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Metabolic fingerprint after acute and under sustained consumption of a functional beverage based on grape skin extract in healthy human subjects†

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Grape-derived polyphenols are considered to be one of the most promising ingredients for functional foods due to their health-promoting activities. We applied a HPLC-MS-based untargeted metabolomic approach in order to evaluate the impact of a functional food based on grape skin polyphenols on the urinary metabolome of healthy subjects. Thirty-one volunteers participated in two dietary crossover randomized intervention studies: with a single-dose intake (187 mL) and with a 15-day sustained consumption (twice per day, 187 mL per day in total) of a functional beverage (FB). Postprandial (4-hour) and 24-hour urine samples collected after acute consumption and on the last day of sustained FB consumption, respectively, were analysed using an untargeted HPLC-qTOF-MS approach. Multivariate modelling with subsequent application of an S-plot revealed differential mass features related to acute and prolonged consumption of FB. More than half of the mass features were shared between the two types of samples, among which several phase II metabolites of grape-derived polyphenols were identified at confidence level II. Prolonged consumption of FB was specifically reflected in urine metabolome by the presence of first-stage microbial metabolites of flavanols: hydroxyvaleric acid and hydroxyvalerolactone derivatives. Overall, several epicatechin and phenolic acid metabolites both of tissular and microbiota origin were the most representative markers of FB consumption. To our knowledge, this is one of the first studies where an untargeted LC-MS metabolomic approach has been applied in nutrition research on a grape-derived FB.

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Introduction

Plant phytochemicals are considered to be one of the most important health-promoting compounds present in our diet.[1] Therefore, due to their strong bioactive potential, it is proposed that phytochemical extracts from fruits and vegetables serve as a principal component in a variety of nutraceuticals, *e.g.* in functional foods.[2] Over the recent decades, the concept of functional food, food designed to allow consumers to eat enriched foods close to their natural state,[2] has attracted much interest and popularity, since it was proposed as a novel therapeutic approach to prevent or attenuate diet-related diseases such as obesity, diabetes, atherosclerosis, and neurodegeneration.[3]

Grape and grape-derived food products are well-known sources of bioactive compounds, which are mainly represented by a wide range of polyphenols, and, therefore, they are among the most commonly used in the development of functional foods.[4,5] In general, grape and grape-derived products are some of the most important natural sources of polyphenols in our diet, and their dietary consumption is directly related to various beneficial health effects.[6] Grape skin, being an edible part of the berry, has an important antioxidant capacity due to its high polyphenol content, among which the most abundant are flavonoids, anthocyanins, proanthocyanidins, procyanidins, phenolic acids and stilbenes.[6,7] Consequently, extracts from grape skin are first-choice candidates for the develop-

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ment of functional foods and nutraceuticals to be used as an alternative treatment in the management of several diet-related diseases.[4,5,8]

Current progress in the development and implementation of omics' technologies has opened new perspectives in nutrition research,[9] *e.g.* food metabolome analysis,[10] and, consequently, they have become widely applied in nutraceutical investigation.[11] Today, metabolomics is being applied successfully in quality control,[12] in metabolism evaluation[13,14] and also in the assessment of the beneficial effects of functional foods.[11,15] In the latest perspective, untargeted metabolomics is foreseen as one of the most promising approaches for understanding the complex relationship between diet and human health due to its potential for data-driven exploration.[16] Recently, in a couple of studies, an untargeted metabolomic approach was applied in the evaluation of the impact of grape-derived food and extract consumption on human urinary metabolome using NMR technology alone[17] or in combination with GC-MS and LC-MS/MS approaches.[14,18] The results from these studies suggested that the metabolic fate of grape-derived polyphenols and other bioactive compounds could be clearly perceived by examining overall urinary metabolome profiles.[14,18,19] However, due to the limitations of the NMR technical approach, mainly related to its relatively low sensitivity (μmol–mmol range),[20] just a few compounds could be identified and related to the dietary consumption of grape-derived products.[14,17] Another methodological approach extensively used in untargeted metabolomics, mass spectrometry coupled with liquid chromatography (LC-MS), offers highly sensitive, reproducible and quasi-quantitative metabolite analyses.[20,21] Therefore, this technique has become widely applied in nutritional research.[22] However, so far, no studies using an LC-MS untargeted metabolomic approach have been performed in human intervention studies with grape-derived products or functional foods.

The objective of the present study was to elucidate the impact of acute and sustained consumption of a functional beverage based on grape skin extracts on the urinary metabolome (*i.e.* food metabolome) of healthy subjects by applying an untargeted HPLC-MS metabolomic approach.

Experimental

Chemicals and reagents

The following chemicals were obtained commercially: L-phenylalanine, 1-methyl-L-histidine, L-tryptophan, 20-deoxyguanosine, glycochenodeoxycholic acid, (−)-epicatechin and procyanidin B2 (Sigma-Aldrich, St Louis, MO), 4-hydroxyhippuric acid (PhytoLab GmbH & Co., KG) and naringenin (Extrasynthèse, Genay, France). HPLC-grade acetonitrile and formic acid were purchased from Scharlau Chemie S.A. (Barcelona, Spain). Ultrapure water (Milli-Q) was obtained from a Milli-Q Gradient A10 system (Millipore, Bedford, MA).

