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

Article

Supplemental Material

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.

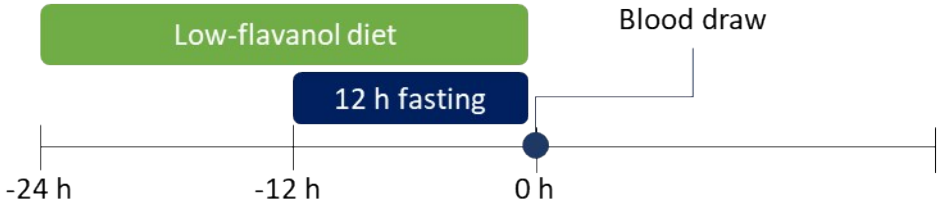
Volunteers were recruited for three different studies that aimed at collecting plasma samples for method validation and application. These studies included: i) sample collection for method validation; ii) Sample collection for method application after single acute intake of flavanol- and methylxanthine-containing 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

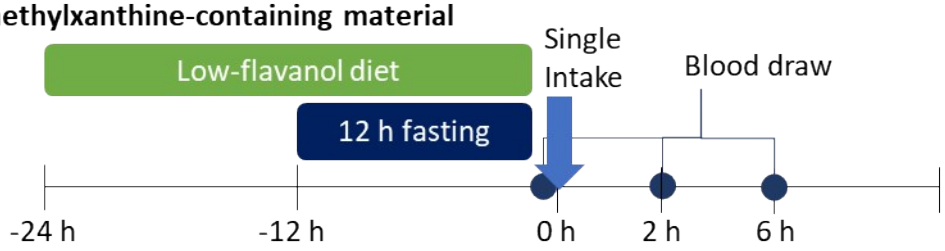
Parameters	Studies		
		Method application	
	i. Method validation	ii. Single acute intake of flavanol- and methylxanthine-containing material	iii. Daily intake of flavanol- and methylxanthine-containing material.
n (f/m)	7 (4/3)	7 (4/3)	7 (3/4)
Age (y)	37±7	33±6	46±9
Weight (Kg)	69±9	74±12	79±13
Body-mass 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.

