Research Article

Metabolic signature of a functional high-catechin tea after acute and sustained consumption in healthy volunteers through 1H NMR-based metabolomic analysis of urine

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ABSTRACT

Functional tea beverages have emerged as a novel approach to achieve health benefits associated with tea. The use of metabolomics may improve the evaluation of their consumption and their effects beyond self-reported questionnaires. The current study aimed to explore the urinary signature of the exposure to a functional high-catechin tea (HCT) using an untargeted NMR-based metabolomics approach. Ten male volunteers participated in a dietary crossover randomized intervention study. Individuals consumed a HCT or a control beverage during 28 days. Multilevel partial least squares discriminant analysis (ML-PLS-DA) was used for paired comparisons across the crossover design. A further univariant crossover-model was performed to assess the significant changes. The acute intake of HCT resulted in the excretion of theanine, gallate, epicatechin and epigallocatechin, as well as higher levels of 3-methyl-2-oxovalerate. The sustained consumption of the HCT exhibited the excretion of pyrogallol, higher succinate levels and a lower excretion of 2-hydroxyisobutyrate. After the repeated exposure to HCT, the acute intake of HCT exhibited a new performance of food metabolome compounds mentioned above. In conclusion, the present work settled known regular tea biomarkers, and novel urinary signatures. Such signatures may be potential biomarkers and/or reflect certain benefits of functional tea beverages.

KEYWORDS

tea; catechins; theanine; metabolomics; NMR; biomarkers

INTRODUCTION

Tea (*Camellia sinensis*) is a common beverage with high consumption per capita worldwide.[1] The chemical composition of tea is affected by the fermentation process. The different types of tea are classified into green and white tea (unfermented), oolong tea (semi-fermented), black tea (fully fermented) and pu-erh tea (post-fermented).[2,3] Green tea is rich in several catechins (flavan-3-ols), being the most abundant polyphenols in this beverage, whereas, theaflavins and thearubigins are abundant in black tea.[4] Furthermore, epigallocatechin gallate (EGCG) is the most important catechin in green tea but also other catechins and their epimeric forms are found such as epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), catechin (C), gallocatechin (GC), catechin gallate (CG), and gallocatechin gallate (GCG).[5]

Advances in ready-to-drink teas have been developed to innovate with brewing requirements of premium leaf while responding as a suitable way for fortification that can add more health benefits to these functional drinks.[6,7] In addition, there is an increasing use of polyphenols and functional beverages due to the high demands for polyphenol-rich consumables.[8] There are numerous studies concerning its benefits for health[9,10] finding that most of the observed health-effects are attributed to its polyphenols.[11] Concretely, the U.S. Food and Drug Administration approved the health claims for green tea consumption against risk of breast and prostate cancers.[12] There are also several studies relating tea consumption with benefits on other chronic diseases, such as cardiovascular, metabolic and neurodegenerative diseases, but showing lack of consistency in the levels commonly consumed by human populations.[13] The traditional methods to determine the consumption are based on the use of food frequency questionnaires.[14] Regular tea intake is usually measured in the number of cups of tea consumed per day[4] whereby this methodology may provide unpredictability to

epidemiologic studies. Several biomarkers of tea intake have been proposed using mass spectrometry.[15] In acute intervention studies with green tea, EC, EGC and conjugated metabolites are predominant in short-term urine samples.[16,17] Longer exposures to green tea showed the presence of microbial co-metabolites such as certain valerolactones in 24-h urine samples.[18,19] These metabolites were associated with tea in cross-sectional studies.[20,21] A lower number of studies described 1,3-dihydroxyphenyl-2-O-sulfate (pyrogallol sulfate), hippuric acid, 4-hydroxyhippuric acid and gallic acid[22–24] as main tea biomarkers using ^{1}H-NMR-based metabolomics. Nevertheless, these metabolites are not highly specific for tea since they were found after other food sources such as coffee, cocoa, wine, fruits or vegetables.[25–28] The application of metabolomics in the nutrition field may let to discover accurate biomarkers of intake and also may reveal potential modifications in diet-related pathways in healthy individuals likewise in early disease stages.[14,29] Metabolomics allows a global description of metabolites that gives detailed information on metabolic pathways and in turn on biological processes, thereby clarifying associations with health benefits and elucidating underlying mechanisms.[30] More concretely, while the biomarkers of intake reflect the dietary exposure, altered endogenous compounds may reveal the mechanistic role of functional foods.[31]

