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Title

Validation of a high-throughput method for the quantification of flavanol and procyanidins biomarkers and methylxanthines in plasma by UPLC-MS§

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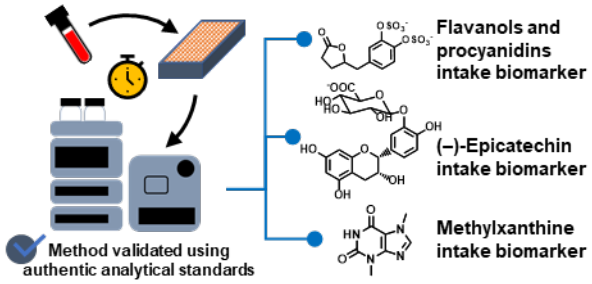
Graphical abstract



New high throughput method for the quantification of flavanols and procyanidin nutritional biomarkers and methylxanthines in plasma.

Abstract

Nutritional biomarkers are critical tools to objectively assess intake of nutrients and other compounds from the diet. In this context, it is essential that suitable analytical methods are available for the accurate quantification of biomarkers in large scale studies. Recently, structurally-related (–)-epicatechin metabolites (SREMs) and 5-(3',4'-dihydroxyphenyl)-γ-valerolactone metabolites (gVLMs) were identified as biomarkers of intake of flavanols and procyanidins, a group of polyphenol bioactives. This study aimed at validating a high throughput method for the quantification of SREMs and gVLMs in plasma along with methylxanthines (MXs), dietary compounds known to interact with flavanol and procyanidin effects. To accomplish this, a full set of authentic analytical standards were used to optimize a micro solid phase extraction method for sample preparation coupled to HPLC-MS detection. Isotopically-labelled standards for all analytes were included to correct potential matrix effects on quantification. Average accuracies of 101%, 93% and 103% were obtained, respectively, for SREMs, gVLMs and MXs. Intra- and inter-day repeatability values were <15%. The method showed linear responses for all analytes (>0.993). Most SREMs and gVLMs had limits of quantifications <5 nM while limits of quantification of MXs were 0.2 μM. All analytes were stable under different tested processing conditions. Finally, the method proved to be suitable to assess SREMs, gVLMs and MXs in plasma collected after single acute and daily intake of cocoa-derived test materials. Overall, this method proved to be a valid analytical tool for high throughput quantification of flavanol and procyanidin biomarkers and methylxanthines in plasma.

1 Introduction

Flavanols, including (−)-epicatechin, and their related oligomers, the procyanidins, are a group of polyphenol bioactives that occur widely in the diet [1-3]. Accumulating epidemiological and intervention studies suggest that the intake of these bioactives mediates beneficial effects on cardiovascular health and cognitive performance [4-10]. Following ingestion, flavanols and procyanidins are metabolized extensively by phase II reactions as well as by the gut microbiome [11-13]. It has recently been established that a combination of structurally-related (−)-epicatechin metabolites (SREMs) formed in the upper gastrointestinal tract is a specific biomarker of (−)-epicatechin intake, while a combination of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone metabolites (gVLMs) is a biomarker of both flavanol and procyanidin intake (Fig. 1) [14, 15]. A number of the main dietary sources of flavanol also contain methylxanthines, such as caffeine (Caf) in tea, and theobromine (Tb) in cocoa-derived products [16, 17]. Among the various biological effects mediated by methylxanthines, they have been shown to mediate interactions with flavanols [18]. The quantification of methylxanthines in biofluids has also been used to assess their habitual intake [19-21], and to assess compliance of test material intake in dietary intervention studies with flavanol-containing cocoa products [5, 22]. In this context, there is a need for high throughput analytical methods that can quantify the recently validated flavanol and procyanidin biomarkers, as well as methylxanthines, in an accurate, precise, and reliable manner. These methods would be valuable tools to further investigate the absorption and metabolism of flavanols and procyanidins and their interaction with methylxanthines, and also to respond to the demands of sample analysis derived from large clinical trials.

Analytical methods for the quantification of metabolites derived from polyphenols bioactives have undergone significant improvements over the years, and LC-MS is now the preferred approach to the quantitative analysis of polyphenol metabolites [23-26]. However, substantial inaccuracies can be incurred when inappropriate analytical standards are used with LC-MS methodology for the quantification of polyphenol metabolites [27]. Recently, a rapid LC-MS method for the quantification of SREMs and gVLMs in urine was validated [14, 15] and methods for the quantification of methylxanthines in different biofluids have also been validated [28]. However, single platform methods for the targeted analysis of flavanol and procyanidin biomarkers and methylxanthines in plasma have, to date, not been developed.

This study aimed at developing and validating a LC-MS method for high throughput quantification of SREMs, gVLMs and methylxanthines in plasma. To accomplish this a micro-solid phase extraction (μSPE) approach for sample preparation based on HLB resin was utilized [29, 30]. Samples were analyzed via UPLC-MS using appropriate authentic reference compounds and isotopically labeled standards to correct for matrix effects.

2 Materials and methods

2.1 Analytical standards

De novo chemically synthesized analytical standards for SREMs and gVLMs were obtained from Analyticon (Analyticon Discovery, Rockville, MD) based on previously described methods [31-35]. SREMs and gVLMs included (−)-epicatechin-3'-glucuronide (EC-3'GlcUA), (−)-epicatechin-3'-sulfate (EC-3'S), 3'-methoxy-(−)-epicatechin-5-sulfate (3'Me-EC-5S), 5-(4′-hydroxyphenyl)-γ-valerolactone-3'-sulfate (4′OH-VL-3'S), and 5-(4′-hydroxyphenyl)-γ-valerolactone-3'-glucuronide (4′-OH-VL-3'-GlcUA). Stable isotopically labeled standards

(ISTDs) of SREMs and gVLMs were also obtained by de novo chemical synthesis from Analyticon and included: [$^{2}H_2/^{2}H_3$](–)-EC-3'GlcUA, (50:50 mix) and [$^{13}C_2$,$^{2}H_2$]4′OH-VL-3'S. Analytical standards for Tb and Caf were obtained from MilliporeSigma (Millipore Sigma, St. Louis, MO, USA). ISTD for MXs were obtained from Toronto Research Chemicals (North York, ONT, Canada) and included [$^{2}H_6$]Tb and [$^{13}C_3$]Caf. Nomenclature for the valerolactones is based on the proposals of Kay et al. (2020) [36] and chemical structures of selected analytes and ISTD are presented in Fig. 1.

2.2 Stock solutions

Stock solutions of EC-3'S, EC-3'GlcUA, and 3'Me-EC-5S were prepared by dissolving ~10 mg aliquots in 70% ethanol in water to obtain individual 10 mM solutions. 4′-OH-VL-3'S and 4′OH-VL-3'G were similarly prepared to obtain individual 20 mM solutions. Tb, and Caf were prepared by dissolving approximately 5 mg of material in 80% methanol in water slightly heated to help dissolution, yielding 10 mM solutions. ISTD solutions were prepared by dissolving approximately 5 mg aliquots in 70% ethanol in water to obtain 10 mM solutions for [$^{2}H_2/^{2}H_3$]EC-3′-GlcUA and [$^{13}C_2$,$^{2}H_2$]4′-OH-VL-3′S. Solutions for [$^{2}H_6$]Tb and [$^{13}C_3$]Caf were prepared by dissolving pre-weighed material with 80% methanol in water to yield 10 mM solutions. All stock solutions were stored at –80°C prior to use.

2.3 Accuracy assessment

Stock solutions were diluted to prepare 5 working solutions (WS1-5) ranging from 0.1 to 10 μM for SREMs and gVLMs and from 10 to 1000 μM for MXs (see Table S1 in on-line the Supplementary Information). These concentrations were chosen to represent potential concentrations of SREMs and gVLMs found in fasting plasma following daily intake of

flavanols and procyanidins, and after acute flavanol and procyanidin intake [13, 22, 37]. All working solutions were prepared in an aqueous solution of 0.1% formic acid on the day of use.

Plasma samples using EDTA as anticoagulant were collected from apparently healthy male adults (n = 7) after 24 h on a low-flavanol diet and following a 12 h fast to minimize the presence of SREMs and gVLMs following a study protocol approved by the Institutional Review Board at the University of California Davis in accordance with the Helsinki Declaration of 1975 as revised in 1983 (Further details on the study are presented in Supplementary Study Design). Informed consent was obtained from all participants. All samples were combined to generate a single pooled plasma sample, divided into aliquots, and stored at −80°C prior to use. Pooled plasma samples were analyzed for the presence of SREMs, gVLMs and MXs. While SREMs and gVLMs were absent in the pooled plasma, MXs were detected mainly as Cf. To assess accuracy, SREM- and gVLM-free pooled plasma samples (350 µL) were added to 10 µL of WS1-5 (n = 4 per level) to generate plasma samples (PS1-5) with SREM and gVLM concentrations ranging from 2.86 to 286 nM for and MX concentrations ranging from 0.286 to 28.6 µM (Table S2). A blank plasma sample (P0) was included to correct for the possible presence of MXs.

Plasma samples were quantified using standard curves prepared in SREM- and gVLM-free pooled plasma samples that had previously taken through the sample µSPE preparation process. In addition, a second standard curve was prepared using the µSPE elution solvent (dimethylformamide [DMF]:methanol, 7:3, v/v) as a matrix to assess if it would be possible to dispense with the use of plasma for standard curve preparation. The

comparison of the signal of the analytes from these two standard curves was also used to calculate the matrix effect on the quantification of analytes as Matrix effect (%)=(signal $_{\text{std curve in plasma}}$/signal $_{\text{std curve in solvent}}$ −1)x100%. In this context, 0% represents no deviation due to matrix effect, positive percentages show ion enhancement, and negative percentages show ion suppression.

2.4 Precision assessment

Plasma samples (n = 60) were collected 1-6 h after intake of different cocoa flavanol-containing test materials from healthy male adults. These plasma samples were pooled and then spiked with urine collected between 12-24 h after intake of cocoa test materials by healthy male adults (n = 2) to prepare three sets of quality control (QC) plasma samples. SREMs, gVLMs and MXs were quantified in these three QC samples and the results obtained are presented in Table S3.

Intra-day precision (repeatability) was determined by relative standard deviation on concentrations ($\%RSD_{\text{intra-day}}$) of analytes in the three QC samples analyzed in triplicate. Inter-day precision (repeatability) was determined by relative standard deviation on concentrations ($\%RSD_{\text{inter-day}}$) of analytes in the three QC samples analyzed on three days, spanning 25 total days between analyses. Analyte quantification was carried out as described in Sections 3.7-3.10, using standard curves prepared in SREM- and gVLM-free plasma.

2.5 Linearity, limit of quantification and limit of detection assessment

Linearity was measured through the coefficient of determination of nine individual calibration curves prepared in a SREM- and gVLM-free plasma matrix (Table S4). Limit of

quantification (LoQ) was determined for individual analytes by the ratio of 10 times the standard deviation of <LoQ samples.

