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Original Article

Urine metabolomics shows an induction of fatty acids metabolism in healthy adult volunteers after supplementation with green coffee (*Coffea robusta* L.) bean extract

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ABSTRACT

Background and objective: Green coffee bean extract is used as herbal medicine or supplement for weight reduction and obesity. The active constituents are considered caffeine and chlorogenic acid (CGA) derivatives. The mode of action of CGA is still unclear and can be related to peroxisome proliferator-activated receptor α (PPAR-α) and liver X receptor Rα (LXR-α). Metabolomics may be an innovative tool for the description and discovery of the multiple target nature of such phytocomplex.

Methods: 24 h urine samples were collected once a week from ten healthy adult volunteers consuming daily 400 mg of dry Green coffee bean extract (GCBE, 4.9% of chlorogenic acid) each day for 30 days (5 harvesting days, considering also the first day of supplementation). Urine samples were analyzed by LC-QTOF using both untargeted and targeted approaches. The latter was used to monitor two urinary markers of oxidative stress (allantoin, 8-OHdG).

Results: Metabolomics analysis (PLS-DA) revealed changes in urine composition before and during the treatment with GCBE. Markers related to treatment were metabolites related to polyphenol administration as hippuric acid, benzoic acid derivatives, dihydroferulic and dihydrosinapic acid sulphate, but also carnitine derivatives and dicarboxylic acids. On the other hand, no changes in the levels of allantoin and 8-OHdG were observed.

Conclusion: This preliminary study showed the possible usefulness of metabolomics approach in the evaluation of GCBE consumption in healthy subjects. The observed changes in urinary composition can be related to the catabolism of GCBE constituents and to induced fatty acid metabolism, mainly related to carnitine derivatives. This latter result could be considered, at least in part, as a further proof of the mode of action of green coffee extract.

Introduction

Nowadays, overweight and obesity are considered important health concern, especially in the western countries (Nguyen and El-Serag, 2010), being amongst the biggest medical problems of the 21st century (Kużbicka and Rachoń, 2013). In fact, obesity is known to contribute to comorbid conditions that may become life threatening such as diabetes, hypertension, dyslipidemia or respiratory problems (Buchanan and Beckett, 2013). Multiple factors contribute to obesity, including genetics, bad dietary habits and lack of sufficient physical activity (Kużbicka and Rachoń, 2013; Booth et al., 2012). Moreover, excess weight can lead to metabolic syndrome, a combination of obesity, dyslipidemia, impaired glucose tolerance and hypertension (Deen, 2004; Quick and Kiefer, 2013), which could lead to other severe health problems such type 2 diabetes and cardiovascular disease (CVD) (Eckel et al., 2005). Several weight management strategies can be used. As an example, moderate overweight could be overcome starting from changing dietary habits and increasing physical activity. The use of herbal medicines and food supplements associated to physical activity and a balanced diet is also frequently adopted as weight management strategy (Yang et al., 2017; Gaullier et al., 2005), and the so called nutraceuticals are largely used for their reported health-promoting

Abbreviations: GCBE, Green coffee bean extract; CGA, Chlorogenic acid; PPAR-α, Peroxisome proliferator-activated receptors α; 8-OHdG, 8-Hydroxydeoxyguanosine; LC, Liquid Chromatography; MS, Mass Spectrometry; UPLC, Ultra Performance Liquid Chromatography; LOD, Limit of Detection; LOQ, Limit of Quantification; DAD, Diode Array Detector; ESI, Electrospray Ionization; PCA, Principal Component Analysis; PLS-DA, Partial Least Squares – Discriminant Analysis; QC, Quality Control; QTOF, Quadrupole time-of-flight mass spectrometer; RT, Retention Time; SRM, Single reaction monitoring; TQD, Tandem quadrupole mass spectrometer; USP, United States Pharmacopeia; VIP, Variable Importance on Projection

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and/or disease-preventing properties (Sut et al., 2016). However, concerning such products literature data are contradictory, showing both their efficacy (Quick and Kiefer, 2013; Shimoda et al., 2006; Onakpoya et al., 2011) and inefficacy (Pittler and Ernst, 2004). Natural products as fucoxantines and carotenoids have been recently considered as possible treatments for obesity (Miyashita and Hosokawa, 2014; Maeda, 2015), along with other classes of natural products, such as polyphenols (Poddar et al., 2011; Ríos-Hoyo et al., 2015).

One popular natural product used in the recent years for moderate overweight treatment is the green coffee beans extract (GCBE, *Coffea robusta* L.), which is produced from coffee beans that have not been roasted (Thom, 2007). Roasting can cause partial degradation of the phenylpropanoid esters of quinic acid (as chlorogenic acids, CGA) during high temperature treatments (Farah and Donangelo, 2006). CGA are considered the main constituents of GCBE (Farah and Donangelo, 2006). Although the content of CGA may vary according to several factors as species and cultivar, degree of maturation, agricultural practices, climate and soil, the amounts of total CGA in regular green coffee beans, on dry matter basis, may vary from 4 to 8.4 % for *Coffea arabica*, and from 7 to 14.4 % for *Coffea robusta*, with some hybrids presenting intermediate levels (Farah and Donangelo, 2006). The extract also contains caffeine, that together with CGA are considered to exert potential weight loss activity (Shimoda et al., 2006). The effects of consumption of GCBE observed in animal models and in humans are related to modulation of glucose metabolism, inhibition of fat accumulation, weight reduction and alteration of body fat distribution, exerted by CGA (Shimoda et al., 2006). Moreover, other proposed mechanisms are the reduction of the intestinal absorption of glucose (Shimoda et al., 2006) and the inhibition of the enzymatic activity of hepatic glucose-6-phosphatase (Shimoda et al., 2006; Arion et al., 1997). More recently, in an *in vivo* study CGA of green coffee were demonstrated to improve the blood lipid metabolism in rats by alleviating the levels of fatty acids and triglycerides and modulating the multiple factors in liver through AMP-activated protein kinase (AMPK) pathway, showing a possible mode of action of this ingredient in the management of obesity (Sudeep et al., 2016). The presence of caffeine can be considered useful because of its stimulant effect, hence it increases energy expenditure in humans, contributing to weight loss effects of coffee (Dulloo et al., 1989). Furthermore, other proposed mechanisms for caffeine effect on weight loss include increased thermogenesis, which consequently enhances lipolysis and lipid metabolism (Dulloo et al., 1989; Greenberg et al., 2006).

Food supplements containing GCBE are increasing in use to control weight gain, but the efficacy of these products is still under debate, due to contradictory data published in literature. In a review of 2013, Buchnan and Beckett indicated that a limited number of clinical studies were published related to the efficacy of GCBE, and that in most of them the clinical significance of the weight loss was minimal (Buchanan and Beckett, 2013). The same authors underlined the significant limitation of the reviewed studies as lack of blinding, direct comparisons, safety assessment, lack of comprehensive endpoints, very low sample size, and not inclusion of lifestyle modifications (Buchanan and Beckett, 2013). On the other hand, other papers reported moderate effects of GCBE on weight loss (Quick and Kiefer, 2013; Shimoda et al., 2006; Onakpoya et al., 2011). Revuelta–Iniesta and coll. compared the effects of a 2-weeks green coffee and black coffee consumption in 20 healthy subjects, observing that systolic blood pressure were significantly reduced after green coffee, as well as body mass index and abdominal fat, with no changes in energy intake. Furthermore, cortisol/cortisone ratio in urine was reduced after green coffee, suggesting that green coffee can play a role in reducing cardiovascular risk factors overall (Revuelta-Iniesta and Al-Dujaili, 2014).