Therefore, the present study was conducted to determine the metabolic fingerprint of a functional green tea high in catechin polyphenols on the urinary metabolome after both acute and sustained consumptions.

MATERIALS AND METHODS

Subjects and study design

Ten healthy male volunteers between 25 and 44 years old with a body mass index (BMI) of 23.0±2.0 kg/m^2 (mean ± SD) participated in a randomized, double-blind,

placebo-controlled, crossover clinical trial (Figure 1). Exclusion criteria included caffeine intoxication, intake of catechins supplements, serious illness (such as heart disease, kidney disease or diabetes) and food allergies. The study protocol was approved by the Human Research Ethics Committee of Biological Science Laboratories of the KAO Corporation (ref: 507-20131218). This clinical trial was registered with the International Standard Randomized Controlled Trial Number (ISRCTN) 15516017.

During the washout and the study periods, the subjects were forbidden to consume coffee and tea beverages other than test drinks. Subjects were not allowed to consume alcoholic beverages and to practice exercise from 2 days before the beginning of the intervention. After a 14-day washout period, subjects were asked to consume a functional high-catechins tea beverage (hereafter HCT) containing 187 mg/100 ml of catechins (KAO Corporation, Japan) or a control beverage (caffeine-containing beverage). The daily dose of caffeine was similar between the intervention and control groups (see Table S1, Supporting Information). Then, the participants consumed the corresponding beverage every day for the next 28 days (period I). The same procedure was repeated switching the individuals between the groups (period II) after a second 14-day washout period, in accordance with the crossover design. To analyse acute consumption, urine samples collected during the first 4 postprandial hours after intake were obtained on the first day of the intervention. Fasting urine samples were collected on the first and last days of each period for the analysis of sustained consumption. Finally, in order to analyse the repeated exposures reflecting the habitual intakes to this drink, urine samples collected during the first 4 postprandial hours after intake were collected during the final day of each period. All urine samples were stored in aliquots at −80 °C prior to analysis.

115 **Sample preparation and data acquisition**

116 Sample processing and data acquisition were performed as previously described.[27] Both
117 urine and beverage samples were thawed, vortexed and centrifuged at 13,200 rpm for 5
118 min. The supernatant (600 μL) from each sample was mixed with an internal standard
119 solution [120 μL, consisting of 0.1% TSP (3-(trimethylsilyl)-proprionate-2,2,3,3-d_4,
120 chemical shift reference), 2 mM of sodium azide (NaN_3, bacteriostatic agent) and 1.5 M
121 KH_2PO_4 in 99% deuterium water (D_2O)]. The optimized pH of the buffer was set at 7.0,
122 with a potassium deuteroxide (KOD) solution, to minimize variations in the chemical
123 shifts of the NMR resonances. This mixture was transferred to a 5 mm NMR tube.

124 Urinary spectra were acquired on a Varian-Inova-500 MHz NMR spectrometer with
125 presaturation of the water resonance using a NOESYPRESAT pulse sequence. During
126 the acquisition, the internal temperature was kept constant at 298 K. An exponential
127 window function was applied to the free induction decay (FID) with a line-broadening
128 factor of 0.3 Hz prior to the Fourier transformation. For each sample, a total of 128
129 scans were recorded into 32 K data points with a spectral width of 14 ppm, an
130 acquisition time of 2 s and a relaxation delay of 5 s. HCT spectra were acquired on a
131 Bruker Avance III 400 MHz NMR spectrometer equipped with a cryoprobe with
132 presaturation of the water resonance using a NOESYPRESAT pulse sequence. The
133 internal temperature was kept constant at 298 K and the sample was processed with a
134 line-broadening factor of 0.3 Hz, 64 scans with a spectral width of 15 ppm, an
135 acquisition time of 3 s and a relaxation delay of 5 s.