2.6 Stability assessment

Plasma samples (n = 36) were collected between 1-6 h after intake of different cocoa flavanol-containing test materials from apparently healthy male adults. These samples were randomly divided into three groups (n = 12 per group) to test stability of analytes in samples under three different conditions, including storage of samples i) in the autosampler for 2 and 4 h at 4°C (n = 12), ii) on the benchtop for 6 h at 23°C (n = 12), and iii) for 14 days at –80°C (n = 12). Each sample was divided in two aliquots. One aliquot was analyzed immediately by UHPLC-MS/MS, while a second aliquot of the same sample was analyzed after completing the storage conditions described above. Stability was assessed by comparing the concentration of analytes before and after storage by paired t-test (two-tailed, significance $p<0.05$). Data were expressed as the relative change to the concentration determined before storage.

2.7 Method application

Method application was tested under two different scenarios. The first scenario evaluated applicability of the method in the context of studies aiming at assessing absorption and metabolism of flavanols, procyanidins and methylxanthines after acute single intake of test materials. To accomplish this, plasma samples were collected from apparently healthy volunteers (n = 7) before and 2 h and 6 h after single intake of a cocoa-derived test drink containing 624 mg of flavanols and procyanidins, including 76 mg of (–)-epicatechin, 140 mg of Tb and 20 mg of Caf. For this study, volunteers followed a low flavanol diet for 24 h

prior intake of test material. The second scenario evaluated applicability of the method in the context of studies aiming at investigating daily/habitual intake of flavanols, procyanidins and methylxanthines. To accomplish this, plasma samples were collected from apparently healthy volunteers (n = 7) before and 3 weeks after daily intake of cocoa-derived test material delivered in cellulose capsules (n = 2) format and containing 829 mg of flavanols and procyanidins, including 110 mg of (–)-epicatechin, 88 mg of Tb and 19 mg of Caf. For this study, volunteers followed their regular diet before study commencement. Content of flavanols and procyanidins reported in test materials corresponded to cocoa flavanols and procyanidins with a degree of polymerization up to 7, according to Bussy et al. [38-40]. Both study protocols were approved by the Institutional Review Board at the University of California Davis in accordance with the Helsinki Declaration of 1975 as revised in 1983. Informed consent was obtained from all participants (Further details on the study are presented in Supplementary Study Design).

2.8 Sample preparation method

Plasma samples were thawed and centrifuged for 5 min at 8,000 g at 4°C. Next, 400 µL of plasma was placed in a 2 mL 96-well collection plate and 400 µL of 4% phosphoric acid was then added to each plasma sample and mixed thoroughly. Separately, in a vacuum manifold, a µHLB 96-well plate and a 2 mL 96-well collection plate were conditioned by adding 50 µL of 0.1% formic acid in methanol and passing it through with a –250 mm Hg vacuum for 5 min. This was then followed by 200 µL of 0.1% formic acid in water again with a –250 mm Hg vacuum for 5 min. Next, 700 µL of the plasma/phosphoric acid mixture and 10 µL of the ISTD solution were added to the µHLB plate and passed through the

cartridge with a −250 mm Hg vacuum for 10 min. If needed, an additional 5 min at −400 mm Hg was applied until the entire sample had passed through the cartridge. The washing step that followed consisted of adding 100 µL of 0.1% formic acid in water and applying a −400 mm Hg vacuum for 5 min. For sample preparation validation, different volumes of washing solution were also used. Next, the 2 mL collection plate was exchanged for a 350 µL collection plate to which 30 µL of 0.1% formic acid in water had already been added. Finally, the elution was performed by adding 70 µL of elution solvent and applying a −700 mm Hg vacuum for 5 min. For development of the sample preparation method, elution solvents tested were: i) methanol, ii) acetonitrile and iii) DMF:methanol, (7:3, v/v). Method validation was subsequently conducted using DMF:methanol (7:3, v/v) as the elution solvent.

2.9 UPLC-MS parameters

Aliquots, 30 µL, of each processed plasma sample was loaded into the system for 10 µL injections into an Acquity binary solvent manager and Acquity sample manager (Waters, Milford, MA) and Kinetex 1.7 µm C18 100 x 2.1 mm column (Phenomenex, Torrance, CA) with column temperature of 25°C and a sample temperature of 4°C. An 8 min gradient was implemented with solvent A consisting of 2.5 mM ammonium formate in water and solvent B consisting of acetonitrile:methanol (9:1, v/v) (Table S5). HPLC eluate was passed to a Quattro Micromass MS via an electrospray interface (Waters, Milford, MD). The system was set to detect via multiple reaction monitoring (MRM) in both negative (SREMs and gVLMs) and positive ionization modes (MXs). Specific tune parameters are detailed in Table S6 and

MRM parameters can be found in Tables S7 and S8. A depiction of a chromatogram of all analytes detected is presented in Fig. S1.

2.10 Standard curve

Stock solutions were diluted to prepare eight working standard solutions of SREMs, gVLMs, and MXs as well as one ISTD mixture. These working standard solutions were diluted to prepare a suitability test solution and a seven point standard curve by combining 10 µL of the corresponding working standard solutions, 10 µL of ISTD solution, 10 µL of 0.1% formic acid in water, and 70 µL of SREM- and gVLM-free plasma samples previously taken through the sample preparation process. The concentration of suitability test solution and standard curve points are detailed in Table S4. Standard curves were processed at the beginning and at the end of the injection sequence. Suitability test solution was used to determine if SREMs and gVLMs were detectable. In case analytes were not detectable, a MS cleanup was performed.

2.11 Data analysis

Analytes were integrated using QuaLynx (Waters). All concentrations were calculated as the ratio of the area counts of each analyte relative to their corresponding ISTD, as follows: i) 4′OH-EC-3'GlcUA, 4′OH-EC-3'S, and 3'Me-EC-5S were expressed as a ratio to [$^{2}H_{2}$/$^{2}H_{2}$](–)-EC-3'GlcUA, ii) 4′OH-VL3'S and 4′-OH-VL-3'GlcUA were expressed as a ratio to [$^{13}C_{2}$,$^{2}H_{2}$]4′OH-VL-3'S; and iii) Tb and Caf were expressed as a ratio to [$^{2}H_{6}$]Tb and [$^{13}C_{3}$]Ca, respectively.

3. Results and discussion

3.1 µSPE method development

Development of the µSPE sample preparation method included the evaluation of different elution solvents including i) methanol, ii) acetonitrile, and iii) a DMF:methanol (7:3, v/v) mixture. The results obtained showed that methanol was not strong enough to elute all SREMs and gVLMs present in the sample from the HLB resin (Table 1). Slightly improved recoveries were obtained with acetonitrile, but the overall values were still low. The use of DMF:methanol (7:3, v/v) as elution solvent yielded much better recoveries, and thus was the selected solvent for further development and validation. These results were consistent with a previously developed sample preparation method also based on HLB resin that required a combination of DMF:methanol for optimal elution [29, 30]. HLB resin presents polyvinylpyrrolidone structures that are known to establish strong H-bond interactions with polyhydroxylated compounds such as flavanols and procyanidins [41]. DMF can disrupt H-bonds interactions between analytes and polyvinylpyrrolidone structures [41], which would explain the higher recoveries of SREMs and gVLMs achieved with HLB resin and DMF compared to other solvents such as methanol [42].

Recoveries of MXs were also investigated. Analysis of the solvents resulting from the washing steps of the sample preparation method provided evidence of the presence of Tb, suggesting that the interaction of Tb and HLB resin was milder than with other analytes, including Caf. It could be argued that the lower hydrophobicity of Tb compared to Caf, and the inability of Tb to establish strong H-bond interactions with the resin bed, unlike flavanols and procyanidin metabolites, explains the lower recoveries of Tb under the

conditions tested. As a result, the washing conditions for the sample preparation method were optimized to minimize possible losses of Tb during these steps, using different volumes of washing solvents (Fig. 2). While omitting the washing step yielded the highest recoveries for Tb, the cleanup of the sample was not optimal as evidenced by HPLC-UV trace of chromatograms (Fig S2). The 100 µL washing solvent proved to be an acceptable compromise between Tb recoveries and sample cleanup. Thus, the method was successfully optimized to maintain recoveries of Tb close to 60%, supporting the notion that the performance of the method regarding Tb quantification is an acceptable tradeoff for the high recoveries and cleanup of the samples achieved for the quantification of SREMs, VLMs and Caf.

3.1 Accuracy and matrix effect

The accuracies determined showed respective average values of 101%, 93% and 103% for SREMs, gVLMs and MXs when using a standard curve prepared in post-extracted SREM- and gVLM-free plasma (Table 2). Similar accuracies values were observed when using a standard curve prepared in elution solvent (Table 3). These results suggest that, different from the previously validated methods for polyphenol metabolite quantification [24, 25, 42], the assessment of SREMs, gVLMs and MXs in plasma using the current method could be done in elution solvent, and that the set of ISTD used effectively corrected any potential deviations in the quantification. The possibility of preparing standard curves in elution solvent represent a significant benefit of the current method as this further supports a high throughput performance by allowing a higher number of samples analyzed per plate in exchange of adding blank samples for standard curve preparation.

To specifically assess the matrix effect on the quantification of analytes, the signal of the analytes in standard curves prepared in post-extracted plasma and elution solvent were compared (Fig. 3). Overall, average matrix effect on SREMs and gVLMs was below 5%, further suggesting an efficient sample cleanup through the μ-SPE method of these analytes. The quantification of MXs, however, showed a more pronounced matrix effect with ion enhancement between 10%-25%.

3.2 Precision

Precision was investigated as the intra- and inter-day repeatability in three different QC samples, showing values <15% (Table 4). Importantly, the precision of Tb showed levels similar to the other analytes, suggesting that the lower recoveries for this analyte did not affect precision.

3.3 Linearity and limits of detection and quantification

The coefficient of determinations for the analysis of all analytes in standard curves prepared in a plasma matrix showed values >0.993 (Table 5). LoQs for SREMs and gVLMs were lower than 5 nM in plasma, except for 3'Me-EC-5S, with a value of 8 nM (Table 5). LoQ for MXs were set at 0.2 μM (Table 5).

3.4 Stability

Stability was assessed considering three different conditions: i) autosampler for 2 and 4 hours at 4°C (n = 12), ii) benchtop for 6 hours at 23°C (n = 12), and iii) freezer for 14 days at -80°C (n = 12). While there was a statistically significant changes in the levels of SREMs (mainly driven by 3'Me-EC-5S) in samples stored in the autosampler for 4 h and of MXs in samples stored on the benchtop for 6 h, this reduction only represented 4% and 3 %,

respectively (Table 6). Thus, the results obtained showed that all analytes were highly stable under the conditions tested (Table 6).