Other papers focused their attention only on CGA, without studying the effects of a treatment with a complete green coffee extract. For example, Cho and coll. compared the effects of treatment with CGA and caffeic acid (0.02% w/w) in obese mice (Cho et al., 2010). Both caffeic acid and CGA significantly lowered body weight, visceral fat mass and plasma leptin and insulin levels, compared to the high-fat control group. They also reported lowered triglyceride (in plasma, liver and heart) and cholesterol (in plasma, adipose tissue and heart) levels. Both treatments significantly inhibited fatty acid synthase, 3 hydroxy-3-methylglutaryl CoA reductase and acyl-CoA: cholesterol acyltransferase activities, while they increased fatty acid β-oxidation activity and peroxisome proliferator-activated receptors α (PPAR-α) expression in the liver compared to the high-fat group (Cho et al., 2010). Hence, despite the large diffusion of GCBE, studies are still needed to fully understand the possible mode of action and the role that this plant extract may have as treatment of obesity and its clinical relevance.

The evaluation of the effects and of the possible mode of action of phytoconstituents is a challenge due to complex composition and possible multiple mode of action, and metabolomics may play a role as a new approach in the study of bioactive plant ingredients (Sut et al., 2016; Wolfender et al., 2013). Metabolomics-based studies may offer new opportunity in the evaluation of the possible efficacy, mode of action and safety related to the use of GCBE. Overall, urinary metabolomics can be a suitable approach, because sample collection is non-invasive and allows long-term studies, furthermore markers of oxidative status may be measured in urines offering the opportunity to study also the redox effect of GCBE administration (Il'yasova et al., 2012). The green coffee extract may present some effect in the weight management but the evaluation of its effects and mode of action may be difficult due to the complex phytochemical composition and due to the multiple possible molecular targets of its constituents. For this reason, a metabolomics approach was used in the present pilot study. Ten healthy adult with normal body mass index (BMI), no metabolic, cardiovascular overweight or obesity problems assumed 400 mg of dry GCBE daily for 30 days. The 24 h urinary samples were collected weekly, and analyzed by LC-MS. Multivariate data analysis approaches were applied and also targeted analysis were also performed to measure oxidative stress urinary biomarkers, namely allantoin and 8 hydroxydeoxyguanosine (8-OHdG), in order to assess the potential antioxidant activity of GCBE *in vivo*. To the best of our knowledge this is the first report describing the effects on healthy subjects of green coffee by metabolomics approach.

Materials and methods

Materials

The supplement containing a standardized dry GCBE (*Coffea robusta* L.) was purchased from a local market. The names of product and supplier are note reported to avoid any conflict of interests. The powder extract used in the experiment was a homogeneous batch and it was formulated in gelatin capsules, each containing 200 mg of product. Standard chlorogenic acid (product number: C3878), allantoin (product number: 05670) and 8-OHdG (product number: H5653) were purchased from Sigma Aldrich (Milan, Italy). HPLC-grade acetonitrile and formic acid were purchased from Sigma Aldrich (Milan, Italy), as well as deuterated methanol used in NMR analysis. Deionized water used in HPLC and UPLC analyses was filtered through a Milli-Q system equipped with a 0.22 μm cut-off filter (Millipore).

Chemical characterization of dry GCBE

Chemical characterization of dry GCBE was performed by ^{1}H NMR analysis and by HPLC-DAD-MS^{n}. For NMR exploratory analysis of GCBE, a Bruker Avance III spectrometer operating at 400 MHz was used. Briefly, 150 mg of dried extract were weighted in Eppendorf tube and 1 ml of deuterated methanol was added. The suspension was sonicated for 5 min then the tube was centrifuged at 13,000 rpm. The supernatant was transferred to a NMR tube and used for the ^{1}H NMR analysis. For the chromatographic analysis, the system was composed of an Agilent 1260 chromatographic system with 1260 autosampler,

column oven and a 1260 diode array detector. Out of the column a "T" union was fitted, splitting the flow identically to the diode array and to a Varian 500-MS ESI-IT mass spectrometer (MS). An Agilent Eclipse XDB-C18 column (3.5 μm, 3.0 × 150 mm) was used as stationary phase. Acetonitrile (A) and 0.1% formic acid in water (B) were used as mobile phase. Gradient elution was as follows: 5 min, 10% A; 30 min, 100% A; 100% A isocratic up to 35 min. Re-equilibration time was 5 min. Flow rate was 125 μl/min and injected volume was 10 μl. The MS parameters were as follows: spray chamber temperature, 50 °C; nebulizer gas pressure, 25 psi; drying gas pressure, 25 psi; drying gas temperature, 350 °C; needle voltage, ± 4500 V; spray shield voltage, 600 V. Mass spectra were acquired both in negative (for phenols characterization) and positive (for caffeine and trigonelline characterization) mode in the spectral range 150–2000 Da. Compounds were identified studying their fragmentation spectra obtained using the turbo data dependent scanning (tdds®) function of the instrument, and comparing the spectra with literature (Clifford et al., 2003, 2005, 2006, 2008). For quantitative purposes, a DAD detector was used and chlorogenic acid and caffeine were used as standard compounds. Calibration curves were built in the range 1–100 μg/ml for chlorogenic acid (at 330 nm) and 2.5–85 μg/ml for caffeine (at 254 nm), and all calibration solutions were analyzed in triplicate. The obtained linear curves were: $y = 35.61x + 0.052$ ($R^2 = 0.9998$) and $y = 5.23x + 0.011$ ($R^2 = 0.9998$) for chlorogenic acid and caffeine, respectively. Limit of detection (LOD) and limit of quantification (LOQ) were determined following US Pharmacopeia methods. LOD = 0.5 μg/ml and 1.5 μg/ml and LOQ = 1.5 μg/ml and 5.0 μg/ml, for chlorogenic acid and caffeine respectively.

Human pilot trial methodology

Ten healthy adult volunteers (five women and five men, 31 ± 10 years, BMI 23.2 ± 2.6) were enrolled for the study and informed consent was obtained. The protocol was conducted in compliance with the Declaration of Helsinki and the International Conference on Harmonization Guidelines. All subjects were non-smoking and normally active. No medication was taken during the study (wash out period: 7 days). They were required to maintain a low-polyphenol diet for 7 days before product consumption, free of green vegetables, fruits, fruit juices and red wine, to reduce as much as possible the amounts of polyphenols and related metabolites excreted in urines. One day before the beginning of the experiment volunteers collected control urine, and nothing except water could be consumed for 8 h prior to GCBE consumption. Then, volunteers took daily 400 mg of dry GCBE (2 capsules) each day for 30 days and they collected 24 h urine once a week during the experiment (5 harvesting days, considering also the first day of supplementation). The dose was established on the basis of the doses of green coffee extract that are commonly introduced in food supplements. Urine was centrifuged at 5000 rpm for 10 min, sampled in 50 ml vials and stored at −80 °C until analysis. The body weight of volunteers was monitored before the starting of the experiment and during treatment.