Human intervention study

Thirty-one healthy adult volunteers (15 men and 16 women, aged 25–55 years) participated in a double-blind, placebo-controlled, randomized crossover intervention study with a drink containing grape skin extract. The study protocol was approved by the Ethics Committee of the Hospital Universitario La Paz, Madrid. The detailed study design was reported earlier.[23] Briefly, one week prior to and during the 46 days of the study the participants maintained a low-polyphenol diet. For the acute study, after a seven-day washout period, the subjects consumed 187 mL of the FB (functional beverage), containing a grape skin extract, or the CB (control beverage), as a placebo, in randomized order together with a standard breakfast consisting of white bread, cheese and yoghurt. After that, they consumed the corresponding beverage twice a day (at lunch and dinner), 187 mL per day in total, for the next 15 days. The same procedure was repeated after a second seven-day washout period according to the crossover design.[23] On the last day of each washout and intervention period, 24-hour urine samples were collected from volunteers. For the acute study, urine samples collected during the first four postprandial hours were used. All urine samples were stored in aliquots at −80 °C prior to analysis.

Both the placebo (CB) and the intervention (FB) beverages had the same macronutrient composition of carbohydrates (sugars), 10 g L^{-1}, and energy, 40 kcal L^{-1}. The FB contained a grape extract preparation obtained from red grape skins using alcohol extraction under different temperature conditions. Therefore, due to the grape skin extract component, the FB contained hydroxycinnamic (4280 μg L^{-1}) and hydroxybenzoic (83 mg L^{-1}) acids, anthocyanins (16 mg L^{-1}), flavanols (96 mg L^{-1}) and stilbenes (5.7 mg L^{-1}), whereas the CB contained no polyphenols.[23] The FB had a total polyphenol index (I_{280}) of 116 (arbitrary units), which is much higher than an average wine index.[24]

LC-MS metabolomics analysis

The sample preparation was conducted according to a previously published methodology.[25–27] HPLC-MS analysis was performed using an Agilent 1200 Series Rapid Resolution HPLC system coupled to a hybrid quadrupole TOF QSTAR Elite (Applied Biosystems/MDS Sciex) according to the protocol for untargeted metabolomic analysis established in our group.[25–27] Thus, chromatographic separation was performed using a RP 18 Luna 5 μm, 50 × 2.0 mm column (Phenomenex, Torrance, CA) in mobile phases consisted of (A) 0.1% HCOOH and (B) ACN 0.1% HCOOH, at a flow rate of 600 μL min^{-1} with the following linear gradient elution (t, %B): (0, 1), (4, 20), (6, 95), (7.5, 95), (8, 1), (12, 1). q-TOF spray parameters were set as previously described[27] and full data acquisition was performed in the scan range 70–700 *m*/*z* in positive ionization mode. LC-MS data were acquired in three successive equilibrated batches. In addition, samples and sequences of injections were randomized in order to avoid possible bias. Four types of quality control (QC) were analysed along with samples

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in each batch in order to monitor the stability and functionality of the system: QC1 – Milli-Q water; QC2 – 5 μg mL^{-1} of exogenous standard mixture consisting of 4-hydroxyhippuric acid, epicatechin, procyanidin B and naringenin; QC3 – 5 μg mL^{-1} of endogenous standard mixture consisting of methyl-L-histidine, deoxyguanosine, glycochenodeoxycholic acid, phenylalanine and tryptophan; and QC4 – about 10% of all samples were reinjected within the same batch. The QC data were used in order to monitor the system's stability and functionality, guaranteeing the reliability of the acquired data.[25,27] Raw LC-MS data are deposited at the laboratory of Biomarkers & Nutrimetabolomic Research Group, Nutrition & Food Science Department, Pharmacy School, University of Barcelona, and can be available upon request.

Data preprocessing

LC-MS raw data were processed using MarkerView™ 1.2.1 software (Applied Biosystems, MDS Sciex, Toronto, Ontario, Canada). Peak detection was performed using a minimum spectral peak width of 1 ppm, a minimum RT peak width of 3 scans, a noise threshold of 5 and a subtraction multiple factor of 1.5. The retention time tolerance was 0.1 min and the mass tolerance was 0.05 Da.

Multivariate analysis (MVA) of data

The MVA was performed using SIMCA-P+ 13.0 software (Umetrics, Umea, Sweden). The data set was log-transformed and Pareto-scaled before unsupervised principal component analysis (PCA), and supervised statistical modelling by simple and orthogonal partial least squares discriminant analysis (PLS-DA and OPLS-DA, respectively). The PCA was used to gain an overview of the quality of the data acquisition step, while the PLS-DAs were applied for the exploration of the differences in metabolomes among the sample groups. OPLS separates the system variation in X (LC-MS data) into two parts, one that is linearly correlated (predictive) and one that is uncorrelated (orthogonal) to Y. This separation facilitates the model interpretation as the variation between classes that can be interpreted from the predictive component, and the variation within classes can be interpreted from the orthogonal component.[28] The type of beverage (CB *vs.* FB) and time of urine collection (washout *versus* intervention) were used as categorical Y variables in both the PLS-DA and OPLS-DA. The quality of the supervised models was evaluated by the goodness-of-fit parameter (R^2X), the proportion of the variance of the response variable that is explained by the model (R^2Y_{cum}) and the predictive ability parameter ($Q_{cum}{}^2$), which was calculated by a seven-round internal cross-validation of the data using a default option of the SIMCA-P+ 13.0 software. The OPLS-DA models were tested for overfitting with an ANOVA based on the cross-validated predictive residuals (CV-ANOVA)[29] using SIMCA software, while the PLS-DA model was tested by the permutation test (n = 200).[30]