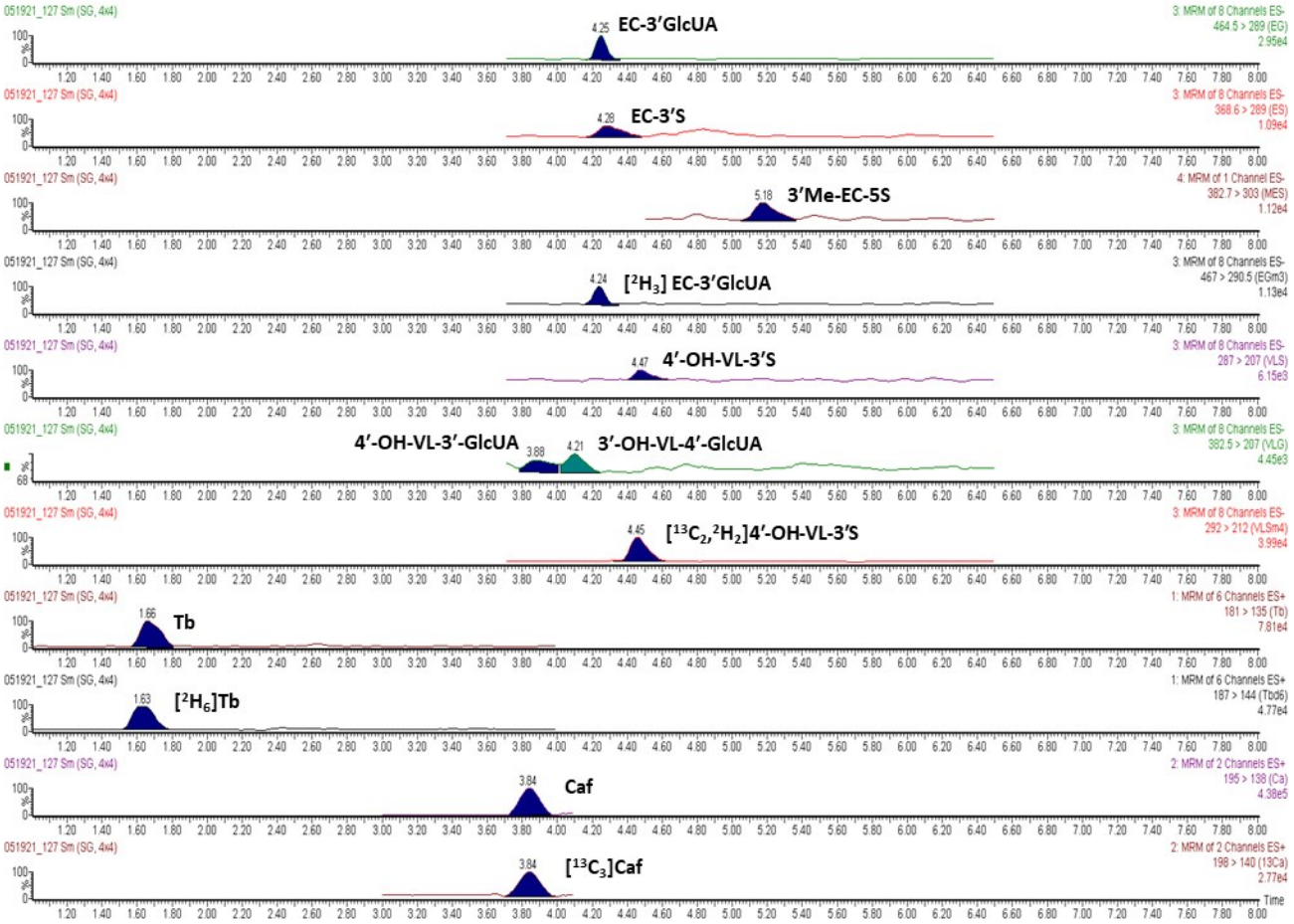


Figure S1: Chromatogram traces of a mixture of analytical standards, including (−)-epicatechin-3'-glucuronide (EC-3'GlcUA); (−)-epicatechin-3'-sulfate (EC-3'S); 3'-methoxy-(−)-epicatechin-5-sulfate (3'Me-EC-5S), 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-sulfate (4'-OH-VL-3'S); 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-glucuronide (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.

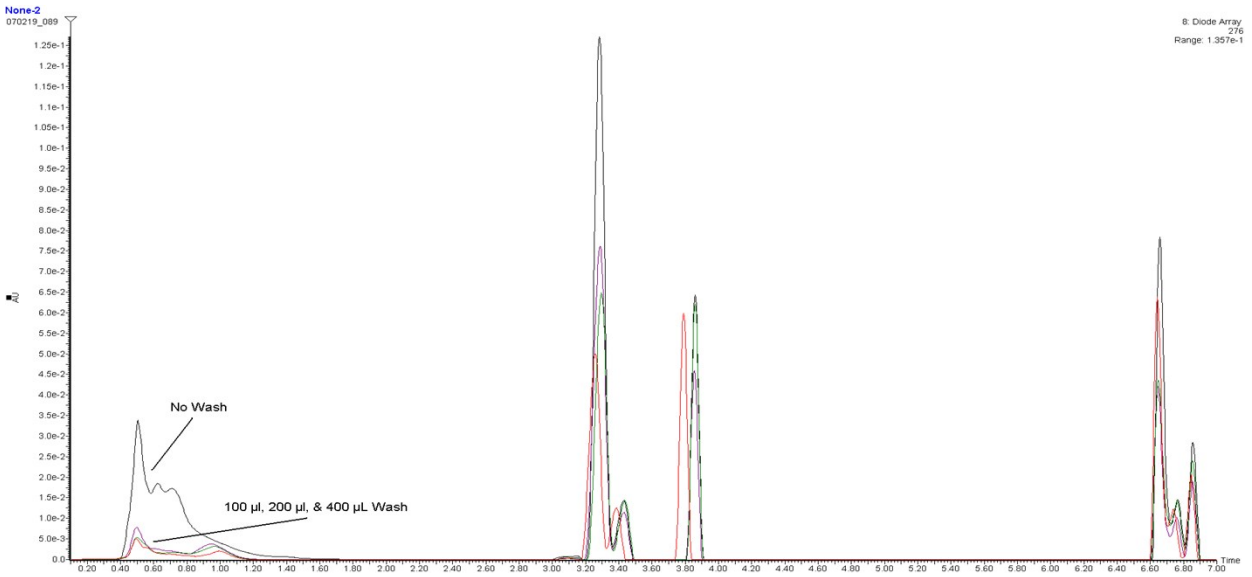


Figure S2: UV-chromatogram traces of plasma samples prepared with different volumes of washing solution. The differences among these chromatogram traces depict the amount of material loaded to the column and removed with the different washing conditions tested.