UPLC-QTOF analysis of 24 h urine output using C-18 stationary phase

To obtain a metabolite profiling of urine samples, an Agilent 1290 Infinity UPLC system equipped with a Waters Xevo G2 Q-TOF MS was employed. The detector was equipped with an ESI ionization source and was operating both in positive and negative modes. The sampling cone voltage was adjusted to 40 V, the source offset at 80 V. The capillary voltage was adjusted to 3 KV. The nebulizer gas used was N_2 at a flow rate of 800 L/h. The desolvation temperature was 450 °C. The mass accuracy and reproducibility were maintained by infusing lock mass (leucine–enkephalin, $[M+H]^+$ = 556.2771 m/z; $[M-H]^-$ = 554.2620 m/z) thorough lockspray at a flow rate of 20 μl/min. Centroided data were collected for each sample in the mass range 50–1200 Da, and the *m/z* value of all acquired spectra was automatically corrected during acquisition based on lock mass. MS^e experiment was simultaneously performed to collect structural information, setting the collision energy to 30 V. An Agilent Zorbax Rapid Resolution High Definition (RRHD) SB-C18 column (2.1 mm x 50 mm, 1.8 μm) was used as stationary phase. The mobile phase was composed of solvent A (acetonitrile with 0.1% formic acid) and solvent B (water with 0.1% formic acid). Linear gradients of solvents A and B were used, as follows: 0 min, 2% A; 1 min, 2% A; 8 min, 55% A; 10 min, 55% A; 10.5 min, 98% A; 11.5 min, 98% A; 12 min, 2% A and isocratic for 1 min. The flow rate was 300 μl/min and the injection volume was 1 μl. Collected urine samples were centrifuged at 13,000 rpm for 10 min and then they were randomized and directly injected (1 μl) in the UPLC. Quality control samples (QC, n = 12) were used to monitor the instrument performance. They were prepared mixing 100 μl of each urine sample and they were centrifuged at 13,000 rpm for 10 min. Sequence of injection comprising pool, control and treated samples was randomized to avoid any bias due to samples order.

LC–MS analysis of 24 h urine output using C-3 stationary phase

A specific LC-ESI-MS method was developed on a C-3 stationary phase to better separate polar and smaller molecular weight metabolites. An Agilent 1260 LC system equipped with a Varian 500-MS IT MS was employed. The detector was equipped with an ESI ionization source and was operating in positive mode. Needle voltage was adjusted to 4.5 kV, spray shield voltage to 600 V and capillary voltage to 750 V. The nebulizer gas pressure was 50 psi and drying gas (400 °C) pressure was 400 psi. Centroided data were collected in the mass range 50–1200 Da. MS^n spectra were acquired using the tdds® utility.

An Agilent Zorbax RRHD 300SB-C3 column (2.1 mm x 100 mm, 1.8 μm) was used as stationary phase. The mobile phase was composed of solvent A (acetonitrile with 0.1% formic acid) and solvent B (water with 1% formic acid) and the gradient used was: 0 min, 2% A; 1 min, 2% A; 20 min, 90% A; 23 min, 90% A; 25 min, 2% A. The flow rate was 150 μl/min and the injection volume was 2 μl. Pre-processing of urine samples was the same previously described and QC samples (n = 12) were used to monitor instrument performance.

Statistical data analysis

Centroided and integrated UPLC-MS data were processed by MarkerLynx Applications Manager version 4.1 (Waters) to generate a multivariate data matrix. Method for data deconvolution, alignment and peak detection was created. The parameters used were retention-time range 1–10 min, mass range 50–1200 Da, mass tolerance 0.01 Da. Noise elimination level was set to 6.00, minimum intensity was set to 15% of base peak intensity, maximum masses per retention time (RT) was set to 6 and, finally, RT tolerance was set to 0.01 min. Isotopic peaks were excluded from analysis. A list of the ion intensities of each peak detected was generated, using retention time and the *m/z* data pairs as the identifier for each ion. The resulting three-dimensional matrix contains arbitrarily assigned peak index (retention time-*m/z* pairs), sample names (observations), and ion intensity information (variables). Variables having more than 30% of missing data in both groups were excluded. Data were mean-centered, Pareto scaled and log-transformed before Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA). Using SIMCA 13 (Umetrics, Umea, Sweden) platform, N-fold full cross-validation (N = 7) and permutation test on the responses (500 random permutations) were performed, in order to avoid over-fitting and prove the robustness of the obtained models (Peron et al., 2017).

Biomarkers were identified calculating the molecular formula by high-resolution *m/z* values of deprotonated pseudomolecular ions ($[M-H]^-$), using MassLynx Elemental Composition tool and searching for both the calculated molecular formula and accurate *m/z* in freely available web databases (*i.e.* Human Metabolome Database, Metlin, MassBank). Possible candidates were then screened comparing the MS^e

fragmentation spectra for variables identification.

LC-MS centroided data were converted using ACD Spectrus 2014 software and then processed using MZmine Software v. 2.0. Chromatogram deconvolution, alignment of peaks, peak integration, isotopic grouping and normalization were performed. Variables having more than 30% of missing data in both groups were excluded and a list of the ion intensities of each peak detected was generated, using retention time and the *m/z* data pairs as the identifier for each ion. Data were mean-centered, Pareto scaled and log-transformed before PCA and PLS-DA, using SIMCA 13 (Umetrics, Umea, Sweden) platform. N-fold full cross-validation and permutation test on the responses were performed using the same software, as previously described. Biomarkers were identified studying their MS^n spectra and comparing fragmentation patterns with literature data.

Targeted analysis of allantoin and 8-OHdG in 24 h urine samples

Allantoin and 8-OHdG were quantified in urine samples using an Agilent 1260 HPLC system equipped with a Varian 320 TQD MS detector. The stationary phase was a Phenomenex Kinetex EVO C-18 column (2.1 mm x 100 mm, 5 μm). The detector was equipped with an ESI source and was operating in positive mode. The operating parameters used were as follows: nebulizing gas (N_2) pressure, 50.0 psi; needle voltage, 4.5 kV; capillary voltage, 600.0 V; drying gas temperature, 380 °C; drying gas pressure, 25.0 psi. The mobile phase was composed of solvent A (acetonitrile with 0.1% formic acid) and solvent B (water with 0.1% formic acid). Linear gradients of A and B were used, as follows: 0 min, 5% A; 1 min, 5% A; 8 min, 90% A; 10 min, 90% A; 11 min, 5% A and isocratic for 1 min. The flow rate was 200 μl/min and the injection volume was 10 μl. For quantitative purposes, the fragmentation of allantoin $[M+H]^+$ ion was monitored (*m/z* 159 → 116) and a standard titration curve obtained eluting 1 - 100 ng/ml allantoin solutions in water (y = 6234.3x; R^2 = 0.9923) was used. Limit of detection (LOD = 1 ng/ml) and limit of quantification (LOQ = 5 ng/ml) were determined following USP guidelines. For LOD, samples with known concentration of allantoin were analyzed and minimum concentration at which analyte could be reliably detected was established. On the other hand, LOQ was established by visual evaluation. For 8-OHdG quantification, the fragmentation of 8-OHdG $[M+H]^+$ ion was monitored (*m/z* 284 → 168) and a standard titration curve obtained eluting 1 - 100 ng/ml 8-OHdG solutions in water (y = 53429x; R^2 = 0.9981) was used. Limit of detection (LOD = 0.3 ng/ml) and limit of quantification (LOQ = 1 ng/ml) were determined following USP guidelines. Before analyses, urine samples were centrifuged at 13,000 rpm for 5 min and supernatants were directly injected in the instrument.

