RESEARCH ARTICLE

The metabolic fate of red wine and grape juice polyphenols in humans assessed by metabolomics

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The metabolic impact of polyphenol-rich red wine and grape juice consumption in humans was studied using a metabolomics approach. Fifty-eight men and women participated in a placebo-controlled, double-crossover study in which they consumed during a period of 4 wk, either a polyphenol-rich 2:1 dry mix of red wine and red grape juice extracts (MIX) or only a red grape juice dry extract (GJX). 24-h urine samples were collected after each intervention. 1H-NMR spectroscopy was applied for global metabolite profiling, while gas chromatography-mass spectrometry (GC-MS) was used for focused profiling of urinary phenolic acids. Urine metabolic profiles after intake of both polyphenol-rich extracts were significantly differentiated from placebo using multilevel partial least squares discriminant analysis (ML-PLS-DA). A significant 35% increase in hippuric acid excretion ($p<0.001$) in urine was measured after the MIX consumption compared with placebo, whereas no change was found after GJX consumption. GC-MS-based metabolomics of urine allowed identification of 18 different phenolic acids, which were significantly elevated following intake of either extract. Syringic acid, 3- and 4-hydroxyhippuric acid and 4-hydroxymandelic acid were the strongest urinary markers for both extracts. MIX and GJX consumption had a slightly different effect on the excreted phenolic acid profile and on endogenous metabolite excretion, possibly reflecting their different polyphenol composition.

Keywords:
Cardiovascular health / Gut microbiota / Flavonoids / Metabolism / Phenolic acids

1 Introduction

Flavonoids (polyphenols) are a major source of antioxidants in our diet and are abundant in fruits, some vegetables, cocoa and in plant-derived beverages like red wine and tea. Epidemiological studies have shown that a polyphenol-rich diet is associated with reduced risk of cardiovascular disease (for review see [1]). Moderate consumption of red wine in particular has been shown to have a positive effect on cardiovascular disease risk, which cannot be merely attributed to its ethanol content [2]. In particular for polyphenol-rich red wine and grape juice [3–9], tea [10, 11] and chocolate [12–16] several intervention studies support the beneficial effects on cardiovascular function-related parameters like vasodilation, blood pressure, insulin resistance and plasma lipids.

The polyphenol composition of red wine and grapes is complex and mainly consists of proanthocyanidins, anthocyanins, flavan-3-ols and phenolic acids. It is increasingly becoming clear that in humans and animals the bioavailability of many dietary polyphenols is low, which is particularly true for proanthocyanidins and anthocyanins present in grape and red wine. Dietary polyphenols undergo extensive phase II biotransformation in the small intestine and liver

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Abbreviations: CMV, cross model validation; **FDR,** false discovery rate; **GAE,** gallic acid equivalent; **GJX,** red grape juice dry extract; **MIX,** polyphenol-rich 2:1 dry mix of red wine and red grape juice extracts; **ML-PLS-DA,** multilevel partial least squares discriminant analysis; **RP,** rank product; **sIRP,** signed logarithm of rank product; **TSP,** 3-(trimethylsilyl)propionic acid-d_4

during which polyphenol aglycones are conjugated to glucuronide, sulphate and/or methyl-moieties to facilitate their elimination from the body. Therefore, the parent polyphenol aglycone compounds have poor bioavailability and thus it is unlikely that they reach sufficiently high concentrations in the peripheral circulation and tissues *in vivo* to be physiologically relevant [17]. Moreover, a major fraction of the dietary polyphenols persists to the colon, where it is extensively degraded by gut microbiota into simpler phenolic compounds. Although, these ring fission metabolites tend to have lower antioxidant activity [18], the circulating levels of microbial phenolic acid metabolites are generally much more abundant than the native intact polyphenols from the diet. In recent years a few studies have addressed the bioavailability of polyphenols in animals and humans and have stressed the importance of microbial metabolism of polyphenols [19–21]. Nevertheless, there is still limited understanding of the role and importance of gut microbial metabolism of dietary polyphenols in humans *in vivo*.

Studying the metabolic fate of foods and beverages with complex phytochemical ingredients is particularly challenging, because the exact composition and active molecules are usually unknown, and because the absorption and metabolism of these phytochemicals in humans may be highly variable. Most studies to date have been limited by an *a priori* assumption of metabolites that may be absorbed. Novel omics-type technologies start off with a more comprehensive, less biased analytical approach, which could offer valuable new insight. Metabolomics entails a combination of broad analytical profiling of small-molecule metabolites, usually involving NMR spectroscopy or hyphenated chromatography-MS and multivariate statistical analysis to establish group differences. Metabolomics has proved to be a useful approach in plant [22–24] and microbial process science [25] and offers new opportunities in disease diagnosis [26, 27] and assessment of drug efficacy and toxicity [28]. Metabolomics is also considered a promising tool in nutrition [29, 30] and a number of human nutrition studies employing metabolomics technology have been published to date [31–38]. Metabolomics applications in human nutritional intervention studies may often be more challenging than drug (-toxicity) and/or disease diagnostic studies. This is because metabolic effects induced by a nutrition intervention tend to be small relative to the intrinsic metabolic variability associated with diet and lifestyle backgrounds as well as the genetic and gut microbial make-up of the healthy population [39].

In the current human study we investigated the urinary excretion of phenolic acids of microbial origin following a 4-wk intake of two different polyphenol-rich grape juice and wine extracts. We also studied changes in other urinary metabolites that could signal an impact of grape polyphenol intake on (metabolic) health status. A comprehensive metabolomics approach was taken, which included global metabolic profiling of urine metabolites by NMR spectroscopy as well as focused profiling of phenolic acids by GC-MS. This integrated approach is essential to understand absorption and metabolism of polyphenol-rich foods and their potential biological activity in humans.

