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Impact of Short-Term Intake of Red Wine and Grape Polyphenol Extract on the Human Metabolome

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ABSTRACT: Red wine and grape polyphenols are considered to promote cardiovascular health and are involved in multiple biological functions. Their overall impact on the human metabolome is not known. Therefore, exogenous and endogenous metabolic effects were determined in fasting plasma and 24 h urine from healthy male adults consuming a mix of red wine and grape juice extracts (WGM) for 4 days in a placebo-controlled, crossover study. Syringic acid, 3-hydroxyhippuric acid, pyrogallol, 3-hydroxyphenylacetic acid, and 3-hydroxyphenylpropionic acid were confirmed as the strongest urinary markers of WGM intake. Overall, WGM had a mild impact on the endogenous metabolism. Most noticeable were changes in several amino acids deriving from tyrosine and tryptophan. Reductions in the microbial metabolites *p*-cresol sulfate and 3-indoxylsulfuric acid and increases in indole-3-lactic acid and nicotinic acid were observed in urine. In plasma, tyrosine was reduced. The results suggest that short-term intake of WGM altered microbial protein fermentation and/or amino acid metabolism.

KEYWORDS: *wine, grape, polyphenols, phenolic acids, metabolomics, local false discovery*

INTRODUCTION

Polyphenols present in grapes and red wine are considered to promote beneficial effects on coronary heart disease (CHD) and atherosclerosis.[1,2] Regular consumption of red wine polyphenols may in part explain the "French paradox", the apparent compatibility of a high-fat diet with a low mortality from CHD.[3] The evidence from human intervention studies, however, is still limited and inconclusive.[4]

The poorly understood health effects of wine and grape consumption, particularly in a healthy population, may depend on the complex composition of the wine/grape polyphenols and their bioavailability.[5,6] Polyphenols present in grapes and red wine are composed of flavonoids, such as anthocyanins and flavan-3-ols, and nonflavonoids, such as stilbenes (e.g., resveratrol) and gallic acid.[2,5] Flavan-3-ols predominate in wine, with oligomeric and polymeric proanthocyanidins (condensed tannins) typically comprising 25−50% of total phenolic constituents.[7]

The biological activity of wine and grape polyphenols in vivo depends on their bioavailability, that is, on whether they are sufficiently absorbed and enter the blood circulation and tissues. Polyphenols are rapidly and extensively metabolized in the intestine and liver. The major modifications of the parent compounds are methylation, glucuronidation, and sulfation.[8] Resveratrol, catechins, and gallic acid are typical compounds that are directly absorbed.[5] The circulating levels of their conjugates in plasma, however, are low, rarely exceeding 1 μM after the consumption of 10−100 mg of a monomeric compound.[9] The majority of polyphenols reach the colon, where they are deconjugated by bacterial glycosidases, glucuronidases, and sulfatases and further fermented to a wide range of low molecular weight phenolic acids. Thus, the gut microbiota plays a key role in the bioavailability of polyphenols.[5,9,10]

From current scientific evidence it becomes apparent that polyphenols may act through multiple mechanisms. Flavan-3-ols have been reported to act as antioxidant, anticarcinogen, anti-inflammatory, antimicrobial, antiviral, and neuron-protective agents.[11] Similarly, resveratrol has numerous targets through which it could exert a variety of health benefits including antiatherogenic, anti-inflammatory, and anticancer effects.[12] The role of gut microbial mediated phenolic metabolites has been less investigated.[13] It is assumed that the gut microbiota can transform polyphenols to more active and health-promoting derivatives.[9,14] Moreover, it can already be anticipated that the multiple interactions between polyphenol metabolites and their targets are affected by diverse genotypes and environmental influences as well as lifestyle and dietary habits.

This complexity may be unraveled using novel metabolite profiling techniques that are able to provide a comprehensive overview on various metabolic processes. Metabolomics in nutritional research holds great potential to assess the metabolic response to dietary interventions,[15] to identify novel biomarkers for food intake,[16] to characterize human

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metabolic phenotypes to dietary modulation,[17] and to capture the gut microbial−host cross-talk.[18,19] The metabolic effects of phytochemical intake in humans have been investigated in several studies using nuclear magnetic resonance (NMR)- or liquid chromatography coupled with mass spectrometry (LC-MS)-based metabolomics techniques.[20−24] Overall, these studies have shown that the intake of phytochemicals induced changes in both exogenous and endogenous metabolites, particularly in urine.[25]

In the current work, we first identified the most abundant gut microbial derived degradation products from a mixture of red wine and grape juice extract (WGM) in urine using GC-MS-based profiling of phenolics, similar to our previous study.[26] The verification of exogenous metabolites of polyphenol mixtures is an important step to identify new markers of exposure and thus to understand the metabolism of phytochemicals in relation to human health.[6,16] Furthermore, we applied semiquantitative broad metabolite profiling (GC-MS and LC-MS/MS) of the lipid and polar phases (four-phase profiling) of urine and plasma, respectively, to assess the overall effect of a 4 day intake of WGM on the human metabolism. This "untargeted", hypothesis-free approach was chosen to initially get a holistic view of the extent to which red wine and grape juice polyphenols affect the endogenous human metabolism. In contrast to our previous study using NMR spectroscopy,[26] we explored MS-based profiling methods in the current study, because these methods are more sensitive and thus allow detection of a wider range of metabolites. In addition, quantitative, targeted profiles of catecholamines and steroids were measured considering that components in red wine have been implicated in dopamine turnover[27] and steroid metabolism.[28]

MATERIALS AND METHODS

Study Design. A placebo-controlled, randomized, full crossover trial with four treatments was conducted with approval by the medical ethical committee of the University of Wageningen and under the ICH-GCP guidelines at the Consumer Center at Unilever R&D, Vlaardingen, The Netherlands. The total duration of this trial was 5 weeks consisting of five consecutive 5 day treatment periods with 2 day washout periods between. In the present study, only a subset of the trial was taken into consideration, including the placebo treatment and the treatment consisting of a mix of red wine and WGM in gelatin capsules. The placebo consisted of microcrystalline cellulose (Avicel PH101; FMC Biopolymer, Philadelphia, PA, USA), and the polyphenol mix was composed of 870 mg dry weight Provinols red wine extract (Seppic, France) and 540 mg dry weight Mega-Natural Rubired grape juice extract (Madera, CA, USA). This amount corresponds to ca. 630 mL of red wine and grape juice. The approximate composition has been reported previously.[26] The amount was distributed over six capsules and was taken with 200 mL of water. Subjects consumed the product 1 h before breakfast on five consecutive days, starting on Monday.

