time 19 and 24 min and molecular ions of 479 (MCG19 and MCG24) matched metabolites in the rat plasma. Glucuronidation products of (presumed) 4′-*O*-methyl-C did not match any of the compounds found in plasma. Figure 3 summarizes the matches between the synthetic (methyl)-Cs and plasma-derived metabolites, and indicates the diagnostic major fragmentation products of the molecular ions.

Structural characterization of C and EC metabolites. We have previously reported the structural characterization of 3′-*O*-methyl-EC-5-*O*-glucuronide (*7*). To elucidate the structures of CG14, CG18, MCG19 and MCG24, 2D NMR experiments using HSQC

(heteronuclear single quantum coherence spectroscopy), HMBC (heteronuclear multiple bond correlation spectroscopy), and NOESY (nuclear Overhauser effect spectroscopy) were performed. The spectral data (chemical shifts) for the four compounds are shown in Table 1, and ^{1}H, HSQC and HMBC, and NOESY data are shown in Supplemental Figures 2 to 8. The glucuronic acid moiety was assigned to the 7 position on (+)-C for CG-14 and MCG-19 based on the presence of a HMBC cross peak between the anomeric proton (H-1″) and C-7 (Supplemental Figure 7). The assignment on CG-18 and MCG-24 was to the 5 position due to a HMBC cross peak between H-1″ and C-5 (Supplemental Figure 4). The methyl group was assigned to the 3′ position on MCG-19 and MCG-24 due to a NOESY cross peak between H-2′ and the methoxy group, confirming the identity of the precursor as 3′-*O*-methyl-C (Supplemental Figure 8). Figure 4 summarizes the structures of the four C metabolites, and its caption provides further description of how the positions of methylation and glucuronidation were assigned.

Several previous studies have reported the presence of PAC metabolites in blood plasma after feeding animals with PA preparations or foods rich in PACs (*10-13*). The metabolites were partially identified as sulfonated, glucuronidated and/or methylated derivatives of EC and C (*11, 14-16*), with most studies focusing on the EC derivatives. Definitive structures, particularly with respect to the position of glucuronidation, were not systematically assigned for many of the biologically relevant metabolites. The present systematic approach to the synthesis and identification of predominant plasma-derived metabolites of flavan-3-ols present in plasma of rodents dosed with GSPE provides a blueprint for the development of improved methods for the evaluation of bioavailability of plant polyphenols, as well as a source of standard target molecules for evaluating modes of action.

A Cross-Validated Analytical Method for Determining Plasma Levels of C and EC Metabolites. LC-MS using an Agilent 6460 Triple Quadrupole system was selected as the analytical method to determine the levels of C and EC metabolites in samples of plasma from rats fed GSPE, due in part to the sensitive and selective nature of this method as well as the broad application of this method in the literature (*17-19*). Initially, the linear dynamic range for each molecule (C, EC, two C-glucuronides, two methyl-C-glucuronides and one methyl-EC -glucuronide) was determined for concentrations ranging over 4 orders of magnitude (pM-μM), with lowest concentrations in the 250-650 pM range. The linear dynamic range was observed to be excellent (Table 2) and provides the ability to quantify these compounds across a range of concentrations typical in biological fluids and tissues. Metabolites were extracted from plasma by solid phase extraction as described in Materials and Methods. Measurement of effects of matrix and plasma concentration (Supplemental Table 1), and intraday and interday variability (Supplemental Tables 2 and 3) revealed the method to be highly robust and efficient at limiting ion suppression or other matrix effects commonly reported, but rarely accounted for, in analysis of biological samples (*20-22*). As expected, there was a greater matrix effect (ME) at lower concentrations. The % ME when measuring C ranged from -7.84% at 500 nM to -20.7% at 50 nM. The ME for MCG24 ranged from -13.5% at 500 nM to -23.0% at 50 nM. Finally, the ME for CG18 ranged from -13.1% at 500 nM to -15.7% at 50 nM. Since metabolites appear in rodent plasma at approximately 300-400 nM after repeated dosing of GSPE, using the SPE procedure results in underestimating metabolites by about 13%.