One limitation of this stability assessment is that randomization of samples to the different conditions tested was not effective. While there were significant differences in average concentration of analytes among the set of samples selected for assessing stability under the different conditions (Table 6), the range of concentrations were still comparable. Furthermore, as these samples were obtained from volunteers after consuming cocoa flavanol-containing test materials, these results are representative for stability under expected concentration of these analytes in plasma. Thus, the results obtained suggest that the differences in the concentration of analytes due to ineffective randomization do not affect the notion that SREMs, VLMs and MXs are stable under the conditions tested.

3.5 Method application

The method was initially tested in plasma samples collected before and after single intake of a cocoa-derived test drink in healthy volunteers that followed a low-flavanol diet for 24 h prior initiation of the study. Consistent with this study design, SREMs and gVLMs concentrations were below LoQ in baseline plasma samples (Fig. 4A). Tb and Caf were present in baseline samples as no dietary restrictions were included for the intake of methylxanthine-containing foods and beverages. After single test drink intake, SREM, gVLM, Tb and Caf significantly increased compared to baseline levels (Fig 4A.). Concurring with previous studies [11-13], SREM and gVLM concentrations reached the highest concentrations in plasma 2 h and 6 h after single test drink intake, respectively. Tb

concentrations were higher than Caf, which was expected given the higher content of Tb than Caf in the test drink (Fig. 4B).

The method was also tested for the analysis of plasma samples collected from healthy volunteers following their regular diet and after daily intake of a cocoa-derived test material for 3 weeks. In this context, SREMs, gVLMs, Tb and Caf were detected in baseline samples, suggesting that volunteers regularly consumed flavanol-, procyanidin- and methylxanthine-containing foods and beverages as part of their diet. SREMs, gVLMs, Tb and Caf concentrations significantly increased after 3 weeks (Fig. 4C and 4D), suggesting compliance of volunteers with the daily intake of the test material during this period. Taken together, these results demonstrate the suitability of the method to assess plasma concentration of SREM, gVLM, Tb and Caf in the context of studies following different test material intake paradigms. Furthermore, while the method was tested with plasma samples obtained from healthy volunteers, it is anticipated that this methodology could be applied to assess SREMs, gVLMs and MXs in plasma from patients.

4 Conclusion

In this study, a method for the simultaneous quantification of SREMs, gVLMs and MXs in plasma was developed and validated. Along with high accuracy, the method showed very good precision, linearity, limits of detection and stability. One of the key strengths of the protocol lies in the use of appropriate authentic analytical standards as well as a series of isotopically-labelled reference compounds [27] that permitted a reliable assessment of accuracy. The method proved to be suitable to assess SREMs, gVLMs and methylxanthines in plasma. As such, it is expected that it will be useful for advancing investigations into the

absorption and metabolism of flavanols and procyanidins as well as quantifying SREMs, gVLMs and MXs as biomarkers to assess habitual intake in epidemiological studies. In the context of large clinical trials, the method represents a critical tool to determine compliance with the intake of flavanols and procyanidins in test materials, proof of absorption and strengthening causality assessments between the effects investigated and the intake of test materials. This information will be essential to continuing to build data on the nutritional relevance of flavanols and procyanidins and thus, support the development of dietary recommendations for the general public.

Authors contributions

Author contributions were as follows: Conceptualization (JIO, GGCK, AC, HS), Data curation and Formal Analysis (RF, JIO), Funding acquisition (HS, JO), Investigation (JO, GGCK, AC), Project administration (JO), Resources (JO, HS), Supervision (JO, HS), Visualization (JO), Writing – original draft (JO, RF), Writing – review & editing (AC, GGCK, HS).

Conflict of interest

J.I.O. and H.S. are employed by Mars Inc., a company engaged in flavanol research and flavanol-related commercial activities. A.C. is a consultant for Mars Inc. G.G.C.K. has received an unrestricted research grant from Mars, Inc.

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Abbreviations

Structurally-related (−)-epicatechin metabolites; (SREMs,); (−)-epicatechin-3′-glucuronide (EC-3′GlcUA); (−)-epicatechin-3′-sulfate (EC-3'S); 3′-methoxy-(−)-epicatechin-5-sulfate (3'Me-EC-5S); 5-(phenyl)-γ-valerolactone metabolites (gVLMs,); 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate (4′-OH-VL-3′S); 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide (4′-OH-VL-3′-GlcUA); 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-glucuronide (4′-OH-VL-3′-GlcUA), methylxanthines (MX); theobromine (Tb), caffeine (Caf); stable isotopically labeled standards (ISTD); limit of detection (LoD); limit of quantification (LoQ); quality control (QC), micro-solid phase extraction (μSPE)

References

1. V. Neveu, J. Perez-Jimenez, F. Vos, V. Crespy, L. du Chaffaut, L. Mennen, C. Knox, R. Eisner, J. Cruz, D. Wishart and A. Scalbert, Phenol-Explorer: an online comprehensive database on polyphenol contents in foods, *Database (Oxford)*, 2010, **2010:bap024.**, 10.1093/database/bap1024. Epub 2010 Jan 1098.
2. S. Bhagwat, D. B. Haytowitz and J. M. Holden. USDA database for the flavonoid content of selected foods. Release 3.1. 2014. Accessed: 19 April 2021. Available from: https://www.ars.usda.gov/arsuserfiles/80400525/data/flav/flav_r03-1.pdf.
3. D. Haytowitz, X. Wu and S. Bhagwat. USDA database for the proanthocyanidin content of selected foods. Release 2.1. 2018. Accessed: 18 April 2021. Available from: https://www.ars.usda.gov/ARSUserFiles/80400535/Data/PA/PA02-1.pdf.
4 G. Gratton, S. R. Weaver, C. V. Burley, K. A. Low, E. L. Maclin, P. W. Johns, Q. S. Pham, S. J. E. Lucas, M. Fabiani and C. Rendeiro, Dietary flavanols improve cerebral cortical oxygenation and cognition in healthy adults, *Sci Rep.*, 2020, **10**, 19409. doi: 19410.11038/s41598-19020-76160-19409.
5. M. M. McDermott, M. H. Criqui, K. Domanchuk, L. Ferrucci, J. M. Guralnik, M. R. Kibbe, K. Kosmac, C. M. Kramer, C. Leeuwenburgh, L. Li, D. Lloyd-Jones, C. A. Peterson, T. S. Polonsky, J. H. Stein, R. Sufit, L. Van Horn, F. Villarreal, D. Zhang, L. Zhao and L. Tian, Cocoa to Improve Walking Performance in Older People With Peripheral Artery Disease: The COCOA-PAD Pilot Randomized Clinical Trial, *Circ Res.*, 2020, **126**, 589-599. doi: 510.1161/CIRCRESAHA.1119.315600. Epub 312020 Feb 315614.
6. J. I. Ottaviani, A. Britten, D. Lucarelli, R. Luben, A. A. Mulligan, M. A. Lentjes, R. Fong, N. Gray, P. B. Grace, D. H. Mawson, A. Tym, A. Wierzbicki, N. G. Forouhi, K. T. Khaw, H. Schroeter and G. G. C. Kuhnle, Biomarker-estimated flavan-3-ol intake is associated with lower blood pressure in cross-sectional analysis in EPIC Norfolk, *Sci Rep.*, 2020, **10**, 17964. doi: 17910.11038/s41598-17020-74863-17967.
7. K. Ried, P. Fakler and N. P. Stocks, Effect of cocoa on blood pressure, *Cochrane Database Syst Rev.*, 2017, **4:CD008893.**, 10.1002/14651858.CD14008893.pub14651853.
8. R. Sansone, A. Rodriguez-Mateos, J. Heuel, D. Falk, D. Schuler, R. Wagstaff, G. G. Kuhnle, J. P. Spencer, H. Schroeter, M. W. Merx, M. Kelm and C. Heiss, Cocoa flavanol intake improves endothelial function and Framingham Risk Score in healthy men and women: a randomised, controlled, double-masked trial: the Flaviola Health Study, *Br J Nutr.*, 2015, **114**, 1246-1255. doi: 1210.1017/S0007114515002822. Epub 0007114515002015 Sep 0007114515002829.
9. R. P. Sloan, M. Wall, L. K. Yeung, T. Feng, X. Feng, F. Provenzano, H. Schroeter, V. Lauriola, A. M. Brickman and S. A. Small, Insights into the role of diet and dietary flavanols in cognitive aging: results of a randomized controlled trial, *Sci Rep.*, 2021, **11**, 3837. doi: 3810.1038/s41598-41021-83370-41592.
10. Y. Sun, D. Zimmermann, C. A. De Castro and L. Actis-Goretta, Dose-response relationship between cocoa flavanols and human endothelial function: a systematic review and meta-analysis of randomized trials, *Food Funct.*, 2019, **10**, 6322-6330. doi: 6310.1039/c6329fo01747j.
11. G. Borges, J. I. Ottaviani, J. J. J. van der Hooft, H. Schroeter and A. Crozier, Absorption, metabolism, distribution and excretion of (−)-epicatechin: A review of recent findings, *Mol Aspects Med.*, 2018, **61,** 18-30, 10.1016/j.mam.2017.1011.1002. Epub 2017 Nov 1019.
12. D. Del Rio, A. Rodriguez-Mateos, J. P. Spencer, M. Tognolini, G. Borges and A. Crozier, Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases, *Antioxid Redox Signal.*, 2013, **18**, 1818-1892. doi: 1810.1089/ars.2012.4581. Epub 2012 Aug 1827.