136 Spectra were manually phased, baseline corrected and calibrated (TSP, 0.0 ppm) using
137 TopSpin software (version 3.0, Bruker, BioSpin, Germany). Spectra data were bucketed
138 in intelligent bucketing domains of 0.005 ppm with ACD/NMR Processor 12.0 software

(Advanced Chemistry Development, Toronto, Canada). The water signal and the noise regions above 9.5 ppm and below 0.5 ppm were excluded from the analysis.

Data processing and statistical analysis

Data from (i) acute intervention, (ii) sustained intervention and (iii) acute intervention after the repeated exposure were submitted separately to MetaboAnalyst 3.0 for normalization purposes.[32] Data were row-wise normalized by the sum of the intensities of the spectra and column-wise normalized using cube root transformation and Pareto scaling.

Datasets were then imported to R software version 3.1.2.[33] Principal component analysis (PCA) was performed on baseline samples to acquire an overview of individuals and to detect potential carryover effects. After performing the differences between baseline and intervention data of each volunteer (Δ data), multilevel partial least squares discriminant analysis (ML-PLS-DA)[34] was used on Δ data for paired comparisons of HCT versus control beverage using the 'mixOmics' R-package.[35] The "leave-one-subject-out" cross-validation was used to assess the models and the classification error rate was calculated by comparing the actual class with the predicted one. Significant variables were obtained based on the lowest classification error rate and on loading scores from the first latent variable.[27] Absolute values of loading scores were ranked and the top 5% of total features were selected as long as those variables were kept at the lowest classification error rate. These features were employed to model a sparse ML-PLS-DA. Signals from the sparse ML-PLS-DA were ranked based on the absolute values of their loading scores termed loading rank, LR.[27]

The univariate crossover model[36,37] of Δ data was also performed between groups to assess the statistical significances. In addition to individual effect, the period (I and II) and the sequence (from control to test interventions, or from test to control

interventions) followed by each volunteer were considered in the crossover model for further correction on carryover effects. Statistical significance was considered at a *p-value* <0.05.

Metabolite identification

Metabolite identification were performed using Chenomx NMR Suite Professional Software package (version 8.1; Chenomx Inc, Edmonton, Canada) and by comparing NMR spectral data to those available in databases such as the Human Metabolome Database (http://www.hmdb.ca), along with the existing NMR-based metabolomics literature. Further, a Pearson's correlation test was performed to assess correlations between signals of the same metabolite.

RESULTS AND DISCUSSION

^{1}H NMR profiling of the HCT

Metabolic profiling of the HCT beverage was carried out for further exploration of the urinary signatures coming from the functional beverage. The identified compounds with the corresponding chemical shifts are summarized in Table S2. Several compounds naturally present in tea, susceptible to be found in urine, were identified in the HCT. EC, EGCG and EGC are frequently reported in regular green tea beverages although in lower concentrations (Table S3).[38] The characteristic signals corresponding to gallate and theanine, which were previously described in tea beverages,[3,39] were observed on the HCT spectra and subsequently found in the urine in the present study (see below). Other compounds such as caffeine, theobromine, catechol and quinate were also identified in the HCT as previous described in regular tea.[39]