2 Materials and methods

2.1 Human intervention study

Fifty-eight adult human volunteers (33 men and 25 women, age range: 18–70 years) with mild hypertension (systolic blood pressure: 130–179 mmHg, diastolic blood pressure $\leq$100 mmHg, body mass index 18–32 kg/m^2) participated in a double-blind, randomised cross-over nutrition intervention study with two placebo-controlled treatment legs. Mildly hypertensive volunteers were selected, because the primary objective of this study was to establish effects on blood pressure and vascular function, but it should be noted that the current report focuses only on the metabolism and bioavailability aspects of the nutritional supplements used. The study was run at Unilever R&D Vlaardingen and was approved by an independent external medical ethical committee at the University of Wageningen. All participating subjects gave their informed consent to the study. The study design consisted of a 2-wk run-in period followed by a 4-wk first treatment period, crossed-over with no washout period in between to the second 4-wk treatment period. During the entire study period, the subjects were asked to minimise changes in their habitual diet and were requested to avoid alcohol (a maximum of two glasses *per* day was allowed) and products with a high salt content as much as possible. Furthermore, they had to refrain from using vitamin-, mineral- and other supplements as well as foods containing plant sterols and stanols from the screening till the end of the study. In one leg of the double cross-over study, volunteers ($n = 29$) received capsules with a 2:1 polyphenol-rich mix of dry red wine and dry red grape juice extracts (MIX) and placebo capsules containing microcrystalline cellulose (Avicel PH101, FMC Biopolymer) in a randomised cross-over manner. In the other leg of the study, a different group of volunteers ($n = 29$) received capsules with a red grape juice extract (GJX) and the placebo capsules again in a randomised cross-over manner. At the end of the treatment periods 24-h urine samples were collected, weighed and stored at -80°C. Also, fasted blood samples were collected in heparinised tubes, plasma samples were prepared by centrifugation and were then immediately stored at -80°C.

The MIX consisted of a 2:1 mixture of red wine extract (Provinols^{TM}, Seppic, France) and Rubired GJX (Mega-Natural^{TM}, Polyphenolics, USA) and was chosen to have a particularly rich composition containing a wide spectrum of different wine and grape polyphenols. The total polyphenol dose of both the MIX and the GJX supplements was 800 mg gallic acid equivalents (GAEs) *per* day, as determined by Folin–Ciocalteu analysis. Both the wine extract and GJXs consisted for more than 80% of polyphenols, as determined by NMR analysis, the majority of which were polymeric

proanthocyanidins. The approximate polyphenol composition of the red wine extract and GJXs was determined by liquid chromatography using commercially available standards and is given in Table 1. It should be noted that only 20–30% of the wine and GJX compositions was identified. The major fraction in both extracts consists of oligomeric proanthocyanidins.

2.2 NMR profiling

Frozen urine samples were thawed at room temperature. To 450 μL of urine, 250 μL of phosphate buffer (pH 7, containing 0.01 mg/mL 3-(trimethylsilyl)propionic acid-d_4 (TSP) and 20% D_2O) was added. After vortexing, the mixture was centrifuged in an Eppendorf centrifuge at 9000 rpm for 15 min at room temperature. An aliquot of 650 μL of the clear urine supernatant was transferred to a 5-mm NMR tube.

One-dimensional ^{1}H NMR spectra of urine samples were acquired on a Bruker Avance 600 NMR spectrometer using a one-dimensional NOESY pulse sequence with pre-saturation of the intense water resonance during the relaxation delay of 3 s and the mixing time of 0.15 s. A 5-mm Triple Axis Inverse resonance probe was used, which was tuned to detect ^{1}H resonances at 600.13 MHz. The internal probe temperature was set to 300 K. The spectral data were obtained in 32 K data points, a relaxation delay of 3 s and 128 transients. An exponential window function was applied to the free induction decay with a line-broadening factor of 0.5 Hz prior to the Fourier transformation.

The Fourier transformed NMR data (samples and standards solutions) were phase- and baseline corrected and referenced to the TSP resonance at 0.0 ppm. The NMR spectra were automatically reduced to discrete regions of equal width (0.02 ppm) and the integral of each region was determined using AMIX (Analysis of Mixtures, Bruker, Karlsruhe, Germany). For urine, the region from 4.3–6.0 ppm was excluded from analysis to remove variations in the suppression of the water resonance and variations in the urea signal. The resulting binned data sets were then imported into Microsoft Excel software. Urine profiles were normalised to the integral of the TSP peak and multiplied by the volume of the 24-h urine sample to correct for dilution effects. Finally, the normalised data were imported into Matlab (Version R2008a, The MathWorks, USA) for multivariate data analysis.

Table 1. Approximate composition of the red wine and grape juice extracts

Ingredients	Red wine (% w/w)	Grape juice (% w/w)
Water	6.5	6.5
Fat	<0.1	<0.1
Protein	7.9	2.3
Ash	1.91	0.94
Fibres	1.1	1
Carbohydrates	0.43	4.96
Identified phenolic acids	(mg/g)	(mg/g)
Caffeic acid	1.27	0.30
p-Coumaric acid	0.68	0.46
Ferulic acid	0.07	0.09
Gallic acid	1.46	2.02
Protocatechuic acid	0.62	0.30
p-Hydroxybenzoic acid	0.20	0.08
Vanillic acid	0.39	0.19
syringic acid	1.02	1.00
Caftaric acid	0.60	0.18
Coutaric acid	0.79	0.09
Fertaric acid	0.52	0.30
Ellagic acid	0.24	0.19
Chlorogenic acid	0.03	0.03
Total identified phenolic acids	**7.88**	**5.22**
Identified polyphenols	(mg/g)	(mg/g)
Anthocyanins	21.50	225.80
Catechins+procyanidin Di/trimers	45.18	0.40
Flavonols	4.82	9.48
Stilbenes	0.97	0.15
Total identified polyphenols and phenolic acids[a)]	**80.35**	**241.04**

Please note: The MIX extract used in this study is a polyphenol-rich 2:1 w/w dry mixture of red wine extract and red grape juice extract.

a) The remaining unidentified fraction contains primarily uncharacterised oligomeric proanthocyanidins.