Results

Characterization of the phytoconstituents of dry GCBE

As preliminary analysis, the dried extract was extracted in deuterated methanol and ^1H NMR was acquired (Fig. 1). Signals supporting the presence of caffeic acid derivatives were detected, namely the pair of doublets at δ 7.57 and 6.28 (J = 16.7 Hz) ascribable to *trans* double bond, the aromatic signals at δ 7.06 (d, 1.8 Hz) and the partially overlapped signals at δ 6.85 and 6.80 (Bajko et al., 2016). Signals supporting the presence of quinic acid (δ 4.20; 3.85; 2.18 and 2.00) were also detected (Bajko et al., 2016), as well as the signals ascribable to caffeine (δ 7.87 and methyl groups at 3.37, 3.55 and 3.91).

Further data were obtained using LC-DAD-ESI-MS^n in negative ionization mode. Twelve different phenolic compounds, mainly caffeoylquinic and feruloyl-quinic acids derivatives (Table 1 and Fig. 2) were identified on the basis of their MS^n spectra and comparison with literature (Clifford et al., 2003, 2005, 2006, 2008). Quantification was obtained using a diode array detector and chlorogenic acid as reference compound. The most abundant constituents were 5-caffeoylquinic acid (5-CQA, 4.93 ± 0.50 w/w) and feruloylquinic acid (FQA, 1.059 ± 0.04 w/w), while hexahydroxydiphenoyl (HHDP) hexoside was the less abundant (0.089 ± 0.01 w/w).

Positive ionization mode was used to quantify caffeine and trigonelline (Table 2). Caffeine amount was 0.593 ± 0.02 % w/w, trigonelline one was 0.308 ± 0.04 % w/w.

Urine analysis by UPLC-QTOF untargeted metabolomics (C-18 stationary phase)

An untargeted approach was used to explore the changes in urine composition related to the consumption of GCBE. At first attempt, we developed a chromatographic method using a C-18 column as stationary phase. Exemplificative chromatograms are reported in Fig. 3. At first attempt, LC-MS data were analyzed by explorative PCA, in order to detect the presence of outliers and to assess method robustness by the analysis of QC samples. The models showed that 24 h urine samples collected from different subjects were partially grouped in different clusters. The PCA models for “positive” and “negative” datasets showed grouping of QC samples in tight clusters at the origin of the axes, confirming the robustness of the chromatographic method and of the metabolic profiling platform used (data not shown) (Vorkas et al., 2015).

The same datasets were further processed using supervised method, namely PLS-DA, using controls and urines collected during treatment as groups (Figs. 4 and 5). The model built using data in positive acquisition mode was characterized by 3 components and R^2 = 0.69 and $Q^2_{7\text{-fold}}$ = 0.46. Similarly, the model built using the data acquired in negative ion mode was characterized by 3 components and R^2 = 0.65 and $Q^2_{7\text{-fold}}$ = 0.39. The two datasets (positive and negative) were composed by 4600 and 3000 variables, respectively, among whom significant descriptive ones for treatment were selected from loading plots depending on their Variable Importance on Projection (VIP) value. Only variables having VIP > 1 were considered. Two sets of 22 and 4 measured variables were detected as relevant in the explanation of the effects of treatment, from “positive” and “negative” datasets, respectively, and were tentatively identified based on their HR *m/z* value and considering their R.T. and fragmentation spectra (Table 3).

Benzoic acid, hippuric acid, 4 hydroxybenzoic acid, quinic acid and dihydrocaffeic acid sulfate amounts were increased in urines collected during treatment, compared to controls, and they can be considered as markers of consumption of CGA, along with isoferulic acid, *p*-coumaric acid, 3(3 hydroxyphenyl)propanoic acid and dihydrosinapic acid (Del Rio et al., 2010; Farah et al., 2008), whose amounts were increased in urines after supplement consumption. Significant traces of intact CGA were not present in urines after treatment, indicating that those compounds are extensively metabolized by gut microbiota and only derived catabolites could be detected. Other significant variables were tentatively identified as medium-chain (C8-C10) acyl-carnitines, whose amounts in urines were increased with intake of GCBE. Acyl carnitines are involved in the metabolism of fatty acids. Further compounds, namely 3 hydroxysuberic acid (3 hydroxyoctanedioic acid) and 3 hydroxyadipic acid (3 hydroxyhexanedioic acid) are metabolite derived from the ω-oxidation of 3 hydroxy fatty acids and the β-oxidation of longer-chain 3 hydroxy dicarboxylic acids (Tserng, 1991). They are normally found in blood and urine and their amounts are increased in ketoaciduria (Verhaeghe et al., 1992), so in conditions that may be related to starvation.

Urine profiling using LC-MS untargeted metabolomics (C-3 stationary phase)

The same urine samples were analyzed by LC-MS using a C-3 column as stationary phase, in order to have different chromatographic

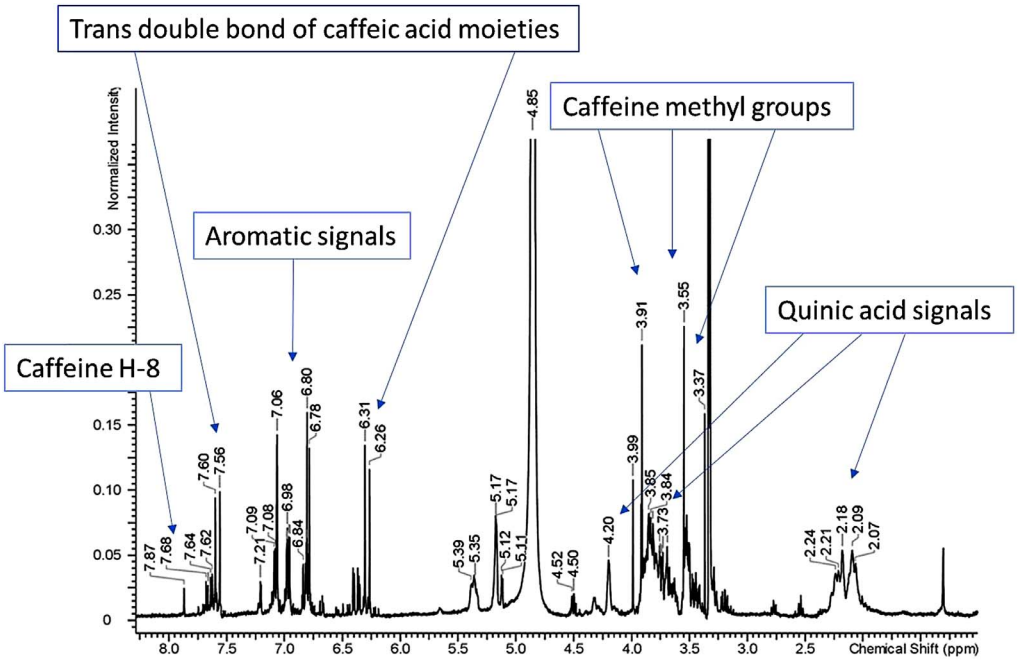

Fig. 1. ^{1}H NMR spectrum of the GCBE.