13. J. I. Ottaviani, G. Borges, T. Y. Momma, J. P. Spencer, C. L. Keen, A. Crozier and H. Schroeter, The metabolome of [2-^{14}C](–)-epicatechin in humans: implications for the assessment of efficacy, safety, and mechanisms of action of polyphenolic bioactives, *Sci Rep.*, 2016, **6,** 29034, 10.1038/srep29034.
14. J. I. Ottaviani, R. Fong, J. Kimball, J. L. Ensunsa, A. Britten, D. Lucarelli, R. Luben, P. B. Grace, D. H. Mawson, A. Tym, A. Wierzbicki, K. T. Khaw, H. Schroeter and G. G. C. Kuhnle, Evaluation at scale of microbiome-derived metabolites as biomarker of flavan-3-ol intake in epidemiological studies, *Sci Rep.*, 2018, **8**, 9859. doi: 9810.1038/s41598-41018-28333-w.
15. J. I. Ottaviani, R. Fong, J. Kimball, J. L. Ensunsa, N. Gray, A. Vogiatzoglou, A. Britten, D. Lucarelli, R. Luben, P. B. Grace, D. H. Mawson, A. Tym, A. Wierzbicki, A. D. Smith, N. J. Wareham, N. G. Forouhi, K. T. Khaw, H. Schroeter and G. G. C. Kuhnle, Evaluation of (-)-epicatechin metabolites as recovery biomarker of dietary flavan-3-ol intake, *Sci Rep.*, 2019, **9**, 13108. doi: 13110.11038/s41598-13019-49702-z.
16. M. E. Alanon, S. M. Castle, P. J. Siswanto, T. Cifuentes-Gomez and J. P. Spencer, Assessment of flavanol stereoisomers and caffeine and theobromine content in commercial chocolates, *Food Chem.*, 2016, **208**, 177-84, 10.1016/j.foodchem.2016.1003.1116. Epub 2016 Apr 1011.
17. M. S. El-Shahawi, A. Hamza, S. O. Bahaffi, A. A. Al-Sibaai and T. N. Abduljabbar, Analysis of some selected catechins and caffeine in green tea by high performance liquid chromatography, *Food Chem.*, 2012, **134**, 2268-2275. doi: 2210.1016/j.foodchem.2012.2203.2039. Epub 2012 Mar 2228.
18. R. Sansone, J. I. Ottaviani, A. Rodriguez-Mateos, Y. Heinen, D. Noske, J. P. Spencer, A. Crozier, M. W. Merx, M. Kelm, H. Schroeter and C. Heiss, Methylxanthines enhance the effects of cocoa flavanols on cardiovascular function: randomized, double-masked controlled studies, *Am J Clin Nutr.*, 2017, **105**, 352-360. doi: 310.3945/ajcn.3116.140046. Epub 142016 Dec 140021.
19. C. Cao, D. A. Loewenstein, X. Lin, C. Zhang, L. Wang, R. Duara, Y. Wu, A. Giannini, G. Bai, J. Cai, M. Greig, E. Schofield, R. Ashok, B. Small, H. Potter and G. W. Arendash, High Blood caffeine levels in MCI linked to lack of progression to dementia, *J Alzheimers Dis*, 2012, **30**, 559-572. doi: 510.3233/JAD-2012-111781.
20. M. C. Cornelis, T. Kacprowski, C. Menni, S. Gustafsson, E. Pivin, J. Adamski, A. Artati, C. B. Eap, G. Ehret, N. Friedrich, A. Ganna, I. Guessous, G. Homuth, L. Lind, P. K. Magnusson, M. Mangino, N. L. Pedersen, M. Pietzner, K. Suhre, H. Volzke, M. Bochud, T. D. Spector, H. J. Grabe and E. Ingelsson, Genome-wide association study of caffeine metabolites provides new insights to caffeine metabolism and dietary caffeine-consumption behavior, *Hum Mol Genet.*, 2016, **25**, 5472-5482. doi: 5410.1093/hmg/ddw5334.
21. G. F. Crotty, R. Maciuca, E. A. Macklin, J. Wang, M. Montalban, S. S. Davis, J. I. Alkabsh, R. Bakshi, X. Chen, A. Ascherio, G. Astarita, S. Huntwork-Rodriguez and M. A. Schwarzschild, Association of caffeine and related analytes with resistance to Parkinson disease among LRRK2 mutation carriers: A metabolomic study, *Neurology.*, 2020, **95**, e3428-e3437. doi: 3410.1212/WNL.0000000000010863. Epub 2020 Sep 30
22. J. I. Ottaviani, M. Balz, J. Kimball, J. L. Ensunsa, R. Fong, T. Y. Momma, C. Kwik-Uribe, H. Schroeter and C. L. Keen, Safety and efficacy of cocoa flavanol intake in healthy adults: a randomized, controlled, double-masked trial, *Am J Clin Nutr.*, 2015, **102**, 1425-1435. doi: 1410.3945/ajcn.1115.116178. Epub 112015 Nov 116174.
23. D. Achaintre, A. Gicquiau, L. Li, S. Rinaldi and A. Scalbert, Quantification of 38 dietary polyphenols in plasma by differential isotope labelling and liquid chromatography electrospray ionization tandem mass spectrometry, *J Chromatogr A.*, 2018, **1558**, 50-58, 10.1016/j.chroma.2018.1005.1017. Epub 2018 May 1019.
24. R. M. de Ferrars, C. Czank, S. Saha, P. W. Needs, Q. Zhang, K. S. Raheem, N. P. Botting, P. A. Kroon and C. D. Kay, Methods for isolating, identifying, and quantifying anthocyanin

metabolites in clinical samples, *Anal Chem.*, 2014, **86**, 10052-10058. doi: 10010.11021/ac500565a. Epub 502014 Oct 500510.
25. M. Dominguez-Fernandez, Y. Xu, P. Young Tie Yang, W. Alotaibi, R. Gibson, W. L. Hall, L. Barron, I. A. Ludwig, C. Cid and A. Rodriguez-Mateos, Quantitative assessment of dietary (poly)phenol intake: a high-throughput targeted metabolomics method for blood and urine Samples, *J Agric Food Chem.*, 2021, **69**, 537-554. doi: 510.1021/acs.jafc.1020c07055. Epub 02020 Dec 07029.
26. P. Mena, L. Bresciani, N. Brindani, I. A. Ludwig, G. Pereira-Caro, D. Angelino, R. Llorach, L. Calani, F. Brighenti, M. N. Clifford, C. I. R. Gill, A. Crozier, C. Curti and D. Del Rio, Phenyl-gamma-valerolactones and phenylvaleric acids, the main colonic metabolites of flavan-3-ols: synthesis, analysis, bioavailability, and bioactivity, *Nat Prod Rep.*, 2019, **36**, 714-752. doi: 710.1039/c1038np00062j.
27. J. I. Ottaviani, R. Y. Fong, G. Borges, H. Schroeter and A. Crozier, Use of LC-MS for the quantitative analysis of (poly)phenol metabolites does not necessarily yield accurate results: Implications for assessing existing data and conducting future research, *Free Radic Biol Med.*, 2018, **124,** 97-103, 10.1016/j.freeradbiomed.2018.1005.1092. Epub 2018 Jun 1012.
28. A. S. Ptolemy, E. Tzioumis, A. Thomke, S. Rifai and M. Kellogg, Quantification of theobromine and caffeine in saliva, plasma and urine via liquid chromatography-tandem mass spectrometry: a single analytical protocol applicable to cocoa intervention studies, *J Chromatogr B Analyt Technol Biomed Life Sci.*, 2010, **878**, 409-416. doi: 410.1016/j.jchromb.2009.1012.1019. Epub 2009 Dec 1021.
29. T. Unno, Y. M. Sagesaka and T. Kakuda, Analysis of tea catechins in human plasma by high-performance liquid chromatography with solid-phase extraction, *J Agric Food Chem*, 2005, **53**, 9885-9889.
30. J. I. Ottaviani, T. Y. Momma, G. K. Kuhnle, C. L. Keen and H. Schroeter, Structurally related (-)-epicatechin metabolites in humans: assessment using de novo chemically synthesized authentic standards, *Free Radic Biol Med.*, 2012, **52**, 1403-1412. doi: 1410.1016/j.freeradbiomed.2011.1412.1010. Epub 2011 Dec 1423.
31. E. S. Mull, M. Van Zandt, A. Golebiowski, R. P. Beckett, P. K. Sharma and H. Schroeter, A versatile approach to the regioselective synthesis of diverse (-)-epicatechin-beta-D-glucuronides, *Tetrahedron Letters*, 2012, **53**, 1501-1503.
32. P. K. Sharma, M. He, J. Jurayj, D.-M. Gou, R. Lombardy, L. J. Romanczyk, Jr. and H. Schroeter, Enantioselctive Syntheses of Sulfur Analogues of Flavan-3-Ols, *Molecules*, 2010, **15**, 5595-5619.
33. P. K. Sharma, M. He, L. J. Romanczyk, Jr. and H. Schroeter, Synthesis of [2-C-13, 4-C-13]-(2R,3S)-catechin and [2-C-13, 4-C-13]-(2R,3R)-epicatechin, *J Labelled Comp Radiopharm* 2010, **53**, 605-612.
34. M. Zhang, G. E. Jagdmann, Jr., M. Van Zandt, P. Beckett and H. Schroeter, Enantioselective synthesis of orthogonally protected (2R,3R)-(-)-epicatechin derivatives, key intermediates in the de novo chemical synthesis of (-)-epicatechin glucuronides and sulfates, *Tetrahedron-Asymmetry*, 2013, **24**, 362-373.
35. M. Zhang, G. E. Jagdmann, Jr., M. Van Zandt, R. Sheeler, P. Beckett and H. Schroeter, Chemical Synthesis and Characterization of Epicatechin Glucuronides and Sulfates: Bioanalytical Standards for Epicatechin Metabolite Identification, *J Nat Prod,* 2013, **76**, 157-169.
36. C. D. Kay, M. N. Clifford, P. Mena, G. J. McDougall, C. Andres-Lacueva, A. Cassidy, D. Del Rio, N. Kuhnert, C. Manach, G. Pereira-Caro, A. Rodriguez-Mateos, A. Scalbert, F. Tomas-Barberan, G. Williamson, D. S. Wishart and A. Crozier, Recommendations for standardizing nomenclature for dietary (poly)phenol catabolites, *Am J Clin Nutr.*, 2020, **112**, 1051-1068. doi: 1010.1093/ajcn/nqaa1204.

37. D. Del Rio, A. Rodriguez-Mateos, J. P. Spencer, M. Tognolini, G. Borges and A. Crozier, Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases, *Antioxid Redox Signal*, 2013, **18**, 1818-1892.
38. U. Bussy, G. Hewitt, Y. Olanrewaju, B. R. May, N. Anderson, J. I. Ottaviani and C. Kwik-Uribe, Single-laboratory validation for the determination of cocoa flavanols and procyanidins (by degree of polymerization DP1-7) in cocoa-based products by hydrophilic interaction chromatography coupled with fluorescence detection: First Action 2020.05, *J AOAC Int*, 2020, DOI: 10.1093/jaoacint/qsaa132.
39. U. Bussy, B. R. May, Y. Olanrewaju, G. Hewitt, N. Anderson, A. Crozier, J. I. Ottaviani and C. Kwik-Uribe, Reliable, accessible and transferable method for the quantification of flavanols and procyanidins in foodstuffs and dietary supplements, *Food Funct*, 2020, **11**, 131-138.
40. U. Bussy, J. I. Ottaviani and C. Kwik-Uribe, Evolution of cocoa flavanol analytics: impact on reporting and cross-study comparison, *Food Funct*, 2021, **12**, 3433-3442.
41. E. Haslam, *Practical polyphenolics : from structure to molecular recognition and physiological action*, Cambridge University Press, Cambridge, UK ; New York, NY, 1998.
42. R. P. Feliciano, E. Mecha, M. R. Bronze and A. Rodriguez-Mateos, Development and validation of a high-throughput micro solid-phase extraction method coupled with ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry for rapid identification and quantification of phenolic metabolites in human plasma and urine, *J Chromatogr A.*, 2016, **1464**, 21-31, 10.1016/j.chroma.2016.1008.1027. Epub 2016 Aug 1011.

