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Structurally-related (−)-epicatechin metabolites in humans: assessment using *de novo* chemically synthesized authentic standards

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Abstract

Accumulating data suggest that diets rich in flavanols and procyanidins are beneficial for human health. In this context, there has been a great interest in elucidating the systemic levels and metabolic profiles at which these compounds occur in humans. While recent progress has been made, there still exist considerable differences and various disagreements with regard to the mammalian metabolites of these compounds, which in turn is largely a consequence of the lack of availability of authentic standards that would allow for the directed development and validation of expedient analytical methodologies. In the present study, we developed a method for the analysis of structurally-related flavanol metabolites using a wide range of authentic standards. Applying this method in the context of a human dietary intervention study using comprehensively characterized and standardized flavanol- and procyanidin-containing cocoa, we were able to identify the structurally-related (−)-epicatechin metabolites (SREM) postprandially extant in the systemic circulation of humans. Our results demonstrate that (−)-epicatechin-3′-β-D-glucuronide, (−)-epicatechin-3′-sulfate, and a 3′-*O*-methyl(−)-epicatechin-5/7-sulfate are the predominant SREM in humans, and further confirm the relevance of the stereochemical configuration in the context of flavanol metabolism. In addition, we also identified plausible causes for the previously reported discrepancies regarding flavanol metabolism, consisting to a significant extent of inter-laboratory differences in sample preparation (enzymatic treatment and sample conditioning for HPLC analysis) and detection systems. Thus, these findings may also aid in the establishment of consensus on this topic.

Keywords

epicatechin, metabolism, flavanols, polyphenols, flavonoids

List of abbreviations

BW: body weight; SPE: solid-phase extraction; UVD: ultraviolet absorbance detection; FLD: fluorescence detection; ECD: electrochemical detection; MS: mass spectroscopy; aSL: aryl-sulfatase; βGL: β-glucuronidase; UGT: UDP-glucuronosyltransferase; SREM: structurally-related (−)-epicatechin metabolites; SULT: sulfotransferase; COMT: catechol O-methyl transferase.

Introduction

Flavanols and their oligomeric derivatives, the procyanidins, belong to a subclass of flavonoids widely present in the human diet; particularly in food and beverages like tea, wine, cocoa, apple, plums, pomegranate and berries [1-3]. Accumulating data from medical anthropological [4], epidemiological [5-7], and dietary intervention studies [8-15] support the notion that the consumption of diets rich in flavanols and procyanidins decrease the incidence of morbidity and mortality from cardiovascular diseases. This concept has been further substantiated by recent studies demonstrating that the presence of individual flavanols, such as (−)-epicatechin, in food can, at least in part, be causally linked to the beneficial vascular effects observed after consumption of flavanol and procyanidin-containing foods [16-18]. In this context, there is an increasing interest in elucidating the mechanisms by which the consumption of these compounds, in particular (−)-epicatechin, mediates the observed effects [19]. However, ingested (−)-epicatechin is metabolized into a wide range of metabolites, including structurally-related (−)-epicatechin metabolites (SREM), which maintain an intact flavanol ring, and ring-fission metabolites, originating from (−)-epicatechin breakdown by gut microbiome. Therefore, it is essential to elucidate the specific chemical structures and levels of (−)-epicatechin metabolites that are systemically present in humans, as these metabolic derivatives may represent the molecules actually eliciting the effects observed after (−)-epicatechin consumption [17, 18]. In this context, and considering that the vascular effects observed after (−)-epicatechin intake are temporally and quantitatively correlated with SREM in circulation [17, 18], establishing the chemical structure of this particular group of metabolites is critical.

There are several studies that reported on the absorption and metabolism of (−)-epicatechin in humans and other mammalian species [17, 20-24]. Thus far, it has been clearly established that ingested (−)-epicatechin is extensively metabolized into SREM

by O-methylation, O-sulfonation, O-glucuronidation, and combinations thereof [25-28]. Consequently, the potential postprandial profile of SREM is of considerable broadness with regard to the properties of its individual constituents in terms of chemical structure, molecular charge, molecular mass, lipophilicity, acidity, chemical reactivity, protein-binding capacity, and others. Thus, it may not be surprising that there exist discrepancies in the literature with regard to the precise structure and abundance of the main SREM systemically present in humans [17, 21, 23]. While several factors can be postulated to contribute to these discrepancies (e.g. food matrix effects on absorption, intake amount-dependent differences, inter-individual variations, etc.), at this point in time, a significant part of the underlying causes for this issue may essentially lie in methodological differences, such as sample preparation and chromatographic analyses. Current sample preparation and conditioning methods vary greatly across different studies [24, 25, 29-33], as do the chromatography and detection systems [23, 33, 34]. Consequently, when considering the broadness of the potential SREM, the wide-ranging inter-laboratory differences in sample preparation and analytical methods may represent a key contributing factor for the substantial differences with regard to the reported levels and relative abundance of particular metabolites. This argument is further supported when considering that none of the currently published methods of either sample preparation or chromatography was validated against an array of authentic standards that reflects the postprandial SREM in terms of its broad range of physicochemical properties. Consequently, the numerical values for systemic levels of SREM that are currently reported in the literature carry a significant burden of uncertainty. On the surface this issue may seem of a somewhat specialized technical nature, but on the contrary, the correct identification of (−)-epicatechin metabolites and their consistent and accurate measurement across different investigator groups is essential not only for characterizing and elucidating the potential metabolites driving (−)-epicatechin-mediated

bioactivities in humans, but for meaningfully assessing dietary intake, investigating cause-effect relationships with regard to potential health benefits, and thus for ultimately translating our collective knowledge into recommendations for primary and secondary prevention, dietary guidelines, and public health.

In the present study, using a wide range of *de novo* chemically synthesized authentic (−)-epicatechin metabolite standards, we developed a method to assess the postprandial profile of SREM extant in the systemic circulation of humans. This methodology was subsequently applied in the context of a controlled dietary intervention study in healthy male adult volunteers. The results obtained were evaluated and discussed in the context of previous data.

Materials and Methods

Materials

Authentic, chemically de novo synthesized (−)-epicatechin metabolite standards, including 3′-*O*-methyl(−)epicatechin, 4′-*O*-methyl(−)epicatechin, and the ammonium salts of (−)-epicatechin-4′-sulfate, (−)-epicatechin-3′-sulfate, (−)-epicatechin-5-sulfate, (−)-epicatechin-7-sulfate, (−)-epicatechin-7-β-D-glucuronide, (−)-epicatechin-3′-β-D-glucuronide, 3′-*O*-methyl(−)-epicatechin-7-β-D-glucuronide, 4′-*O*-methyl(−)-epicatechin-5-β-D-glucuronide, 4′-*O*-methyl(−)-epicatechin-7-β-D-glucuronide, 4′-*O*-methyl(−)-epicatechin-3′-β-D-glucuronide, and 3′-*O*-ethyl(−)epicatechin (recovery standard) were provided by Mars Incorporated (Hackestown, NJ). (−)-Epicatechin and sulfatase and β-glucuronidase enzymes were purchased from Sigma (Saint Louis, MI). Water, N,N-dimethylformamide, methanol and acetonitrile HPLC grade were purchased from Fisher (Pittsburgh, PA).