2.3 GC-MS profiling

To 500 μL urine, 50 μL 1.5 M sodium acetate and 30 μL of β-glucuronidase (1500 units in 150 mM sodium acetate) was added and the samples were incubated at 50°C for 2 h. After cooling the samples down to room temperature, 65 μL of 1 M hydrochloric acid and an internal standard (50 μL of 100 μg/mL *trans*-cinnamic acid-d_6 in 1:1 v/v methanol/water) were added. The samples were kept at 4°C for 10 min after which 2 mL of ethyl acetate was added. Then, they were vortexed for 30 s and centrifuged at 3000 × *g* for 10 min. The supernatants were transferred and the extraction was repeated twice. The combined extracts were dried under a stream of nitrogen at 40°C. Samples were further dried by the subsequent addition and evaporation of 1 mL of dichloromethane. After addition of 100 μL of *N,O-Bis* (trimethylsilyl)trifluoroacetamide containing 10% trimethylchlorosilane, the samples were incubated at 90°C for 30 min. After cooling down to room temperature, 400 μL of hexane was added and the samples were vortex-mixed. Aliquots of 200 μL of the derivatised samples were transferred to GC vials and analyzed by GC-MS. Derivatised samples were analyzed on a Waters GCT TOF mass spectrometer (Waters, Etten-Leur, The Netherlands) equipped with an Agilent 6890

gas chromatograph and an Agilent 7683 autosampler (Agilent Technologies, Amstelveen, The Netherlands). The compounds were separated on a fused silica VF-17ht column [30 m x 0.25 mm, df = 0.10 μm] (Varian BV Scientific Instruments, Middelburg, The Netherlands) with helium as the carrier gas at a flow rate of 1 mL/min. The sample (1 μL) was injected into a CIS-4 liquid-nitrogen-cooled split/splitless injector (Gerstel, Mülheim an der Ruhr, Germany) at a split ratio of 20:1. The injector used a temperature gradient from 55°C (held for 0.05 min) to 300°C (held for 5 min) at 8°C/s. The temperature program of the column oven was as follows: 0–1 min isothermal at 45°C; 1–6.5 min at 10°C/min to 100°C; 6.5–26.5 min at 7.5°C/min to 250°C; 26.5–29 min at 10°C/min to 300°C, where it was held for 6 min. The interface and source temperature of the mass spectrometer were 250 and 180°C, respectively. Mass spectra were recorded in EI mode from 6 to 30 min at a scan time of 0.3 s, an inter-scan time of 0.1 s and a scan range of *m/z* 50–600. The mass spectrometer was tuned with 2,4,6-tris(trifluoromethyl)-1,3,5-triazine and calibrated with perfluorotributylamine.

2.4 GC-MS pre-processing

MS-based metabolomics typically requires initial alignment of the data, to correct for peak shifts in subsequent profiles when they are collected over prolonged periods of time. Visual inspection of the TIC chromatograms measured in this study showed very good alignment for the majority of the signals, owing to the design of the measurement scheme and relatively short measurement period [40]. Therefore, and to simplify and speed up analysis, simple binning of the TICs prior to multivariate data analysis was preferred in this case over more sophisticated alignment algorithms that attempt to align molecular fragments using both retention time and *m/z* dimensions. GC-MS raw data were pre-processed using metAlign™ software (Rikilt, Wageningen, http://www.rikilt.wur.nl/UK/services/MetAlign+download/), typically only for baseline correction, noise elimination and conversion of the raw data to NetCDF format. The resulting data were then imported into AMIX software (Bruker), which was used to segment the TICs in equally sized bins of 0.02 min. Individual binned TICs were normalised to the peak of the internal standard *trans*-cinnamic acid-d_6 at 13.22 min and corrected for the total volume of the 24-h urine sample.

2.5 Data analysis

2.5.1 Model significance testing

Multilevel partial least squares discriminant analysis (ML-PLS-DA) was used for the pair-wise comparison of MIX *versus* placebo and for GJX *versus* placebo. ML-PLS-DA is an extension of ordinary PLS-DA and its use in metabolomics analysis of intervention studies was described recently by van Velzen *et al.* [41]. The method has been implemented as a set of home-written Matlab routines, which are available online (http://www.bdagroup.nl/index.php/downloads). ML-PLS-DA has the advantage that it allows separation of the within-subject variation from the inter-subject variation in metabolite profiles, and can also exploit the cross-over design of intervention studies [41]. This is extremely useful in human nutritional intervention studies since, typically, inter-individual variability caused by genetic, dietary, lifestyle and environmental factors can obscure nutrition-related metabolic effects. First, the inter-individual variation in the NMR and GC-MS data was separated from the intra-individual variation. Then, the intra-individual data was mean-centred and autoscaled and regressed against the intervention class labels (−1 for placebo samples, +1 for treated samples) using PLS-DA. The ML-PLS-DA models were extensively validated using a fivefold cross model validation (CMV) scheme, which is an extension of normal cross-validation and contains an additional variable selection loop [42]. The prediction error of the models was expressed in two different ways. The parameter Q^2 indicates how good the model is able to predict the class membership. The second parameter is the false discovery rate (FDR), which is the proportion of misclassified samples found in a permutation test, in which class labels of the samples are randomly permuted [43, 44]. Thus, an FDR lower than 0.5 will indicate a prediction that is better than chance, *i.e.* a stronger class prediction model. To obtain stable prediction errors, the average Q^2 and FDR values were calculated from 20 independent CMV rounds. The statistical significance of these model parameters was established, again using a permutation test. In this permutation test, repeated CMVs were performed on the data with randomly permuted class labels. The Q^2 and FDR values from 1000 permutations were collected and represented as a H_0-distribution of no-effect. The statistical significance (*p*-value) of Q^2 and FDR was assessed from the difference between the original Q^2 and FDR values and the chance distribution.

2.5.2 Variable selection

To select which NMR or GC-MS variables contribute most to the treatment effect, rank products (RPs) were calculated during the CMV process [45, 46]. Twenty CMV rounds were used, and after each round the NMR or GC-MS peaks were ranked according to the magnitude of the PLS-DA regression coefficient. The ranks of each CMV round were multiplied to give the RP, where the lowest RP-values correspond to peaks/variables that have the strongest discriminative power. RPs were validated in a 1000-fold permutation test. Only variables with an average RP-value in the lowest ten percentile after permutation testing were considered significant ($\alpha = 0.10$).