The Horwitz ratio (HorRat), a well-recognized normalized performance parameter that provides a measure of laboratory precision, was calculated for individual metabolites based on the equations described by Horwitz and Albert (*23*). HorRat values ranging from 0.13 to 1.29

indicated the high reproducibility of this method. While lower values of the HorRat (0.13-0.21) do fall outside of the generally accepted range, it is important to note that these were for minor metabolites (C-7-*O*-glucuronide, 3′-*O*-Me-C-7-*O*-glucuronide and 3 ′-*O*-Me-EC-7-*O*-glucuronide). Comparatively, values for major metabolites identified in this method, (C-5-*O*-glucuronide, 3 ′-*O*-Me-C-5-*O*-glucuronide) were 1.29 and 0.51 respectively. Overall these data are consistent with that of a highly reproducible analytical method.

Calculated LOD (defined as 3:1 signal:noise) ranged from 1.03-3.41 fmol on column for metabolites of C and EC compared to 55.5 and 48.4 pmol on column for the parent compounds C and EC, respectively. This suggest the potential for more efficient ionization of metabolites relative to parent compounds. These values translated to LOQs of 2.1-10.6 fmol on-column or ~11-53 fmol per mL of plasma for metabolites.

Finally, the analyses were repeated at a second site, using the same column and separation parameters, but a different mass spectrometer (see Materials and Methods). The C and EC metabolites in the plasma were resolved by HPLC with very similar profiles to those recorded previously (compare Supplemental Figure 9 and Figure 3). Calibration with authentic standards resulted in absolute values and standard deviations that closely matched the results from the first site analyses, with the exception of MCG24 (3′-*O*-methyl-C-5-*O*-glucuronide) which was determined based on calibration with the corresponding 7-*O*-glucuronide (Supplemental Table 4). It was observed that the C and EC metabolites were unstable in thawed plasma samples, as recently reported elsewhere (*24*), such that reproducible results were only obtained if the samples were injected onto the HPLC within a few hours. Samples left at room temperature were almost totally degraded after 3 days.

Advantages and Disadvantages of the Present Approach. Alternative approaches for identifying metabolites of flavan-3-ols in biological fluids have either generated standard compounds by chemical synthesis (*9*), or attempted to identify the compounds directly from the fluids (*14-16, 25*); most of these previous approaches have only unequivocally identified a sub-set of the metabolites present. A recent study has reported the chemical synthesis and full characterization of a complete range of EC glucuronides and sulfates (*24*). Such compounds will be of great value for future studies on bioavailability, although their chemical synthesis is complex, requiring, as starting point, the availability of a complete series of orthogonally protected EC derivatives.

The present study has combined a semi-synthetic/biosynthetic scheme with matching of compounds to authentic metabolites. Although the glucuronosyl transferase enzymes are not completely regiospecific, their mammalian origin means that they can generate physiologically relevant metabolites, and the matching of synthetic compounds to plasma metabolites prior to final purification means that only those metabolites that are physiologically relevant are pursued. Furthermore, an enzymatic strategy has advantages for the facile introduction of radiolabel, for example in the methyl (from S-adenosyl-L-methionine) or glucuronide (from UDP-glucuronic acid) moieties, should labeled compounds be required for mechanism of action studies or analytical internal standards. The limiting steps from a cost perspective are the price of the enzymes, which cannot be recycled, and the cost of UDP-glucuronic acid. Now that standards of all glucuronidated metabolites are available for subsequent identification of products, this problem could be circumvented by a total biological synthesis using recombinant enzymes expressed in *E. coli*, as we have previously shown for synthesis of flavonol and isoflavone glucosides (*26*). It should be noted that the present work does not address the sulfated