Tables

Table 1: Recovery of structurally- related (–)-epicatechin metabolites (SREMs) and 5-(phenyl)-γ-valerolactone metabolites (gVLMs) in plasma using different elution solvents. Data are expressed as mean values ± SD in percentages (n=4).

SREMs/gVLMs[a]	Recovery (%)		
	Methanol	Acetonitrile	DMF:MeOH (7:3. v/v)
EC-3'S	0 ± 0	21 ± 18	84 ± 8
EC-3'GlcUA	35 ± 9	34 ± 22	92 ± 9
3'Me-EC-5S	5 ± 2	41 ± 23	91 ± 9
4′OH-VL-3'S	13 ± 5	43 ± 32	99 ± 7
4′OH-VL-3'GlcUA	87 ± 7	32 ± 30	101 ± 10

[a](–)-epicatechin-3′-sulfate (EC-3′S); (–)-epicatechin-3′-glucuronide (EC-3′-GlcUA); 3'-methoxy-(−)-epicatechin-5-sulfate (3'-Me-EC-5S); (4′-hydroxyphenyl)-γ-valerolactone-3'-sulfate (4′OH-VL-3'S); 5-(4′-hydroxyphenyl)-γ-valerolactone-3'-glucuronide (4′-OH-VL-3'-GlcUA)

Table 2: Accuracy for the quantification of structurally- related (–)-epicatechin metabolites (SREMs), 5-(phenyl)-γ-valerolactone metabolites (gVLM)s and methylxanthines (MXs) in plasma using a standard curve prepared in SREMs- and gVLMs-free plasma. Data are expressed as mean values ± SD in percentages (n=4).

Spike level[a]	Accuracy (%)				
	PS5 (High)	PS4 (Mid)	PS3 (Low)	PS2 (>LLOQ)	PS1 (<LLOQ)
SREM/gVLM concentration	286 nM	143 nM	28.6 nM	14.3 nM	2.86 nM
EC-3'S	107 ± 4	101 ± 4	105 ± 3	105 ± 8	97 ± 6
EC-3'GlcUA	91 ± 7	92 ± 6	89 ± 4	105 ± 5	97 ± 5
3'Me-EC-5S	103 ± 6	103 ± 4	109 ± 6	103 ± 2	104 ± 4
4′OH-VL-3'S	89 ± 10	93 ± 10	91 ± 6	90 ± 4	96 ± 5
4′OH-VL-3'GlcUA	91 ± 4	91 ± 5	92 ± 6	94 ± 4	100 ± 4
MX concentration	28.6 µM	14.3 µM	2.86 µM	1.43 µM	0.283 µM
Tb	70 ± 16	89 ± 12	101 ± 5	100 ± 2	110 ± 8
Caf	103 ± 5	103 ± 5	96 ± 3	106 ± 2	n.d.

[a](–)-epicatechin-3′-sulfate, (EC-3′S); (–)-epicatechin-3′-glucuronide (EC-3′-GlcUA); 3'-methoxy-(−)-epicatechin-5-sulfate (3'-Me-EC-5S); 5-(4′-hydroxyphenyl)-γ-valerolactone-3'-sulfate (4′OH-VL-3'S) 5-(4′-hydroxyphenyl)-γ-valerolactone-3'-glucuronide (4′OH-VL-3'-GlcUA); theobromine, (Tb); caffeine (Caf)

n.d.: not determined as baseline levels of MX in SREM- and gVLM-free plasma prevented assessment.

Table 3: Accuracy for the quantification of structurally- related (–)-epicatechin metabolites (SREMs), 5-(phenyl)-γ-valerolactone metabolites (gVLMs)sand methylxanthines (MXs) in plasma using a standard curve prepared in elution solvent. Data are expressed as mean values ± SD in percentages (n=4).

Spike Level	Accuracy (%)				
	PS5 (High)*	PS4 (Mid)*	PS3 (Low)*	PS2 (>LLoQ)*	PS1 (<LLoQ)*
SREMs/gVLMs concentration	286 nM	143 nM	28.6 nM	14.3 nM	2.86 nM
EC-3'S	102 ± 4	110 ± 5	99 ± 3	110 ± 9	86 ± 6
EC-3'GlcUA	89 ± 7	86 ± 6	98 ± 4	109 ± 5	100 ± 5
3'Me-EC-5S	102 ± 6	105 ± 4	110 ± 6	98 ± 2	93 ± 4
4′OH-VL-3'S	88 ± 10	87 ± 9	82 ± 6	87 ± 4	98 ± 5
4′OH-VL-3'GlcUA	89 ± 4	93 ± 5	80 ± 5	94 ± 4	82 ± 4
MXs concentration	28.6 µM	14.3 µM	2.86 µM	1.43 µM	0.283 µM
Tb	85 ± 20	95 ± 13	93 ± 5	105 ± 2	111 ± 8
Caf	116 ± 6	118 ± 6	104 ± 4	125 ± 3	n.d.

[a](–)-epicatechin-3′-sulfate (EC-3′S) (–)-epicatechin-3′-glucuronide (EC-3′-GlcUA) 3'-methoxy-(–)-epicatechin-5-sulfate (3'-Me-EC-5S); 5-(4′-hydroxyphenyl)-γ-valerolactone-3'-sulfate (4′OH-VL-3'S) 5-(4′-hydroxyphenyl)-γ-valerolactone-3'-glucuronide (4′-OH-VL-3'-GlcUA); theobromine, (Tb); caffeine (Caf).

n.d.: not determined as baseline levels of MX in SREM- and gVLM-free plasma prevented assessment.

Table 4: Intra-day and inter-day precision of analyte quantification. Data are expressed as percentage relative standard deviation (RSD) (n = 3).

Analytes[a]	QC-α		QC-β		QC-γ	
	Intra-day RSD (%)	Inter-day RSD (%)	Intra-day RSD (%)	Inter-day RSD (%)	Intra-day RSD (%)	Inter-day RSD (%)
EC-3'S	11	14	9	9	3	6
EC-3'GlcUA	6	6	5	6	8	8
3'Me-E-5S	4	11	4	7	4	9
SREMs	7	10	6	7	5	8
4′OH-VL-3'S	6	7	3	12	4	12
4′OH-VL-3'GlcUA	4	6	2	9	7	12
gVLMs	5	6	3	11	6	12
Tb	1	4	3	6	3	13
Caf	4	6	4	6	5	9
MXs	3	5	4	6	4	9

[a](–)-epicatechin-3′-sulfate (EC-3′S); (–)-epicatechin-3′-glucuronide (EC-3′-GlcUA), 3'-methoxy-(–)-epicatechin-5-sulfate (3'-Me-EC-5S); structurally-related (–)-epicatechin metabolites (SREMs), 5-(4′-hydroxyphenyl)-γ-valerolactone-3'-sulfate (VL-3'S); 5-(4′-hydroxyphenyl)-γ-valerolactone-3'-glucuronide (VL-3'-GlcUA); 5-(phenyl)-γ-valerolactone metabolites (gVLMs); theobromine, (Tb); caffeine (Caf); methylxanthines (MX).

Table 5: Linearity, limit of quantification (LoQ) and limit of detection (LoD) for targeted structurally-related (−)-epicatechin metabolites (SREMs), 5-(phenyl)-γ-valerolactone metabolites (gVLMs) sand methylxanthines (MXs). Data are expressed as plasma concentrations, after correcting for the concentration factor resulting from the sample preparation method.

SREMs/gVLMs[a]	Range (nM)	Linearity (R^2)	LoQ (nM)	LoD (nM)
EC-3'S	7.14–714	0.997	4	1
EC-3'GlcUA	7.14–714	0.993	3	1
3'Me-EC-5S	14.3–714	0.994	8	3
4′OH-VL-3'S	7.14–714	0.994	1	1
4′OH-VL-3'GlcUA	7.14–714	0.993	2	1
MXs[b]	Range (μM)	Linearity (R^2)	LoQ (μM)	LoD (μM)
Tb	0.57-57	0.995	0.2	0.1
Caf	0.57-57	0.993	0.2	0.1

[a](−)-epicatechin-3′-sulfate (EC-3′S); (−)-epicatechin-3′-glucuronide (EC-3′-GlcUA), 3'-methoxy-(−)-epicatechin-5-sulfate (3'-Me-EC-5S). 5-(4'-hydroxyphenyl)- γ -valerolactone-3′-sulfate, 4'OH-VL-3'S; 5-(4′-hydroxyphenyl)- γ -valerolactone-3-glucuronide (4′OH-VL-3'-GlcUA).

[b]theobromine, Tb; caffeine, Caf.

Table 6: Concentration of structurally- related (–)-epicatechin metabolites (SREMs), 5-(phenyl)- γ -valerolactone metabolites (gVLM)s and methylxanthines (MXs) in processed plasma samples before and after storage in different conditions.

	Autosampler (4°C)			Bench (room temp. 23°C)		Freezer (-80°C)	
	0 h	2 h	4 h	0 h	6 h	0 h	14 days
SREMs							
Average concentration (nM; max-min)	169 (338-33)	168 (326-32)	164 (324-32)	58 (108-9)	59 (110-9)	92 (198-10)	91 (201-9)
Relative concentration (%, ± SD)	–	99 ± 5	96 ± 3	–	101 ± 5	–	99 ± 8
p (Paired t-test)[a]	–	0.287	0.003	–	0.375	–	0.798
gVLMs							
Average concentration (nM; max-min)	580 (1002-252)	569 (906-253)	557 (986-229)	517 (2072-18)	516 (2226-19)	302 (1263-21)	301 (1300-19)
Relative concentration (%, ± SD)	–	99 ± 5	99 ± 5	–	101 ± 6	–	92 ± 7
p (Paired t-test) [a]	–	0.265	0.636	–	0.943	–	0.434
MXs							
Average concentration (μM; max-min)	16.2 (34.2-1.0)	16.2 (33.4-1.1)	16.4 (34.3-1.0)	29.3 (73.7-2.4)	30.2 (76.4-2.6)	23.2 (43.6-1.4)	22.6 (42.0-1.4)
Relative concentration (%, ± SD)	–	101 ± 4	101 ± 4	–	103 ± 3	–	99 ± 9
p (Paired t-test) [a]	–	0.958	0.286	–	0.019	–	0.471

[a] Paired t-test was performed comparing concentration of analytes before and after incubation under the different conditions tested.