Markers were then given a positive or negative sign, depending on whether there was an average increase or

decrease following the intake, respectively. In order to visualise the markers corresponding to the ML-PLS-DA models a RP plot was made, similar to the regression coefficients plots in ordinary PLS models. For visualization purposes the signed logarithm of the rank product (slRP) was calculated, *i.e.* slRP = sign × (max(logRP)–logRP), where logRP is the logarithm of the rank product and max(logRP) is the maximum value of all logRPs. The value of sign is +1 for variables that increased on average after treatment, −1 for decreasing variables. Variables with an RP that failed to reach the significance threshold of $\alpha = 0.10$ were assigned a sign value of 0.

In addition to the multivariate method of variable selection, a univariate variable selection was performed, using the Wilcoxon signed rank test. The combination of multivariate and univariate approaches was used to minimise loss of potentially interesting variables [47]. Variables were selected if they exceeded the experimental noise level (NMR: 2% and GC-MS: 0.2%; relative to max. peak intensity) and if the Wilcoxon test was significant at $\alpha = 0.35$. Fold changes for each metabolite and for each individual were calculated and the median fold change for each metabolite is reported.

2.5.3 Quantification of hippuric acid levels

Hippuric acid levels in urine were determined from the peak integral of the aromatic hippuric acid resonance at 7.83 ppm, relative to that of the internal standard TSP and are expressed in g/24 h. Differences in urinary hippuric acid concentrations between the various treatments were assessed using a two-sided paired *t*-test with $p < 0.05$ considered statistically significant.

3 Results

3.1 NMR profiling of urine

Twenty-four-hour urine samples were collected after 4 wk of intake of two different polyphenol-rich grape extracts (MIX and GJX) and after placebo intake. High resolution ^{1}H NMR spectroscopy was used to provide global metabolite profiles of urine, including the most abundant degradation products of dietary polyphenols as well as urinary metabolites derived from endogenous human metabolism. Hippuric acid levels in 24-h urine were quantified by NMR spectroscopy, as an indicator of gut microbiota-mediated degradation of dietary polyphenols. Urinary hippuric acid levels after each treatment period are given in Fig. 1. After 4-wk intake of the MIX, hippuric acid levels in 24-h urine samples increased significantly ($p = 0.001$) compared with placebo levels, namely from 0.99 to 1.32 g/24 h (*i.e.* a 35% increase). Hippuric acid levels following GJX intake were not significantly different from the placebo levels for this group of volunteers ($p = 0.13$). Results were not different when corrected for creatinine (data not shown). The hippuric acid levels in the two placebo groups appeared slightly different, but this was not significant ($p = 0.16$). It should be noted that the subjects in these two groups were not the same. Probably the slightly different hippurate levels reflect a high inter-individual biological variability, which may be related to both background diet and gut microbial diversity and activity. The stronger hippuric acid excretion after MIX consumption than after GJX consumption is remarkable given that the total polyphenol content in the two extracts was equal.

ML-PLS-DA [41] was used to differentiate the 24-h urine NMR profiles after the MIX and GJX treatments from their respective placebo profiles. The ML-PLS-DA model differentiating the urine NMR profiles after MIX intake from the placebo-1 profile was validated by means of CMV and permutation testing and was found to have significant prediction ability with a Q^2 of 0.05 ($p = 0.05$) and FDR of 0.25 ($p < 0.001$). The NMR profiles after GJX intake could not be differentiated from the placebo-2 profiles. With a Q^2 of −0.36 ($p = 0.65$) and FDR of 0.42 ($p = 0.15$) the ML-PLS-DA model for the GJX intervention was not significant. Figure 2 shows a typical urine NMR profile after MIX intake along with the RP plot resulting from ML-PLS-DA model. The RP plot displays the slRP, which expresses the discriminating power and directions of each (NMR-) variable to the ML-PLS-DA model, *i.e.* positive peaks increasing following polyphenol-rich intake. In general, most changes in phenolic compounds may be expected to be visible in the aromatic region of the NMR metabolite profile (*i.e.* between 6 and 9 ppm). In line with the NMR quantification of hippuric acid shown in Fig. 1, hippuric acid (characterised by three major signals from chemically distinct aromatic protons at 7.83, 7.64 and 7.55 ppm) made a significant positive contribution ($p < 0.001$) to the separation of MIX and placebo. The strongest contribution was actually made by the two peaks of 4-hydroxyhippuric acid at 7.76 an

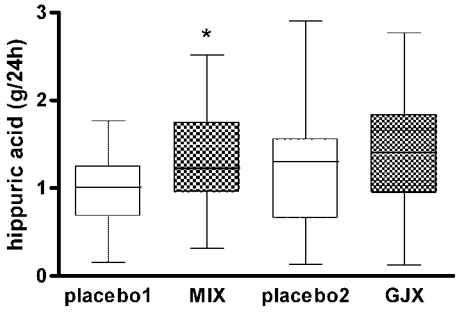

Figure 1. Box-whisker plot of hippuric acid levels in 24-h urine for two groups of volunteers after taking polyphenol-rich supplements or a placebo for a period of 4 wk. Group 1 took a mix of wine and GJX or placebo-1, while group 2 took a GJX or a placebo-2. The difference between mix and placebo-1 was significant ($p < 0.001$). Total polyphenol content of the grape supplements was 800 mg GAEs.

6.98 ppm (Wilcoxon $p<0.001$). A small but significant contribution was made by trigonelline at 9.15 ppm (Wilcoxon $p=0.02$). However, most of the other NMR peaks from aromatic metabolites that were elevated after MIX intake (Fig. 2B) could not be identified so far using available databases.

ML-PLS-DA analysis also showed several significant changes in the aliphatic part of the NMR spectrum (0–4.5 ppm), although these contributed less to the model than the aromatic compounds. Among others, MIX intake caused a significant increase in urinary citrate (2.8–3 ppm) and in a singlet signal at 3.29 ppm, which was assigned to betaine. Most other significant changes in aliphatic urine metabolites as indicated in Fig. 2 could not be identified. The ML-PLS-DA model differentiating between urine NMR profiles of GJX and placebo-2 intake was not significant; therefore, no markers for intake of the GJX are reported here.

Since the levels of phenolic acids in urine are generally too low for detection by NMR profiling, a focussed profiling method has been set up using GC-MS, specifically for more sensitive detection and identification of phenolic acid excretion in urine.