366 derivatives that also arise from mammalian metabolism of flavan-3-ols, particularly in humans
367 (*27*).
368
369 **■ASSOCIATED CONTENT**
370 * Supporting Information
371 Supplemental Figure 1. Glucuronidation of catechin by mouse microsomes in the presence of
372 UDP-glucuronic acid, and comparison of major products to metabolites of catechin in plasma
373 from rats fed GSPE.
374 Supplemental Figure 2. ^{1}H-NMR spectrum of catechin glucuronide CG-18.
375 Supplemental Figure 3. ^{1}H-NMR spectrum of methyl catechin glucuronide MCG-24.
376 Supplemental Figure 4. HSQC and HMBC spectra of CG-18 and MCG-24.
377 Supplemental Figure 5. ^{1}H-NMR spectrum of methyl catechin glucuronide MCG-19.
378 Supplemental Figure 6. ^{1}H-NMR spectrum of catechin glucuronide CG-14.
379 Supplemental Figure 7. HSQC and HMBC spectra of CG-14 and MCG-19.
380 Supplemental Figure 8. NOESY spectra of methylcatechin glucuronide MCG-19.
381 Supplemental Figure 9. Chromatographic separation and identification of C/EC metabolites for
382 each mass transition in the second site validation experiment.
383 Supplemental Table 1. Target assessment (plasma dilutions) and matrix effects for quantitation
384 of catechin and epicatechin metabolites in rat plasma by LC-MS.
385 Supplemental Table 2. Intraday variability for quantitation of catechin and epicatechin
386 metabolites in rat plasma by LC-MS, n = 5.
387 Supplemental Table 3. Interday variability for quantitation of catechin and epicatechin
388 metabolites in rat plasma by LC-MS. Experiments were performed over 5 days, n = 17.

Supplemental Table 4. Quantitation of catechin and epicatechin metabolites in rat plasma (n mol/l) by LC-MS in the second site validation experiment, n = 5.

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■ABBREVIATIONS USED

GSPE, grape seed polyphenol extract; PAC, proanthocyanidin; C, (+)-catechin; EC, (-)-epicatechin; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; UGT, uridine diphosphate glycosyltransferase; CV, column volume; SPE, solid phase extraction; DI, deionized; LC-TOF-MS, liquid chromatography- time of flight mass spectrometry; NMR, nuclear magnetic resonance; SRM, selective reaction monitoring; HSQC, heteronuclear single quantum coherence

spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; LOD, limit of detection; LOQ, limit of quantitation.

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■FIGURE CAPTIONS

Figure 1. Flavan-3-ol derivatives in rodent plasma. **A**, **B**. Representative mass spectra of unknown metabolites in rodent plasma obtained on a LC/TOF system with a recording range of 100-1000 m/z. The mass spectrum of **A** is consistent with the mass of methyl-C/EC glucuronide, while that of **B** is consistent with C/EC glucuronide. C. Structure of 2,3-*cis*-flavan-3-ols.

Epicatechin: R1 = R2 = R3 = OH; 3′-*O*-methyl-epicatechin-5-*O*-β-D-glucuronide: R1 = glucuronic acid; R2 = OMe; R3 = OH.

Figure 2. *O*-Methylation of epicatechin (A) and catechin (B) by reaction with iodomethane. HPLC traces show the multiple products formed, with the 3′- and 4′-*O*-methyl derivatives identified by comparison to authentic standards.

Figure 3. Chromatographic separation and identification of C/EC metabolites for each mass transition. Peak identification: (a) CG14, C-glucuronide; (b) CG18, C-glucuronide; (c) MCG24, 3′-*O*-methyl-C-glucuronide; (d) MCG19, 3′-*O*-methyl-C-glucuronide; (e) 3′-*O*-methyl-EC-5-*O*-glucuronide.

Figure 4. Structures assigned to CG14, CG18, MCG19 and MCG24. The glucuronic acid moiety was able to be assigned to the 7 position on (+)-catechin for CG-14 and MCG-19 based on the presence of a HMBC cross peak between the anomeric proton (H-1″) and C-7. The chemical shift of C-7 was determined by a HMBC cross peak between H-8 and C-7. Further support of this connectivity was provided by the absence of a cross peak between H-1″ and C-5 (chemical shift determined by a cross peak between H-4 and C-5). The assignment on CG-18 and MCG-24 was to the 5 position due to a HMBC cross peak between H-1″ and C-5. The methyl group was assigned to the 3′ position on MCG-19 and MCG-24 due to a NOESY cross peak between H-2′ and the methoxy group. HMBC: ⟶ NOESY: ←–→

TABLES

524 **Table. 1.** NMR spectral data (chemical shifts) for CG14, CG18, MCG19 and MCG24.