Table 1
Polyphenolic constituents of GCBE. Analysis was performed by HPLC-MS/MS in negative ion mode.

R.T. (min)	Compound	$[M-H]^-$ (*m/z*)	Fragments (*m/z*)	% (w/w)
9.3	3-CQA	353	191 85	0.593 ± 0.02
11.5	4-CQA	353	191 85	0.308 ± 0.04
11.8	5-CQA	353	173 93	4.93 ± 0.50
12.1	F pentoside	367	193 134 149	0.177 ± 0.01
12.7	Caffeic acid[a]	179	135	0.422 ± 0.06
13.5	FQA	367	191 85 111 127	1.059 ± 0.04
14.6	HHDP hexoside	481	301 257	0.089 ± 0.01
14.9	3,4-diCQA	515	353 335 191 179 173	0.520 ± 0.04
15.4	1,5-diCQA	515	353 191 179 173	0.304 ± 0.04
15.8	3,5-diCQA	515	353 191 173	0.437 ± 0.04
16.2	3-C-5-FQA	529	367 335 173 193 111 93	0.162 ± 0.01
16.8	3-F-4-CQA	529	353 367 173 93	0.150 ± 0.01
			Total amount	**9.15**

(R.T. = Retention Time; QA = quinic acid; diCQA = dicaffeoyl quinic acid; C = caffeoyl; F = feruloyl).

[a] Identification was confirmed comparing with reference compounds.

Table 2
Amounts of trigonelline and caffeine in GCBE. Analysis was performed by HPLC-MS/MS in positive ion mode.

R.T. (min)	Compound	$[M+H]^+$ (*m/z*)	Fragments (*m/z*)	% (w/w)
2.6	Trigonelline	138	94 92	0.308 ± 0.04
12	Caffeine	195	138 110 83	0.593 ± 0.02
			Total amount	**1.01**

(R.T. = Retention Time).

behavior. Also in this case, an untargeted approach was used. Explorative PCA model did not show discrimination between "treated" samples and controls. The same model showed grouping of QC samples in tight clusters at the origin of the axes, confirming the robustness of the chromatographic method and of the metabolic profiling platform used (data not shown) (Vorkas et al., 2015). To observe differences between control urine samples and ones collected during supplement consumption, the same dataset was processed using PLS-DA. The resulting model (characterized by 2 components and $R^2 = 0.69$ and $Q^2_{7\text{-fold}} = 0.32$) can emphasize the differences in urine samples due to GCBE intake (the score plot is reported in Fig. 6). Four variables were selected as significantly discriminating treated urines from controls (Table 4), and they were tentatively identified studying their

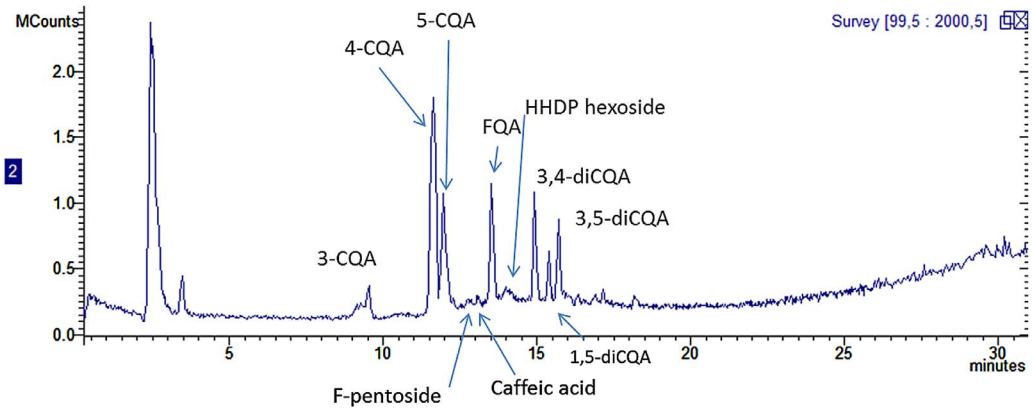

Fig. 2. Representative chromatogram obtained from HPLC-MS/MS analysis of GCBE in negative ion mode.

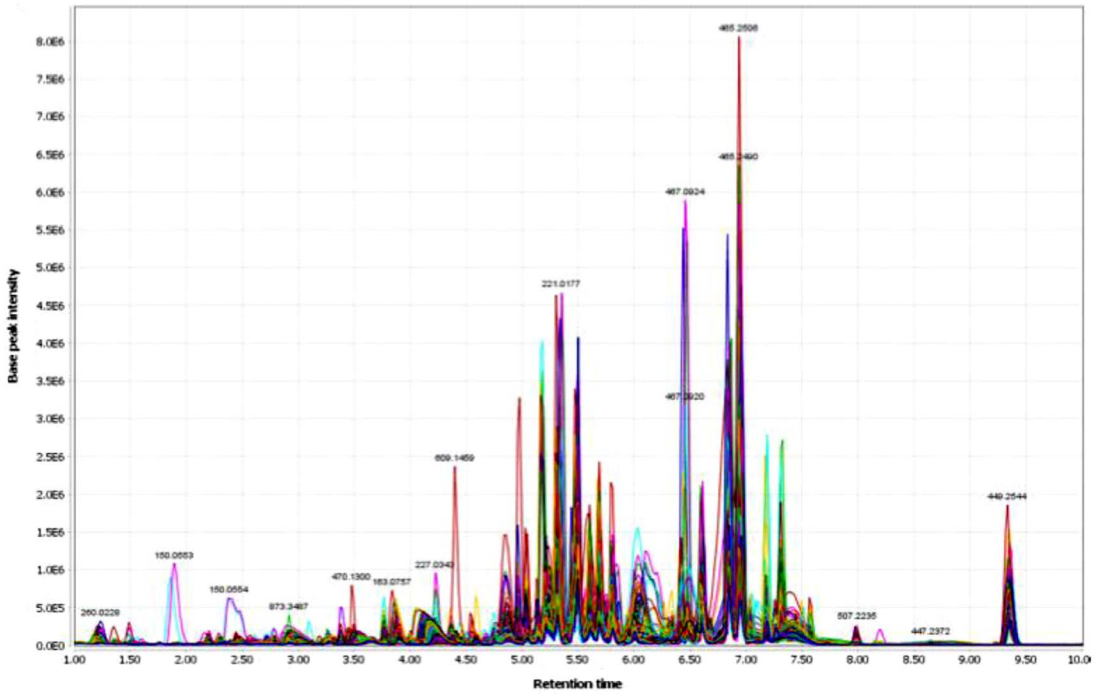

Fig. 3. Exemplificative chromatograms obtained from LC-MS metabolomics profiling of urines collected after consumption of GCBE and control samples. Chromatograms were re-built using MZmine software.

fragmentation spectra and by comparison with web databases and literature data. Along with two acyl-carnitine derivatives (O-adipoylcarnitine and decanoylcarnitine), the other two variables were tentatively identified as acetone and vanillic acid. Carnitine derivatives and acetone are related to fatty acids metabolism, while vanillic acid could be related to intestinal catabolism of CGA.