Potential markers of interest were extracted from the S-plot, a scatter plot that graphically combined the covariance and correlation loading profiles resulting from the constructed OPLS models.[31] Pairwise discrimination between the FB and CB classes for acute and sustained chronic consumption of beverages was performed by two distinct S-plots and differential features were chosen based on their correlations in each data set. Only mass features showing correlation coefficients ($|p(\text{corr})|$) in both S-plots of ≥0.5[27] were included in the list of candidate markers explaining the separation between FB and CB interventions.

Metabolite identification

Markers contributing to the discrimination between diets were identified through a multiple-step procedure.[26,32] First, a two-way hierarchical clustering analysis using the Pearson distance and Ward's method (PermutMatrix 1.9.3.0 software)[33] was applied to both sets of differential signals obtained from the S-plots. Mass features and urine samples were clustered simultaneously in order to group the observations by their similarities. The mass features corresponding to the same metabolite – protonated molecules, ^{13}C isotopes, adducts and in-source generated fragments – were identified as clusters based on a heat map of intensities and of a correlation matrix.[34] Second, identification of compounds was performed on the basis of their exact mass (±5 mDa of accepted mass difference), which was compared to those registered in an in-house database[35] that mainly focused on the food metabolome, and in freely available external databases: the Human Metabolome Database (HMDB; http://www.hmdb.ca) and the MetLin database.[36] In addition, the in-source fragmentation behaviour of compounds, represented as ion clusters aggregated by two-way HCA, was used as complementary information to identify the metabolites.[37] Finally, the metabolites with the two highest levels of confidence identity, levels I and II (when at least two orthogonal physicochemical properties, such as mass, fragmentation or retention time, corresponded with authentic standards or with public/in-house spectral libraries, respectively),[38] were taken into further consideration. The biological relevance of the identified metabolites was evaluated based on information extracted from earlier published reports accessed through free search engines.

Results

Data acquisition quality

PCA analysis of the three QC classes revealed a clear separation between distinct control samples belonging to QC1, QC2 and QC3 (Fig. 1S(A), ESI†). Neither carry-over nor apparent clustering due to the sequence of injections were detected. No artificial trends took place in the study samples during data acquisition, since QC4 replicates were tightly clustered in the PCA score plots (Fig. 1S(B), ESI†). The quality control results were in agreement with criteria recently proposed for metabolomics studies,[25,27] suggesting that the overall quality of the acquired data was good.

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Analysis of urinary profiles in acute and midterm sustained consumption studies

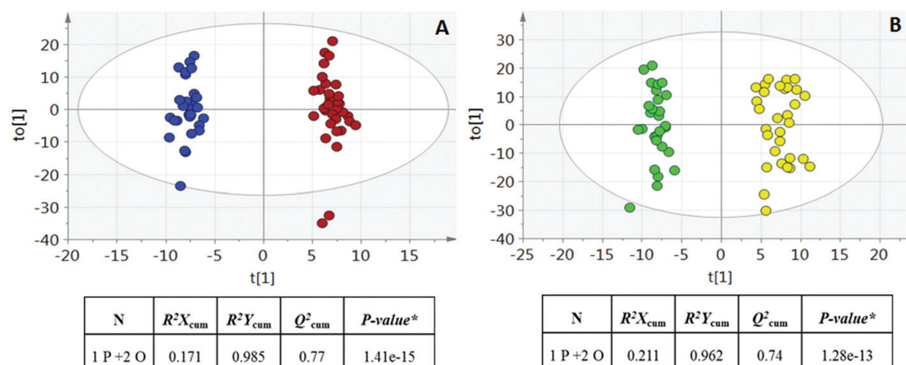
To assess the intervention-dependent changes in the urinary metabolome in both consumption studies, an OPLS-DA using two classes, FB and CB, was applied separately to the acute and sustained studies' data sets. The robust, high-quality 1 + 2 OPLS-DA models were obtained with regard to the separation of samples by the type of beverage consumption for both studies (Fig. 1A and B). Both OPLS-DA models were further applied for metabolite discriminant analysis.

The metabolic profiles of 4-hour postprandial urine samples and 24-hour urine samples collected on the last day of the one-week washout and of the 15-day sustained intervention periods were assessed using PLS-DA modelling. In contrast to both acute and sustained FB consumption, where changes in urinary metabolic profiles were observed, no important alterations in urinary metabolic profiles caused by any intervention with the CB were noticed (Fig. 2S(A), ESI†). The differences in 24-hour urinary metabolome between washout and under sustained FB intervention were similar to those observed in comparative analysis between the FB and CB beverages (Fig. 2S(B), ESI†).

The S-plots[31] associated with the OPLS-DA were generated to reveal the statistically significant metabolites responsible for FB consumption. The differential mass feature selection using a reliability cut-off of $p(\text{corr}) \geq |0.5|$, indicating a significant relation to the consumption of the FB (upper-right quadrants) or CB (lower-left quadrants) beverages, is presented in Fig. 2A and B, for acute and prolonged intervention studies, respectively.