Assessment of multi- and univariate approaches

PCA did not indicate the presence of carryover effects (Figure S1). There was no sign difference between the control group and the HCT group on baseline samples in terms of period and sequence. The differences between the control group and the HCT group resulted in three ML-PLS-DA models on the Δ data, with a minimum classification error rate of 0.1 for the sustained intervention and 0.0 for both the first acute study and the second acute study after the sustained treatment. The lowest estimated classification error rate remained for a range between the 2^{nd} and the 39^{th} variable after the first acute intervention, whereas in the sustained intervention the minimum estimated classification rate was from the 7^{th} to the 10^{th} variable. For the second acute study after the prolonged HCT consumption, the classification error rate was kept to 0.0 during the first 93 variables. Hence, three sparse ML-PLS-DA models were constructed with the selected variables. Discriminant features projected in the first component were ranked according the loading score values and set in the LRs.

The statistical univariate crossover models revealed that a total of 8 compounds were significantly different between HCT and control beverages. As expected, there was a strong agreement between uni- and multivariate analyses performed separately, in which lowest *p*-values matched with the highest loading scores, and thus, lowest loading rank, as it is shown in Table 1. Figure 2 shows box plots of the metabolites levels for HCT and control groups.

Urinary compounds from the food metabolome

After the punctual intake (single dose) of HCT, several compounds coming from the beverage were found in the 4h-urine. A prominent signal at δ2.04 and a triplet at δ1.12 were originally assigned to theanine (Table 1). Then, a triplet at δ3.79 and a multiplet at δ3.18 were also correlated between the signals corroborating the identification. Theanine is a nonproteinogenic amino acid present in green tea[40] and the mushrooms

213 *Xerocomus badius*[41] that improves memory and attention.[42] Interestingly, this
214 compound was also identified in the HCT used in the study (Table S2). Therefore
215 theanine may display a possible direct excretion from tea (Figure 3). Van der Pijl and
216 co-workers found maximum plasmatic concentrations of theanine after 50 min of the
217 intake of 25, 50, and 200 mg L-theanine provided via an aqueous solution or black tea.[43]
218 Similar results were observed by Scheid and co-workers finding a maximum plasma
219 concentration of theanine at 0.8 h after capsules and green tea intake; also found in the
220 urine of participants collected within the interval 3-24 h after intake.[44] In consonance
221 with these findings, we identified theanine in urine sample collected within the first 4 h,
222 entailing an early excretion of this potential biomarker. Gallate and EC were also
223 detected in 4h-urine and also the HCT beverage (Figure 3) denoting the successful
224 absorption and excretion of those compounds as suggested by Rhodes and co-authors.[45]
225 Nevertheless, although the methylated, glucuronide and sulfate catechins forms are
226 frequently detected by mass spectrometry,[19,46,47] they were not detected in the present
227 study. EGC was weakly detected in urine in spite of being strongly distinguished
228 through the singlet δ6.59 in the beverage. In contrast, urinary EGC was firmly detected
229 by other authors using liquid chromatography.[16,17,21] Interestingly, ECG and EGCG,
230 which were identified in the HCT beverage, were not detected in the urine of
231 participants. While Del Rio et al., (2010) and Stalmach et al., (2009) described the
232 presence of unmetabolized EGCG and ECG in plasma after tea ingestion, neither EGCG
233 nor ECG were easily detected in the urinary metabolome.[19,48,49] This fact implicates that
234 the unmetabolized forms of EGCG and ECG probably are not suitable urinary
235 biomarkers of tea intake. We tentatively assigned pyrogallol (sulfate) to the doublet
236 shown at δ6.59 and signals at δ7.05 based on the previous work of the authors Daykin,
237 2005; and Van Dorsten, 2006.[22,23] This metabolite is derived from the cleavage of the 3-