A total of 35 healthy males (18−69 years; nonsmokers) participated in this trial. Each treatment started on a Monday (day 1) and ended on a Friday (day 5). On Thursday morning subjects started to collect urine for 24 h. On Friday morning, thus, after a 4 day long treatment period, subjects handed in their 24 h urine, and blood samples were withdrawn in the fasted state (t = 0 h). Additional physiological data recorded on day 5 after the final polyphenol dose are not discussed in this study.

On Wednesday and Thursday, subjects received standardized dinners low in polyphenols. Furthermore, subjects were instructed to repeat the diet that they consumed on Thursday of the first treatment period. In this way, each subject had the same dietary background before and after the WGM treatment and thus was his own control. From Tuesday evening on, subjects were restricted in their background diet with respect to polyphenol intake. The subjects were not allowed to consume polyphenol-rich foods (e.g., tea, coffee, fruit juices, beer, red wine, chocolate, onions, kale, broccoli, applesauce). They also consumed as little fruits and vegetables as possible. Furthermore, they were instructed to refrain from drinking alcohol-containing beverages and eating fish.

Sample Collection. All individual urine samples were collected by the subject over a period of 24 h in acid-containing containers (23 g of dried metaphosphoric acid (Sigma-Aldrich) in 2.5 L containers) to prevent bacterial growth. The samples were combined, and three aliquots of 10 mL each were stored at −20 °C. Nine milliliters of blood was drawn by venapuncture from the antecubital vein in tubes containing lithium heparin as anticoagulant. The blood samples were stored on ice for no longer than 35 min. Subsequently, plasma was separated by centrifugation (1500g, 10 min, 4 °C), aliquoted in three 1.5 mL samples, and stored at −80 °C.

GC-MS-Based Profiling of Phenolics in Urine. GC-MS-based profiles of phenolics were recorded as previously described.[26] The samples were run in several batches. However, all samples from a respective subject were analyzed in one batch. Phenolic acids in urine were identified using GC-MS spectra of commercially available standard compounds (Sigma-Aldrich). A target set of 18 phenolic acids was selected for semiquantitative analysis and identified by a specific retention time (rt)−m/z pair: pyrogallol (12.79−*239*), *trans*-cinnamic acid (12.95−*205*), 3-hydroxyphenylacetic acid (13.89−*281*), 4-hydroxyphenylacetic acid (14.36−*179*), 4-hydroxybenzoic acid (14.16−*267*), isovanillic acid (14.56−*257*), 3-hydroxyphenylpropionic acid (15.62−*310*), vanillic acid (16.14−297), homovanillic acid (16.23−*326*), 3,4-dihydroxyphenylacetic acid (17.00−*384*), 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (17.34−*267*), 3-methoxy-4-hydroxyphenylpropionic acid (17.88−*340*), vanillylmandelic acid (17.73−*297*), syringic acid (17.96−*327*), 3,4-dihydroxyphenylpropionic acid (18.50−*398*), ferulic acid (20.37−*338*); 3-hydroxyhippuric acid (21.06−*193*), and 4-hydroxyhippuric acid (21.98−*193*). A home-written script in Matlab (Matlab 2009R, Mathworks, USA; available upon request) was used for initial detection of phenolic acid-specific peaks in the extracted ion chromatogram and for visual inspection and semiautomatic peak integration. All peaks were first normalized to the internal standard *trans*-cinnamic acid-d_6 (12.95−*211*) (Sigma-Aldrich) to correct for analytical variation such as batch-to-batch variations. They were also normalized to the 24 h urine creatinine concentration, as determined by ^{1}H NMR spectroscopy, to correct for variable urine volumes.

Hippurate by NMR Spectroscopy. NMR-based metabolite profiles of urine were recorded as previously described.[26] Hippuric acid levels in urine were determined from the peak integral of its aromatic signal at 7.83 ppm, relative to the peak integral of creatinine at 3.05 ppm.

Broad Metabolite Profiling. The broad metabolite profiling comprised the combined approach of untargeted GC-MS- and LC-MS/MS-based metabolite profiling together with targeted profiling of catecholamines and steroids. Due to budgetary reasons, this broad metabolite profiling approach was performed on only a subset of samples including urine and plasma from 21 randomly selected subjects.

Three types of mass spectrometry analyses were applied to all urine and plasma samples. GC-MS (Agilent 6890 GC coupled to an Agilent 5973 MS-System, Agilent, Waldbronn, Germany) and LC-MS/MS (Agilent 1100 HPLC-System coupled to an Applied Biosystems API4000 MS/MS-System (Applied Biosystems, Darmstadt, Germany)) were used for broad profiling, as described by van Ravenzwaay et al.[29] Solid phase extraction (SPE)-LC-MS/MS (Symbiosis Pharma (Spark, Emmen, The Netherlands) coupled to an Applied Biosystems API4000 MS/MS-System) was used for the determination of catecholamine and steroid levels. In plasma, 234 metabolites fulfilled the quality criteria for relative quantification, and absolute quantification was performed for an additional 17 metabolites. From a total of 251 metabolites, 161 were known metabolites and 90 were not chemically identified with sufficient certainty (i.e., thus considered

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in the present study to be unknown analytes). In urine, 256 metabolites fulfilled the quality criteria for relative quantification. Of these, 159 were known metabolites, and 97 analytes were unknown analytes. Technical reference samples were measured in parallel with the study samples to allow the relative quantification of metabolites in the study samples. These technical reference samples were generated by pooling aliquots of the urine or plasma from all study samples. A relative quantification for each metabolite was obtained by normalizing peak intensity in the study samples to the median peak intensity of the corresponding metabolite in the technical reference samples measured in the same batch.

For the four-phase metabolite profiling by GC-MS and LC-MS/MS, proteins were removed from plasma samples (60 μL) by precipitation using acetonitrile. Subsequently, polar and nonpolar plasma fractions were separated for both GC-MS and LC-MS/MS analysis by adding water and a mixture of ethanol and dichloromethane. For GC-MS analyses, the nonpolar fraction was treated with methanol under acidic conditions to yield the fatty acid methyl esters derived from both free fatty acids and hydrolyzed complex lipids. The polar and nonpolar fractions were further derivatized with *O*-methylhydroxyamine hydrochloride (20 mg/mL in pyridine, 50 μL) to convert oxo groups to *O*-methyloximes and subsequently with a silylating agent (MSTFA, 50 μL) before GC-MS analysis.[30] For LC-MS/MS analyses, both fractions were reconstituted in appropriate solvent mixtures. High-performance LC (HPLC) was performed by gradient elution using methanol/water/formic acid on reversed phase separation columns. Mass spectrometric detection technology was applied as described in U.S. patent 7196323, which allows targeted and high-sensitivity multiple reaction monitoring profiling in parallel to a full screen analysis.