Comparative analysis of metabolome alteration due to acute and sustained interventions

The comparison of metabolome modifications induced by both acute and sustained FB intake was performed and aggregation of m/z features are represented on a Venn diagram (Fig. 3). The mass features positively affected by FB intake ($p(\text{corr}) \geq 0.5$) were classified into three groups: specifically related to acute consumption, specifically related to sustained consumption, and a common group. In general, about 70% of acute consumption markers were also detected in samples from the sustained study, whereas 44% of markers were exclusively related to the prolonged FB consumption in contrast to about 30% for the acute consumption.



N	R^2X_{cum}	R^2Y_{cum}	Q^2_{cum}	*P-value**
1 P +2 O	0.171	0.985	0.77	1.41e-15

N	R^2X_{cum}	R^2Y_{cum}	Q^2_{cum}	*P-value**
1 P +2 O	0.211	0.962	0.74	1.28e-13

Fig. 1 Score plots of orthogonal partial least squares discriminant (OPLS-DA) modelling for acute (A) and 15-day sustained (B) intervention studies with control (samples marked as blue and green cycles, respectively) and functional (samples marked as red and yellow cycles, respectively) beverages. N – number of components in OPLS-DA modelling; O – orthogonal component; P – predictive component; R^2X_{cum} and R^2Y_{cum} are the cumulative modelled variation in the X and Y matrix, respectively; $Q_{cum}{}^2$ is the cumulative predicted variation in the Y matrix; the P-value from CV-ANOVA, based on the cross-validated predictive residuals, is listed for both models (significant result indicates a valid model).

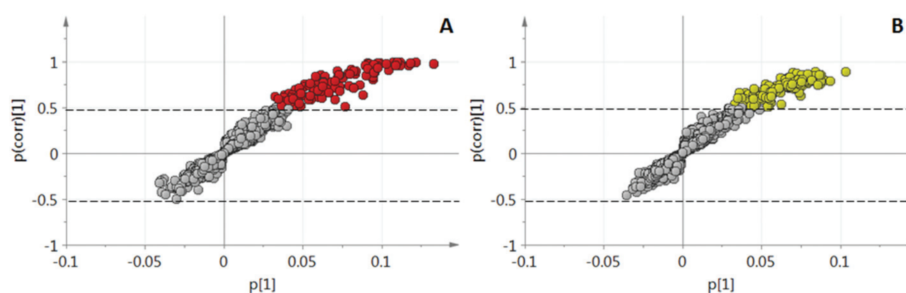


Fig. 2 S-plots associated with OPLS-DA score plots for data obtained from urine LC-MS analysis for acute (A) and sustained (B) intervention studies on the consumption of both (FB and CB) beverages. Dashed lines represent $p(\text{corr}) \geq |0.5|$ cut-offs used for the selection of putative markers coloured in red and yellow for acute and sustained consumptions, respectively.

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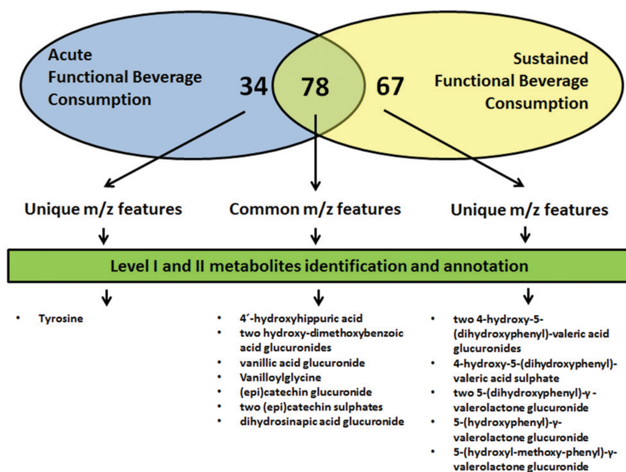

Fig. 3 Diagram showing the overlap among the *m/z* features and those metabolites which were identified and annotated according to the levels I and II of the confidence of identity[38] in both acute and sustained intervention studies with functional beverage (FB). The corresponding listed metabolites' mass features are presented in Table 1.

Characterization and identification of FB consumption biomarkers

A number of differential compounds unique and common to acute and sustained consumption studies detected as clusters composed of different kinds of ions were identified. Eighteen metabolites, identified at least with confidence level II, are summarized in Table 1 according to their main characteristics: retention time, detected mass and postulated identification. The identity of 4-hydroxyhippuric acid was confirmed comparing its mass-chromatographic behaviour with the authentic standard present in QC2 (Fig. 3S, ESI†). The rest of the metabolites were annotated at confidence level II. All eighteen metabolites from both studies had a higher relative abundance in the samples related to the consumption of the FB. These metabolites were considered in relation to their metabolic origin: as host (represented principally by phase II metabolites of polyphenols originally present in the grape skin) and microbial (compounds derived from grape polyphenols due to the catabolic activities of intestinal microbiota). With regard to the differences in metabolic profiles observed between acute and prolonged consumption of the FB, the sustained consumption was clearly associated with the presence of metabolites of an exclusively microbial origin.[27] On the other hand, only one postprandial-specific metabolite was identified, while other metabolites were common for both sample types (Table 1).