238 O-gallate groups.[58] These authors also suggested other metabolites such as hippuric acid
239 as major urinary metabolite; however, this was not observed in our study. Pyrogallol
240 (sulfate) was significant according the crossover model (Table 1). Nevertheless, the
241 statistical power of the multivariate approach was not strong due to the presence of this
242 metabolite only in certain individuals. This inter-individual variation in the excretion
243 may be related to the colonic microbiota response of participants.[59] Therefore, the
244 variability in the biological effects of tea consumption are related to the presence or the
245 absence of certain microorganisms and their metabolites in the gut.[60] Lastly, the
246 punctual intake of the beverage after a prolonged exposure seated the findings of the
247 first acute intake, replicating short-terms biomarkers of intake such as theanine, EC and
248 gallate. However, in the fasted urine collected after the sustained exposure, none of
249 these compounds were found to be statistically significant in any participant entailing
250 the return to baseline levels within the 24 h after the last intake. In fact, most excretions
251 of the flavan-3-ol metabolites occur within the first 12 h after intake.[50] Therefore, as we
252 expected, these metabolites were newly observed in 4h-urine after a new intake.
253 Overall, the present findings confirm known intake biomarkers coming from the food
254 metabolome, but also propose new potential compounds that should be further studied
255 as novel biomarkers of intake.

256 **Endogenous and microbial metabolites**

257 Regarding endogenous compounds, mainly metabolites from tricarboxylic acid (TCA)
258 cycle and microbiota activity were altered. After the intake of the HCT, 3-methyl-2-
259 oxovalerate, which is a product of isoleucine metabolism, was statistically increased in
260 4h-urine after both punctual and prolonged consumption. Isoleucine is metabolized to
261 succinate via the methylmalonyl-CoA[51] in TCA cycle. Urinary succinate was also raised
262 after prolonged consumption in both 4h- and 24h-urine samples. Similar studies giving

a single dose of a rich-polyphenol food such as coffee and wine exhibited higher urinary levels of 3-methyl-2-oxovalerate and succinate.[27,52] The branched-chain keto-acid 3-methyl-2-oxovalerate was also the strongest predictive biomarker for type 2 diabetes and impaired fasting glucose,[53] while succinate was found to be increased after the consumption of regular green tea and green tea with ascorbate in rats.[54] The fact that nutritional studies included healthy volunteers may point to a strong impact on energy regulation by polyphenols even in short-term,[55] especially observed in tea.[56,57] Therefore, the increased levels of 3-methyl-2-oxovalerate and succinate reflect this regulation by the functional tea rather than an illness situation in the present study. After the sustained consumption of HCT, several compounds were related to microbiota metabolism of catechins. The crossover model revealed a decrease of the singlet δ1.36 in the HCT group, identified as 2-hydroxyisobutyrate. Li and co-workers used combination of spectroscopic, microbiomic, and multivariate statistical tools to model the microbial–host connections and showed this compound associated to the presence of Faecalibacterium prausnitzii in the colon.[61] This metabolite was associated to the microbial degradation of undigested proteins in the gastrointestinal tract.[61] The compound was also reported as discriminant between obese and lean individuals demonstrating functional differences in the microbiome metabolic activity.[62] The urinary metabotype of obese individuals was characterized by a higher concentration of 2-hydroxyisobutyrate and lower levels of hippuric acid, trigonelline and xanthine. In this sense, lower excretion of 2-hydroxyisobutyrate may be associated to a gut microbiota modulation by the constituents present in the functional green tea and might be the responsible of certain health benefits in terms of obesity and Crohn disease.[63] In summary, the present endogenous metabolites might be connected with certain benefits derived of functional beverages.

Our study shows evidence for metabolic changes by a functional beverage composed by higher concentrations of catechins than a regular green tea. Unlike the expected results, theanine was a prominent urinary biomarker, beyond the catechins present as food metabolome compounds from the functional beverage. The HCT also had an impact on the endogenous metabolome. Certain metabolites connected to TCA cycle and to the microbial activity were found to be altered in urine suggesting some of the connections to the described health benefits by tea. In conclusion, we not only seated the presence of tea markers but also present metabolic changes based on endogenous alterations. theanine as intake biomarker as well as different biomarkers of effect.