3.2 GC-MS profiling of urine

We recently described a validated method for profiling of low molecular weight polyphenol metabolites in urine, which was based on liquid–liquid extraction and GC-TOF-MS analysis [40]. This profiling method focuses specifically on the phenolic acids that result from degradation of polyphenols by gut microbiota. Figure 3A shows representative GC-MS TIC chromatograms of urine following the MIX treatment. ML-PLS-DA of the GC-MS profiles of urinary phenolic acids was able to differentiate between the MIX and placebo intervention and between the GJX and placebo intervention. The ML-PLS-DA model distinguishing between MIX and placebo was validated by CMV and permutation testing with a Q^2 of 0.60 ($p<0.001$) and an FDR of 0.04 ($p<0.001$). The ML-PLS-DA model distinguishing between GJX and placebo was also significant with a Q^2 of 0.00 ($p=0.05$) and an FDR of 0.15 ($p<0.001$). The RP plots associated with both ML-PLS-DA models are given in Figs. 3B and C. They indicate which urinary (phenolic) metabolites contribute significantly to the model. The impact of the GJX on urinary phenolics excretion was very similar to that of the MIX. The retention time peaks with positive slRP indicate (phenolic) metabolites that are excreted at higher levels after polyphenol intake. GC-MS variables that were significantly elevated following the polyphenol-rich intervention according to RP analysis (multivariate) and also passed a not so stringent univariate Wilcoxon rank test ($p<0.35$) were identified using the NIST02 library if possible.

The library search allowed the identification of a considerable number of potentially interesting phenolic acids. Consumption of the MIX induced significantly elevated urinary excretion of more than 32 different identified compounds, 18 of which were positively identified as phenolic acids. Consumption of the GJX also induced significantly elevated urinary excretion of over 43 different compounds, 19 of which were positively identified as phenolic acids. There were also a number of significant

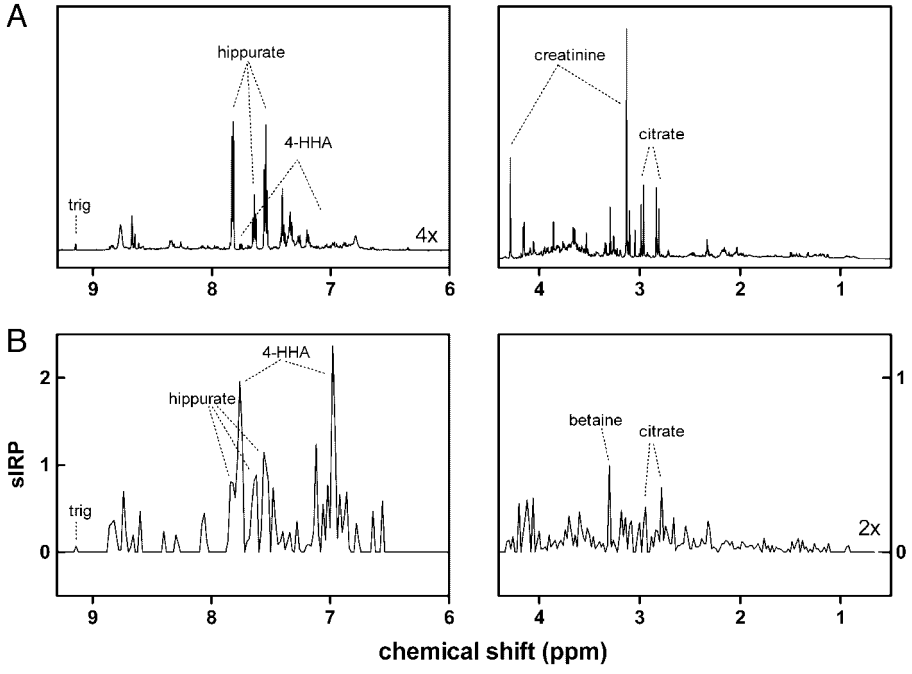

Figure 2. NMR spectroscopy-based global metabolite profiling of 24-h urine following a 4-wk consumption of a polyphenol-rich supplement or placebo. (A) a representative NMR metabolite profile of urine after intake of the grape juice/wine MIX, showing spectral regions for the aromatic metabolites (6–10 ppm) and for aliphatic (mostly endogenous) metabolites (0–4.5 ppm). (B) Marker selection based on RP analysis of the ML-PLS-DA model differentiating the urinary NMR metabolite profiles of the red wine/grape juice MIX from placebo. slRP is centered and is plotted against the NMR chemical shift.