For the lipid phase, the broad profiling technology determines, for example, fatty acid concentrations after acid/methanol treatment, which is essential for derivatization preceding GC-MS analysis. As a consequence, complex lipids are hydrolyzed to components of the lipid backbone (i.e., glycerol) and fatty acids. Hence, the concentration of a fatty acid determined by this procedure represents the sum of its occurrence in free and in lipid-bound form. Components of the backbone can be recognized by the term ("lipid fraction") added to the metabolite name. As an example "glycerol, lipid fraction" represents glycerol liberated from complex lipids; in contrast, "glycerol, polar fraction" represents glycerol that had been present originally in the biological sample.

For urine analysis, a photometric creatinine analysis according to the method of Jaffé[31] was performed prior to polar MS analysis, and samples were diluted to reach the same creatinine concentration. The polar fraction was analyzed by both GC-MS and LC-MS/MS.

Use of the term "minor" (or "additional") in Figure 3 indicates that quantification can be affected by the co-occurrence of metabolites exhibiting identical characteristics in the analytical methods. Literature data or comparison with alternative methods (e.g., LC-MS/MS, GC-MS) suggests that such metabolites are present at minor levels only.

Steroids and their related metabolites were measured by online SPE-LC-MS/MS. Catecholamines and their related metabolites were measured by online SPE-LC-MS/MS, as described by Yamada et al.[32] For plasma, quantification was performed using stable isotope-labeled standards. In the case of urine samples, conjugated derivatives of steroids were enzymatically cleaved prior to analyses using a β-glucuronidase (EC 3.2.1.31) and an arylsulfatase (EC 3.1.6.1) from *Helix pomatia*. The relative quantification of catecholamines and steroids in urine was performed by normalization to pool levels as described for profiling.

Data Analysis of Broad Metabolite Profiles. A robustified statistical analysis was applied on paired data representing per individual the log 10-transformed ratios of the metabolite levels measured after the polyphenol intake to those measured after the placebo intake. The robustification included checks on the number of missing values per subject and for sample mix-up as well as univariate outlier detection for the log ratios of the metabolites. In urine an additional standardization was performed by dividing all log ratios of one subject by the robustified average standard deviation of all log ratios of this subject. In doing so, metabolite changes were down-weighted for those subjects showing overall strong changes between the two measurements. The robustified data set of log ratios was then further analyzed by paired *t* tests. To account for multiple testing, local false discovery rates were calculated for each metabolite.

To check for sample mixup a total least-squares regression was performed to estimate the strength of the relationship (R^2) of the metabolite composition of any two treatment samples, any two placebo samples, and any pair of placebo and treatment sample. The intraindividual correlation was then compared to the interindividual correlations. In metabolomic analysis and in nutritional intervention studies, the within-correlation is typically much larger than the between-correlations. A sample mixup was considered to be likely, if the within-correlation for a specific subject was at the size of the between-correlations. The urinary profiles of one subject were excluded from the analysis due to a likely sample mismatch: The R^2 value in the total least-squares regression for these two metabolite profiles was even lower than most intersubject R^2 values (subject's R^2 = 31%, intersubject R^2 = median 41%, quartiles, 32%, 50%) and much smaller than all of the other intrasubject R^2 values (median R^2 = 77%, second smallest R^2 = 59%). Two subjects were removed from the analysis of urine metabolites because the percentage of missing values in the paired analysis was high (>60% of metabolites compared to a mean of 5% for the others). For plasma samples, no mixup of samples was detected and no exclusion of subjects due to too many missing values was necessary.

The total least-squares regression R^2 values were also used to get an indication of the overall strength of between-treatment, within-treatment, and within-individual relationships of the metabolite profiles.

For univariate outlier detection, subjects with treatment-to-placebo log ratios beyond the 2.5*interquartile range of the log ratios calculated for all subjects and for the same metabolite were marked as outliers, such that they could be excluded for any statistical analysis that was sensitive to this type of outlier.

The local false discovery rate procedure was applied on the *p* values from the robustified paired *t* tests. First, the *p* values were transformed such that under the null hypothesis each of them would follow a standard normal distribution. The so-called local false discovery rate (locfdr) procedure was based upon the assumption that most metabolites were not affected by the treatment. Therefore, their transformed *p* values could be used to estimate a common empirical null distribution that is typically different from the standard normal distribution. If all metabolites would change independently from each other, the empirical null distribution would be standard normal. Due to the strong interdependencies of the metabolites, the empirical null distribution can become skewed, have larger or smaller variance, and can even be shifted in the mean.[33] The observed empirical distribution of all metabolites was considered to be a mixture of this empirical null distribution and some non-null distribution. By estimating both, the locfdr was calculated for each metabolite on the basis of its *p* value. Locfdr gives the estimated probability that there is actually no treatment effect on this metabolite. The procedure and its relationship to other strategies coping with the multiple testing problem in *omics type of data are described elsewhere.[33]

In this study, metabolites with a locfdr < 1 were considered to be statistically interesting. This threshold is higher than the threshold of 0.2, which is conventionally used.[33] The applied threshold of 1 is the strictest criterion possible to avoid false-negative discovery rates, or, in other words, the weakest possible criterion for false-positive discovery rates. Due to the subtle effects expected in human nutritional interventions, any metabolite was considered to be statistically interesting insomuch as there was an estimated positive probability that it was affected by the treatment. This statistical analysis was performed in Matlab (Matlab 2009R, Mathworks, USA) in combination with R package locfdr version 1.1-6 and included known and unknown metabolites.

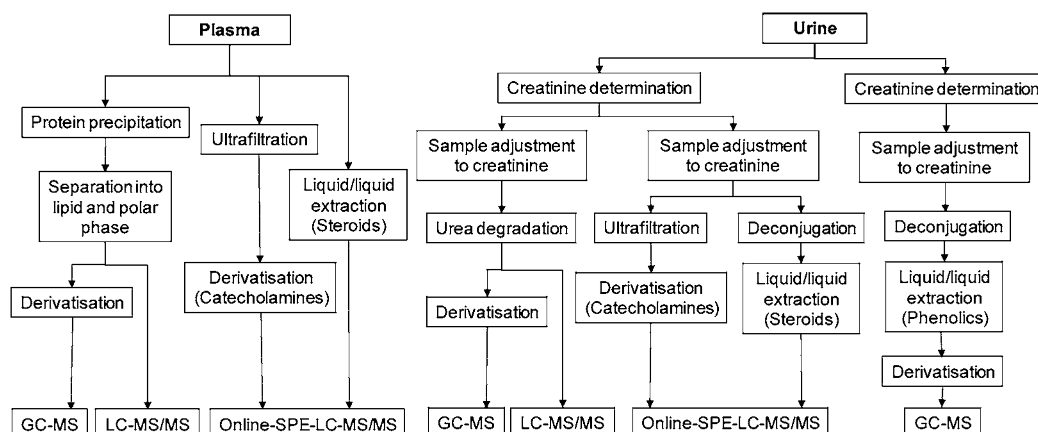

Figure 1. Scheme of metabolite profiling approach.