ABBREVIATIONS

BMI, body mass index; C, catechin; CG, catechin gallate; EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; GC, gallocatechin; GCG gallocatechin gallate; HCT, high catechin tea; FID, free induction decay; ISRCTN, International Standard Randomized Controlled Trial Number; KOD, potassium deuteroxide; LR, loading rank; ML-PLS-DA, multilevel partial least squares discriminant analysis; NMR, Nuclear Magnetic Resonance; PCA, Principal component analysis; TCA, tricarboxylic acid; TSP, (3-(trimethylsilyl)-proprionate-2,2,3,3-d_4.

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AUTHOR CONTRIBUTIONS

312 The authors' responsibilities were as follows — F.M-G: wrote the manuscript; F.M-G,
313 E.V-L and A.S-P: design and conducted the statistical analysis; F.M-G, M.G-A, and
314 R.V-F: conducted the research; F.M-G and R.V-F: performed the samples analyses;
315 F.M-G, M.G-A, E.V-L, A.S-P, K.M., T.H., A.S and CA-L: provided critical revision of
316 the manuscript; K.M., T.H., and A.S. designed and conducted the intervention study,
317 CA-L: had primary responsibility for the final content of the manuscript; and all
318 authors: read and approved the final manuscript.

319 The authors report no financial interests or potential conflicts of interest.

320 **SUPPORTING INFORMATION**

321 Table S1. Composition of beverages used in the study. Table S2. Compounds identified
322 in the HCT beverage by NMR spectroscopy. Table S3. Composition of catechins of the
323 HCT used in the study and the reported composition in regular green tea. Figure S1.
324 Principal component analysis (PCA) of baseline points in control and HCT groups in