Discussion

The current study shows that acute and 15-day sustained consumption of a FB based on grape skin extracts produces significant changes in urinary metabolome. Using an LC-MS-based untargeted approach with subsequent multivariate analysis, we identified a range of metabolites that are chiefly related to the metabolism of grape skin polyphenols, a principal active component of the tested FB.

Metabolite 1 (Table 1) was annotated as tyrosine, based on its LC-MS behaviour. It was the only discriminative metabolite related specifically to acute intervention with an FB. This non-essential amino acid was earlier also described as an important contributor to the 6-hour postprandial urinary metabolome changes provoked by acute consumption of cocoa powder.[27] According to the phytochemical database of Dr Duke (http://www.ars-grin.gov/duke/plants.html), grape berries can contain about 120–617 ppm of tyrosine. Therefore, it could have been extracted along with other polyphenols and consumed within the FB at much more elevated concentrations, thus appearing excreted in 4-hour postprandial urine. Nevertheless, the endogenous origin of the detected tyrosine must also be considered.

Elevated excretion of 4-hydroxyhippuric acid (Table 1, metabolite 2) after consumption of the functional beverage was noticed in both acute and under prolonged consumption of FB. Recently, elevated 4-hydroxyhippurate was detected by untargeted NMR, along with its 3-hydroxyl isoform, in 24-hour urine samples after grape-derived product consumption.[18] Consumption of other polyphenol-rich foods, such as tea[39] and cocoa,[40] was also related to its elevated excretion, which was associated mainly with the microbial metabolism of consumed proanthocyanidins and flavanols. On the other hand, whether directly from the grape skin[41] or released from pelargonidin,[42] 4-hydroxybenzoic acid may directly undergo conjugation with glycine in the liver under its absorption in the small intestine,[43] forming 4-hydroxyhippuric acid. Thus, this may explain its postprandial appearance after FB consumption. Therefore, we relate 24-hour excretion of 4-hydroxyhippuric acid under chronic consumption to both *p*-hydroxybenzoic glycination and microbial catabolic activities.

Common to acute and sustained consumption of FB, metabolites 3 and 6 (Table 1) were annotated as glucuronides of hydroxy-dimethoxybenzoic acid based on their LC-MS behaviour. Their mass fragmentation patterns suggest to us that they are conjugates of syringic and dimethylgallic acids, however no specific assignment could be made due to the lack of standards. Syringic acids could directly derive from the grape extract due to their relative abundance in the berry skin,[44] or could originate in the body as microbiota catabolic products[45] derived from corresponding *O*-methylated flavonoid syringetin and anthocyanidin malvidin, normally abundant in red grapes.[46] Therefore, syringic acid may be glucuroconjugated directly upon absorption or later after its release by microbiota, and thus may be available in the body during the first postprandial hours and over a prolonged time after ingestion. Earlier, syringic acid was reported to be elevated after sustained consumption of grape-derived food.[14,18,47] With regard to dimethylgallic acid, very little information is available on this metabolite in relation to polyphenol metabolism. Thus, the 3,4-dimethyl derivative of gallic acid was proposed as a putative marker of black tea consumption,[48] in contrast to its

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Table 1 Metabolites which are positively correlated with acute and sustained consumption of a functional beverage (FB)