Sample preparation for the identification and quantification of individual (−)-epicatechin metabolites in human plasma

The sample preparation method was adapted from Unno et al. [32] and further developed. 1 ml of plasma was spiked with 50 µl of a solution containing 10 µM 3′-*O*-ethyl(−)-epicatechin (recovery standard) and diluted with 2 ml of 3.4% (w/v) of phosphoric acid. Thereafter, samples were loaded onto solid phase extraction (SPE) cartridges (Oasis HLB 60 mg, 3 cc) previously conditioned with 1 ml of N,N-dimethyl formamide (DMF):methanol (7:3) and 0.5% (v/v) acetic acid in water. The washing steps consisted of 3 ml of 0.5% (v/v) acetic acid in water, 1 ml of water:methanol:acetic acid (80:20:0.5) and 1 ml of 0.5% (v/v) acetic acid in acetonitrile. For elution, cartridges were dried and eluted with the addition of 1 ml of DMF:methanol (7:3) twice. The eluate was

collected in tubes containing 200 µl of 0.5% (v/v) acetic acid in methanol. The total volume was reduced to approximately 50 µl using a speedvac concentrator (Thermo Electron Corp., SPD131DDA-115) at 2-3 mmHg of pressure and refrigerated vapor trap (Thermo Electron Corp., RVT4101-115) working at -100°C . Samples were then mixed with a solution containing 75 pmols of catechol and 300 pmols of resorcinol (internal standards) and analyzed by HPLC within 24 h.

To determine the extraction efficiency (i.e. recovery) of (−)-epicatechin metabolites, plasma samples were spiked with increasing concentration of the (−)-epicatechin metabolites to generate final concentrations ranging from 10 to 1000 nM. These samples were analyzed applying the method described above. For comparison purposes, plasma samples spiked with select (−)-epicatechin metabolites were analyzed using a different sample conditioning method which was based on a protein precipitation with methanol as described elsewhere [35].

Sample preparation for the quantification of (−)-epicatechin metabolites in human plasma using differential hydrolysis.

To assess the presence of those (−)-epicatechin metabolites for which we do not have standards and to further compare the results obtained using the direct quantification of individual metabolites, we quantified (−)-epicatechin metabolites in plasma using differential hydrolysis. This method is based on the treatment of plasma samples with aryl-sulfatase (aSL) and β-glucuronidase (βGL) enzymes that specifically hydrolyze (−)-epicatechin sulfates and glucuronides, respectively, giving rise to (−)-epicatechin, 3′-*O*-methyl(−)epicatechin and 4′-*O*-methyl(−)epicatechin that are later quantified in the samples. Using this approach it is possible to identify the following groups of (−)-epicatechin metabolites: (−)-epicatechin glucuronides, 3′-*O*-methyl(−)-

epicatechin glucuronides, 4′-*O*-methyl(−)-epicatechin glucuronides, (−)-epicatechin sulfates and 3′-*O*-methyl(−)-epicatechin sulfates and 4′-*O*-methyl(−)-epicatechin sulfates. To select suitable aSL and βGL enzymes to be used in this study, we compared the extent of hydrolysis of surrogate O-glucuronidated and O-sulfonated metabolites (4-nitrophenyl-β-D-glucuronide and nitrocatechol sulfate, respectively) in plasma using aSLs and βGLs from: i) *Helix pomatia* (13.1 kIU/ml of βGL and 0.32 kIU/ml of aSL), ii) agglutinin-free *Helix pomatia* (9.0 kIU/ml of βGL and 0.04 kIU/ml of aSL), iii) *E. coli* (9.8 kIU/ml of βGL) and *Abalone entrails* (0.14 kIU/ml of aSL), and iv) *Patella vulgate* (22.1 kIU/ml of βGL and 0.39 kIU/ml of aSL) (Fig. 1). The results obtained demonstrated that that aSL and βGL from *Helix pomatia* exerted a complete hydrolysis of the surrogate metabolites. In addition, these enzymes were capable of hydrolyzing a series of authentic (−)-epicatechin metabolite standards, including (−)-epicatechin-4′-sulfate, (−)-epicatechin-7-β-D-glucuronide, 3′-*O*-metyl(−)-epicatechin-7-β-D-glucuronide, 4′-*O*-methyl(−)-epicatechin-5-β-D-glucuronide, 4′-*O*-metyl(−)-epicatechin-7-β-D-glucuronide and 4′-*O*-metyl(−)-epicatechin-3′-β-D-glucuronide (data not shown). Importantly, β-glucuronidase from bovine liver, while effective in catalyzing the complete hydrolysis of O-glucuronidated metabolites, O-sulfates remained unaffected by these enzymes.

The quantification of (−)-epicatechin, 3′-*O*-methyl(−)epicatechin, and 4′-*O*-methyl(−)epicatechin in plasma was determined in 0.5 ml of plasma that was spiked with 50 µl of a solution containing 10 µM 3′-*O*-ethyl(−)-epicatechin (recovery standard), acidified with 50 µl of 1.2 M acetic acid and incubated for 40 min at 37°C in the presence of 5,000 IU of β-glucuronidase and at least 150 IU of aSL from Helix Pomatia or 5,000 IU of βGL from bovine liver. Thereafter, samples were put on ice, diluted with 1.3 ml of 0.5% (v/v) acetic acid in water and centrifuged for 15 min at 16,500xg at 4°C. Finally,

samples loaded onto SPE cartridges following the same protocol as for the determination of individual metabolites, with the exception of the second washing step that was done using 1 ml of water:methanol:acetic acid (70:30:0.5).

To determine the extraction efficiency (i.e. recovery) of (−)-epicatechin, 3′-*O*-methyl(−)epicatechin and 4′-*O*-methyl(−)epicatechin, plasma samples containing aSL and βGL from *Helix pomatia* were spiked with increasing concentrations of the above metabolites to generate final concentrations ranging from 10 to 1000 nM. These samples were incubated for 40 min at 37°C and analyzed using the method described above. For comparison purposes, plasma samples spiked with the same compounds were analyzed using a different sample conditioning method, which was based on a protein precipitation with methanol as described elsewhere [35].

Chromatographic conditions for the quantification of (−)-epicatechin metabolites

(−)-Epicatechin and its related metabolites were resolved and quantified using an Agilent HPLC 1100 series equipped with a quaternary pump, autosampler, column and sample thermostat, UV/Vis (UVD) and fluorescence detectors (FLD) and an ESA Coulochem Array Detector (ECD), model 5600A equipped with a cell model 6210 with an array of 4 electrodes. Chromatography was based on a Phenomenex Luna C-18(2) column (150 x 4.6 mm, 3 µm particle size) with guard column. The separation and resolution of compounds was achieved using the elution program as described by Ottaviani et al. [35]. The detection of (−)-epicatechin and its related metabolites was done following the traces of fluorescence at 276 nm excitation and 316 nm emission, UV absorption at 280 nm and oxidation at 225 mV, 350 mV, 425 mV and 550 mV.

Identification of individual metabolites using HPLC-MS/MS and enzymatic hydrolysis

Peaks designated as potential SREMs were initially identified based on a retention time comparison with authentic (−)-epicatechin metabolite standards, fluorescence emission spectra (from 301 to 400 nm), UV-absorbance spectra (from 200 to 400 nm), the ratio of fluorescence- vs. UV intensity, and electrochemical detection (at 225 mV, 350 mV, 425 mV and 550 mV). To further substantiate the identity of the metabolites detected, samples from different subjects were analyzed using the chromatographic conditions described above, and the putative SREM peaks were collected. An aliquot of the collected fractions was incubated in the presence of aSL or βGL enzymes, and the products of hydrolysis were analyzed by HPLC as described above. Other aliquots of collected fractions were analyzed by HPLC-MS/MS, using an Agilent HPLC 1100 series LC equipped with a Metachem Polaris C18-A column (4.6x250 mm, 3 µm particle size) coupled to a AB Sciex API 4000 QTrap mass spectrometer with a turbo ion spray source in negative ion mode. AB Sciex Analyst version 1.5 software was used for data analyses. The chromatographic conditions consisted of a linear gradient from A to B at constant flow rate of 0.5 ml/min starting with 100% A for 1 min, continuing with a linear increase of B up to 100% in 25 min and maintaining 100% B for 1 more min; where A was 10 mM ammonium acetate in water:acetonitrile (95:5) and B was 10 mM ammonium acetate in water:acetonitrile (5:95). The identification of (−)-epicatechin metabolites was conducted with the following *m*/*z* transition: 369→289 (epicatechin sulfates); 465→289 (epicatechin glucuronides), 383→289 and 383→303 (O-methylepicatechin sulfates); 479→113 (O-methylepicatechin glucuronides), 305→109 (O-methylepicatechin) and 289→109 (epicatechin). The ion spray and orifice voltages, temperature, and collision energy were set at -4200 V, -65 V, 500°C, and -36 V, respectively. The collision gas (nitrogen) was maintained at the medium setting. Data acquisition was conducted using multiple reaction monitoring (MRM) with a 75 ms dwell time per transition.