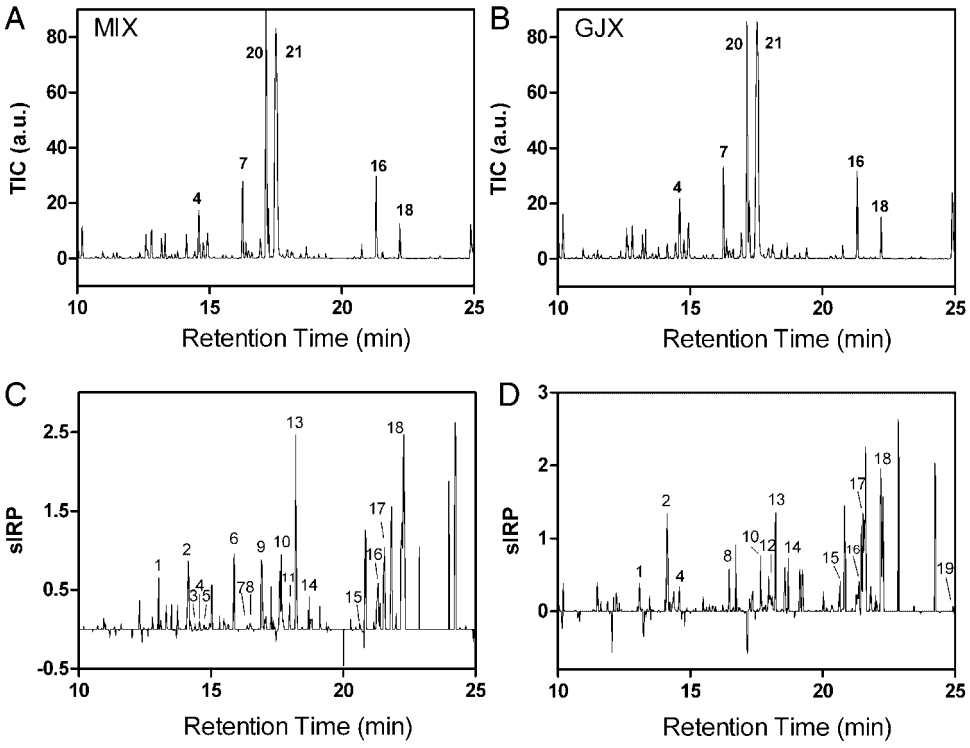

Figure 3. GC-MS-based focused profiling of phenolic acids in 24-h urine following 4-wk intake of a polyphenol-rich supplement or placebo. (A) Mean total-ion-chromatogram of urine after intake of grape juice/wine MIX and (B) mean total-ion-chromatogram of urine after intake of only GJX. (C) Marker selection based on RP analysis of the ML-PLS-DA model differentiating the urinary NMR metabolite profiles of the red wine/grape juice MIX from placebo. (D) Marker selection based on RP analysis of the ML-PLS-DA model differentiating the urinary NMR metabolite profiles of the GJX from placebo. sIRP is centered and is plotted against the chromatographic retention time. Peak assignments: 1. Pyrogallol, 2. 3-Hydroxyphenylacetic acid, 3. 4-Hydroxybenzoic acid, 4. 4-Hydroxyphenylacetic acid, 5. 4-Methoxy-3-hydroxybenzoic acid (isovanillic acid), 6. 3-Hydroxyphenylpropionic acid, 7. 3-Methoxy-4-hydroxybenzoic acid (vanillic acid), 8. 3-Methoxy-4-hydroxyphenylacetic acid (homovanillic acid), 9. Hippuric acid (-diTMS), 10. 3-(3-Hydroxyphenyl)-3-hydroxypropionic acid, 11. 3-Methoxy-4-hydroxymandelic acid (vanilmandelic acid), 12. 3-Methoxy-4-hydroxyphenylpropionic acid (dihydroferulic acid) 13. 4-Hydroxy-3,5-dimethoxybenzoic acid (syringic acid), 14. 3,4-Dihydroxyphenylpropionic acid, 15. Ferulic acid, 16. 3-Hydroxyhippuric acid, 17. 4-Hydroxymandelic acid, 18. 4-Hydroxyhippuric acid, 19. Phenylacetylglutamine, 20. Citric acid, 21. Hippuric acid (-monoTMS).

GC-MS peaks that could not yet be identified with the NIST library and phenolic reference standards used.

A list of all identified phenolic acids that were elevated in urine after the MIX and GJX treatments is given in Table 2 together with their logRPs from the ML-PLS-DA model. Results from the univariate Wilcoxon rank test and median fold changes in each metabolite are shown for comparison.

As presented in Table 2, most of the excreted phenolic acids identified after the MIX and GJX treatments were the same. However, their relative importance was different, and there were some minor discrepancies. The urinary phenolic acids that gave the strongest contribution to both the MIX and GJX treatments, as assessed form their RPs (logRP), were 3- and 4-hydroxyhippuric acid, syringic acid, 3-hydroxyphenylacetic acid, 4-hydroxymandelic acid and vanilmandelic acid. In addition, hippuric acid, 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylpropionic acid and 1,2,3-trihydroxybenzene were stronger markers for the MIX intake, whereas 4-hydroxyphenylacetic acid, homovanillic acid, dihydroferulic acid and phenylacetylglutamine were stronger markers for GJX consumption.

As pointed out above, some of the significant urinary metabolite changes found by GC-MS were not identified as phenolic acids. These were identified using the NIST library as non-aromatic hydroxylated mono-, di- or tricarboxylates, which are mostly common to urine (data not shown). As an example, and potentially in line with the NMR detection of citrate, some of the significantly elevated urine metabolites detected by GC-MS were identified as citric acid cycle intermediates, including isocitrate, which was elevated after both MIX (logRP: 2.52; $p<0.001$) and GJX intake (logRP: 2.89; $p=0.003$), and *cis*-aconitate (logRP: 2.98; $p=0.028$), which was elevated after GJX intake only. However, it should be emphasised that the current urine sample preparation and GC-MS profiling method was specifically focussed on and validated for phenolic acids only. Due to the nature of the profiling approach, organic acids with similar polarity will be detected, but their recovery and reproducibility would require further validation, beyond the scope of

Table 2. Significantly elevated phenolic acids in 24-h urine after 4-wk consumption of polyphenol-rich MIX or GJX

Peak ID	RT (min)	Metabolite ID *(synonym)*	Mix				GJX			
			Log RP	Lowest RP rank	Wilcoxon *p*	Fold change	LogRP	Lowest RP rank	Wilcoxon *p*	Fold change
1	13.00	1,2,3-Trihydroxybenzene *(pyrogallol)*	2.68	33	4.90E−04	1.51	3	339	3.00E−02	1.18
2	14.14	3-Hydroxyphenylacetic acid	2.19	25	3.80E−07	2.87	1.72	13	1.90E−04	1.54
3	14.40	4-Hydroxybenzoic acid	2.99	292	6.10E−02	1.37	3.01	367	5.50E−03	1.21
4	14.56	4-Hydroxyphenylacetic acid	2.96	232	5.10E−02	1.4	2.73	74	4.20E−03	1.2
5	14.82	4-Methoxy-3-hydroxybenzoic acid *(isovanillic acid)*	3.04	511	5.10E−02	1.36	3.06	1011	4.60E−02	1.05
6	15.86	3-Hydroxyphenylpropionic acid	2.16	21	1.40E−05	1.51	2.99	307	3.20E−02	1.34
7	16.38	3-Methoxy-4-hydroxybenzoic acid *(vanillic acid)*	3.01	340	2.10E−01	1.48	3.04	561	5.80E−02	0.94
8	16.46	3-Methoxy-4-hydroxyphenylacetic acid *(homovanillic acid)*	3.03	263	1.50E−03	1.49	2.49	41	2.80E−04	1.37
9	16.90	Hippuric acid (-diTMS)	2.18	24	4.90E−04	2.18	3.04	674	1.40E−01	1.16
10	17.62	3-(3-Hydroxyphenyl)-3-hydroxypropionic acid	2.81	98	1.80E−03	1.37	2.95	195	4.90E−03	1.27
11	17.96	3-Methoxy-4-hydroxymandelic acid *(vanilmandelic acid)*	2.73	77	1.50E−03	1.39	2.58	49	7.60E−05	1.36
12	18.00	3-Methoxy-4-hydroxyphenylpropionic acid *(dihydroferulic acid)*	3.03	467	9.80E−02	1.45	2.85	124	1.40E−03	1.45
13	18.20	4-Hydroxy-3,5-dimethoxybenzoic *acid (syringic acid)*	0.6	3	6.50E−10	3.01	1.71	11	3.00E−07	1.59
14	18.72	3,4-Dihydroxyphenylpropionic acid	2.65	61	1.50E−05	1.62	3.02	437	1.80E−01	1.19
15	20.62	Ferulic acid	2.99	287	1.70E−02	1.52	2.93	199	1.20E−04	1.37
16	21.32	3-Hydroxyhippuric acid	2.47	37	2.20E−06	1.77	2.86	84	2.80E−03	1.33
17	21.56	4-Hydroxymandelic acid	2.02	18	1.50E−09	6.61	1.71	12	4.20E−08	2.13
18	22.20	4-Hydroxyhippuric acid	1.91	12	4.50E−09	2	1.1	5	3.00E−07	1.8
19	24.92	Phenylacetylglutamine					2.99	319	9.30E−02	1.7