Figures

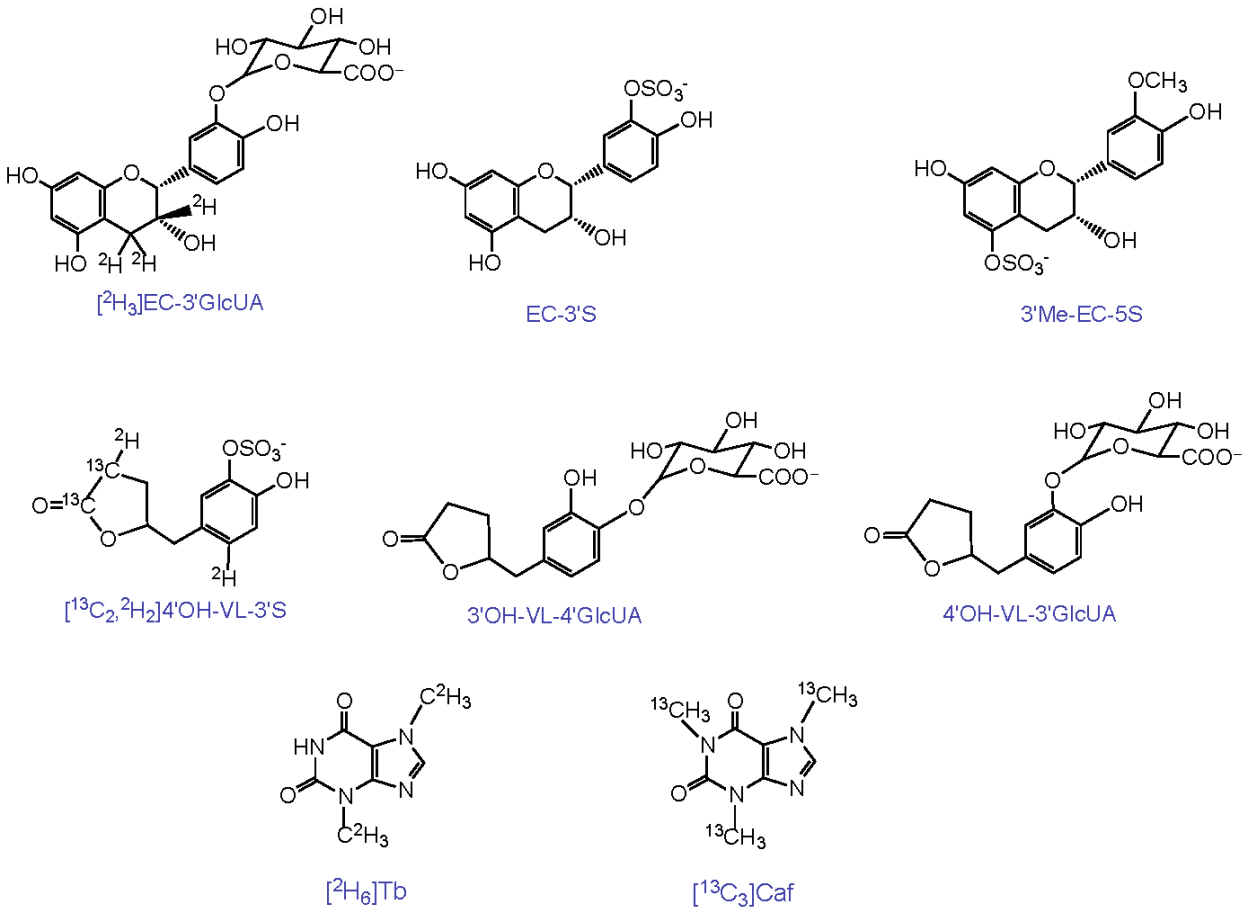

Fig. 1. Chemical structure of selected analytes and labelled internal standard., [$^{2}H_3$](−)-epicatechin-3'-glucuronide ([$^{2}H_3$]EC-3'GlcUA); (−)-epicatechin-3'-sulfate (EC-3'S); 3'-methoxy-(−)-epicatechin-5-sulfate (3'Me-EC-5S), [$^{13}C_2$,$^{2}H_2$](5-(4'-hydroxyphenyl)-γ-valerolactone-3'-sulfate ([$^{13}C_2$,$^{2}H_2$]4'-OH-VL-3'S); 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-glucuronide (4'-OH-VL-3'-GlcUA); 5-(3'-hydroxyphenyl)-γ-valerolactone-4'-glucuronide (3'-OH-VL-4'-GlcUA); [$^{2}H_6$]theobromine ([$^{2}H_6$]Tb) and [$^{13}C_3$]caffeine ([$^{13}C_3$]Caf).

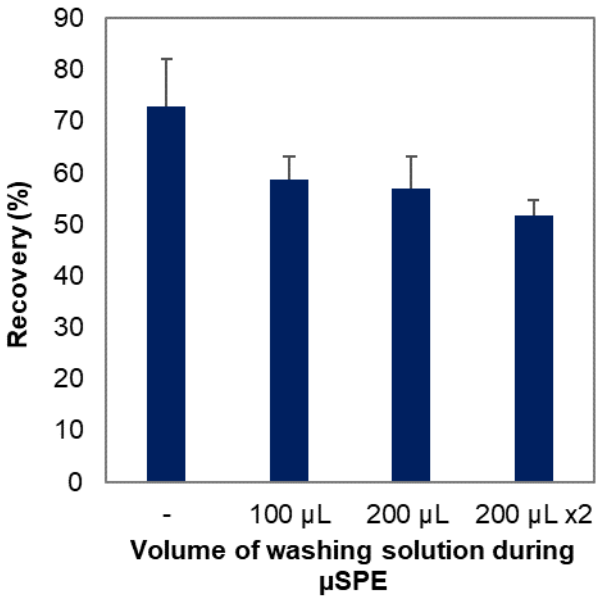

Fig. 2. Recovery of theobromine (Tb) from plasma using different volumes in the washing step. Data are expressed as mean values ± SD in % (n = 6).

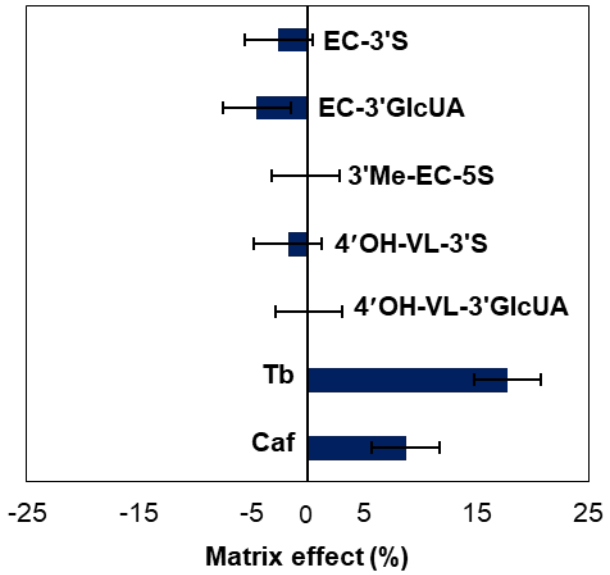

Fig. 3. Calculated matrix effect of processed plasma across the five calibration concentrations. Data are expressed as mean ± SD (n = 2). (–)-epicatechin-3′-sulfate (EC-3′S); (–)-epicatechin-3′-glucuronide (EC-3′-GlcUA); 3'-methoxy-(−)-epicatechin-5-sulfate (3'Me-EC-5S); 5-(4′-hydroxyphenyl)-γ-valerolactone-3'-sulfate (4′OH-VL-3'S); 5-(4′-hydroxyphenyl)-γ-valerolactone-3'-glucuronide (4′OH-VL-3'-GlcUA); theobromine (Tb); caffeine, (Caf).

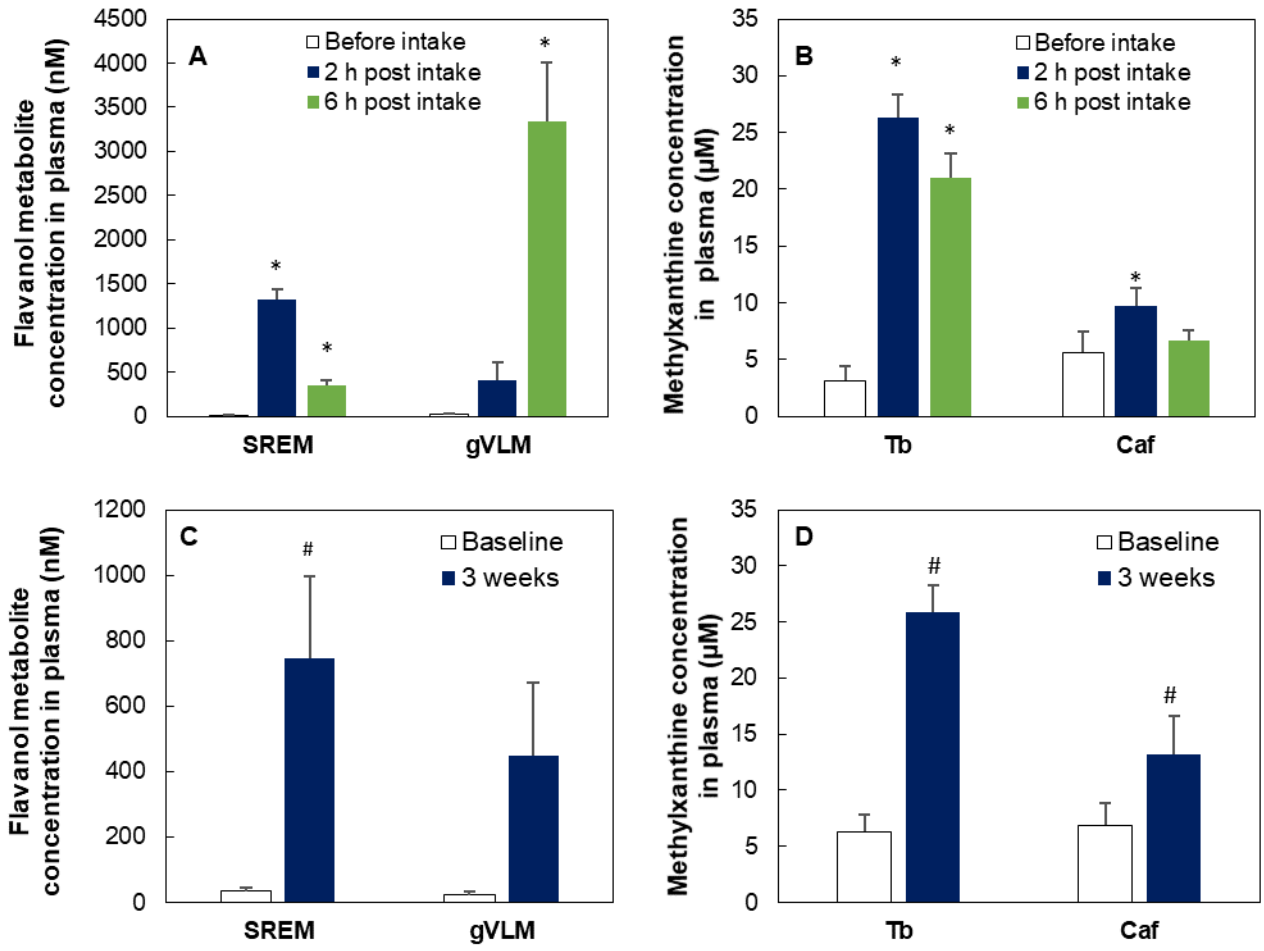

Fig. 4. Plasma concentration of structurally related (–)-epicatechin metabolites (SREMs), 5-(phenyl)-γ-valerolactone metabolites (gVLM,) theobromine (Tb) and caffeine, (Caf) after single acute intake (A, B) and daily intake (C, D) of cocoa flavanol-and methylxanthine-containing test materials in healthy volunteers. Data are expressed as mean ± SEM (n = 7). *p<0.05 vs. baseline; ANOVA repeated measures, Bonferroni post hoc; #p<0.05 vs. baseline, Paired t-test.