RESULTS

In the current study, we assessed the exogenous and endogenous metabolic effects of a 4 day long intake of WGM in healthy male adults. Only male subjects were recruited to limit the interindividual variability. Considering the different polyphenol composition of red wine and grape juice with red wine being rich in proanthocyanidins and grape juice in anthocyanins, we used a mixture of red wine and grape juice extracts to cover a broad range of polyphenols. Figure 1 displays our comprehensive metabolite profiling approach. Untargeted GC-MS-based profiling of phenolic metabolites was used to identify various gut microbiota-mediated markers of WGM in urine. Hippuric acid, the most abundant phenolic acid in urine, was measured using NMR spectroscopy, because the concentrations tend to be underestimated using MS detection due to saturation effects. After the 4 day intake of WGM, 14 phenolic metabolites were increased in 24 h urine (Figure 2). More than 2-fold increases were found for syringic acid, 3-hydroxyhippuric acid, pyrogallol, 3-hydroxyphenylacetic acid, and 3-(3-hydroxyphenyl)propionic acid. In comparison, other phenolic acids such as ferulic acid and *trans*-cinnamic acid and (derivatives of) vanillic acid showed only slight increases. These differences became particularly apparent when the effects per individual were compared, thus, by calculating the ratios of concentrations obtained after the WGM and placebo treatments. This indicates a significant interindividual variation in the gut microbial bioconversion of polyphenols and highlights the importance of a crossover design, in which each subject serves as his own control.

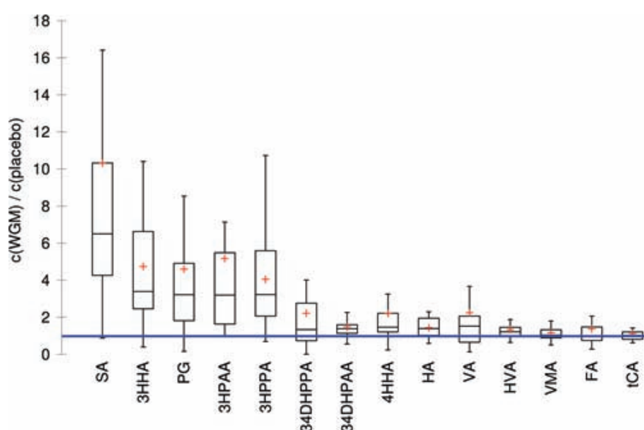

Figure 2. Summary of exogenous metabolites in urine with increased concentrations after the WGM intake when compared to placebo. The metabolites were identified from GC-MS-based profiles of phenolics. The box plots of fold changes [c(WGM)/c(placebo)] are displayed. In the box plots, the 0.1%ile, 0.25 quartile, median, 0.75 quartile, 0.9%ile, and average values are represented. SA, syringic acid; 3HHA, 3-hydroxyhippuric acid; PG, pyrogallol; 3HPPA, 3-(3-hydroxyphenyl)-propionic acid; 34DHPPA, 3-(3,4-dihydroxyphenyl)propionic acid; 34DHPAA, 3,4-dihydroxyphenylacetic acid; 4HHA, 4-hydroxyhippuric acid; HA, hippuric acid; VA, vanillic acid; HVA, homovanillic acid; VMA, vanillylmandelic acid; FA, ferulic acid; tCA, *trans*-cinnamic acid.

The broad four-phase metabolite profiling (GC-MS and LC-MS/MS) together with quantitative targeted profiling of catecholamines and steroids delivered data for a total of 256 and 249 metabolites in urine and plasma, respectively. In each biofluid 159 compounds were identified. Table 1 lists the number of metabolites per compound class and thus demonstrates the broad coverage of our metabolite profiling approach. The total least-squares regression analysis showed that the overall metabolite composition was not significantly affected by the WGM intake: A general effect on the metabolite composition that can be attributed to WGM and that exceeded

Table 1. Number of Known Metabolites per Compound Class Measured in Urine and Plasma

compound class	urine	plasma
amino acids	17	20
amino aicd derivatives	20	14
carbohydrates and related metabolites	32	10
glycolipids	2	
metabolites involved in energy metabolites	9	8
nucleobases and related metabolites	8	2
vitamins (cofactors and related metabolites)	8	7
catecholamines and other monoamines	15	11
steroids	7	8
miscellaneous compounds	41	14
group of cholesterol, bile acids, and fatty alcohols		6
fatty acids		20
glycerides		6
glycolipids		3
phospholipids		21
sphingolipids		9
phenolic metabolites[a]	18	

[a]Derived from GC-MS-based profiling of phenolics.