Metabolite cluster	RT (min)	Detected mass (m/z^+)	Assignation	Identification	Elemental formula	Level[a] of identification	Biological relevance to intervention	Acute	Sustained
1	0.47	182.0833	$[M + H]^+$	Tyrosine	$C_9H_{11}NO_3$	II	FB or endogenously derived tyrosine	↑	—
2	2.87	196.0598 121.0274 122.0297	$[M + H]^+$ $[M + H\text{-}Glyc]^+$ $^{13}C[M + H\text{-}Glyc]^+$	4-Hydroxyhippuric acid	$C_9H_9NO_4$	I	Hydroxybenzoic acid and/or microbial polyphenol metabolism	↑	↑
3	3.15	392.1206 199.0618 155.0687	$[M + NH_4]^+$ $[M + H\text{-}Gluc]^+$ $[M + H\text{-}Gluc\text{-}CO_2]^+$	Hydroxy-dimethoxybenzoic acid glucuronide[b]	$C_{15}H_{18}O_{11}$	II	Hydroxybenzoic acid and/or microbial polyphenol metabolism	↑	↑
4	3.28	345.0833 362.1113 169.0499 125.0585	$[M + H]^+$ $[M + NH_4]^+$ $[M + H\text{-}Gluc]^+$ $[M + H\text{-}Gluc\text{-}CO_2]^+$	Vanillic acid glucuronide	$C_{14}H_{16}O_{10}$	II	Phenolic acid and polyphenol microbial metabolism	↑	↑
5	3.52	226.0727 227.0753 151.042 123.0439	$[M + H]^+$ $^{13}C[M + H]^+$ $[M + H\text{-}Glyc]^+$ $[M + H\text{-}Glyc\text{-}CO]^+$	Vanilloylglycine	$C_{10}H_{11}NO_5$	II	Phenolic acid and polyphenol microbial metabolism	↑	↑
6	3.62	375.0973 392.1247 199.0624 155.0691 140.0481	$[M + H]^+$ $[M + NH_4]^+$ $[M + H\text{-}Gluc]^+$ $[M + H\text{-}Gluc\text{-}CO_2]^+$ $[M + H\text{-}Gluc\text{-}CO_2\text{-}CH_3]^+$	Hydroxy-dimethoxybenzoic acid glucuronide[b]	$C_{15}H_{18}O_{11}$	II	Hydroxybenzoic acid and/or microbial polyphenol metabolism	↑	↑
7	3.72	420.1501 209.081	$[M + NH_4]^+$ $[M + H\text{-}Gluc\text{-}H_2O]^+$	4-Hydroxy-5-(dihydroxyphenyl)-valeric acid glucuronide	$C_{17}H_{22}O_{11}$	II	Microbial metabolism of flavan-3-ols	—	↑
8	3.88	403.125 420.1523 209.0812 191.0706 149.0561	$[M + H]^+$ $[M + NH_4]^+$ $[M + H\text{-}Gluc\text{-}H_2O]^+$ $[M + H\text{-}Gluc\text{-}H_2O\text{-}H_2O]^+$ $[M + H\text{-}Gluc\text{-}H_2O\text{-}H_2O\text{-}C_2H_2O]^+$	4-Hydroxy-5-(dihydroxyphenyl)-valeric acid glucuronide	$C_{17}H_{22}O_{11}$	II	Microbial metabolism of flavan-3-ols	—	↑
9	4.37	385.1151 407.0959 402.1401 403.1444 209.0819 210.0839 191.0707	$[M + H]^+$ $[M + Na]^+$ $[M + NH_4]^+$ $^{13}C[M + NH_4]^+$ $[M + H\text{-}Gluc]^+$ $^{13}C[M + H\text{-}Gluc]^+$ $[M + H\text{-}Gluc\text{-}H_2O]^+$	5-(Dihydroxyphenyl)-γ-valerolactone glucuronide	$C_{17}H_{20}O_{10}$	II	Microbial metabolism of flavan-3-ols	—	↑
10	4.57	385.113 386.1153 407.0927 402.1382 403.1403 367.1009 209.0813 210.083 191.0698 192.0723	$[M + H]^+$ $^{13}C[M + H]^+$ $[M + Na]^+$ $[M + NH_4]^+$ $^{13}C[M + NH_4]^+$ $[M + H\text{-}H_2O]^+$ $[M + H\text{-}Gluc]^+$ $^{13}C[M + H\text{-}Gluc]^+$ $[M + H\text{-}Gluc\text{-}H_2O]^+$ $^{13}C[M + H\text{-}Gluc\text{-}H_2O]^+$	5-(Dihydroxyphenyl)-γ-valerolactone glucuronide	$C_{17}H_{20}O_{10}$	II	Microbial metabolism of flavan-3-ols	—	↑

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Table 1 *(Contd.)*

Metabolite cluster	RT (min)	Detected mass (m/z^+)	Assignation	Identification	Elemental formula	Level[a] of identification	Biological relevance to intervention	Acute	Sustained
		149.0581	$[M + H\text{-}Gluc\text{-}C_2H_3O_2]^+$						
		131.0468	$[M + H\text{-}Gluc\text{-}C_2H_3O_2\text{-}H_2O]^+$						
		123.0416	$[M + H\text{-}Gluc\text{-}C_2H_3O_2\text{-}C_2H_2]^+$						
11	4.67	467.1165	$[M + H]^+$	(*epi*)Catechin glucuronide[c]	$C_{21}H_{22}O_{12}$	II	Epicatechin metabolism	↑	↑
		291.0894	$[M + H\text{-}Gluc]^+$						
		139.0393	$[M + H\text{-}Gluc\text{-}C_8H_8O_3{}^c]^+$						
12	4.73	399.1325	$[M + H]^+$	5-(Hydroxy-methoxy-phenyl)-γ-valerolactone glucuronide	$C_{18}H_{22}O_{10}$	II	Microbial metabolism of flavan-3-ols	—	↑
		416.159	$[M + NH_4]^+$						
		417.1613	$^{13}C[M + NH_4]^+$						
		223.0982	$[M + H\text{-}Gluc]^+$						
		224.1004	$^{13}C[M + H\text{-}Gluc]^+$						
		205.087	$[M + H\text{-}Gluc\text{-}H_2O]^+$						
		163.0756	$[M + H\text{-}Gluc\text{-}H_2O\text{-}C_2H_2O]^+$						
13	4.85	369.1195	$[M + H]^+$	5-(Hydroxyphenyl)-γ-valerolactone glucuronide	$C_{17}H_{20}O_9$	II	Microbial metabolism of flavan-3-ols	—	↑
		370.1229	$^{13}C[M + H]^+$						
		351.1092	$[M + H\text{-}H_2O]^+$						
		193.0867	$[M + H\text{-}Gluc]^+$						
		194.0895	$^{13}C[M + H\text{-}Gluc]^+$						
		133.0641	$[M + H\text{-}Gluc\text{-}C_2H_3O_2]^+$						
14	5	307.0513	$[M + H]^+$	4-Hydroxy-5-(dihydroxyphenyl)-valeric acid sulphate	$C_{11}H_{14}O_8S$	II	Microbial metabolism of flavan-3-ols	—	↑
		289.0379	$[M + H\text{-}H_2O]^+$						
15	5.17	223.095	$[M + H\text{-}Gluc]^+$	5′-(Hydroxy-methoxy-phenyl)-γ-valerolactone glucuronide	$C_{12}H_{14}O_4$	II	Microbial metabolism of flavan-3-ols	—	↑
16	5.45	371.043	$[M + H]^+$	(*epi*)Catechin sulphate-2	$C_{15}H_{14}O_9S$	II	Epicatechin metabolism	↑	↑
17	5.9	371.0445	$[M + H]^+$	(*epi*)Catechin sulphate-3	$C_{15}H_{14}O_9S$	II	Epicatechin metabolism	↑	↑
18	6.03	420.1493	$[M + NH_4]^+$	Dihydrosinapic acid glucuronide	$C_{17}H_{22}O_{11}$	II	Phenolic acid and polyphenol microbial metabolism	↑	↑
		227.0904	$[M + H\text{-}Gluc]^+$						
		228.0937	$^{13}C[M + H\text{-}Gluc]^+$						
		155.0709	$[M + H\text{-}Gluc\text{-}C_2H_4COO]^+$						
		140.0471	$[M + H\text{-}Gluc\text{-}C_2H_4COO\text{-}CH_3]^+$						