525

526 **Table 2.** Linear dynamic ranges for the quantitation of (methyl)catechin and

527 (methyl)epicacatechin glucuronides by LC-MS/MS.

528

529

530

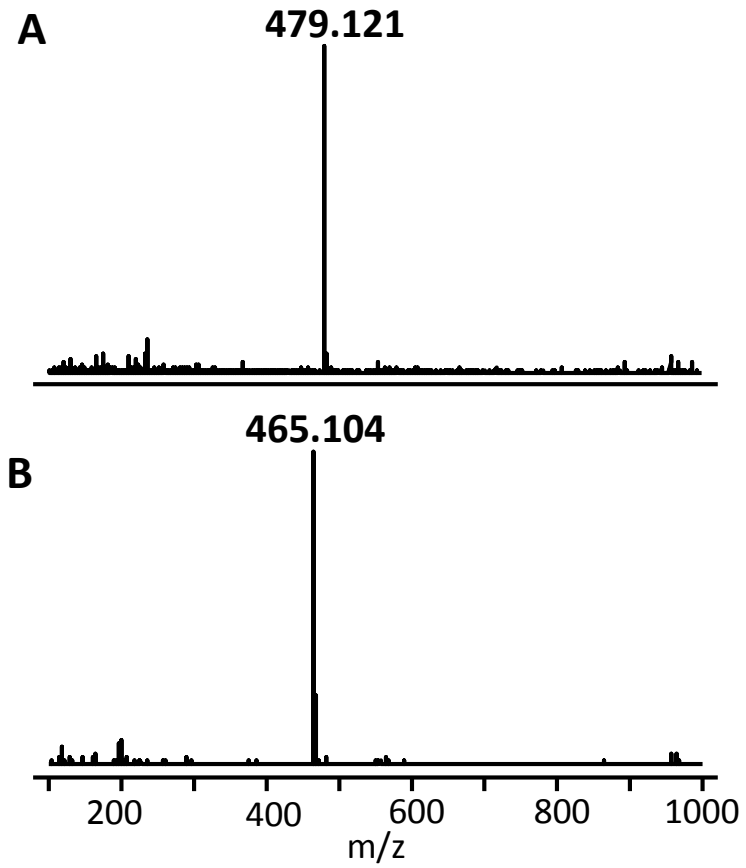
CG-14 Position	Chem. Shift (ppm) ^{1}H	^{13}C	MCG-19 Position	Chem. Shift (ppm) ^{1}H	^{13}C	MCG-24 Position	Chem. Shift (ppm) ^{1}H	^{13}C	CG-18 Position	Chem. Shift (ppm) ^{1}H	^{13}C
2	4.59	82.55	2	4.63	82.54	2	4.62	83.04	2	4.59	82.53
3	3.97	68.17	3	4.01	68.29	3	4.13	66.49	3	4.05	66.31
4a	2.87	28.04	4a	2.90	30.88	4a	2.61	27.08	4a	3.00	28.35
4b	2.53	---	4b	2.54	---	4b	2.17	---	4b	2.60	---
6	6.25	96.94	6	6.22	96.94	6	6.34	97.49	6	6.27	96.76
8	6.15	96.81	8	6.14	96.59	8	6.00	98.08	8	6.01	97.97
2′	6.83	114.86	2′	6.97	111.46	2′	6.96	111.61	2′	6.81	114.83
5′	6.71	120.27	OMe	3.84	55.83	OMe	3.83	56.40	5′	6.70	115.75
6′	6.76	115.62	5′	6.79	115.42	5′	6.77	115.97	6′	6.75	115.50
1″	4.83	102.06	6′	6.83	121.44	6′	6.85	121.21	1″	4.84	102.23
2″	3.45	74.23	1″	4.84	102.03	1″	4.82	102.81	2″	3.48	74.43
3″	3.44	77.27	2″	3.48	74.06	2″	3.50	74.60	3″	3.48	77.43
4″	3.53	72.98	3″	3.43	74.42	3″	3.49	77.94	4″	3.55	73.12
5″	3.77	76.16	4″	3.56	72.65	4″	3.55	73.71	5″	3.81	76.57
			5″	3.87	76.13	5″	3.69	76.59			