Quantification of allantoin and 8-OHdG in 24 h urine samples

The amounts of the two oxidative stress biomarkers were monitored in controls and in urine samples collected during supplementation with GCBE. Data are summarized in Figs. 7 and 8. Amounts of both the markers were not affected by consumption of GCBE, in fact the concentrations monitored did not significantly change along supplementation period, as well as if compared to controls. The average amount of allantoin in control samples was 25.82 ± 7.08 μg/ml, whereas the average concentration in urine collected during treatment was 26.70 ± 6.50 μg/ml. Similarly, the average concentration of 8-OHdG in controls was 1.37 ± 0.40 ng/ml, whereas in treated was 1.38 ± 0.70 μg/ml. Over all, the results show that a regular consumption of a 400 mg dose of GCBE for 30 days does not affect the oxidative status in humans, despite the high content in CGA (9% w/w).

Discussion

Products containing GCBE are frequently used to manage weight gain and to promote weight loss, due to the claimed activity of CGA on fatty acids and glucose metabolisms (Shimoda et al., 2006), and due to the content of caffeine, a well-known stimulant (Dulloo et al., 1989). On the other hand, although the efficacy of a treatment with isolated CGA on weight loss, induction of fatty acids and glucose metabolisms and lipid re-distribution were reported both in animals and humans (Dulloo et al., 1989), the efficacy of GCBE is still under debate. Furthermore, the mode of action of green coffee extract is not fully

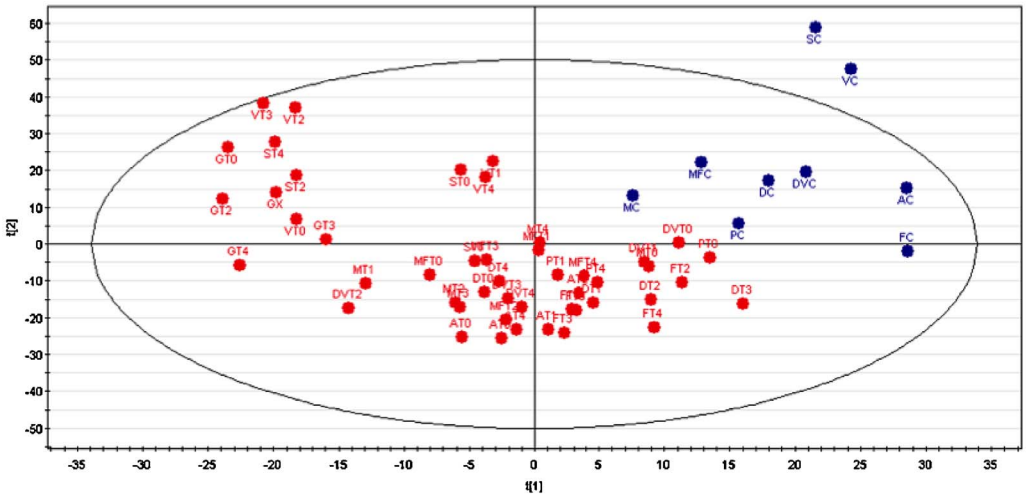

Fig. 4. PLS-DA score plot obtained from UPLC-MS acquired in positive ion mode, using the C-18 stationary phase. Control (blue circles) and treated (red circles) urine samples collected during the experiment are represented in the plot. The acronyms reported in the plot represent sample names. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

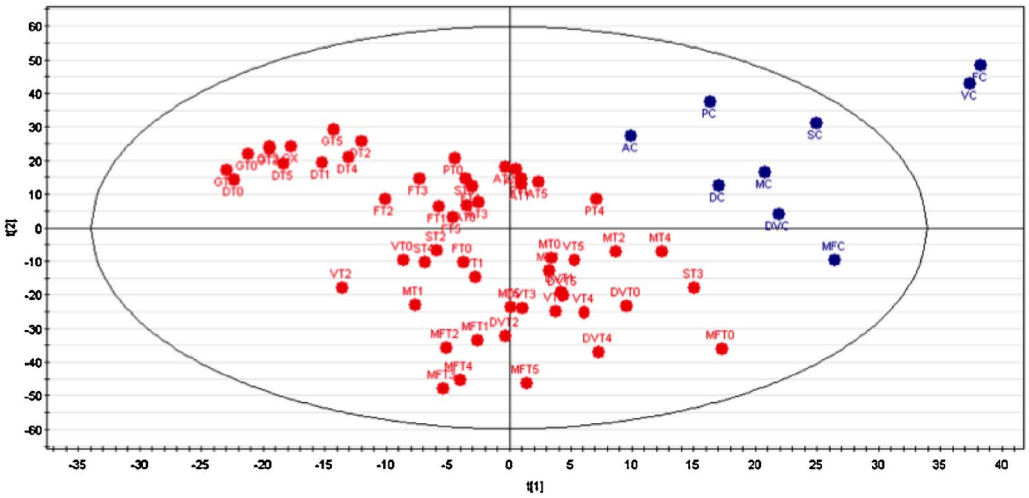

Fig. 5. PLS-DA score plot obtained from UPLC-MS acquired in negative ion mode, using the C-18 stationary phase. Control (blue circles) and treated (red circles) urine samples collected during the experiment are represented in the plot. The acronyms reported in the plot represent sample names. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

understood and the molecular targets that have been up to now discovered for CGA can be considered multiple. For example, CGA derivatives were studied for their antioxidants and antibacterial activities and for their interaction with lipid and glucose metabolism (Arion et al., 1997; Sudeep et al., 2016; Cho et al., 2010; Li et al., 2009; Liang and Kitts, 2015; Liu et al., 2015; Meng et al., 2013; Brower, 1998; Huang et al., 2016, 2015). Metabolomics may offer the opportunity to study natural bioactive compounds, such as the green coffee extracts, with a new holistic approach aimed to assess the global metabolite composition of specific biofluids (Wolfender et al., 2013).

Hence, in this study we monitored the effects of a 30 days supplementation with an oral dose of 400 mg of dry GCBE (containing 9% w/w CGA and 0.5% w/w caffeine) in healthy non-obese volunteers, in order to mimic supplement consumption by healthy consumers. To perform the study, volunteers collected 24 h urine once a week during treatment, and these samples were finally analyzed using a metabolomics approach. Among variables significantly ($p < .01$) describing treatment with GCBE, none of them was identified as intact CGA; on the other hand, other variables, whose amounts were increased in urines during supplementation, were tentatively identified as benzoic acid, hippuric acid, 4 hydroxybenzoic acid, quinic acid, dihydrocaffeic acid sulfate, isoferulic acid, *p*-coumaric acid, 3(3 hydroxyphenyl)propanoic acid, 4 hydroxyphenylacetic acid and vanillic acid. Benzoic acid derivatives and hippuric acid are ascribable to intestinal and microbial degradation of CGAs and other polyphenols (Olthof et al., 2003; Krupp et al., 2012), and produced metabolites are mainly as 4 hydroxyphenylacetic acid (Gao et al., 2006). Also quinic acid, dihydrocaffeic acid sulfate, isoferulic acid, p-coumaric acid, 3(3 hydroxyphenyl)propanoic acid and vanillic acid can be considered as degradation products of CGA coming from catabolism by gut microbiota (Del Rio et al., 2010; Farah et al., 2008; Konishi and Kobayashi, 2004). Overall, the results of our study showed that CGA from dry GCBE were absorbed by intestine, but they were extensively subjected to microbial catabolism and subsequent phase II metabolism (as observed for dihydrocaffeic acid, that

Table 3
Significant urinary variables (p-value < .01) related to consumption of GCBE. Beside Retention Time (R.T., min), experimental HR *m/z* values and tentative identification, variations of the urinary amounts of each metabolite in treated urines (T) compared to controls (C) are reported.