Human Study I: identification and quantification of (−)-epicatechin metabolites in circulation

(−)-Epicatechin-containing test drink: the test drink consisted of a cocoa dairy-based drink containing (−)-epicatechin in addition to other flavanols and procyanidins. The amount of the test compound consumed by each participant was identical in terms of amount per kg of body weight (BW). The test drink was prepared by mixing 0.5 g/kg BW of a low-flavanol cocoa powder, 5 g/kg BW of milk (1% fat) and 22 mg/kg BW of a cocoa extract containing 47.7% (w/w) of flavanols and procyanidins (as determined using the method detailed in [36]. The resulting amount of (−)-epicatechin consumed with the drink was 1.8 mg of (−)-epicatechin/kg of BW. The content of macro-nutrients, caloric load, as well as the amounts of theobromine, caffeine and other flavanols and procyanidins in the drink are listed in table 1. This drink was similar in composition to flavanol-rich drinks that have been reported to induce an acute vascular response in humans after consumption [10, 13]. The cocoa extract (Cocoapro® processed cocoa extract) and the low-flavanol cocoa powder were supplied by Mars Incorporated (Hackettstown, NJ, USA).

Participants: We screened healthy male volunteers between 18 and 35 years of age. Main exclusion criteria were a body mass index (BMI) higher than 30 kg/m^2, a history of cardiovascular, renal or liver disease, gastrointestinal disorders and previous gastrointestinal surgery, smoking during or within the last 5 years from the initialization of the study, current consumption of herbal, antioxidant or vitamin supplements and existing allergies to peanuts and milk. To reduce the contribution of flavanols from the diet, participants were asked to follow a low-flavanol diet on the day before, and during the study days. All volunteers were instructed on how to follow a low-flavanol diet,

receiving a list of suggested foods containing low or negligible amounts of flavanols. In addition, we provided the volunteers with flavanol-free foods for their supper on the day prior to the study day, as well as for all the meals during the study day. Volunteers were asked to restrain from consuming alcohol, coffee or other caffeine-containing beverages, on the day prior to, and during the study visits. Volunteers were asked to fast for 12 h before each study day (water *ad libitum*).

Study design: Upon arrival, volunteers (n=10) received 4 g/kg BW of drinking water to control for the level of hydration. One h later, volunteers were given the test drink and were asked to consume it within 5 min. Venous blood samples were taken using EDTA-containing Vacutainers at 0 h (before intake), and 1 h, 2 h and 4 h after drink ingestion. Plasma was obtained by whole blood centrifugation at 1800xg for 15 min at 4°C, separated into aliquots, and spiked with ascorbic acid (final concentration: 1 mg/ml). Plasma samples were stored at -80°C until analysis.

Volunteers participated in the study on different days, and the study was completed in 4 weeks. This study protocol was approved by the Institutional Review Board of the University of California Davis, and all subjects gave their written informed consent to participate.

Human Study II: comparison of (+) and (−)-epicatechin metabolites in circulation

To compare the profile of SREM after the consumption of (+) and (−)-epicatechin we re-analyzed samples that were originally generated from a study aimed at investigating the effect of flavanol stereochemistry on absorption and O-methylation [35]. While the latter investigation did not concern itself with assessing the O-sulfonated and O-glucuronidated metabolites, a re-probing of the plasma samples collected can provide valuable insights with regard to the stereochemistry-specific aspects of flavanol

sulfonation and glucuronidaton. This re-analysis was limited to samples collected 2 h after the consumption of 1.5 mg/kg of BW of (−)- and (+)-epicatechin in healthy adult humans [n=7]. For further details on the study design, test drink and study group, please refer to [35].

Statistical analysis

The primary test for an effect was a test of the interaction in a two-way repeated measures ANOVA (where the factors were time and the different metabolites). The ANOVA and Tukey P values as well as the Pearson-product motion correlation coefficient (r) were computed with SigmaStat 3.5 (Systat Software Inc, San Jose, CA, USA). P-values of 0.05 or less were considered statistically significant. To compare variances of the recoveries obtained with SPE and protein precipitation, we used the F test for equal variance. $P<0.05$ indicated that variances of both groups are not equal.

Results

Characterization of (−)-epicatechin metabolite standards, sample preparation method and the HPLC detection systems used.

For this study we used a total of 12 authentic (−)-epicatechin metabolite standards. The chemical identity of these standards is supported by an unambiguous *de novo* chemical synthesis and by ^{1}H-NRM, ^{13}C-NMR and mass spectrometric analysis (Supplementary data; supplementary Scheme I, and supplementary Fig. 1-3. Additionally, we present HPLC chromatogram traces demonstrating the resolution and detection of all 12 (−)-epicatechin metabolite standards using UVD, FLD and ECD as described in 'material and methods' (Fig. 2). While all of the metabolite standards can be detected following UV-absorbance at 280 nm, the detectability of these compounds based on FLD and ECD methods varied significantly. For instance, metabolites bearing a free catechol group were detected with high sensitivity and selectivity using ECD at an electrochemical potential of 225 mV (Fig. 2). Conversely, metabolites with a conjugated catechol group were only detected at higher potentials (550 mV). While most metabolite standards were detected using FLD, (−)-epicatechin sulfates exhibited a very low fluorescence signal, and therefore were unquantifiable by this detection method. The optimal conditions identified for the quantification of each of (−)-epicatechin metabolite standards investigated in this study are listed in table 2.

Additionally, this series of (−)-epicatechin metabolite standards were used to determine the extraction efficiency (i.e. recovery) of these compounds from plasma using a solid-phase extraction method, as described in the materials and method section. The recoveries obtained for this series of (−)-epicatechin metabolite standards ranged from 74 to 100% (Table 2). Importantly, these recoveries were higher (pair-t test; $p<0.001$) and less variable (F test; $p<0.05$) than the recoveries obtained by employing a

more commonly used sample conditioning method based on a plasma protein precipitation approach with methanol (Table 2).

Study population

Basic biometric characteristics of the study group participating in Study I are presented in table 3. Baseline flavanol concentrations in plasma were below the limit of detection on each study day, indicating compliance with the flavanol-low diet as detailed in the study protocol. Volunteers reported no adverse events associated with their participation in this study. For details on the study group participating in Study II, please refer to reference [35].

Determination of the identity and concentration of individual (−)-epicatechin metabolites in humans

Oral ingestion of the test drink resulted in the absorption of (−)-epicatechin and the subsequent occurrence of different SREM in plasma (Fig. 3 and 4). Despite the fact that (−)-epicatechin O-glucuronidation could theoretically occur at 5 different sites on the (−)-epicatechin molecule, one single predominant (−)-epicatechin glucuronide was detected in plasma. This metabolite corresponded to epicatechin-3′-β-D-glucuronide (Fig. 3B). The identity of this metabolite was confirmed by further analysis of the collected peak by HPLC-MS/MS (peak detected at m/z 465→289) and differential enzymatic hydrolysis (βGL sensitive; product of hydrolysis: (−)-epicatechin). The maximal plasma concentration of epicatechin-3′-β-D-glucuronide (589±85 nM; $p<0.05$) was reached within 2 h after the consumption of the test drink. Additionally, we also detected the presence of significantly smaller amounts of *O*-methylated epicatechin glucuronides, particularly 4′-*O*-methyl(−)-epicatechin-7-β-D-glucuronide. This

observation remained qualitative, as the levels of this metabolite were below the limit of quantification (~20 nM for this compound).