Table 1

Compound	Range	Regression line	Linearity (R^2)	LOQ (S/N=5) MOC	LOD (S/N=3) MOC
C	5nM-50µM	y = 7E+09x+ 528.31	0.9997	138.9 pmol	55.5 pmol
EC	5nM-50µM	y = 7E+09x+ 689.79	0.9997	106.4 pmol	48.4 pmol
3'OMe-EC-5-*O*-glucr	250pM-2.5µM	y=5E+11x– 3699.8	0.9998	2.05 fmol	1.07 fmol
CG18	500pM-10µM	y = 4E+11x+ 403.99	0.9999	10.6 fmol	3.41 fmol
CG14	500pM-10µM	y=6E+11x– 16601	0.9998	3.68 fmol	1.2 fmol
MCG19	650pM-10µM	y=2E+11x+ 12580	0.9990	5.80 fmol	2.80 fmol
MCG24	500pM-20µM	y=4E+11x– 31920	0.9994	4.70 fmol	1.03 fmol

Table 2

Figure 1


A
479.121
B
465.104
200
400
600
800
1000
m/z

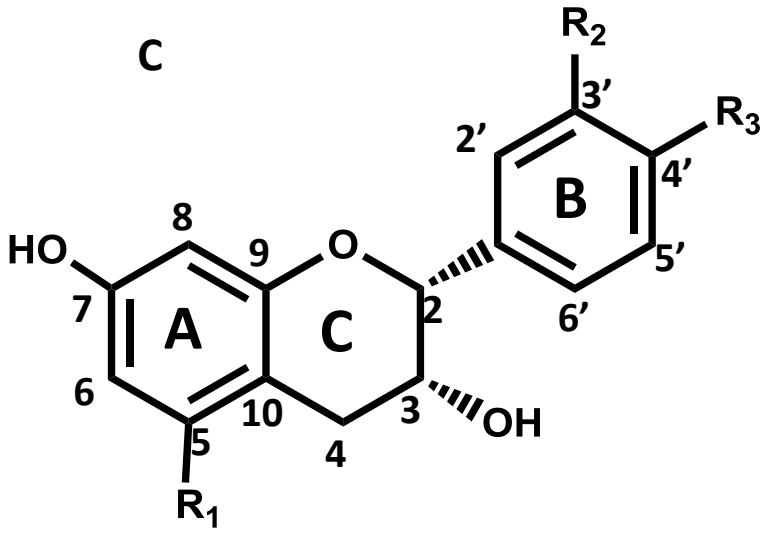

C
R2
3'
R3
2'
4'
B
8
HO
9
O
5'
7
A
C
2
6'
6
10
3
OH
5
4
R1

Figure 2

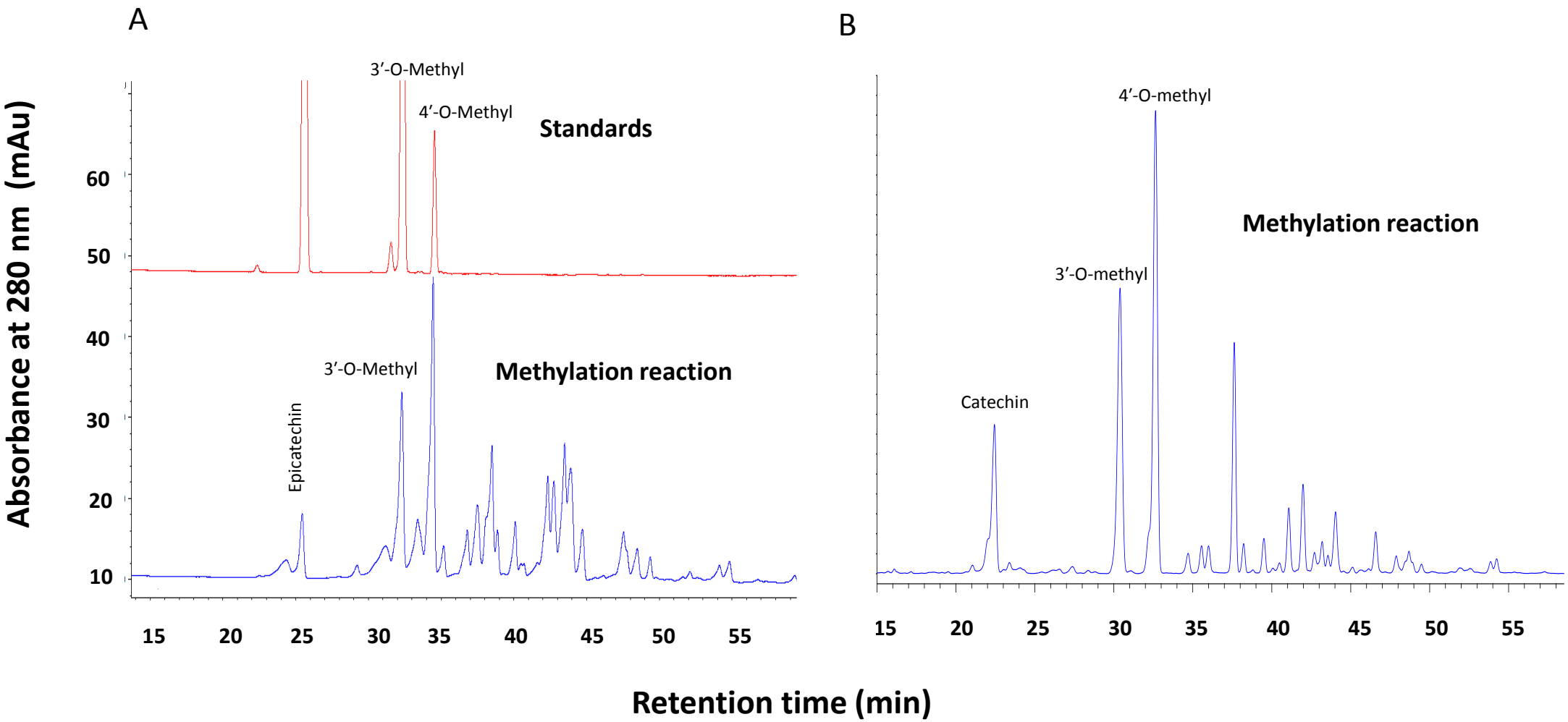

A
B
3′-O-Methyl
4′-O-Methyl
Standards
Methylation reaction
Epicatechin
3′-O-Methyl
4′-O-methyl
Methylation reaction
3′-O-methyl
Catechin
Absorbance at 280 nm (mAu)
10
20
30
40
50
60
15
20
25
30
35
40
45
50
55
Retention time (min)