Gluc – glucuronide residue; Glyc – glycine residue. [a] Levels of identification are reported according to Sumner *et al.*[38] (2007). [b] Either syringic or dimethylgallic acid glucuronide, no specific assignment could be made due to the lack of standards. [c] Fragment of (*epi*)catechin, according to Flamini R.[61] ↑ indicates higher levels after FB consumption in comparison to CB consumption ($p[\text{corr}] \leq 0.05$); — , no changes were observed.

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3,5-dimethyl isoform (syringic acid), suggesting that the two isomers detected in our samples could originate from different ways. However, both of them might also have a common origin related to 3-*O*-methylated gallic acid,[48,49] originating from the corresponding anthocyanin, petunidin, abundant in grape skin[46] or as a minor *O*-methylated metabolite[49] of grape-derived gallic acid. However, due to the limited information available, it is difficult to be conclusive with certainty about the origins of dimethylgallic acid with respect to our intervention.

Metabolites 4 and 5 (Table 1), positively changed in both postprandial and after prolonged FB consumption urine samples, were annotated as vanillic acid glucuronide and vanilloylglycine, respectively, based on their LC-MS characteristics. Recently, vanilloylglycine was detected in 24-hour urine samples after regular dealcoholized red wine consumption.[47] Both compounds were related to the colonic microbiota metabolism of dietary polyphenols, principally of hydroxycinnamates[50,51] and flavan-3-ols,[27,52] also quite abundant in grape berry skins.[44] However, in our study we have already detected these metabolites in 4-hour postprandial urine samples. This could be explained by the fact that vanillic acid was probably already present in the FB polyphenol extract, as was shown for red grape skin.[44] Thus, we hypothesize that on the one hand, both metabolites could be directly related to the FB polyphenol extract, and on the other, they could be related to the microbial metabolism of more complex grape skin polyphenols, therefore being excreted as glucuronidated and glycinated derivatives of vanillic acid in urine samples obtained both after acute and sustained consumption.

Metabolite 18 (Table 1), common to both acute and sustained FB consumption, was annotated as dihydrosinapic acid glucuronide according to its LC-MS behaviour. It is a metabolite of sinapic acid *via* reduction of the double bond in the cinnamic acid side chain.[50,51] Sinapic acid is among the most abundant hydroxycinnamates found in red grapes and derived products,[53] and recently its increase in 24-hour urine samples was related to consumption of dealcoholized red wine.[47] However, no data on the detection of its hydrated metabolite, dihydrosinapic acid, in relation to dietary consumption of polyphenols, have been reported yet. Recently, it was proposed that reduction activity on cinnamates is not restricted only to intestinal microflora activity, as tissular metabolism[54] might also be involved. Therefore, we postulated that dihydrosinapic acid detected in acute samples might also be generated as a phase I metabolite of sinapic acid, and later, upon prolonged ingestion of an FB, appear in urine as a microbial metabolite as well.

Metabolites 11, 16 and 17 (Table 1) were annotated as a glucuronide and two sulphates of epicatechin, respectively, based on their LC-MS behaviour. The increase of epicatechin in urinary host metabolites during both acute and sustained consumption of FB, was obviously related to the intake of flavan-3-ols, which are the most abundant class of flavonoids present in grape and related products.[46,53] In general, epicatechin phase II metabolites are recognized markers of consumption of flavanol-rich food,[26,27,55,56] especially for postprandial stages. Their relation to the consumption of grape and derived food products was recently reported by our group in a targeted metabolomics study on the consumption of dealcoholized wine.[47] Relatively short excretion times of epicatechin phase II metabolites enable their identification in both of our sample types: 4-hour postprandial and 24-hour urine samples. In contrast, the metabolites 7, 8, 14 and metabolites 9, 10, 12, 15 and 13 (Table 1) were identified as principal discriminant metabolites of prolonged FB consumption. Based on their exact masses, retention times and in-source fragmentation patterns, these compounds were annotated as belonging to first-stage flavan-3-ol microbial metabolism[57] derivatives of hydroxy-(dihydroxyphenyl)-valeric acid (two glucuronides and sulphate), and derivatives of dihydroxyphenyl- (two glucuronides), hydroxy-methoxyphenyl- (two glucuronides) and hydroxyphenyl-valerolactones (one glucuronide), respectively. Earlier, various phase II hydroxyvaleric acid and hydroxyvalerolactone derivatives, including those identified in the present study, were also reported to be markers of consumption of flavanol-rich food such as almond skin,[55] cocoa[26,27] and tea.[52,58] Although grapes, especially red ones, are known to be very rich in flavanols, so far no studies have reported on their microbial metabolites in relation to grape polyphenol consumption. Only recently, our group suggested them as markers of chronic grape-derived product consumption (dealcoholized red wine) using targeted LC-MS analysis,[47] and the present study, applying an untargeted approach, has ratified that the phase II metabolites of microbiota-derived hydroxyvaleric acid and hydroxyvalerolactones could serve as principal markers of prolonged grape skin flavanol consumption.