Electronic supplementary information

Validation of a high-throughput method for the quantification of flavanol and procyanidins biomarkers and methylxanthines in plasma by UPLC-MS

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Supplementary Study design

Healthy male and female adults were recruited by public advertisement in the city of Davis and surrounding areas (California, USA). Exclusion criteria included a body mass index (BMI) higher than 30 kg/m2, blood pressure (BP) higher than 140/90 mmHg, allergies to peanut or cocoa, avoidance of caffeinated food products and beverages, a history of cardiovascular disease (CVD), stroke, renal, hepatic, or thyroid disease, gastrointestinal (GI) tract disorders, previous GI surgery (except appendectomy), the current intake of herbal-, plant- or botanicals-containing dietary supplements, persons following a vegan/vegetarian diet, and those adhering to an uncommon diet or a weight loss program. To determine eligibility, participants were asked to complete health and lifestyle questionnaires, have their height, weight, and in-office BP determined, and to provide a blood sample for complete blood count (CBC), liver panel, lipid panel and metabolic panel assessments. Enrolled participants commenced the study protocol between 1-3 weeks after eligibility was determined. Participants characteristics are shown in Table S0.

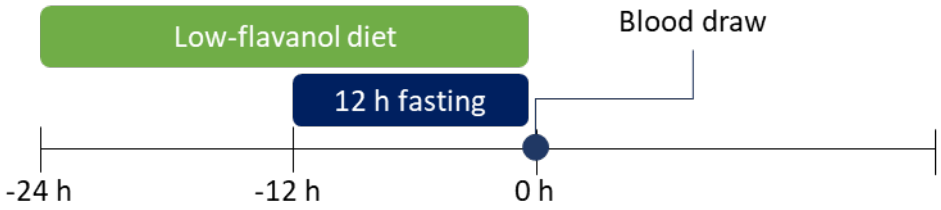
Volunteers were recruited for three different studies that aimed at collecting plasma samples for method validation and application. These studies included: i) sample collection for method validation; ii) Sample collection for method application after single acute intake of flavanol- and methylxanthine-containing material; iii) Sample collection for method application after daily intake of flavanol- and methylxanthine-containing material. A summary of the procedures involved in each study is provided in Scheme I.

Table S0: Characteristics of volunteers participating in the different studies

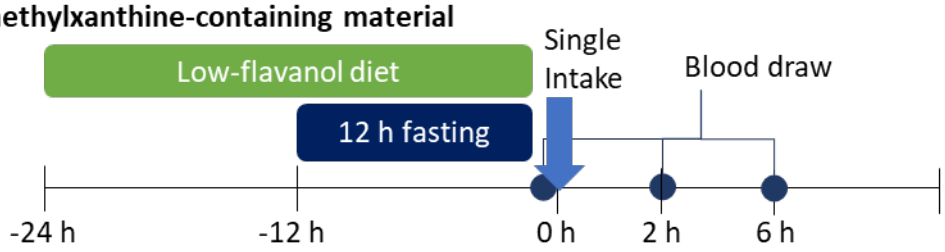
Parameters	Studies		
		Method application	
	i. Method validation	ii. Single acute intake of flavanol- and methylxanthine-containing material	iii. Daily intake of flavanol- and methylxanthine-containing material.
n (f/m)	7 (4/3)	7 (4/3)	7 (3/4)
Age (y)	37±7	33±6	46±9
Weight (Kg)	69±9	74±12	79±13
Body-mass index (Kg/m^2)	23±1	26±3	26±3
Systolic blood pressure (mmHg)	120±11	124±10	122±9
Diastolic blood pressure (mmHg)	76±7	80±7	78±10
Heart rate (bpm)	62±14	61±10	64±5
Total cholesterol (mg/dL)	151±29	188±36	202±40
HDL (mg/dL)	56±13	54±54	52±9

In the case of studies for method validation and for method application after single acute intake of flavanol- and methylxanthine-containing material, volunteers were asked to follow a low flavanol diet 24 h prior sample collection. To accomplish this, volunteers were instructed on how to follow a low-flavanol and procyanidin diet, receiving a list of suggested foods containing low or negligible amounts of flavanols and procyanidins. Volunteers were allowed to consume one 8 oz-cup of coffee the morning prior to the study day but refrained from consuming coffee or other methylxanthine-containing beverages during the rest of the day prior and during study visits. Given that recruited volunteers per inclusion/exclusion criteria were regular consumers of caffeine/theobromine-containing foods and beverages, a longer dietary restriction was deemed unnecessary given the inconvenience this would represent to volunteers. Finally, volunteers were asked to refrain from consuming alcohol the day prior to and during the study visit to. Volunteers were asked to fast for 12 h before each study day (water ad libitum).

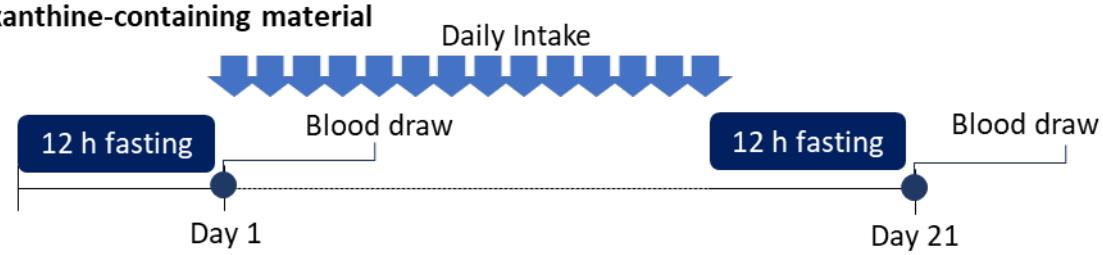
i. Sample collection for method validation



ii. Sample collection for method application after single acute intake of flavanol- and methylxanthine-containing material



iii. Sample collection for method application after daily intake of flavanol- and methylxanthine-containing material



Scheme I: description of study designs for the sample collection for method validation and application.

Table S1: Composition and concentration of working solutions.

	Concentration of working solutions (μM)				
Analytes in each working solution	WS1	WS2	WS3	WS4	WS5
SREMs (EC-3'S, EC-3'GlcUA, 3'Me-EC-5S)[a]	0.1	0.5	1	5	10
gVLMs (4'OH-VL-3'S, 4′OH-VL-3'GlcUA)[b]	0.1	0.5	1	5	10
MXs (Tb, Cf)[c]	10	50	100	500	1000

[a](−)-epicatechin-3′-sulfate, EC-3′S; (−)-epicatechin-3′-glucuronide, EC-3′-GlcUA, 3'-methoxy-(−)-epicatechin-5-sulfate (3'-Me-EC-5S)
[b]5-(4′-hydroxyphenyl)-γ-valerolactone-3'-sulfate, 4′OH-VL-3'S; 5-(4′-hydroxyphenyl)-γ-valerolactone-3'-glucuronide (4′OH-VL-3'-GlcUA). [c]theobromine, Tb; caffeine, Caf.

Table S2: Concentrations of individual structurally- related (−)-epicatechin metabolites (SREMs), 5-(phenyl)- γ -valerolactone metabolites (gVLM) and methylxanthines (MXs) in plasma samples after mixing SREM- and gVLM-free pooled plasma samples with working solutions WS1-5[a].

Analytes	Concentration of analytes in plasma samples (n=4)					
	P0	PS1 (<LLoQ)*	PS2 (>LLoQ)*	PS3 (Low)*	PS4 (Mid)*	PS5 (High)*
Individual SREMs (nM)	–	2.86	14.3	28.6	143	286
Individual gVLMs (nM)	–	2.86	14.3	28.6	143	286
Individual MXs (μM)	–	0.286	1.43	2.86	14.3	28.6

*Concentrations selected based on the levels of SREMs and gVLMs expected in plasma samples collected after overnight fasting; Lowest Level of Quantification (LLoQ).

Table S3: Concentration of structurally- related (–)-epicatechin metabolites (SREMs), 5-(phenyl)-γ-valerolactone metabolites (gVLM) and methylxanthines (MXs) in quality controls samples used to assess precision of the method.

	Concentration		
SREMs/gVLMs[a]	QC-α (nM)	QC-ß (nM)	QC-γ (nM)
EC-3'-GlcUA	17	570	451
EC-3'S	57	2870	1933
3′Me-EC-5S	371	2809	1492
4′OH-VL-3'S	2931	4499	3149
4′-OH-VL-3′GlcUA	286	313	263
MXs[b]	QC-α (μM)	QC-ß (μM)	QC-γ (μM)
Tb	8.09	9.57	4.13
Caf	18.4	18.6	16.4

[a](–)-epicatechin-3′-sulfate (EC-3′S); (–)-epicatechin-3′-glucuronide (EC-3′-GlcUA); 3'-methoxy-(–)-epicatechin-5-sulfate (3'-Me-EC-5S); (4′-hydroxyphenyl)-γ-valerolactone-3'-sulfate (VL-3'S); 5-(4′-hydroxyphenyl)-γ-valerolactone-3'-glucuronide (VL-3'-GlcUA)

[b]theobromine (Tb); caffeine (Caf)

Table S4: Composition and concentration of structurally- related (–)-epicatechin metabolites (SREMs), 5-(phenyl)- γ -valerolactone metabolites (gVLM), methylxanthines (MXs) and internal standards (ISTD) in standard curve and suitability test solution.

Analytes in each working solution[a]	Concentration of standard curve[b]								
	Suitability test solution	Level 1	Level 2	Level 4	Level 5	Level 6	Level 7	Level 8	ISTD solution
SREMs (EC-3'S, EC-3'G, 3'Me-EC-5S; nM)	10	25	50	100	250	500	1000	2500	600
gVLMs (4′OH-VL-3'S, 4′OH-VL-3'GlcUA; nM)	10	25	50	100	250	500	1000	2500	600
MXs (Tb, Caf; µM)	0.8	2	4	8	20	40	80	200	6

[a](–)-epicatechin-3′-sulfate (EC-3′S); (–)-epicatechin-3′-glucuronide (EC-3′-GlcUA); 3'-methoxy-(–)-epicatechin-5-sulfate (3'Me-EC-5S); 5-(4′-hydroxyphenyl)-γ-valerolactone-3'-sulfate (4′-OH-VL-3'S) 5-(4′-hydroxyphenyl)-γ-valerolactone-3'-glucuronide (4′OH-VL-3'-GlcUA); theobromine (Tb); caffeine (Caf)

[b]Working solutions used for preparing standard curve had a 10x concentration compared to concentration of the standard curve listed in the Table. Given the concentration of the sample during sample preparation, the concentrations of the analytes in the standard curve represent 3.5x the concentration detected in plasma (e.g. 25 nM = 7.14 nM)

Table S5 UPLC settings and mobile phase gradient.