Regarding the (−)-epicatechin sulfates, we observed the presence of 3 distinct metabolites in plasma (Fig. 4B). The most abundant compound of this series, (−)-epicatechin-3′-sulfate, reached a maximal plasma concentration of 331±26 nM at 2 h after consumption. The other two sulfates corresponded to (−)-epicatechin-5-sulfate and (−)-epicatechin-7-sulfate, both of which presented significantly lower plasma levels (37±3 nM and 12±1 nM, respectively) as compared to (−)-epicatechin-3′-sulfate. The identity of these (−)-epicatechin derivatives was also verified via the collection of HPLC fractions and further analysis by HPLC-MS/MS (peaks detected at m/z 369→289) and enzymatic hydrolysis (aSL sensitive; product of hydrolysis: (−)-epicatechin). Furthermore, we also observed the presence of at least 4 peaks that were collected by HPLC and analyzed by HPLC-MS/MS (peaks detected at m/z 383→303) and enzymatic hydrolysis (aSL sensitive; product of hydrolysis: 3′-O-methyl(−)-epicatechin [peaks *i* and *ii*] and 4′-*O*-methyl(−)-epicatechin [peaks *iii* and *iv*]). Therefore, the results obtained support the notion that these compounds represent two individual 3′-*O*-methyl(−)-epicatechin sulfates (peaks *i* and *ii*) and two distinct 4′-*O*-methyl(−)-epicatechin sulfates (peaks *iii* and *iv*), respectively (Fig. 4B). Supporting this conclusion, further analyses with authentic 3′-*O*-methyl(−)-epicatechin sulfates standards, demonstrated that sulfonation of the metabolites represented by peaks *i* and *ii* occurred in position O5 and O7, respectively.

Unmetabolized (−)-epicatechin was present in circulation at low concentrations, reaching a maximum of 4±1 nM at 1 h after test drink consumption. No free 3′/4′-*O*-methy(−)-epicatechin was detected.

Corresponding determination of plasma (−)-epicatechin metabolites using differential hydrolysis

Corroborating the results detailed above, the analysis of plasma samples using differential hydrolysis also identified (−)-epicatechin glucuronides and (−)-epicatechin sulfates as major constituents of the metabolome of (−)-epicatechin in humans (Fig. 5A). The levels reached by (−)-epicatechin glucuronides were maximal 2 h after consumption, presenting values of 573±77 nM ($p<0.05$). The concentration of (−)-epicatechin glucuronides as determined by differential hydrolysis was not significantly different from the concentration of (−)-epicatechin-3′-β-D-glucuronide as assessed using authentic standards ($r=0.995$ and a slope of 1.03±0.02; $p<0.001$; Fig. 5B). The group of (−)-epicatechin sulfates reached a maximal plasma level of 301±30 nM at 2 h after consumption that was not significantly different from the sum of (−)-epicatechin-3′-sulfate, (−)-epicatechin-5-sulfate and (−)-epicatechin-7-sulfate as assessed using authentic standards ($r=0.952$ and a slope of 1.12±0.03; $p<0.001$; Fig. 5B). In addition, the use of differential hydrolysis permitted the identification and quantification of two other groups of (−)-epicatechin metabolites, namely the 3′ and 4′-*O*-methyl(−)-epicatechin sulfates. In this case, the maximal levels of 3′-*O*-methyl(−)-epicatechin sulfates were 214±26 nM at 2 h after consumption ($p<0.05$), while the group of 4′-*O*-methyl(−)-epicatechin sulfates presented plasma levels of 51±8 nM ($p<0.05$).

Concurring with the findings using (−)-epicatechin metabolite standards, the levels of 3′ and 4′-*O*-methylated (−)-epicatechin glucuronides represented a negligible fraction of the SREM in humans, presenting values below 20 nM in plasma. No O-sulfonated and O-glucuronidated (−)-epicatechin metabolites were detected in the samples.

Relative abundance of (−)-epicatechin metabolites in humans

Combining the data that emanated from the use of differential hydrolysis with the data obtained using authentic standards, we are able to detect and measure 8 distinct postprandial metabolites of (−)-epicatechin in human plasma. The identity of all 8 metabolites was further supported by HPLC-MS/MS. The most abundant (−)-epicatechin metabolite was (−)-epicatechin-3′-β-D-glucuronide, which accounted for 46±6% of all epicatechin metabolites detected in plasma at 2 h after consumption (Fig. 6). The respective abundance of (−)-epicatechin-3′-sulfate, (−)-epicatechin-5-sulfate, and (−)-epicatechin-7-sulfate was 28±6%, 3.1±0.8% and 1.1±0.3% of all epicatechin metabolites identified (Fig. 6). The group of 3′ and 4′-*O*-methyl(−)-epicatechin-5/7-sulfates presented a relative abundance of 17±2% and 4.3±0.7%, respectively (Fig. 6).

Influence of the stereochemical configuration of epicatechin on its metabolic fate in humans

We also investigated the influence of flavanol stereochemistry on the metabolic profile of the postprandial profile of SREM following the ingestion by humans of (−)-epicatechin and (+)-epicatechin, respectively. As depicted in figure 8, the concentrations as well as relative abundance of individual metabolites detected in plasma were highly dependent on the stereochemical configuration of the epicatechin ingested. While epicatechin-3′-β-D-glucuronide was the only O-glucuronidated metabolite identified in plasma regardless of the epicatechin enantiomer consumed, the maximal plasma concentration of this compound reached 336±75 nM after (−)-epicatechin consumption, but only 13±7 nM after the ingestion of (+)-epicatechin (Fig. 7). Analogously, the concentration detected for epicatechin-3′-sulfate (377±49 nM) after (−)-epicatechin consumption was almost 4 times higher than the concentration detected for the same metabolite after (+)-epicatechin consumption (101±13 nM, $p<0.05$; Fig. 7). In contrast,

epicatechin-5-sulfate, which reached a level of 50±6 nM after (−)-epicatechin intake, was extant at a concentration of 270±29 nM after (+)-epicatechin consumption ($p<0.05$; Fig. 7), representing a 5 fold difference in favor of the (+) enantiomer.

Discussion

In the present study, we developed a method for the analysis of the postprandial profile of SREM in human plasma samples. This method was subsequently employed to assess (−)-epicatechin absorption and metabolism after the consumption of a well characterized and standardized flavanol- and procyanidin-containing cocoa drink. Our work resulted in the identification of the chemical structure, plasma concentration, and relative abundance of the majority of SREM systemically present in humans.

The development and testing of our method using a wide range of *de novo* chemically synthesized (−)-epicatechin metabolite standards resulted not just in novel insights into flavanol metabolism, but also in the identification of important limitations associated with analytical methods in current use. For instance, the wide variation in sample conditioning/preparation methods, including methods of plasma protein precipitation [35], is an important contributing factor to the widely varying yields in extraction efficiencies (recoveries), and therefore also to the differences in the reported relative abundance of individual metabolites. For example, testing a range of currently utilized sample preparation procedures, our data demonstrated differences in the recovery of (−)-epicatechin-3′-β-D-glucuronide that ranged from 28% to 74% (Table 2). In addition, the low fluorescence signal yielded by (−)-epicatechin sulfates, especially evident in the case of (−)-epicatechin-3′-sulfate, could explain why previous investigations that exclusively relied on FLD detection systems were unable to detect, and thus to recognize these metabolites as a significant part of the (−)-epicatechin metabolome in humans [17, 22]. Finally, it is also worth mentioning the considerable limitations associated with the use of aSL and βGL to assess (−)-epicatechin levels and metabolism. In this context, we detected significant differences in the extent of hydrolysis of sulfates depending on the origin of the enzymes utilized (Fig. 1). The limitations of

using aSL and βGL enzymes is also supported by previous reports suggesting that 3′- and 4′-O-methyl(−)-epicatechin sulfates are more resistant to hydrolysis [37]. Consequently, these data indicate that the sole dependency on enzymatic hydrolysis may lead to an underestimation of the circulating levels of (−)-epicatechin metabolites, which should be considered as a limitation of data sets generated by using this methodological approach. Taken together, the results presented here clearly demonstrate that the current ambiguity in the scientific literature with regard to (−)-epicatechin levels and metabolism in mammals can be attributed, at least in part, to the significant differences in various aspects of the analytical methods employed. In this context, we hope that the present study may contribute towards the building of a consensus with regard to the analytical methodology used to assess the identity and concentration of (−)-epicatechin metabolites in humans.