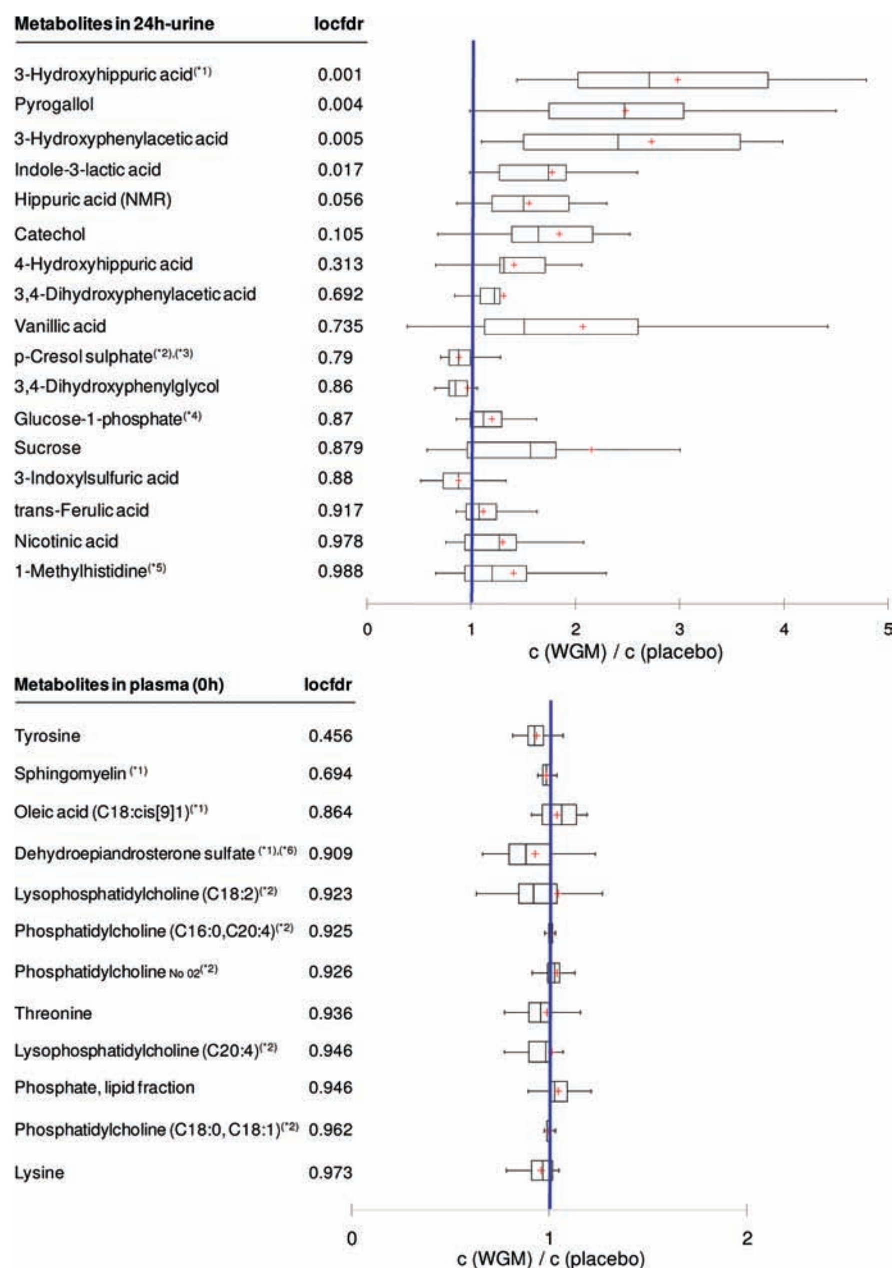

Figure 3. Summary of identified metabolites in urine (upper panel) and fasted plasma (lower panel) after 4 days of dietary intervention that showed statistically interesting differences in concentrations between the WGM and placebo treatments. For each metabolite, the local false discovery rates (locfdr) and the box plots of fold changes [c(WGM)/c(placebo)] are displayed. Metabolites with a locfdr < 1 were considered to be statistically interesting. The weakest possible criterion for false positive discovery rates was applied to account for subtle nutritional effects. In the box plots, the 0.1%ile, 0.25 quartile, median, 0.75 quartile, 0.9%ile, and average values are represented. $^{(*1)}$ Structure annotation is based on strong analytical evidence (combinations of, e.g., chromatography, mass spectrometry, chemical reactions, deuterium-labeling, database and literature search, comparison to similar/homologue/isomeric reference compounds). $^{(*2)}$ Metabolite exhibits identical qualitative analytical characteristics (chromatography and mass spectrometry) compared to a status $^{(*1)}$ metabolite. Further structural and analytical investigations of this metabolite, also in comparison to structurally identified or status $^{(*1)}$ metabolites, are still pending. $^{(*3)}$ (Minor: *m*-cresol sulfate, *o*-cresol sulfate). $^{(*4)}$ (Minor: glucose). $^{(*5)}$ (Minor: 3-methylhistidine). $^{(*6)}$ (Additional: testosterone-17-sulfate).

the interindividual variation was not observed. However, the crossover study design allowed for pairing the data and thus for differentiating on the level of each individual the effect induced by WGM from those induced by the placebo. In doing so, effects induced by the polyphenols are subject only to intraindividual variations, which are usually lower than the interindividual variations.[34]

In urine, the estimation of the local false discovery rates found 17 of 159 known metabolites statistically interesting (upper panel of Figure 3). In fact, the cutoff "<1" for the local false discovery rate coincided well with the conventional univariate cutoff of 0.05 for the p value (locfdr < 1 in comparison to p value < 0.055). Of these 17 statistically interesting metabolites, 9 were phenolic compounds. 3-Hydroxyhippuric acid, pyrogallol, and 3-hydroxyphenylacetic acid showed the highest ranking. These results are in large agreement with those from untargeted GC-MS-based profiling of phenolics. However, the untargeted GC-MS-based profiling

method was targeted to phenolics and hence able to identify more phenolic compounds, such as syringic acid, 3-(3-hydroxyphenyl)propionic acid, 3-(3,4-dihydroxyphenyl)-propionic acid, homovanillic acid, vanillylmandelic acid, and *trans*-cinnamic acid. Unidentified signals in the untargeted profiles and/or a higher selectivity of the GC-MS-based profiling of phenolics may explain the higher coverage of the GC-MS method. On the other hand, catechol was only identified from the broad profiling method. In addition to the phenolic metabolites, changes in a number of other endogenous metabolites were observed: the concentrations of indole-3-lactic acid, glucose-1-phosphate, sucrose, nicotinic acid, and 1-methylhistidine were increased, whereas the concentrations of 3-indoxylsulfuric acid, *p*-cresol sulfate, and 3,4-dihydroxyphenylglycol were decreased.

In fasted plasma, 12 of 161 known metabolites were identified with locfdr values below 1, which were affected by the 4 day intake of WGM (lower panel of Figure 3). Most of these 12 metabolites showed high locfdr values (>0.9) and only small changes of the median values (±5%). The statistically most interesting metabolite changes were reductions in tyrosine (−8%, locfdr of 0.46) and sphingomyelin (−2%, locfdr of 0.69). Furthermore, reductions in threonine, lysine, dehydroepiandrosterone sulfate (−12%), and two lysophosphatidylcholines were notable.