	Amount T *vs* C	VIP	R.T. (min)	HR *m/z*	Tentative identification (adduct ion type)	Database ID
Positive	T > C	8.80	5.42	312.2177	2-trans,4-cis-Decadienoylcarnitine	HMDB13325
	T > C	6.30	3.60	123.0444	Benzoic acid[a]	HMDB01870
	T > C	6.20	5.09	286.2020	2-Octenoylcarnitine	HMDB13324
	T > C	4.50	3.60	202.0479	Hippuric acid ($[M+Na]^+$)	HMDB00714
	T > C	4.20	6.06	314.2332	9-Decenoylcarnitine	HMDB13205
	T > C	4.10	5.86	326.1968	3-Hydroxyoctanoylcarnitine ($[M+Na]^+$)	HMDB61634
	T > C	3.00	2.71	180.0884	3-Hydroxyadipic acid ($[M+NH_4]^+$)	HMDB00345
	T > C	2.70	6.24	338.2329	Decanoylcarnitine ($[M+Na]^+$)	HMDB00651
	T > C	2.50	4.42	139.0393	4-Hydroxybenzoic acid	HMDB00500
	T > C	2.40	5.27	330.2277	6-Keto-decanoylcarnitine	HMDB13202
	T > C	2.20	4.68	274.2018	Heptanoylcarnitine	HMDB13238
	T > C	2.10	2.95	195.0656	Isoferulic acid	HMDB00955
	T > C	2.00	5.73	288.2171	L-Octanoylcarnitine	HMDB00791
	T > C	1.90	5.52	444.3101	Gamma-linolenyl carnitine ($[M+Na]^+$)	HMDB06318
	T > C	1.80	4.55	302.1425	Hydroxyvalerylcarnitine ($[M+K]^+$)	HMDB13132
	T > C	1.70	5.07	374.2537	Dodecanedioylcarnitine	HMDB13327
	T > C	1.65	4.19	184.0969	3-(3-Hydroxyphenyl)propanoic acid ($[M+NH_4]^+$)	HMDB00375
	T > C	1.58	5.72	229.0502	3-Hydroxysuberic acid ($[M+K]^+$)	HMDB00325
	T > C	1.55	3.82	182.0808	*p*-Coumaric acid ($[M+NH_4]^+$)	HMDB41592
	T > C	1.50	4.36	330.2275	6-Keto-decanoylcarnitine	HMDB13202
	T > C	1.45	3.40	328.1588	3-Hydroxyhexanoyl carnitine	HMDB61633
	T > C	1.40	2.94	153.0549	4-Hydroxyphenylacetic acid	HMDB60390
Negative	T > C	2.80	4.25	227.0340	Quinic acid ($[M+Cl]^-$)	HMDB03072
	T > C	2.60	6.04	272.1290	Glutaconylcarnitine	HMDB13129
	T > C	2.32	3.43	207.0663	Dihydrosinapic acid ($[M\text{-}H_20\text{-}H]^-$)	HMDB41727
	T > C	1.85	3.96	261.0064	Dihydrocaffeic acid sulfate	HMDB41721

[a] Identification was confirmed comparing with reference compounds.

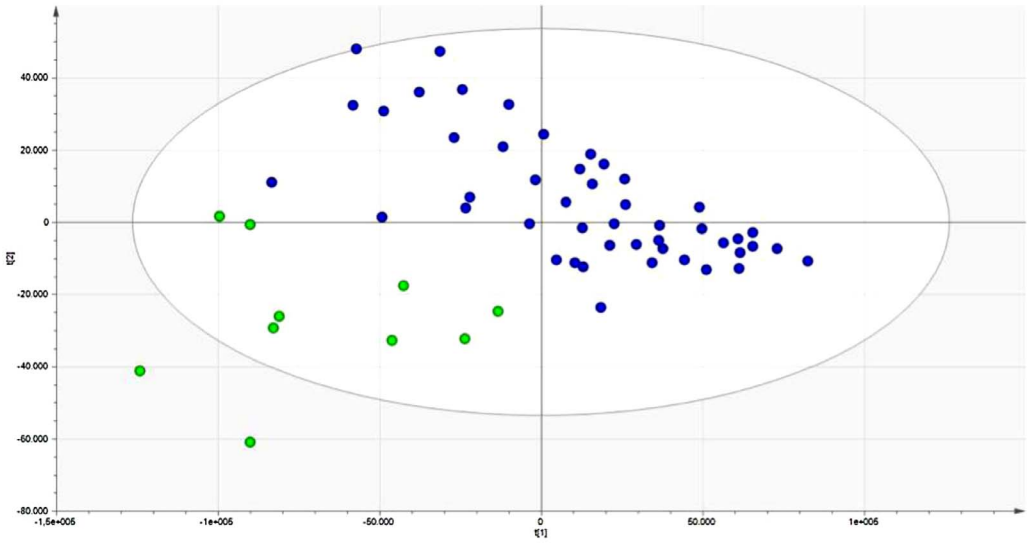

Fig. 6. PLS-DA score plot obtained from HPLC-MS untargeted analysis of urines, using the C-3 stationary phase in positive ion mode. The plot was obtained using SIMCA 13 software. Control (green circles) and treated (blue circles) urine samples collected during the experiment are represented.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Significant urinary variables (p-value < .01) related to consumption of GCBE, detected by LC-MS metbolomics, using a C-3 stationary phase. Beside Retention Time (R.T., min), experimental *m/z* values and tentative identification, variations of the urinary amounts of each metabolite in treated urines (T) compared to controls (C) are reported.

Amount T *vs* C	VIP	R.T. (min)	*m/z*	Tentative identification	Database ID
T > C	3.50	7.26	76.18	Acetone[a]	HMDB00714
T > C	3.12	17.87	312.48	*O*-Adipoylcarnitine	HMDB13325
T > C	2.40	18.98	354.37	Decanoylcarnitine	HMDB01870
T > C	1.80	15.09	266.34	Vanillic acid	HMDB13324

[a] Identification was confirmed comparing with reference compounds.

was detected as sulfate derivative), prior to be excreted with urine. This observation is in agreement with results previously published by other authors, whose reported that, after ingestion of coffee containing 3.395 μmol of caffeoylquinic acids (CQA), only CGA metabolites could be found in significant amounts in urine collected 0–24 h after coffee intake (Monteiro et al., 2007).