The application our method in the context of a human dietary intervention study resulted in the identification, verification, and measurement of 8 individual (−)-epicatechin metabolites, consisting of (−)-epicatechin-3′-β-D-glucuronide, (−)-epicatechin-3′-sulfate, (−)-epicatechin-5-sulfate and (−)-epicatechin-7-sulfate. Moreover, we described the presence of 4 distinct 3′- and 4′-*O*-methyl(−)-epicatechin sulfates, each of which is sulfonated in either position O5 or O7 of the epicatechin molecule.

Our findings demonstrate that (−)-epicatechin-3′-β-D-glucuronide is the most predominant O-glucuronidated metabolic derivative of (−)-epicatechin present in the human circulation. These results are supported by previous quantitative or semi-qualitative observations by Natsume et al. who originally described this metabolite in humans [21], and by more recent qualitative observations of a single O-glucuronidated (−)-epicatechin metabolite present in plasma after the consumption of different (−)-epicatechin-containing products [23, 24, 38]. Unlike O-glucuronidation, (−)-epicatechin

O-sulfonation resulted in a more diverse group of metabolites in humans. While in the present study, a structural identification of 3 distinct (−)-epicatechin sulfates is provided for the first time, Auger et al. previously reported on the presence of 3 O-sulfonated (−)-epicatechin derivatives, the structures of which were not further elucidated [23]. Collectively, these findings substantiate the notion that O-sulfonation is a key conjugating reaction in the metabolism of (−)-epicatechin in humans, resulting in (−)-epicatechin-3′-sulfate as the most abundant derivative of this series, and one of the most abundant (−)-epicatechin metabolites in humans. Furthermore, we clearly demonstrated that (−)-epicatechin O-methylation in humans occurs primarily in position 3′ of the (−)-epicatechin molecule, as is in accordance with the catalytic properties of catechol O-methyl transferase [39]. Finally, we showed that (−)-epicatechin O-methylation is mainly extant in combination with O-sulfonation, and not with O-glucuronidation, as suggested in earlier investigations by our group and others [17, 21, 22, 31, 40]. In this context, it is noteworthy that previous studies describing the presence of O-methyl(−)-epicatechin glucuronides [21] administered (−)-epicatechin in amounts approximately 10 times higher than consumed in the present study (ca. 20 mg/kg of BW vs. 1.8 mg/kg of BW), suggesting the potential for (−)-epicatechin being subjected to different metabolic pathways when consumed in quantities that greatly exceed those relevant in a dietary context. Such a concept would also be supported by previous findings on the metabolism of mono-phenolic compounds in rats that also describe an amount-dependent shift in O-sulfonation and O-glucuronidation [41, 42]. In addition, other studies identifying the presence of O-methyl(−)-epicatechin glucuronides in circulation were conducted in rats [31, 40], also indicating the potential for species-dependent differences in the metabolism of (−)-epicatechin. Taken together, it is tenable to conclude that in humans, the consumption of diet-relevant amounts of (−)-epicatechin leads to the presence in systemic circulation of three predominant and structurally-

related metabolites, including (−)-epicatechin-3′-β-D-glucuronide, (−)-epicatechin-3′-sulfate, and a 3′-*O*-methyl(−)-epicatechin-5/7-sulfate (Fig. 8). Nonetheless, to establish the limits of validity of these findings and to allow their population-based interpretation, future investigations should address inter-individual differences in (−)-epicatechin metabolism due to: a) differences in intake amount, food matrix, and consumption frequency; b) variations in age, sex, race, dietary- and genetic background; and c) the influence of pathophysiological states and medications.

In the context of our investigations into the role of the stereochemical configuration as a determinant of flavanol metabolism, our results were somewhat unexpected. While the intake of (+)-epicatechin resulted almost exclusively in the formation of O-sulfonated derivatives, the main (−)-epicatechin metabolites included both, O-sulfonated and O-glucuronidated derivatives. Moreover, while sulfonation of (+)-epicatechin took place in position O5, (−)-epicatechin was predominantly sulfonated in the O3′ position. Thus, these findings unequivocally demonstrate that the stereochemical configuration of flavanols represents a major aspect determining the resulting structure of flavanol metabolites in humans. These findings go beyond previous results reported by our group that demonstrated stereoisomer-specific differences in the O-methylation of flavanols in humans [35]. In addition, our data demonstrate that the majority of (−)-epicatechin metabolites identified in this study were those with a conjugated catechol ring. As the catechol ring is often referenced as critical element in structure-activity relationships, especially with regard to the putative *in vivo* antioxidant properties of flavanols [43], the fact that this structure undergoes significant metabolic derivatization argues for a critical review of current assumptions in this direction.

Overall, the results from the current study provide a detailed description of the systemic, postprandial profile of SREM in humans, thus identifying tenable candidate compounds that may represent the bioactive principles in a causality chain linking the intake of

flavanol-containing foods and the observed biological effects. Consequently, these data are thus relevant when investigating the potential molecular targets and the mechanisms-of-actions of dietary flavanols and procyanidins *in vivo*.

Acknowledgment

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Figure Legends

Fig. 1: Extent of hydrolysis of O-glucuronidated and O-sulfonated metabolites in plasma. Hydrolysis catalyzed by β-glucuornidase (βGL) and aryl-sulfatase (aSL) enzymes from i) *Helix pomatia*, ii) agglutinin-free *Helix pomatia*, iii) *E. coli* and *Abalone entrails*, and iv) *Patella vulgate*. Data are expressed as mean ± SEM of the percentage (%) of the metabolites hydrolyzed. *p<0.01, significantly different from the control (100% hydrolysis).

Fig. 2: Chromatogram traces of *de novo* synthesized authentic (−)-epicatechin metabolite standards. Detection with UV absorbance (UVD), fluorescence (FLD), and electrochemical detection (ECD) at 225 mV and 550 mV, where [A] is (−)-epicatechin-7-β-D-glucuronide, [B] is 3′-*O*-methyl(−)-epicatechin-7-β-D-glucuronide, [C] is (−)-epicatechin-3′-β-D-glucuronide, [D] is 4′-*O*-methyl(−)-epicatechin-5-β-D-glucuronide, [E] is (−)-epicatechin-5-sulfate, [F] is 4′-*O*-methyl(−)-epicatechin-7-β-D-glucuronide, [G] is 4′-*O*-methyl(−)-epicatechin-3′-β-D-glucuronide, [H] is (−)-epicatechin-7-sulfate, [I] is (−)-epicatechin , [J] is (−)-epicatechin-3′-sulfate, [K] is 3′-*O*-methyl(−)epicatechin, [L] is 4′-*O*-methyl(−)epicatechin, and [RS] is 3′-*O*-ethyl(−)epicatechin. Insert: chemical structure of the authentic (−)-epicatechin metabolites standards used in the present study that include metabolites derived from O-methylation (-CH_3), O-glucuronidation (-$Gluc^-$) and O-sulfonation (-SO_3^-) in four different positions in the molecule O4′ (R_1), O3′ (R_2), O5 (R_3) and O7 (R_4).