Figure 3

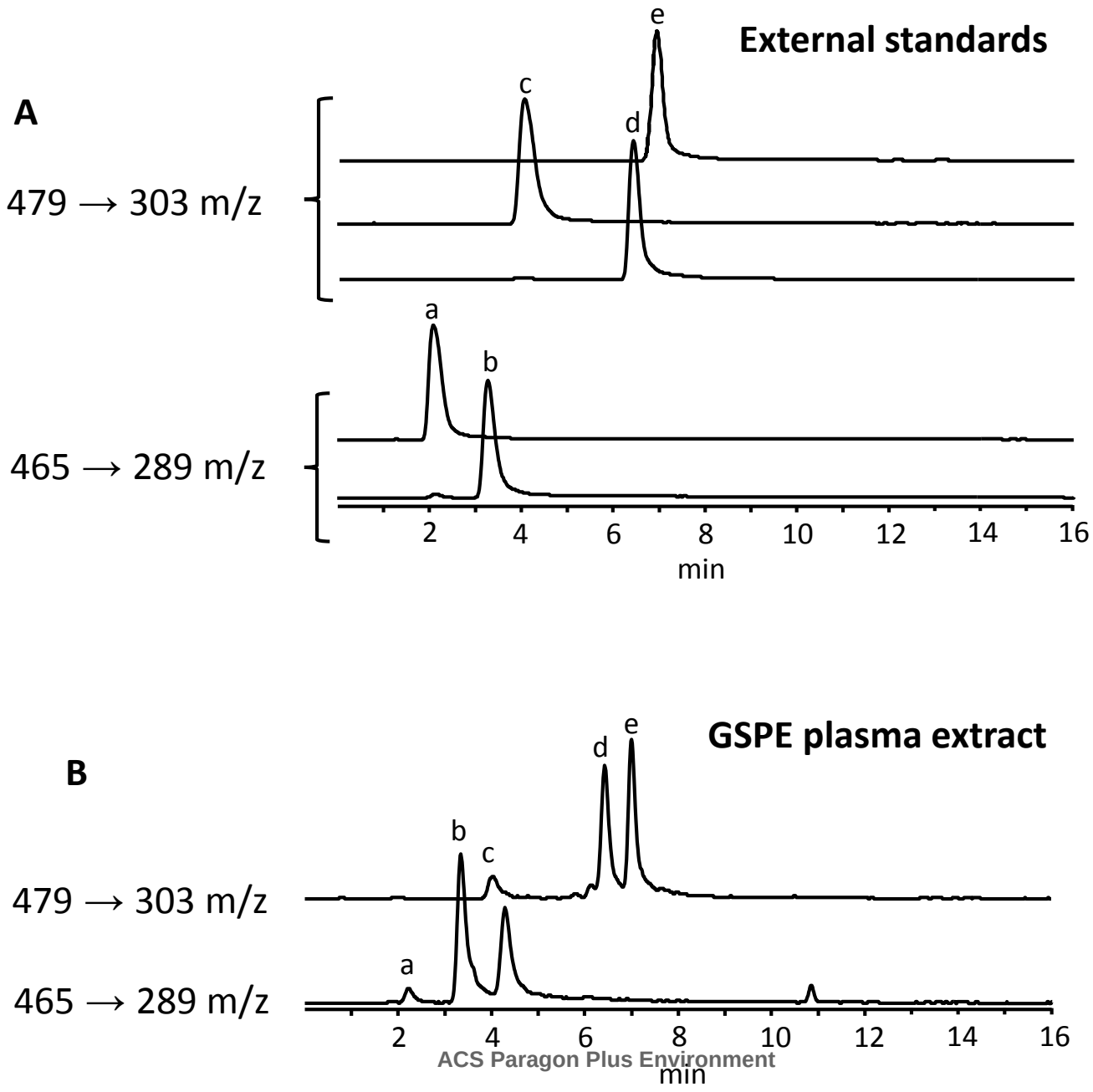
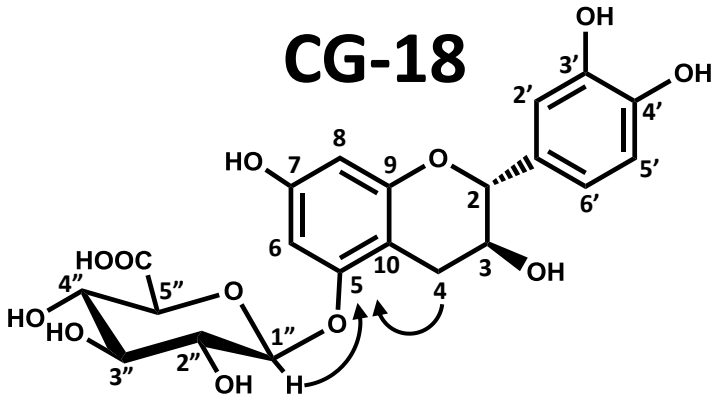