325 both I and II periods of the crossover study.

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530

Table 1. Tentative metabolites detected in urine of the participants in the present study.

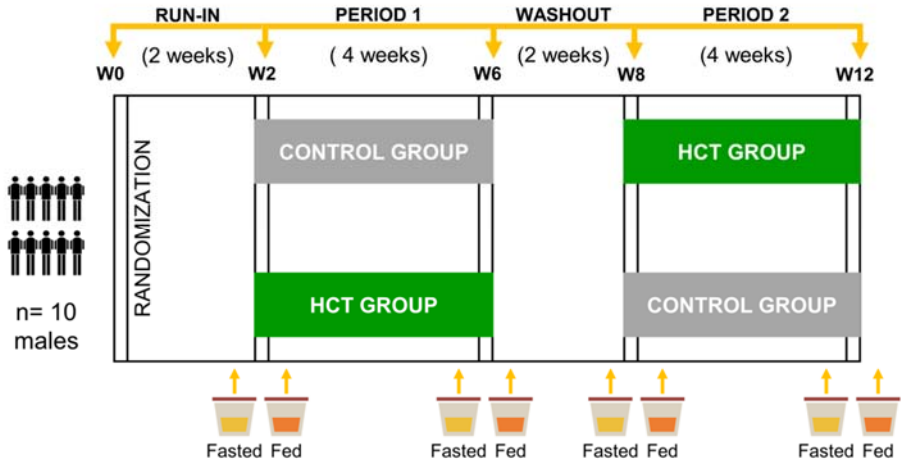
Intervention type	Metabolite	δ (multiplicity)	HCT vs control	*P value*[1]	LR[2]
Acute	3-Methyl-2-oxovalerate	1.10 (d)	↑	4.72×10^{-5}	7,11
		0.89 (t)*		-	-
	Theanine	3.80 (t)*	↑	-	-
		3.18 (m)		5.67×10^{-3}	20
		2.04 (m)		2.77×10^{-6}	1,2,15
		1.12 (t)		3.00×10^{-5}	5,10,24
	Gallate	7.05 (s)	↑	2.11×10^{-2}	-
	EC	7.01 (s)	↑	7.55×10^{-3}	21,29
		6.96 (m)		5.22×10^{-3}	19,22,25
		6.16 (d)		2.18×10^{-5}	4,27,36
		6.12 (d)		1.46×10^{-3}	12,33
		4.36 (s)		2.52×10^{-2}	-
		2.94 (dd)		-	-
		2.78 (dd)		-	-
	EGC	6.50 (s)	↑	2.24×10^{-2}	-
		4.32 (m)*		-	-
		2.93 (m)		-	-
		2.81 (m)		-	-
Sustained	2-Hydroxyisobutyrate	1.36 (s)	↓	$3.60 \times 10\text{-}^{2}$	-
	Succinate	2.41 (s)	↑	1.81×10^{-2}	-
	Pyrogallol (sulfate)	7.05 (m)	↑	4.01×10^{-2}	-
		6.59 (d)		3.81×10^{-3}	-
2nd Acute	3-Methyl-2-oxovalerate	1.10 (d)	↑	3.51×10^{-4}	6,24
		0.89 (t)*		-	-
	Theanine	3.80 (t)*	↑	-	-
		3.18 (m)		-	-
		2.04 (m)		8.14×10^{-6}	1,5,39
		1.12 (t)		4.47×10^{-3}	21,44,55
	Gallate	7.04 (d)	↑	5.94×10^{-3}	26,70,93
	Succinate	2.41 (s)	↑	7.47×10^{-3}	37
	EC	7.01 (s)	↑	3.31×10^{-2}	77,81
		6.95 (m)		3.55×10^{-3}	30,40,52
		6.17 (d)		4.79×10^{-5}	4,7,41
		6.12 (d)		2.67×10^{-3}	14,65
		4.36 (s)		3.88×10^{-2}	-
		2.94 (dd)		1.86×10^{-2}	51
		2.78 (dd)		1.79×10^{-2}	49,53
	Pyrogallol (sulfate)	7.05 (m)	↑	2.10×10^{-2}	54,63,92
		6.59 (d)		2.12×10^{-2}	62,83