Fig. 3: Circulating (−)-epicatechin glucuronides. A) Level of individual (−)-epicatechin glucuronides in plasma after the consumption of a test drink containing 1.8 mg/kg of BW of (−)-epicatechin. Data are expressed as average ± SEM [n=10] in nM. B)

Chromatogram traces (FLD detection) of authentic (−)-epicatechin glucuronide standards and plasma samples obtained before (0 h) and 2 h after the consumption of the test drink, where [A] is (−)-epicatechin-7-β-D-glucuronide, [B] is 3′-*O*-methyl(−)-epicatechin-7-β-D-glucuronide, [C] is (−)-epicatechin-3′-β-D-glucuronide, [D] is 4′-*O*-methyl(−)-epicatechin-5-β-D-glucuronide, [F] is 4′-*O*-methyl(−)-epicatechin-7-β-D-glucuronide and [G] is 4′-*O*-methyl(−)-epicatechin-3′-β-D-glucuronide.

Fig. 4: Circulating (−)-epicatechin sulfates. A) Level of individual (−)-epicatechin sulfates in plasma after the consumption of a test drink containing 1.8 mg/kg of BW of (−)-epicatechin. Data are expressed as average ± SEM [n=10] in nM. B) Chromatogram traces (ECD detection) of authentic (−)-epicatechin sulfate standards and plasma samples obtained before (0 h) and 2 h after the consumption of the test drink, where [E] is (−)-epicatechin-5-sulfate, [H] is (−)-epicatechin-7-sulfate, [I] is (−)-epicatechin, [J] is (−)-epicatechin-3′-sulfate, and where [*i*] and [*ii*] were assigned as 3′-*O*-methyl(−)-epicatechin-5–sulfate and 3′-*O*-methyl(−)-epicatechin-7–sulfate, respectively, and [*iii*] and [*iv*] were assigned as 4′-*O*-methyl(−)-epicatechin-5/7-sulfates.

Fig. 5: Postprandial (−)-epicatechin metabolites in plasma. A) Level of different groups of (−)-epicatechin metabolites in plasma after the consumption of a test drink containing 1.8 mg/kg of BW of (−)-epicatechin. Data are expressed as average ± SEM [n=10] in nM. *significantly different from the levels determined at the rest of the time points within the same group of metabolites, $p<0.05$. B) Correlation between the levels of (−)-epicatechin-3′-β-D-glucuronide and the levels of (−)-epicatechin glucuronides determined with differential hydrolysis in plasma ($r=0.995$, slope of 1.03±0.02; $p<0.001$) and between the

sum of individual (−)-epicatechin sulfates and the levels of (−)-epicatechin sulfates determined with differential hydrolysis in plasma (r=0.951, slope of 1.13±0.02; p<0.001).

Fig. 6: Relative amounts of individual (−)-epicatechin metabolites in plasma. Data represent SREM profile at 2 h after the consumption of a test drink containing 1.8 mg/kg of BW of (−)-epicatechin. *The abundance of these metabolites was calculated according to the concentration determined by differential hydrolysis.

Fig. 7: Stereochemistry-dependent epicatechin metabolism. Level of individual epicatechin metabolites in plasma after the consumption of a cocoa drink containing 1.5 mg/kg of BW of (−)-epicatechin or 1.5 mg/kg of BW of (+)-epicatechin, respectively. Data are expressed as average ± SEM [n=7] in nM. *significantly different from the levels reached after (−)-epicatechin consumption within the same metabolite, p<0.05

Fig. 8: Schematic representation of the main circulating SREM. Based on assessments in healthy males after the consumption of diet-relevant amounts (−)-epicatechin. UGT: UDP-glucuronosyltransferase, SULT: sulfotransferase, COMT: catechol O-methyl transferase.

Tables

Table 1: Composition of the test drink used in study I. Values represent the amounts that would have been consumed by a 75 kg subject.

Nutritional details	Amount received
Calories (kcal)	224
Total fat (g)	19
Saturated fat (g)	3
Cholesterol (mg)	19
Total carbohydrates (g)	29
Dietary fiber (g)	3
Sugars (g)	24
Caffeine (mg)	35
Theobromine (mg)	297
Total flavanols and procyanidins (mg)	1100
(−)-Epicatechin (mg)	138
(±)-Catechin (mg)	19
Procyanidins (dimers to decamers) (mg)	944

Table 2: Recovery of (−)-epicatechin metabolites from plasma samples using SPE (as described in Materials and Method section) and a protein precipitation method [35]. Data are expressed as average ± SEM of the percentage recovered. Additionally, the detection system used for quantification for each metabolite is included. n.q. equals not quantifiable (because of the presence of interfering peaks in the chromatogram introduced via protein precipitation method).

(−)-Epicatechin metabolites	**Method**	**Recovery (%)**		**Detection system**
		Solid-phase Extraction	**Protein Precipitation**	
(−)-epicatechin	Direct quantification of individual metabolites	93±1	76±1	ECD
(−)-epicatechin-7-β-D-glucuronide		90±2	n.q.	ECD/FLD
(−)-epicatechin-3'-β-D-glucuronide		74±1	28±3	FLD
3'-*O*-methyl(−)-epicatechin-7-β-D-glucuronide		86±4	60±1	FLD
4'-*O*-methyl(−)-epicatechin-5-β-D-glucuronide		88±1	59±1	FLD
4'-*O*-methyl(−)-epicatechin-7-β-D-glucuronide		101±1	62±1	FLD
4'-*O*-methyl(−)-epicatechin-3'-β-D-glucuronide		72±2	49±1	FLD
(−)-epicatechin-5-sulfate		85±1	n.q.	ECD
(−)-epicatechin-7-sulfate		84±1	n.q.	ECD
(−)-epicatechin-3'-sulfate		70±1	n.q.	ECD
(−)-epicatechin	Differential hydrolysis	82±1	78±1	FLD
3'-*O*-methyl(−)-epicatechin		73±1	109±3	FLD
4'-*O*-methyl(−)-epicatechin		78±1	103±2	FLD

Table 3: Study I participant characteristics

Parameter (units)	Value
Age (y)	32±1
Weight (kg)	75±8
BMI (kg/m^2)	24±2
Systolic blood pressure (mmHg)	123±9
Diastolic blood pressure (mmHg)	74±8