■ DISCUSSION

In this study, we identified a range of low molecular weight phenolic compounds in urine after a 4 day intake of WGM. The most abundant phenolic metabolites in urine were syringic acid, 3-hydroxyhippuric acid, pyrogallol, 3-hydroxyphenylacetic acid, and 3-hydroxyphenylpropionic acid, showing a >2-fold increase after the WGM intake. Other phenolic metabolites with increased concentrations included 3-(3,4-dihydroxyphenyl)-propionic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxyhippuric acid, hippuric acid, and vanillic acid. Most of these metabolites are known to originate from gut microbial fermentation of the wine and grape polyphenols,[13,26] although it cannot be excluded that some of them originated directly from the WGM itself. In particular, syringic acid, ferulic acid, and vanillic acid have been identified in the WGM.[26] Our results are largely in agreement with the results from our previous study with a similar placebo-controlled, crossover design and using the same WGM.[26] They confirm that a wider set of specific phenolic metabolites rather than one specific marker is characteristic for the intake of wine and grape polyphenols. Nevertheless, some differences between the two studies were observed: the levels of 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, isovanillic acid, 3-(3-hydroxyphenyl)-3-hydroxypropionic acid, dihydroferulic acid, and 4-hydroxyphenylmandelic acid were significantly elevated in the previous study,[26] but not in the current study. Differences in treatment durations (4 weeks versus 4 days), study population (male/female versus male), and dietary control (weak versus strong dietary restrictions) may account for the slightly different set of phenolic metabolites observed in these studies. The verification of results from two different studies is an essential validation step considering that phenolic metabolites are the end products of many food sources of which intake cannot be completely controlled during a longer term nutritional intervention study. It is assumed that the wide range of diverse polyphenols is funnelled into a limited number of microbial metabolites using similar degradation pathways. Syringic acid and vanillic acid are catabolites from anthocyanins known to be present in red wine.[35] Hydroxylated phenylpropionic acids can be converted to hydroxylated and free hippuric acids and are the main fermentation products of many flavonoids.[5,36] 3,4-Dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid have been identified from flavonols such as quercetin[37,38] and from in vitro fermentation of purified procyanidin dimers with human microbiota.[39] Interestingly, the urinary excretion of pyrogallol, a catabolite of catechins[40] and hydrolyzable tannins,[41] has not been widely associated with the intake of wine and grape polyphenols. It has mainly been shown that pyrogallol is present in urine as a sulfate conjugate after the consumption of black tea.[42]

Besides the effects on the exogenous metabolites, we have explored the impact of a 4 day intake of WGM on a wide range of metabolites involved in many different metabolic pathways with the aim to generate new hypotheses on the mechanism of actions of dietary polyphenols. The analysis of local false discovery rates clearly identified metabolite changes characteristic for WGM intake in urine. In general, the effects on endogenous metabolites were weaker than the effects on exogenous metabolites. Nevertheless, an interesting pattern of changes was found involving several amino acid derivatives. Most noticeable were the changes in microbial fermentation products of aromatic amino acids. The levels of nicotinic acid and indole-3-lactic acid were significantly increased in urine. Both metabolites can be produced from tryptophan by the activity of intestinal or gut microbiota. For example, *Bifidobacterium* spp. has been shown to be able to convert tryptophan to indole-3-lactive acid in culture broth.[43] Furthermore, reduced urinary levels of *p*-cresol sulfate and 3-indoxylsulfuric acid were found. Both compounds are direct bacterial metabolites of tyrosine and tryptophan, respectively.[19,44] These results suggest that WGM altered the breakdown of dietary protein in the colon. Putrefactive components are generally considered to cause adverse effects on the host. High levels of urinary *p*-cresol have been associated with various disease conditions such as acute infections, inflammatory bowel disease, and colon cancer.[45] Furthermore, *p*-cresol sulfate and indoxyl-3-sulfate have been identified as uremic toxins, which are poorly cleared with dialysis and have been linked to cardiovascular disease and oxidative injury.[46] Thus, wine and grape polyphenols may be beneficial by reducing colonic protein fermentation or changing microbial amino acid metabolism. Further indications that WGM had an impact on protein and/or amino acid metabolism are the increased levels of urinary 1-methylhistidine, which is known to derive from dietary meat sources,[47] and the reduced levels of tyrosine, threonine, and lysine in fasted plasma. Our findings are consistent with a previous study showing that polyphenols can influence protein digestibility.[48] Furthermore, interactions between polyphenols and plasma proteins are known to modulate the bioavailability and bioactivity of polyphenols.[49,50]

Aside from these changes in amino acids and their derivatives, we have found only little evidence supporting the hypothesis that wine and grape polyphenols affect the catecholamine metabolism. Only the urinary levels of 3,4-dihydroxyphenylglycol, a biochemical index of the norepinephrine turnover,[51,52] were reduced after the WGM intake. The reduction in urinary 3,4-dihydroxyphenylglycol may be related to tyramine, known to be present in red wine and to affect paroxysmal hypertension resulting from release of vesicular norephinephrine.[53]

The metabolite changes in fasted plasma after the 4 day intake of WGM were only marginal. Nevertheless, the cluster of the reductions in sphingomyelin, two lysophosphatidylcholines, and two phosphatidylcholines may indicate that wine and grape polyphenols affect the inflammatory signaling cascade, possibly at the mucosal gastrointestinal barrier.[54] Moreover, WGM also induced reduction in dehydroepinandrosterone sulfate in fasted plasma. This finding is novel, yet difficult to interpret because of the multiple biological functions of this steroid hormone. Further studies are required to confirm these subtle effects and to understand the significance of these metabolite changes.

In summary, the impact of the 4 day intake of WGM on the comprehensive metabolite profiles was small compared to the substantial interindividual variation. These subtle effects may be attributed to the interindividual variation in the gut microbial composition, the short duration of the WGM intake, and/or the complex composition of the WGM. However, owing to the crossover design and the multiparametric approach, clear metabolic changes could be attributed to the WGM. In essence, the 4 day intake of WGM mainly resulted in the urinary elevation of 14 phenolic metabolites originating from gut microbial fermentation of diverse polyphenols present in WGM. The effects of WGM intake on endogenous metabolism were less pronounced. Most noticeable were changes in several amino acid derivatives including bacterial metabolites of tyrosine and tryptophan. These findings led to the hypothesis that wine and grape polyphenols or their microbial metabolites alter colonic protein fermentation and/or amino acid metabolism. Our results encourage follow-up studies elucidating the cross-talk between the gut microbial metabolism and host metabolic response following polyphenol and protein consumption.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Cooper, K. A.; Chopra, M.; Thurnham, D. I. Wine polyphenols and promotion of cardiac health. *Nutr. Res. Rev.* **2004**, *17* (1), 111–130.

(2) Walzem, R. L. Wine and health: state of proofs and research needs. *Inflammopharmacology* **2008**, *16* (6), 265–271.

(3) Renaud, S.; de Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **1992**, *339* (8808), 1523–1526.

(4) Karatzi, K.; Karatzis, E.; Papamichael, C.; Lekakis, J.; Zampelas, A. Effects of red wine on endothelial function: postprandial studies vs clinical trials. *Nutr. Metab Cardiovasc. Dis.* **2009**, *19* (10), 744–750.

(5) Forester, S. C.; Waterhouse, A. L. Metabolites are key to understanding health effects of wine polyphenolics. *J. Nutr.* **2009**, *139* (9), 1824S–1831S.

(6) van Duynhoven, J.; Vaughan, E. E.; Jacobs, D. M.; Kemperman, A.; van Velzen, E. J.; Gross, G.; Roger, L. C.; Possemiers, S.; Smilde, A. K.; Dore, J.; Westerhuis, J. A.; van de Wiele, T. Microbes and Health Sackler Colloquium: Metabolic fate of polyphenols in the human superorganism. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108* (Suppl. 1), 4531–4538.