Significantly elevated metabolites were selected by a rank product analysis during cross model validation of the multilevel PLS-DA models (for details see Section 2). Metabolites with the lowest value of log RP and the lowest rank product ranking have the strongest discriminative power. The Wilcoxon *p*-value gives the significance of the metabolite increase in a Wilcoxon signed rank test. Median fold changes in metabolite levels after treatment are given.

this study. Most of the non-phenolic metabolites did not have low RPs and thus did not contribute importantly to the model.

4 Discussion

This study showed that a 4-wk nutritional supplementation with a mix of red wine and red GJX (MIX) or with only GJX produces significant changes in the urinary global metabolite and phenolic acid profiles. The NMR- and GC-MS-based metabolomics methods provided valuable and complimentary information on polyphenol degradation in the human body and its potential impact on endogenous metabolism. The GC-MS metabolomics approach primarily aimed at identification of a wide range of phenolic acids in urine. There is accumulating literature that consumption of polyphenol-rich foods and beverages, including chocolate, wine, tea and coffee, or the intake of specific polyphenol compound classes, like catechins, proanthocyanidins and chlorogenic acids, result in urinary excretion of phenolic acids derived from microbial metabolism, as reviewed in [19]. Polyphenols, including those present in red wine and grape juice are poorly absorbed in the small intestine, and will enter the colon where they are degraded by colonic bacteria [20, 21, 48–50]. The microbial metabolites actually account for a major fraction of the total amount of absorbed dietary polyphenols. In rats that were fed red wine polyphenols it has been estimated that the fraction of total polyphenols excreted in urine in the form of aromatic acids was almost eight times higher than that of intact catechins [51]. Circulating levels of the intact polyphenols (aglycones), including proanthocyanidins, anthocyanins and catechins rarely exceed 1 μM in plasma [20].

The current study showed that supplementation of the polyphenol-rich mixed wine/grape juice and grape juice only extracts increased urinary excretion of a wide range of low molecular weight phenolic acids, as listed in Table 2. This finding thus supports the critical role of gut microbiota in the metabolism of dietary polyphenols. We cannot exclude, however, that some of the urinary phenolic acids may actually originate to some extent from the ingested

wine or GJX itself. As presented in Table 1, the total phenolic acid content of the extracts was very low (0.5–0.8%), but the ingested dose of the two most abundant phenolic acids, *i.e.* syringic acid and gallic acid could amount 1–2 mg *per* day, respectively. Thus, it is likely that, *e.g.* the urinary increase in syringic acid (see Table 2) originates directly from the extract itself. However, many of the other elevated urinary phenolic acids are in line with gut microbial degradation of polyphenols, as is discussed below.

NMR metabolomics showed elevated excretion of hippuric acid and 4-hydroxyhippuric acid following MIX intake. Hippuric acid (*N*-benzoylglycine) is formed by conjugation of benzoic acid with glycine in the kidney or liver and could be considered the final product of gut microbial metabolism of dietary polyphenols. It was calculated that in this study hippuric acid excretion was elevated by 0.3 g *per* day following intake of 0.8 g GAE of the MIX extract, suggesting that a major portion of the ingested complex polyphenols is excreted in the form of this simple aromatic acid. Increased urinary excretion of hippuric acid has also been observed after black and green tea consumption [33, 52, 53]. Interestingly, hippuric acid levels were significantly elevated following the MIX supplementation, but not after supplementation of the GJX alone. Moreover, the global urine NMR metabolic profile was not significantly affected by GJX intake at all. Since the daily dose of total polyphenols was equivalent for both treatments, this could be related to the slightly different composition of the extracts. In both extracts, oligomeric proanthocyanidins constitute the major polyphenol fraction (70–80%), but the MIX contained higher levels of catechins and procyanidin di- and trimers, whereas the GJX supplement contained higher levels of anthocyanins (Table 1). This could indicate that hippuric acid may originate from monomeric catechins and procyanidin di- and trimers present in the wine extract but may not originate, or at least to a lesser extent, from oligomeric proanthocyanidins and anthocyanins. Interestingly, 3- and 4-hydroxyhippuric acid, 3-hydroxyphenylpropionic acid and 3- and 4-hydroxyphenylacetic acid were all significantly elevated after GJX as well as after MIX consumption (Table 2). These phenolic acids have been reported previously as urinary metabolites following the intake of proanthocyanidin-rich wine or grape seeds [51, 54, 55], but are not specific to wine or grape (juice) consumption.