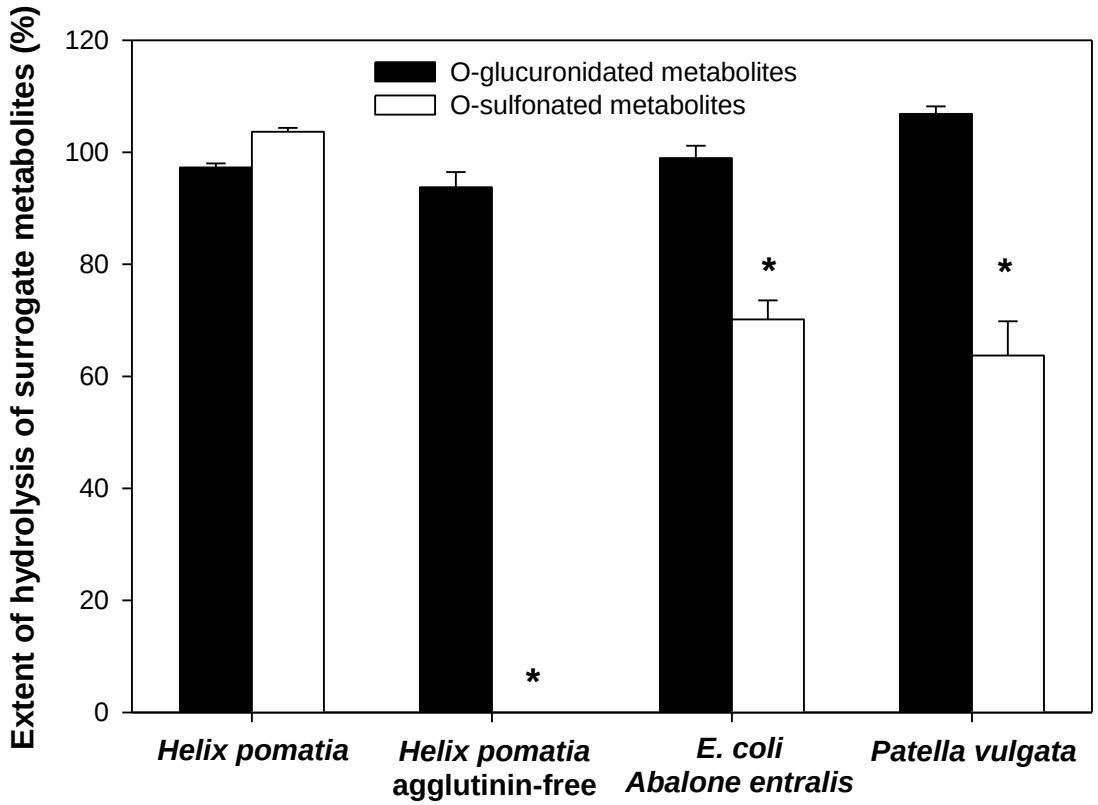


Figure 1

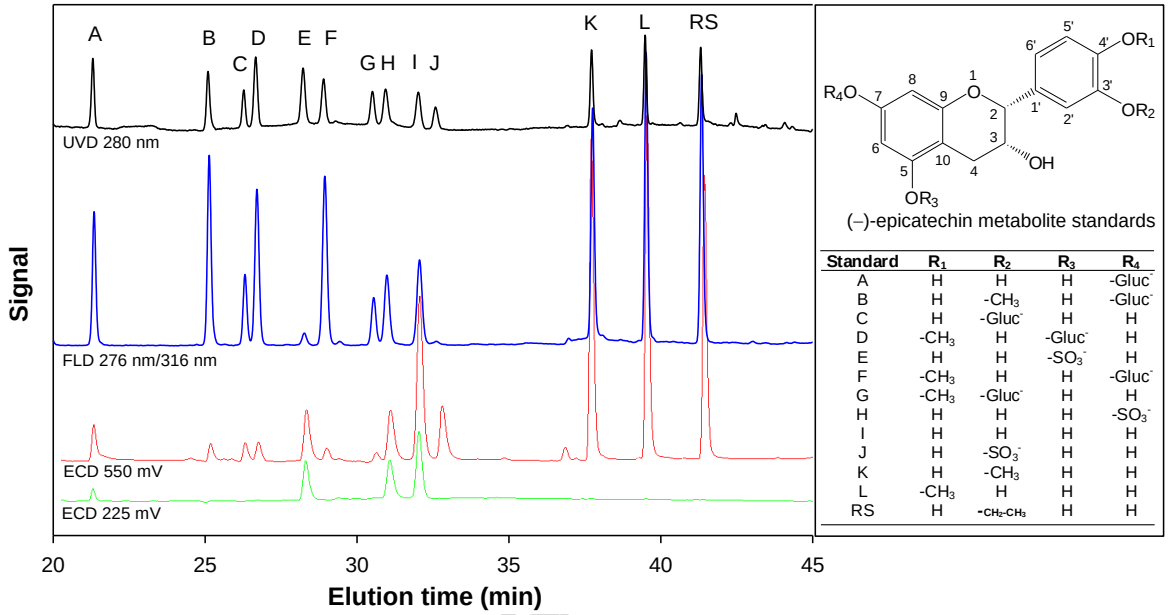


Figure 2

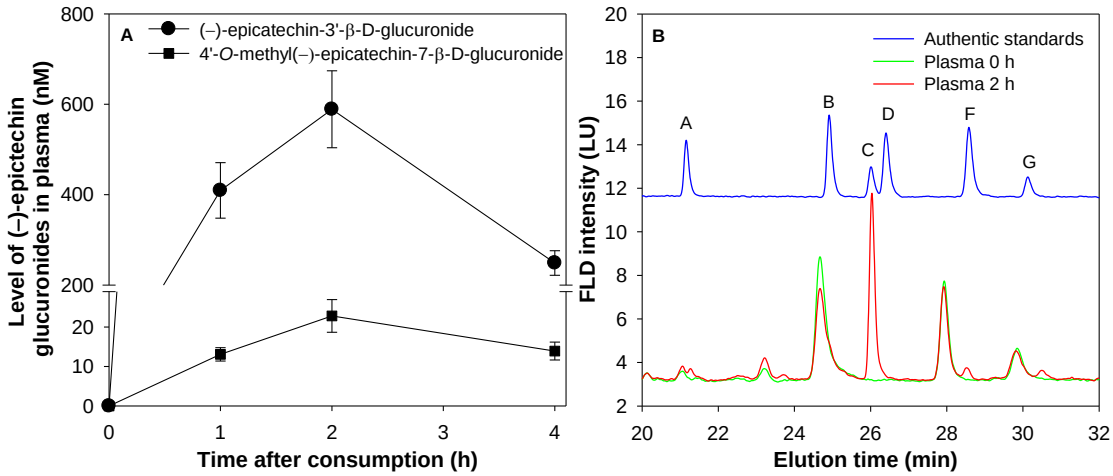


Figure 3

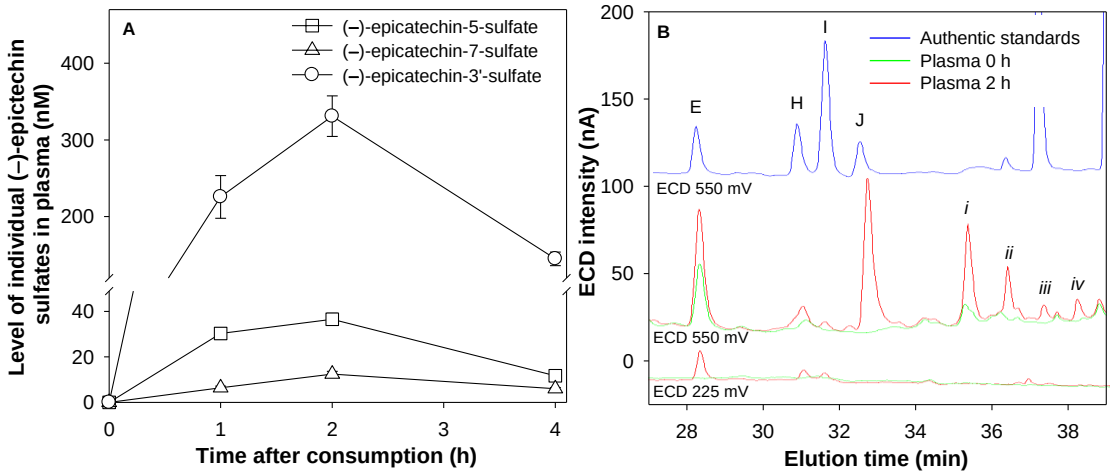


Figure 4

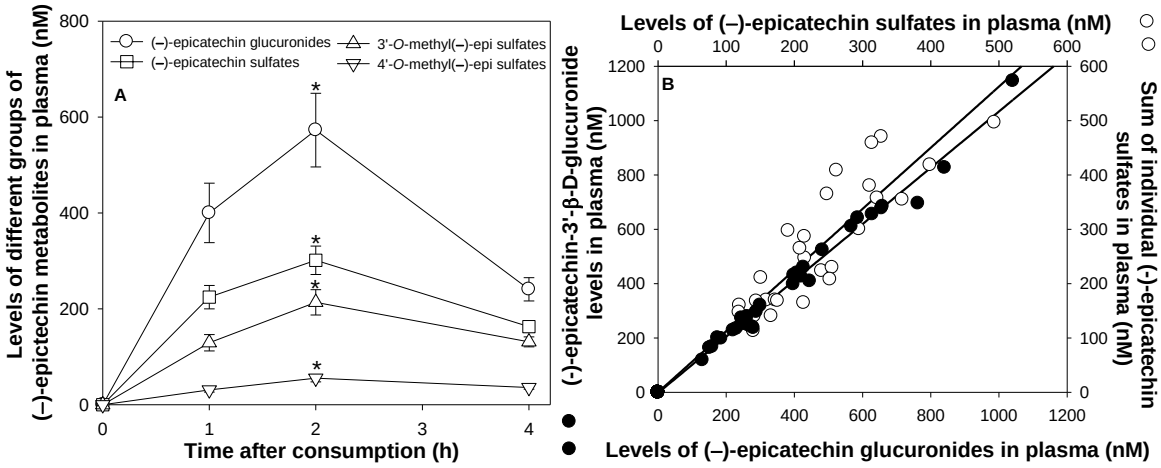


Figure 5

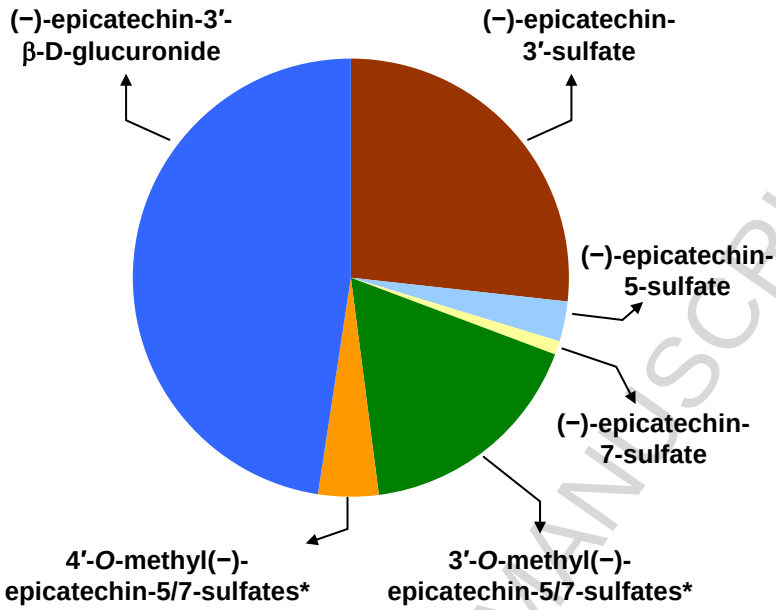
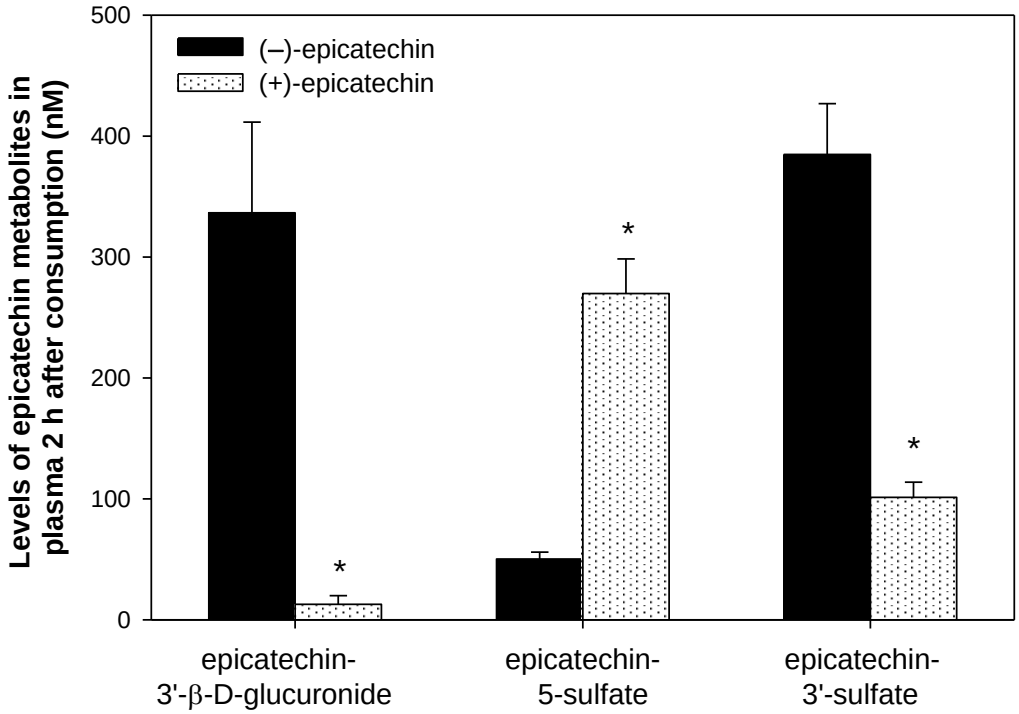


Figure 6



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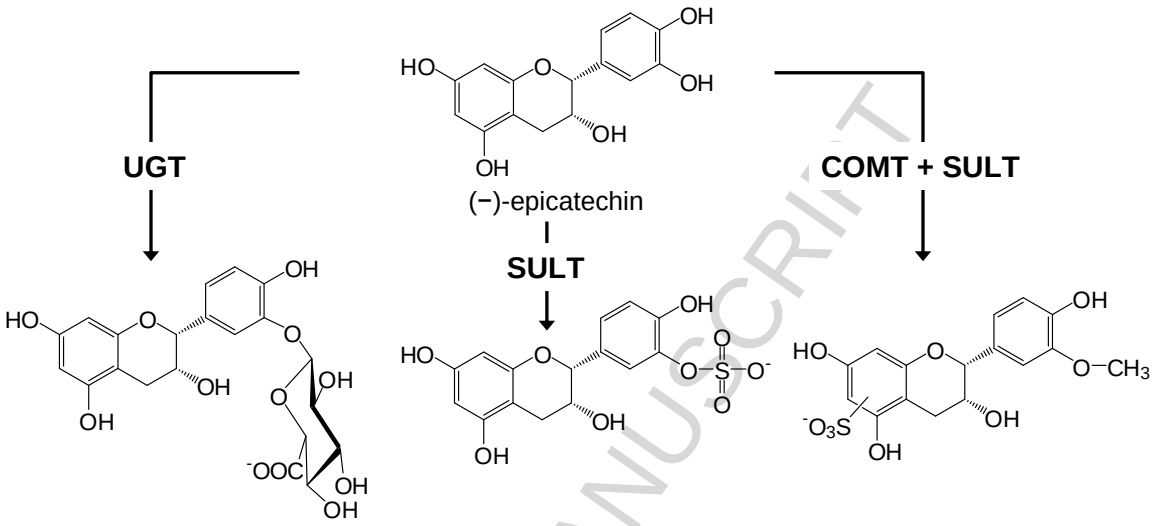