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow rate (ml/min)
0.0	95	5	0.5
2.0	95	5	0.5
3.5	85	15	0.5
6.5	80	20	0.5
6.6	5	95	0.5
7.1	5	95	0.5
8.0	95	5	0.5

Table S6: Mass spectrometer tune parameters for positive and negative ionization modes.

Parameter	Positive Mode	Negative Mode
Capillary (kV)	4.0	3.1
Extractor (V)	3	3
RF Lens (V)	3.0	2.0
LM Resolution 1	13.0	11.0
HM Resolution 1	8.0	10.0
Ion Energy 1	0.5	0.5
LM Resolution 2	10.0	10.0
HM Resolution 2	8.0	8.0
Ion Energy 2	2.0	2.0
Source Temperature (°C)	150	150
Desolvation Temperature (°C)	500	500
Desolvation Flow (L/h)	1000	1000
Cone Flow (L/h)	0	0
Multiplier	900	900

Table S7: Negative ionization MRM transitions, dwell time, cone energy, collision energy, time interval for data collection, and retention time of analytes.

Compound[a]	Parent (*m/z*)	Daughter (*m/z*)	Dwell (s)	Cone (V)	Collision (V)	Time Interval (min)	Retention Time (min)
EC-3'GlcUA	465.5	289	0.25	22	20	3.8 – 4.6	4.2
EC-3'S	369.4	289	0.25	25	20	4.1 – 6.5	4.6
3'Me-EC-5S	383.5	303	0.25	25	22	4.1 – 6.5	4.9
4′OH-VL-3'S	287.1	207	0.005	20	17	3.8 – 4.6	4.2
3'-OH-VL-4'GlcUA	383.5	207.1	0.05	25	25	3.8 – 4.6	3.8
4′OH-VL-3′GlcUA	383.5	207.1	0.05	25	25	3.8 – 4.6	4.1
[$^2H_2$/$^2H_3$]EC-3′-GlcUA	468	291	0.25	22	18	3.8 – 4.6	4.2
[$^{13}C_2$,$^2H_2$]4′-OH-VL-3′S	291.5	211	0.01	25	20	3.8 – 4.6	4.2

[a](−)-epicatechin-3′-sulfate, EC-3′S; (-)-epicatechin-3′-glucuronide, EC-3′-GlcUA, 3'-methoxy-(−)-epicatechin-5-sulfate (3'-Me-EC-5S).
[b]5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate, 4′OH-VL-3'S; 5-(4′-hydroxyphenyl)-γ valerolactone-3′-glucuronide (4′OH-VL-3'-GlcUA); 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-glucuronide (3′OH-VL-4'-GlcUA).

Table S8: Positive ionization MRM transitions, dwell time, cone energy, collision energy, time interval for data collection, and retention time of analyte(s).

Compound[a]	Parent (*m/z*)	Daughter (*m/z*)	Dwell (s)	Cone (V)	Collision (V)	Time Interval (min)	Retention Time (min)
Tb	181.2	134.9	0.4	25	15	1 – 2.25	1.6
Ca	195.2	138	0.005	18	15	3.5 – 4.2	3.8
[$^2H_6$]Tb	187.2	141.1	0.2	25	18	1 – 2.25	1.6
[$^{13}C_3$]Caf	198.5	139.9	0.005	20	15	3.5 – 4.2	3.8

[a]theobromine, Tb; caffeine, Caf.

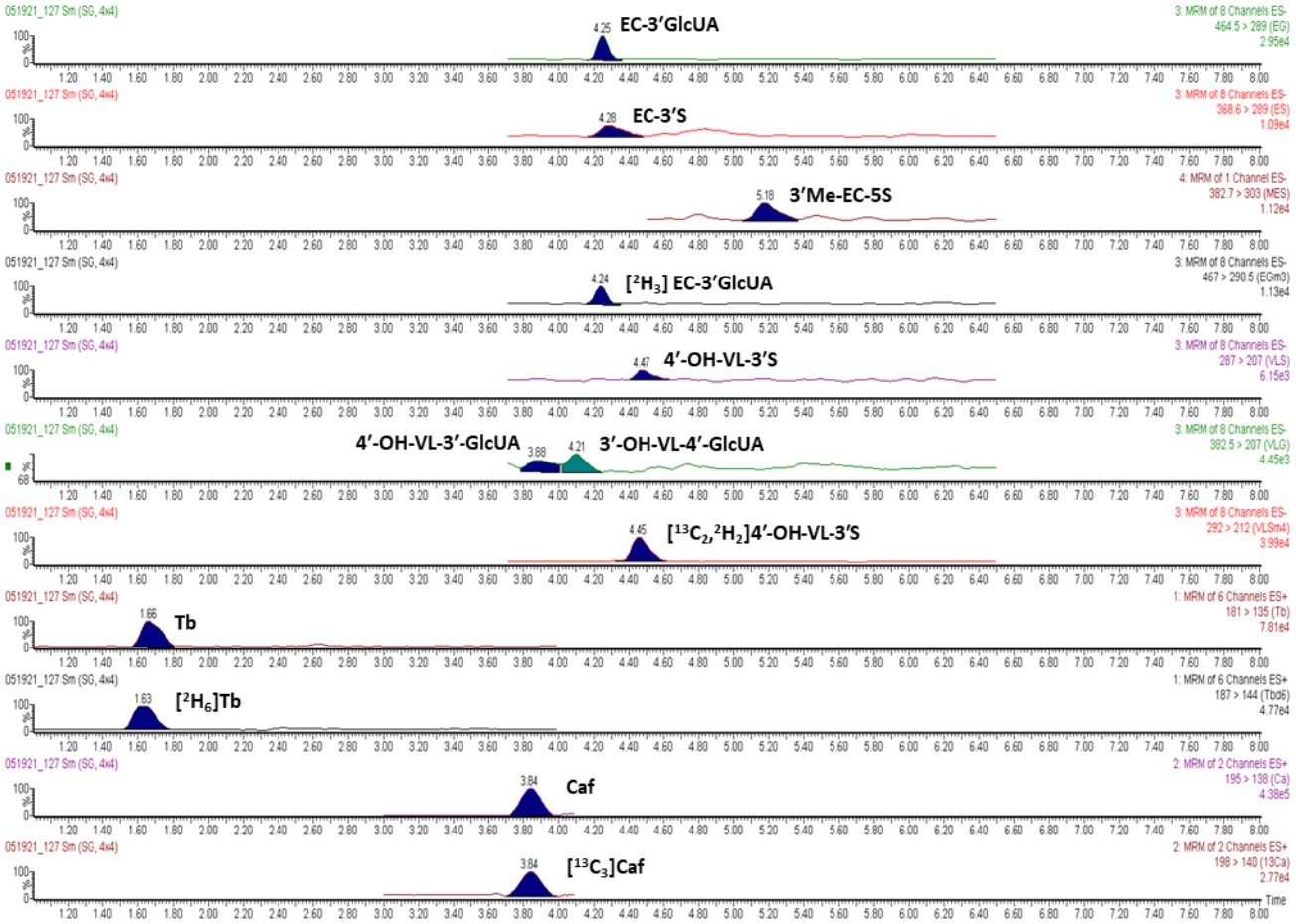


Figure S1: Chromatogram traces of a mixture of analytical standards, including (−)-epicatechin-3'-glucuronide (EC-3'GlcUA); (−)-epicatechin-3'-sulfate (EC-3'S); 3'-methoxy-(−)-epicatechin-5-sulfate (3'Me-EC-5S), 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-sulfate (4'-OH-VL-3'S); 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-glucuronide (4'-OH-VL-3'-GlcUA), 5-(3'-hydroxyphenyl)-γ-valerolactone-4'-glucuronide (3'-OH-VL-4'-GlcUA), theobromine (Tb) and caffeine (Caf). The mixture of analytical standards contained SREMs and and gVLMs at 1 µM an MXs at 80 µM, volume of injection was 5 µL.

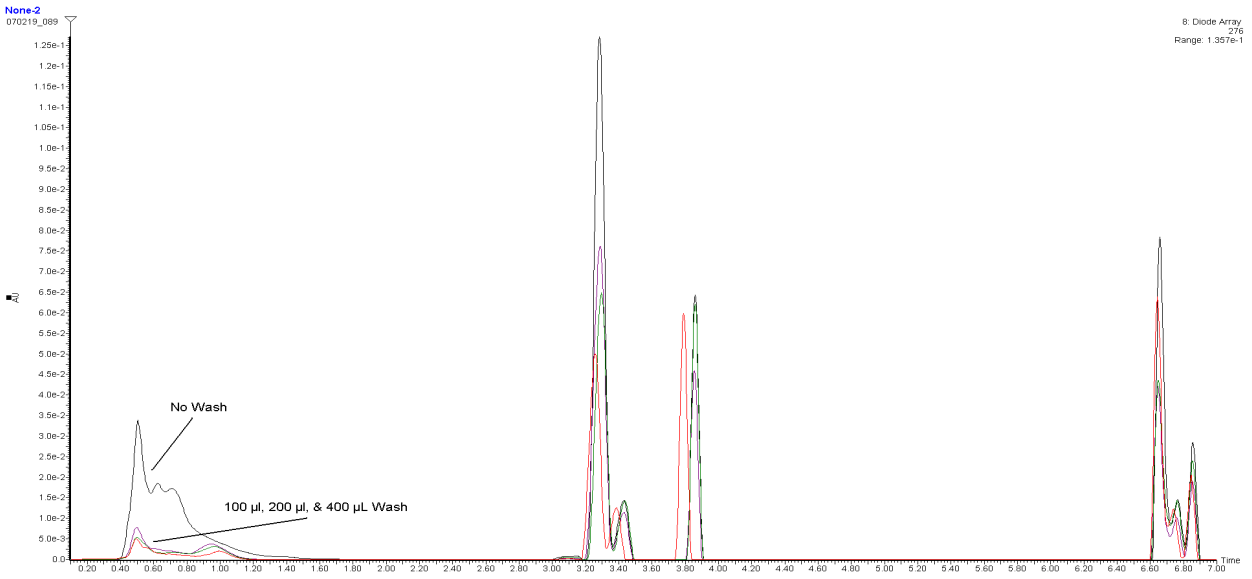


Figure S2: UV-chromatogram traces of plasma samples prepared with different volumes of washing solution. The differences among these chromatogram traces depict the amount of material loaded to the column and removed with the different washing conditions tested.