Other variables significantly describing treatment with GCBE were tentatively identified as endogen metabolites, namely medium-chain (C8-C10) acyl carnitines, two dicarboxylic acids (3 hydroxysuberic acid and 3 hydroxyadipic acid) and acetone. Acyl carnitines are mainly involved in transportation of fatty acids inside the mitochondrion matrix (Sharma and Black, 2009), where they are further catabolized via β-oxidation to produce energy. On the other hand, 3 hydroxy dicarboxylic acids as 3 hydroxysuberic acid and 3 hydroxyadipic acid are derived from the ω-oxidation of 3 hydroxy fatty acids and the subsequent β-oxidation of longer chain 3 hydroxy dicarboxylic acids (Tserng, 1991), while acetone derives from degradation of ketone bodies, whose are produced mainly by 3 hydroxy-3-methylglutaryl-CoA synthase during ketogenesis. Overall, these markers belong to different pathways involved in lipid transportation and metabolism, whose are regulated by receptor PPAR-α, the major regulator of lipid metabolism in the liver (Gervois et al., 2000). Activation of this receptor promotes uptake, utilization, and catabolism of fatty acids by upregulation of genes involved in the transportation, binding and activation of fatty acids, and peroxisomal and mitochondrial fatty acid β-oxidation (Kersten et al., 1999). Purified CGA have been reported to act as agonists of PPAR-α (Meng et al., 2013), but few is known about the activity of the extract of GCBE. Li and coll. examined the effects of isolated CGA on lipid and glucose metabolism in hamsters under a high dietary fat burden, and they studied the role of PPAR-α in these effects (Li et al., 2009). After eight weeks of treatment they observed a reduction of the levels of

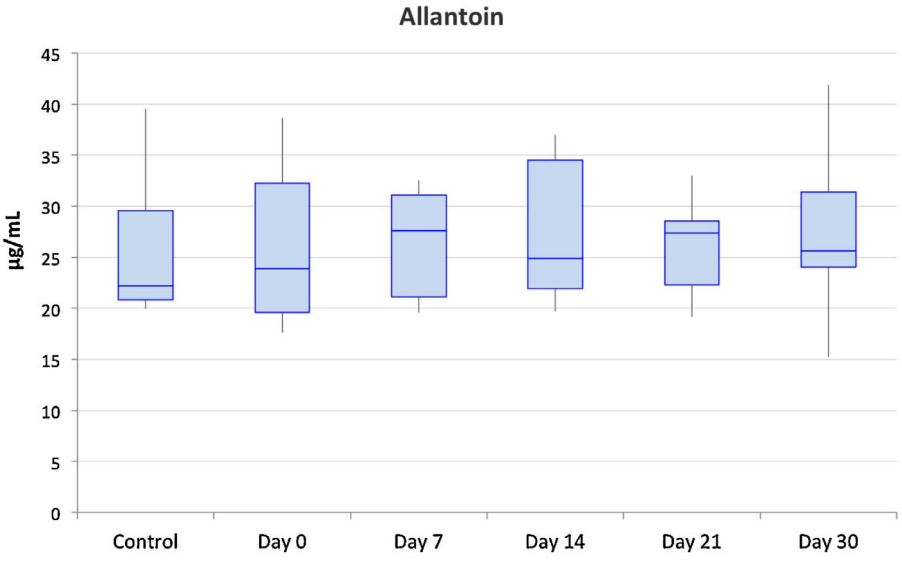

Fig. 7. Allantoin concentration in control urines and during supplementation with GCBE. Day 0 corresponds to the first day of treatment, day 30 to the last.

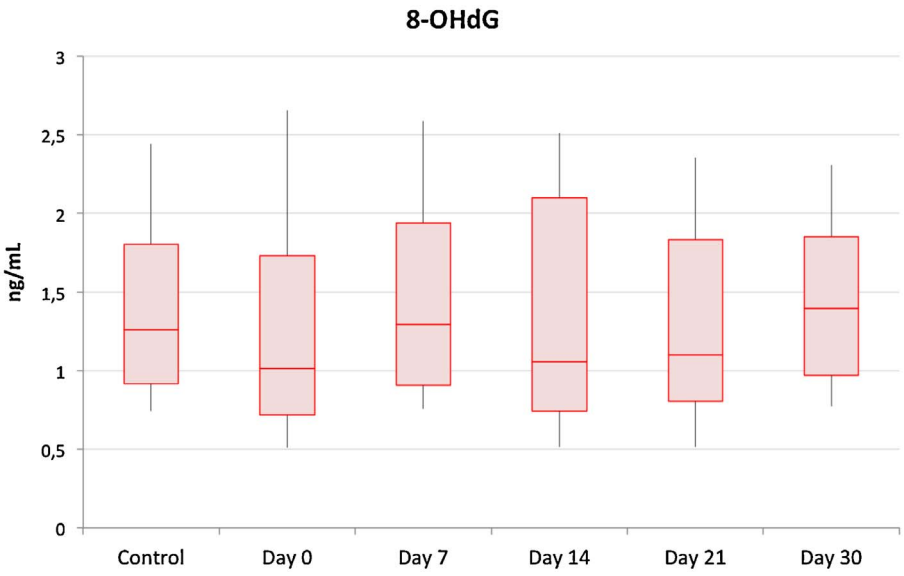

Fig. 8. 8-OHdG concentration in control urines and during supplementation with GCBE. Day 0 corresponds to the first day of treatment, day 30 to the last.

fasting serum triglyceride, free fatty acid, total cholesterol LDL-Cholesterol, HDL-Cholesterol, fasting glucose and insulin. Furthermore, CGA significantly elevated the expression level of mRNA and protein expression in hepatic PPAR-α (Li et al., 2009). Our results showed that, after 30 days consumption of dry GCBE, the amounts of markers related to lipid metabolism in urine were increased, suggesting an induction of fat metabolism, although significant alterations of volunteers body weight were not observed (data not shown). Moreover, these markers were related to different pathways of fatty acids metabolism regulated by PPAR-α, so we postulate that the effects of GCBE could be related to the agonist activity exerted by CGA and CGA-derived metabolites on this receptor. This hypothesis is consistent with previously published data. In fact, 5-CQA was shown to up-regulate PPAR-α mRNA expression in the liver of rats in a dose-dependent manner (Huang et al., 2015).

Due to its chemical composition, green coffee is considered a rich natural source of antioxidants, with CGA being the most active compound (Babova et al., 2016). Previous published studies assessed the antioxidant properties of CGA in animal models (Liang and Kitts, 2015) and of green coffee extracts *in vitro* (Liang and Kitts, 2015; Babova et al., 2016). In our study, we monitored the levels of two urinary markers of oxidative stress, namely allantoin and 8-OHdG. Allantoin is normally present in urine and is formed from uric acid through reactions with oxidative species, so its urinary amounts are increased with increasing oxidative stress (Tolun et al., 2010). On the other hand, 8-OHdG is one of the predominant forms of free radical-induced lesions of DNA. Because oxidative DNA lesion products are reasonably water soluble and excreted into the urine without being further metabolized, urinary 8-OHdG is considered a significant biomarker of oxidative stress (Cooke et al., 2002; Nakajima et al., 2012). Our results showed that the levels of both the markers were not affected by GCBE consumption and this finding suggest that in healthy subject the supplementation of green coffee is not changing levels of urinary markers of oxidative stress.

In conclusion, in this work urine samples of human volunteers collected during 30 days of consumption of 400 mg of GCBE (containing 4.9% of 5-CQA, 0.5 % of caffeine and 0.3% trigonelline) were studied by metabolomics approach. Markers related to treatment were assigned to metabolites belonging to the pathways of fatty acid metabolism, showing an influence of green coffee extract on lipid metabolism. However, during the period of the experiment, the weight of volunteers did not change. Overall, these results could be considered, at least in part, as a further proof of the mode of action of GCBE, and they show that metabolomics-based studies offer new opportunities as new tools for the study of phytochemicals.

Conflict of interest

The authors confirm that there are no conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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