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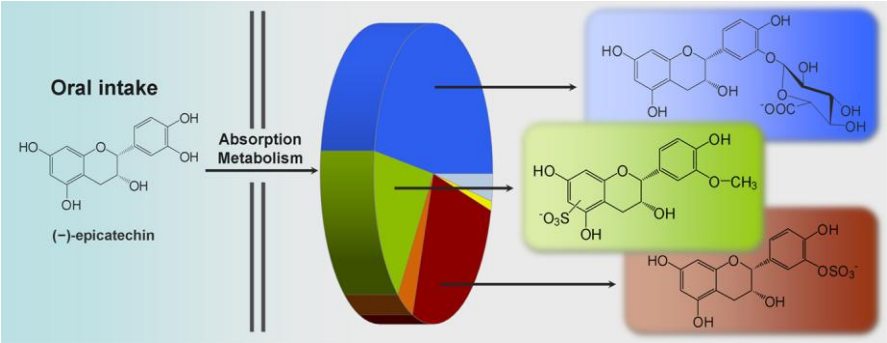

Graphical Abstract

Highlight

- Improved sample preparation- and analytical methods for (-)-epicatechin metabolites
- Novel insights into the uptake and metabolism of flavanols in humans
- Identification & quantification of structurally-related (-)-epicatechin metabolites
- Stereochemical configuration of flavanols affects their metabolism in humans