A
External standards
479 → 303 m/z
465 → 289 m/z
a
b
c
d
e
2
4
6
8
10
12
14
16
min
B
GSPE plasma extract
479 → 303 m/z
465 → 289 m/z
a
b
c
d
e
2
4
6
8
10
12
14
16
min

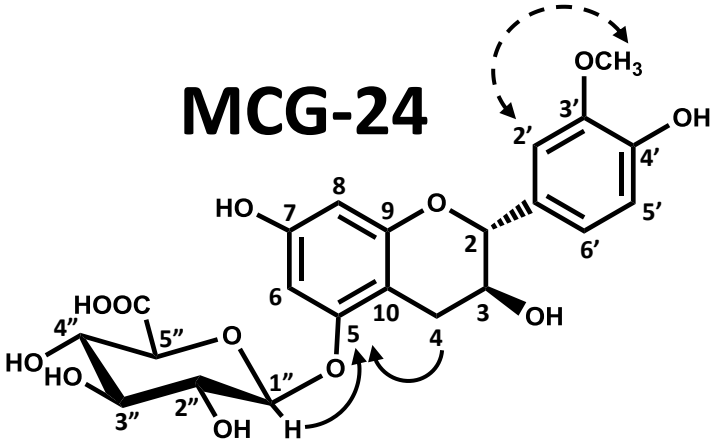
Figure 4

CG-18



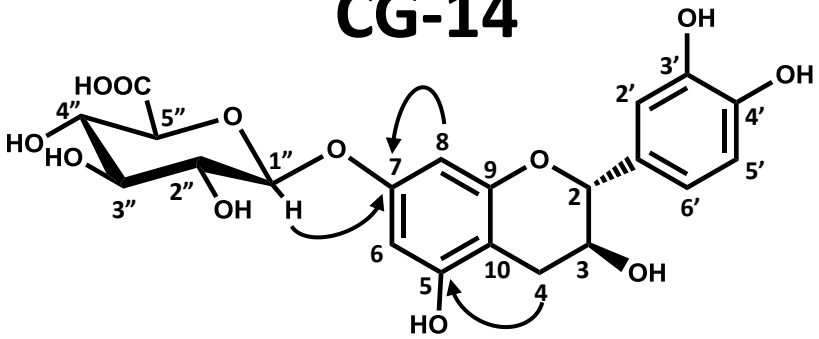
(+)-catechin-5-*O*-β-glucuronide

MCG-24



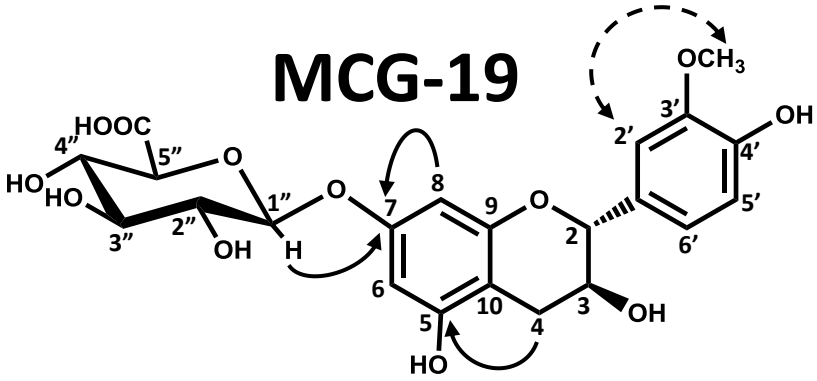
3'-*O*-methyl-(+)-catechin-5-*O*-β-glucuronide

CG-14

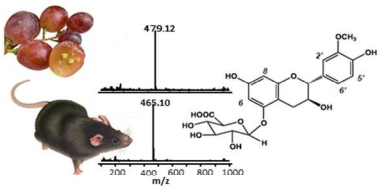


(+)-catechin-7-*O*-β-glucuronide

MCG-19



3'-*O*-methyl-(+)-catechin-7-*O*-β-glucuronide



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