[1]*P-value* of univariate statistical crossover model of differences. [2]Loading rank of ML-PLS-DA (when several LR values per signal, only the three lowest LR per signal were

534 reported). *Signals not considered because a strong overlapping. U: unknown, s: singlet,

535 d: doublet, t: triplet, dd: double doublet, m: multiplet, br s: broad singlet.

536



537
538 **Figure 1.** Schematic representation of the randomized, placebo-controlled and

539 crossover design of the study. HCT, high catechin tea.

540

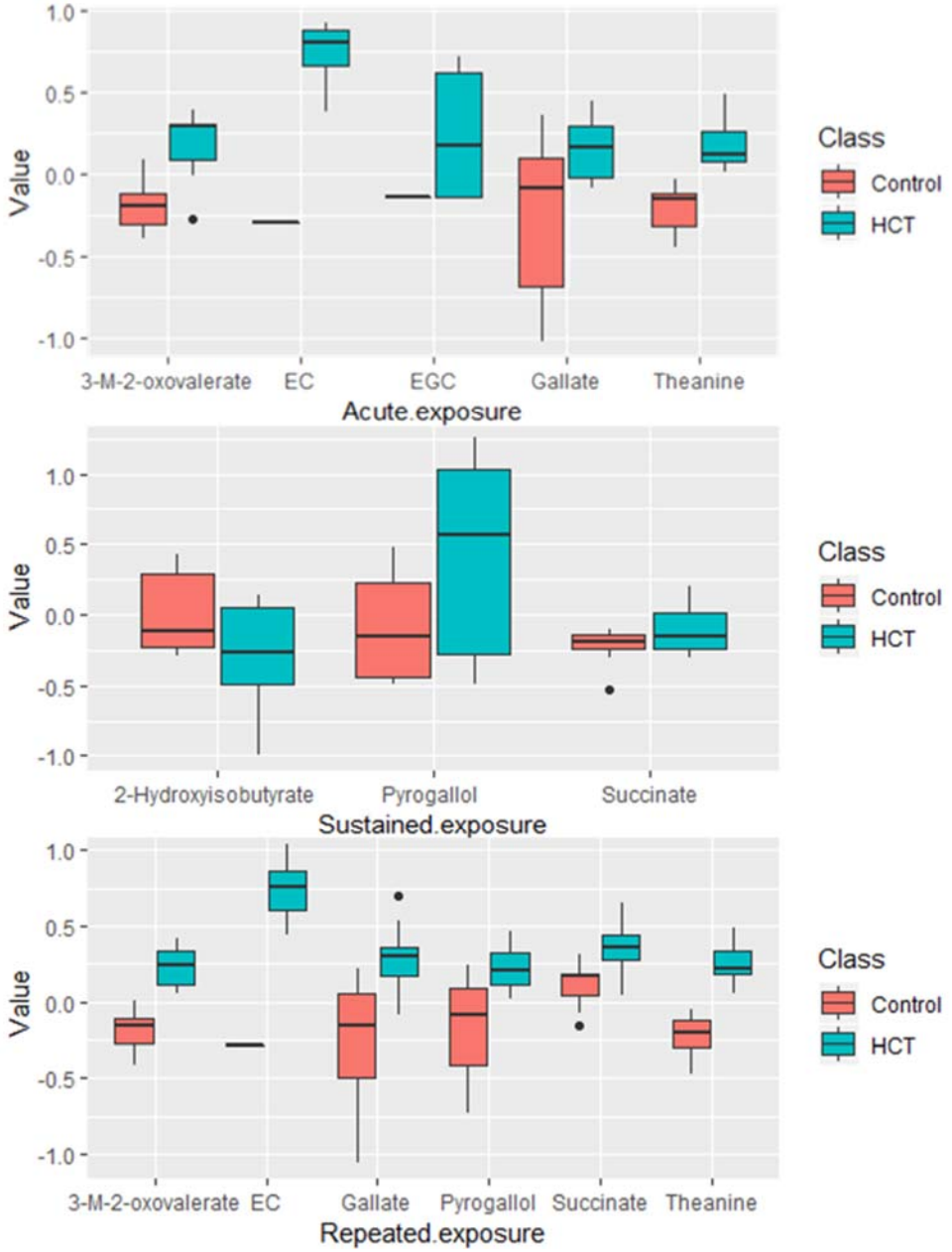
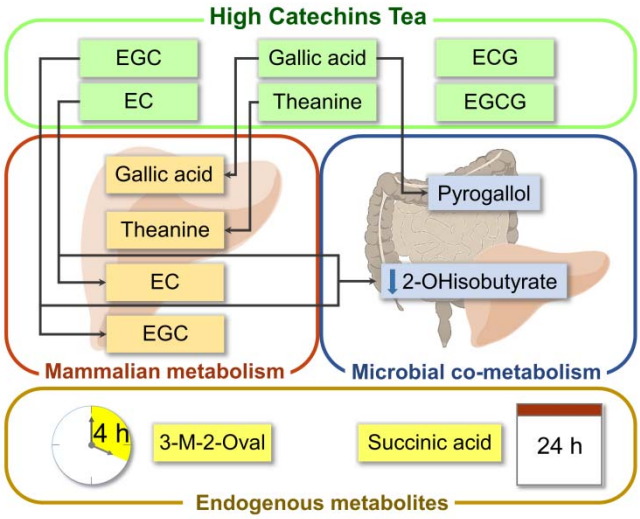


Figure 2. Box plots of urinary metabolites that were significantly different after high catechin tea (HCT) compared with control beverage in acute, sustained and repeated exposures. 3-M-2oxovalerate: 3-methyl-2-oxovalerrate; EC, epicathechin; EGC, epigallocatechin.



546
547 **Figure 3.** Main compounds found in the HCT urine of the participants in both acute and

548 sustained interventions.