(7) Waterhouse, A. L.; Walzem, R. L. Nutrition of grape phenolics. In *Flavonoids in Health and Disease*; Rice-Evans, C. C., Packer, L., Eds.; Dekker: New York, 1998; pp 359–385.

(8) Manach, C.; Donovan, J. L. Pharmacokinetics and metabolism of dietary flavonoids in humans. *Free Radical Res.* **2004**, *38* (8), 771–785.

(9) Scalbert, A.; Morand, C.; Manach, C.; Remesy, C. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomed. Pharmacother.* **2002**, *56* (6), 276–282.

(10) Requena, T.; Monagas, M.; Pozo-Bayón, M. A.; Martín-Álvarez, P. J.; Bartolomé, B.; del Campo, R.; Ávila, M.; Martinéz-Cuesta, M. C.; Peláez, C.; Moreno-Arribas, M. V. Perspectives of the potential implications of wine polyphenols on human oral and gut microbiota. *Trends Food Sci. Technol.* **2010**, *21*, 332–344.

(11) Aron, P. M.; Kennedy, J. A. Flavan-3-ols: nature, occurrence and biological activity. *Mol. Nutr. Food Res.* **2008**, *52* (1), 79–104.

(12) Borriello, A.; Cucciolla, V.; Della, R. F.; Galletti, P. Dietary polyphenols: focus on resveratrol, a promising agent in the prevention of cardiovascular diseases and control of glucose homeostasis. *Nutr. Metab. Cardiovasc. Dis.* **2010**, *20* (8), 618–625.

(13) Selma, M. V.; Espin, J. C.; Tomas-Barberan, F. A. Interaction between phenolics and gut microbiota: role in human health. *J. Agric. Food Chem.* **2009**, *57* (15), 6485–6501.

(14) Halliwell, B.; Rafter, J.; Jenner, A. Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? *Am. J. Clin. Nutr.* **2005**, *81* (1 Suppl.), 268S–276S.

(15) Brennan, L. Session 2: Personalised nutrition. Metabolomic applications in nutritional research. *Proc. Nutr. Soc.* **2008**, *67* (4), 404–408.

(16) Primrose, S.; Draper, J.; Elsom, R.; Kirkpatrick, V.; Mathers, J. C.; Seal, C.; Beckmann, M.; Haldar, S.; Beattie, J. H.; Lodge, J. K.; Jenab, M.; Keun, H.; Scalbert, A. Metabolomics and human nutrition. *Br. J. Nutr.* **2011**, 1–7.

(17) Stella, C.; Beckwith-Hall, B.; Cloarec, O.; Holmes, E.; Lindon, J. C.; Powell, J.; van der Ouderaa, F.; Bingham, S.; Cross, A. J.; Nicholson, J. K. Susceptibility of human metabolic phenotypes to dietary modulation. *J. Proteome Res.* **2006**, *5* (10), 2780–2788.

(18) Nicholson, J. K.; Holmes, E.; Wilson, I. D. Gut microorganisms, mammalian metabolism and personalized health care. *Nat. Rev. Microbiol.* **2005**, *3* (5), 431–438.

(19) Wikoff, W. R.; Anfora, A. T.; Liu, J.; Schultz, P. G.; Lesley, S. A.; Peters, E. C.; Siuzdak, G. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106* (10), 3698–3703.

(20) Llorach, R.; Urpi-Sarda, M.; Jauregui, O.; Monagas, M.; Andres-Lacueva, C. An LC-MS-based metabolomics approach for exploring urinary metabolome modifications after cocoa consumption. *J. Proteome Res.* **2009**, *8* (11), 5060–5068.

(21) Llorach, R.; Garrido, I.; Monagas, M.; Urpi-Sarda, M.; Tulipani, S.; Bartolome, B.; Andres-Lacueva, C. Metabolomics study of human urinary metabolome modifications after intake of almond (*Prunus dulcis* (Mill.) D.A. Webb) skin polyphenols. *J. Proteome Res.* **2010**, *9* (11), 5859–5867.

(22) Solanky, K. S.; Bailey, N. J.; Beckwith-Hall, B. M.; Bingham, S.; Davis, A.; Holmes, E.; Nicholson, J. K.; Cassidy, A. Biofluid ^{1}H NMR-based metabonomic techniques in nutrition research – metabolic effects of dietary isoflavones in humans. *J. Nutr. Biochem.* **2005**, *16* (4), 236–244.

(23) van Dorsten, F. A.; Daykin, C. A.; Mulder, T. P.; van Duynhoven, J. P. Metabonomics approach to determine metabolic differences between green tea and black tea consumption. *J. Agric. Food Chem.* **2006**, *54* (18), 6929–6938.

(24) Walsh, M. C.; Brennan, L.; Pujos-Guillot, E.; Sebedio, J. L.; Scalbert, A.; Fagan, A.; Higgins, D. G.; Gibney, M. J. Influence of acute phytochemical intake on human urinary metabolomic profiles. *Am. J. Clin. Nutr.* **2007**, *86* (6), 1687–1693.