Figure 3 showed that the GC-MS phenolic profiles of urine were able to show a significant impact of MIX intake and a small effect of the GJX intake, which was borderline significant. The elevated phenolic acids as listed in Table 2 revealed additional small differences between the two treatment groups, beyond hippurate. Comparison of the RPs from the ML-PLS-DA models (Table 2) indicated that 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylpropionic acid and pyrogallol (1,2,3-trihydroxybenzene) were stronger markers for the MIX intake, whereas 4-hydroxyphenylacetic acid, homovanillic acid, dihydroferulic acid and phenylacetylglutamine were stronger markers for GJX consumption. The median fold changes gave some indication of the size of the individual metabolite changes, while the statistical significance of the univariate changes were given by the Wilcoxon rank test and were largely supporting the multivariate tests. It should be noted that it is difficult to trace back the phenolic acids to their single parent compounds in the extract, because of the complex and to a large extent unknown composition of the polyphenol extracts.

Interestingly, phenylacetylglutamine, 4-hydroxymandelic acid and 3-methoxy-4-hydroxymandelic acid (vanillylmandelic acid) were also significantly elevated in urine after MIX and/or GJX intake. To our knowledge, they have not been previously reported in relation to dietary polyphenol intake, although they are well-known urinary metabolites. Most likely they are not (microbial) metabolites of dietary polyphenols, but result from the modulation of endogenous biological pathways by polyphenol (-metabolites). Phenylacetylglutamine is formed in the liver by glutamination of phenylacetate, which itself may result from phenylalanine metabolism. 4-hydroxymandelic acid is a natural occurring metabolite of tyramine and of similar biogenic amines. Vanillylmandelic acid as well as homovanillic acid are well-known end-products of catecholamine metabolism. The link between grape/wine polyphenol intake and catechol metabolism is yet unclear and thus further studies are required to elucidate the underlying mechanisms, and if they could contribute to potential neurological or cardiovascular health benefits.

Thus, apart from giving insight into metabolic degradation (catabolism) of grape and wine polyphenols in humans, this metabolomics study has also demonstrated changes in endogenous urinary metabolites that may point to an impact on the host metabolism. The current literature indicates that beneficial effects of polyphenols to cardiovascular health may be the result of improved bioavailability and bioactivity of nitric oxide, which promotes vascular endothelial function, but there are numerous potential molecular mechanisms by which polyphenols may trigger this response [4, 56, 57].

NMR profiling indicated an increase in urinary excretion of citrate after MIX intake. We recently found a similar effect of green tea intake on TCA-cycle intermediates in human urine [33]. This may suggest that grape/wine and/or tea polyphenols could have an impact on mitochondrial TCA cycle turnover, *e.g.* modulating kidney function. Our GC-MS profiling actually suggested additional changes in other urinary TCA cycle intermediates, such as isocitrate (MIX, GJX), *cis*-aconitate (GJX) and oxaloacetate (GJX). Unfortunately, the current GC-MS method was not validated to draw strong conclusions on the changes in non-phenolic compounds, and thus further substantiation of these findings is necessary. Even so, other recent studies have shown effects of the flavonol quercetin on the expression of genes and proteins, which are also related to mitochondrial energy metabolism. Quercetin was shown to increase gene and protein expression in rat tissues in favour

of fatty acid oxidation [58, 59]. Since increased fatty acid oxidation may stimulate the TCA cycle turnover *via* the production of acetyl-CoA, this may be in agreement with the current findings, but clearly more research in this area is needed. NMR metabolomics further indicated that MIX supplementation was associated with elevated excretion of betaine and to a lesser extent also trigonelline. The latter is a known component of coffee, and also a metabolite of niacin (vitamin B3), and is thus most likely associated with the normal dietary intake. Betaine is an important methyl-donor and is an important regulator homocysteine homeostasis, *e.g.* in diabetes [60].

The current study focused on microbial polyphenol metabolites in urine, while the identification of higher molecular weight parent polyphenol compounds and their conjugates was outside the scope of this study. There is still limited understanding of the relative importance *in vivo* of intact polyphenols and -conjugates *versus* gut microbial metabolites of polyphenols. Further studies are needed to determine whether microbial polyphenol metabolites have important systemic effects and can play a role in maintenance of (cardiovascular) health, or if they only have a local impact, *e.g.* at the level of the intestinal wall. It should be noted that intestinal absorption is not absolutely required for polyphenol (-metabolites) to exert their beneficial effects [61]. The levels of intact polyphenols may be high in the stomach and small intestine, whereas phenolic acids can reach high levels in the colon [62] and therefore it is not unlikely that their antioxidant or other beneficial properties help protect the gastrointestinal tract against diseases such as cancer and inflammation. Recently, we also reported a metabolic profiling method for faeces and described the effect of wine/grape MIX consumption on faecal metabolites [63]. The NMR-based profiling method was not sensitive enough to detect polyphenol metabolites in faeces, but it did detect several short chain fatty acids. Notably, MIX supplementation reduced the amount of isobutyrate in faeces, suggesting an impact on gut microbial population or activity. There is increasing awareness that gut microbial metabolism and host mammalian metabolism are intimately linked through exchange of metabolites [39, 64, 65].

Metabolomics was demonstrated here to be a valuable technology for the assessment of nutritional intervention effects in apparently healthy human volunteers. It is a particularly challenging task to study the metabolic fate of complex phytochemical ingredients in the human body, against an extremely variable background of host genetic, lifestyle, dietary and gut microbiota differences. Comprehensive and unbiased analytical methods and rigorous multivariate statistics are therefore required to filter out relevant subtle effects of nutrition. The ML-PLS-DA method used in this study exploits the individual paired data structure inherent to the cross-over design, and is combined with rigorous validation of multivariate models and biomarkers found and thus greatly improves the robustness of metabolic information recovery from nutritional intervention studies [41].

In conclusion, we have shown here that 4-wk consumption of polyphenol-rich wine and grape supplements results in the elevated excretion of a wide range of phenolic acids, which is in line with extensive gut microbial metabolism of grape/wine polyphenols. The metabolomics approach was able to detect marginally different impacts on urine metabolic profiles, following consumption of GJX or a red wine/grape juice MIX, although they only had a slightly different polyphenol composition. Thus, a change in phytochemical composition of the diet can induce shifts in local and/or systemic profiles of phenolic metabolites with potential significance to human health. Metabolomics can play an important role in nutritional intervention studies to help unravel the complex interactions between food bioactives and human metabolism and health.

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