Similarities between urinary metabolome under acute and prolonged intakes of grape skin polyphenols, both as differential mass features (Fig. 2) and identified metabolites (Table 1), were observed. This is in line with the notion that the urine under sustained consumption should also contain compounds related to postprandial metabolism, since the 24-hour urine samples were collected on the last intervention day when the last two doses of the FB had been consumed at lunch and dinner. Differences in the metabolic profiles of the continuous consumption were clearly associated with the presence of metabolites of an exclusively microbial origin (Table 1). According to the study design and analysis, all unique mass features detected under sustained consumption, including unidentified ones, should be related either directly to microbial metabolism or to effects provoked by prolonged grape skin polyphenol consumption.

In contrast to prolonged consumption, we identified only one postprandial-specific metabolite. As we explained earlier, we expected mass features unique to the 4-hour postprandial sample also to be present in 24-hour urine samples, however they could not be detected due to the metabolome-diluting effect. Thus, in this study the acute samples were used for two purposes: (i) to evaluate 4-hour postprandial urinary metabolome after FB intake; and (ii) to contrast those metabolites

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related specifically to 24-hour urine under prolonged grape skin consumption.

The potential health effects of grape polyphenols are of particular interest from the nutraceutical and functional food viewpoints.[4,5,7,8] The mechanisms by which grape polyphenols exert their protective effects are not entirely clear, but they are conditioned by polyphenol bioavailability within the human organism.[53] The detection of polyphenol metabolites in urine following their dietary ingestion approximates their availability and recapitulates their fate in an organism, linking it to their biological effects. Previously, such evaluations were usually studied applying (targeted) analysis that was specifically focused on the main polyphenols or on grape-specific polyphenols.[23,47,59,60] Thus, our previous study characterized in detail the resveratrol metabolism following ingestion of the functional beverage tested in the present study by applying a targeted LC-MS approach.[23] Since, the resveratrol metabolites could not be detected in the present study, probably due to the technical limitation of the applied untargeted approach, the results of the targeted study could be considered as complementary data to our results, confirming the bioavailability of ingested polyphenols. Our study, applying an untargeted LC-MS approach and data-driven analysis, was able to show that after dietary ingestion of grape skin polyphenols in the form of a functional beverage, the main alterations in urinary metabolites were related to polyphenol metabolism. As a result, we were able to assess the specificity of these alterations under sustained consumption with respect to the postprandial period.

Conclusion

In conclusion, our study showed that single and prolonged consumption of a grape skin polyphenol-based FB promoted significant changes in the profiles of urinary low-weight metabolites. To our knowledge, this is the first study to assess the urinary metabolic fingerprint of dietary ingested grape polyphenols applying an untargeted LC-MS approach. Although our study was performed with a limited (for untargeted metabolomics) number of subjects living within close geographical area, the application of the crossover study design and highly sensitive HPLC-qTOF-MS technology enabled us to detect that the principal changes in urinary metabolome were related to the grape skin polyphenol metabolism. Thus, on the one hand, various metabolites of grape skin-derived phenolic acids and epicatechins were mutually identified in 4-hour postprandial and 24-hour urine samples after acute and under prolonged sustained consumption of FB, respectively. These metabolites could be recognized as generic markers of grape skin polyphenol consumption. On the other hand, several first-stage microbial metabolites represented by the phase II metabolites of hydroxyvaleric acid and hydroxyvalerolactone derivatives were also associated with high grape skin flavanol content. They were exclusively present in 24-hour urine samples during sustained consumption of the FB, therefore we would suggest them as principal microbiota-derived markers of prolonged consumption of grape skin polyphenols. However, it should be mentioned that despite the controlled dietary conditions, which were applied in the present study, these metabolites cannot be considered solely as biomarkers of grape skin polyphenol intake since most of them have also been identified after consumption of other sources of flavan-3-ols, such as cocoa, almond,[26,27,55] and tea.[58] Furthermore, we should admit that the use of only positive ionization conditioned our research to discover principally positively ionized markers. This should be reconsidered in future studies, since negative ionization could provide complementary metabolic data. In addition, a multimetabolite marker approach recently introduced in nutrimetabolomics[32] could provide more accurate evaluation of the exposure to grape-derived polyphenols, but this should be specifically tested. Therefore, we would suggest that the health impact of the tested functional beverage consumption, as well as other foods with grape skin-derived components, should be studied considering the complexity of polyphenol metabolism.

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