(25) Manach, C.; Hubert, J.; Llorach, R.; Scalbert, A. The complex links between dietary phytochemicals and human health deciphered by metabolomics. *Mol. Nutr. Food Res.* **2009**, *53* (10), 1303−1315.
(26) van Dorsten, F. A.; Grun, C. H.; van Velzen, E. J.; Jacobs, D. M.; Draijer, R.; van Duynhoven, J. P. The metabolic fate of red wine and grape juice polyphenols in humans assessed by metabolomics. *Mol. Nutr. Food Res.* **2010**, *54* (7), 897−908.
(27) de la Torre, R.; Covas, M. I.; Pujadas, M. A.; Fito, M.; Farre, M. Is dopamine behind the health benefits of red wine? *Eur. J. Nutr.* **2006**, *45* (5), 307−310.
(28) Dolara, P.; Luceri, C.; De, F. C.; Femia, A. P.; Giovannelli, L.; Caderni, G.; Cecchini, C.; Silvi, S.; Orpianesi, C.; Cresci, A. Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. *Mutat. Res.* **2005**, *591* (1−2), 237−246.
(29) van Ravenzwaay, B.; Cunha, G. C.; Leibold, E.; Looser, R.; Mellert, W.; Prokoudine, A.; Walk, T.; Wiemer, J. The use of metabolomics for the discovery of new biomarkers of effect. *Toxicol. Lett.* **2007**, *172* (1−2), 21−28.
(30) Roessner, U.; Wagner, C.; Kopka, J.; Trethewey, R. N.; Willmitzer, L. Technical advance: simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. *Plant J.* **2000**, *23* (1), 131−142.
(31) Cook, J. G. Creatinine assay in the presence of protein. *Clin. Chim. Acta* **1971**, *32* (3), 485−486.
(32) Yamada, H.; Yamahara, A.; Yasuda, S.; Abe, M.; Oguri, K.; Fukushima, S.; Ikeda-Wada, S. Dansyl chloride derivatization of methamphetamine: a method with advantages for screening and analysis of methamphetamine in urine. *J. Anal. Toxicol.* **2002**, *26* (1), 17−22.
(33) Efron, B. Microarrays, empirical Bayes and the two-groups model. *Statistical Sci.* **2008**, *23* (1), 1−22.
(34) van Velzen, E. J.; Westerhuis, J. A.; Van Duynhoven, J. P.; van Dorsten, F. A.; Hoefsloot, H. C.; Jacobs, D. M.; Smit, S.; Draijer, R.; Kroner, C. I.; Smilde, A. K. Multilevel data analysis of a crossover designed human nutritional intervention study. *J. Proteome Res.* **2008**, *7* (10), 4483−4491.
(35) Aura, A. M. Microbial metabolism of dietary phenolic compounds in the colon. *Phytochem. Rev.* **2008**, *7*, 407−429.
(36) Gross, G.; Jacobs, D. M.; Peters, S.; Possemiers, S.; van Duynhoven, J.; Vaughan, E. E.; van de Wiele, T. In vitro bioconversion of polyphenols from black tea and red wine/grape juice by human intestinal microbiota displays strong interindividual variability. *J. Agric. Food Chem.* **2010**, *58* (18), 10236−10246.
(37) Gross, M.; Pfeiffer, M.; Martini, M.; Campbell, D.; Slavin, J.; Potter, J. The quantitation of metabolites of quercetin flavonols in human urine. *Cancer Epidemiol. Biomarkers Prev.* **1996**, *5* (9), 711−720.
(38) Gonthier, M. P.; Cheynier, V.; Donovan, J. L.; Manach, C.; Morand, C.; Mila, I.; Lapierre, C.; Remesy, C.; Scalbert, A. Microbial aromatic acid metabolites formed in the gut account for a major fraction of the polyphenols excreted in urine of rats fed red wine polyphenols. *J. Nutr.* **2003**, *133* (2), 461−467.
(39) Appeldoorn, M. M.; Vincken, J. P.; Aura, A. M.; Hollman, P. C.; Gruppen, H. Procyanidin dimers are metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-γ-valerolactone as the major metabolites. *J. Agric. Food Chem.* **2009**, *57* (3), 1084−1092.
(40) Crozier, A.; Del, R. D.; Clifford, M. N. Bioavailability of dietary flavonoids and phenolic compounds. *Mol. Aspects Med.* **2010**, *31* (6), 446−467.
(41) Diez, M. T.; Garcia, d. M.; Resines, J. A.; Arin, M. J. Determination of phenolic compounds derived from hydrolysable tannins in biological matrices by RP-HPLC. *J. Sep. Sci.* **2008**, *31* (15), 2797−2803.
(42) Daykin, C. A.; van Duynhoven, J. P.; Groenewegen, A.; Dachtler, M.; Van Amelsvoort, J. M.; Mulder, T. P. Nuclear magnetic resonance spectroscopic based studies of the metabolism of black tea polyphenols in humans. *J. Agric. Food Chem.* **2005**, *53* (5), 1428−1434.
(43) Aragozzini, F.; Ferrari, A.; Pacini, N.; Gualandris, R. Indole-3-lactic acid as a tryptophan metabolite produced by *Bifidobacterium* spp. *Appl. Environ. Microbiol.* **1979**, *38* (3), 544−546.
(44) De Preter, V.; Geboes, K.; Verbrugghe, K.; De Vuyst, L.; Vanhoutte, T.; Huys, G.; Swings, J.; Pot, B.; Verbeke, K. The in vivo use of the stable isotope-labelled biomarkers lactose-[^{15}N]ureide and [$^{2}H_4$]tyrosine to assess the effects of pro- and prebiotics on the intestinal flora of healthy human volunteers. *Br. J. Nutr.* **2004**, *92* (3), 439−446.
(45) Jacobs, D. M.; Gaudier, E.; van Duynhoven, J.; Vaughan, E. E. Non-digestible food ingredients, colonic microbiota and the impact on gut health and immunity: a role for metabolomics. *Curr. Drug Metab.* **2009**, *10* (1), 41−54.
(46) Raff, A. C.; Meyer, T. W.; Hostetter, T. H. New insights into uremic toxicity. *Curr. Opin. Nephrol. Hypertens.* **2008**, *17* (6), 560−565.
(47) Dragsted, L. O. Biomarkers of meat intake and the application of nutrigenomics. *Meat Sci.* **2010**, *84* (2), 301−307.
(48) McDougall, G. J.; Stewart, D. The inhibitory effects of berry polyphenols on digestive enzymes. *Biofactors* **2005**, *23* (4), 189−195.
(49) D'Archivio, M.; Filesi, C.; Vari, R.; Scazzocchio, B.; Masella, R. Bioavailability of the polyphenols: status and controversies. *Int. J. Mol. Sci.* **2010**, *11* (4), 1321−1342.
(50) Xiao, J.; Kai, G. A review of dietary polyphenol-plasma protein interactions: characterization, influence on the bioactivity, and structure-affinity relationship. *Crit. Rev. Food Sci. Nutr.* **2012**, *52* (1), 85−101.
(51) Eisenhofer, G.; Kopin, I. J.; Goldstein, D. S. Catecholamine metabolism: a contemporary view with implications for physiology and medicine. *Pharmacol. Rev.* **2004**, *56* (3), 331−349.
(52) Elsworth, J. D.; Roth, R. H.; Redmond, D. E. Jr. Relative importance of 3-methoxy-4-hydroxyphenylglycol and 3,4-dihydroxyphenylglycol as norepinephrine metabolites in rat, monkey, and humans. *J. Neurochem.* **1983**, *41* (3), 786−793.
(53) Goldstein, D. S.; Eisenhofer, G.; Kopin, I. J. Sources and significance of plasma levels of catechols and their metabolites in humans. *J. Pharmacol. Exp. Ther.* **2003**, *305* (3), 800−811.
(54) Schneider, H.; Braun, A.; Fullekrug, J.; Stremmel, W.; Ehehalt, R. Lipid based therapy for ulcerative colitis-modulation of intestinal mucus membrane phospholipids as a tool to influence inflammation. *Int. J. Mol. Sci.* **2010**, *11* (10), 4149−4164.