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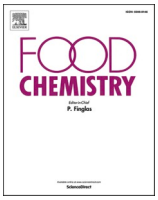

Exploring biomarkers of regular wine consumption in human urine: Targeted and untargeted metabolomics approaches

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ABSTRACT

The epidemiological assessment of wine consumption usually has been obtained using self-reporting questionnaires. In this study, two metabolomic approaches, targeted and untargeted, were applied to 24-h urine samples from a cohort of La Rioja (Spain) (aged 52–78), comparing moderate and daily wine consumers (20 males and 13 females) without diet intervention, versus non-consumers (8 males and 35 females). Results showed that the non-targeted metabolomics approach has allowed for the annotation of sixteen compounds in 24-h urine samples from regular wine-consumers that were not detected in the urine of non-wine consumers. Additionally, the targeted metabolomics approach showed a wide range of phenol metabolites, mainly hepatic phase-II conjugates, whose concentration was significantly higher in the urine of wine consumers. As a novelty, this study focuses on discovering the main urinary biomarkers of regular wine consumption involving free-living volunteers, without dietary intervention or restrictions that might alter their regular behaviors and lifestyles.

1. Introduction

Moderate wine consumption has been positively correlated with beneficial health effects, including the prevention of cardiovascular diseases, anti-inflammatory, antioxidant and antiatherogenic effects, among others (Fraga et al., 2010; Rasouli et al., 2017; Esteban-Fernández et al., 2018; Buljeta et al., 2023). The beneficial effect of wine seems to be ascribed to its polyphenolic content (Esteban-Fernández et al., 2018; Hrelia et al., 2023) together with the "Mediterranean way of drinking" that means a moderate wine consumption mainly with meals (Giacosa et al., 2016). After wine intake, polyphenols undergo several metabolic modifications and are extensively metabolized by the liver and gut microbiota (Del Rio et al., 2013; Esteban-Fernández et al., 2018) before being excreted in urine. According to the literature, a limited number of compounds have been proposed as good biomarkers of moderate wine consumption, including gallic acid, 4-*O*-methylgallic acid, dihydroxyphenyl valerolactones, ethylgallate, epicatechin, phenolic acids and mainly resveratrol metabolites (Zamora-Ros et al., 2009; Urpi-Sarda et al., 2015; Motilva et al., 2016; Mosele & Motilva, 2021). Additionally, Regueiro et al. (2014) reported an increase in tartaric acid in urine after the consumption of wine, suggesting that urinary tartaric acid may be a sensitive and specific biomarker of wine consumption.

Metabolomic strategies are commonly categorized into two approaches, untargeted and targeted metabolomics. Over the last decade, the significance of untargeted metabolomics has increased with promising results in biomarker seeking in a wide range of field, including dietary studies (Esteban-Fernández et al., 2018; Lacalle-Bergeron et al., 2023; Low et al., 2019; Rebholz et al., 2018), drug discovery (Alarcón-Barrera et al., 2022), plant biology (Carreno-Quintero et al., 2012) and biomedical research (Yang et al., 2022; You et al., 2019; Yu et al., 2017). This cutting-edge technology could be extremely useful in corroborating food consumption when self-reported dietary assessment methods are used, especially in the case of wine, given that individuals may be reluctant to report customary drinking levels accurately for a range of social reasons (Regueiro et al., 2014). Untargeted metabolomics can

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reveal a large number of signals, and is useful for exploring samples and discovering new biomarkers (Peron et al., 2020). The existence of prior knowledge of the sample is not a requirement, which is one of the major advantages of this approach (Schrimpe-Rutledge et al., 2016). Liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) is one of the most suitable techniques for untargeted metabolomics (Odriozola & Corrales, 2015), LC enables the separation of a wide range of metabolites with a variety of stationary phases available and HRMS allows the exact mass identification of compounds with high sensitivity and selectivity to confirm the molecular formula of compounds with errors lower than 5 ppm. In contrast, targeted metabolomics is focused on the identification of well-defined groups of metabolites (Leborgne et al., 2022). Every parameter of the analysis (ionization energy, selection of the *m/z*, etc.) can be customized and optimized for the detection of each compound. Meanwhile, in untargeted metabolomics, a compromise must be reached to optimize the conditions of the experiment, since every compound will need different parameters and there is no knowledge of the compounds present in the sample. Subsequently, combined use of both metabolomics strategies could be promising for exploring the compounds present in the samples.

To date, most of the works that have studied the effect of polyphenols on health are based on diet intervention studies (Annuzzi et al., 2014; Belda et al., 2021; Esteban-Fernández et al., 2018; Esteban-Fernández et al., 2020; Noad et al., 2016; Vetrani et al., 2020). Thus phenol intake biomarkers can be identified accurately mainly by targeted metabolomics. Moreover, the appropriate use of specific food biomarkers in clinical and epidemiologic studies requires the previous validation in the free-living population (Zamora-Ros et al., 2009). A limitation of studies without diet intervention is related to inter-individual variability. Consequently, drawing conclusions might be an arduous task.

Based on this background, we hypothesize that untargeted metabolomics may serve as an effective tool for identifying new biomarkers associated with wine consumption, as it facilitates the qualitative identification and relative quantification of thousands of endogenous metabolites in biological samples. Prior knowledge of the sample is not a requirement, which is one of the major advantages of this approach. Additionally, urine samples have drawn increasing attention in biomarker discovery because, compared with other biofluids, such as plasma and serum, they can be obtained non-invasively. In this study, targeted and untargeted metabolomics were applied to study the metabolite profile of 24-h human urine samples, comparing those from regular wine consumers and from non-consumers (aged 52–78) in a cohort in La Rioja (Spain). The targeted metabolomics approach was performed (LC-triple-quadrupole) for supervised analysis and quantification of the wine phenolic metabolites excreted in the 24-h urine samples. Untargeted metabolomics was performed by UPLC-QTOF-MS combining RP and HILIC. As a novelty, this study focuses on discovering the main urinary biomarkers of regular wine consumption involving free-living volunteers without dietary intervention or restrictions that could influence their usual behaviors and lifestyles.

2. Materials and methods

2.1. Chemicals and reagents

LC-MS grade acetonitrile (ACN) and LC-MS grade ammonium formiate were from Carlo Erba (Sabadell, Barcelona, Spain). Formic acid eluent additive for the LC-MS grade was purchased from VWR (Radnor, Pennsylvania, USA). Methanol (HPLC grade), acetic acid (HPLC grade), and hydrochloric acid were purchased from Scharlab Chemie (Sentmenat, Catalonia, Spain). The ultrapure water was supplied by a MilliQ system (Millipore Corp, Bedford, MA, USA). Leucine-enkephalin (mass-axis calibration) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). The analytical standards used in targeted metabolomics are described in Supplementary Material (Supplementary Material Text S1). Stock solutions of standard compounds were prepared by dissolving each compound in methanol at a concentration of 1000 mg/L, which were then stored in dark flasks at −20 °C.

2.2. Human subjects

One hundred and three participants aged 52–78 were recruited from February 2021 to September 2022 in the Hospital San Pedro in Logroño, La Rioja (Spain). All participants signed informed consent statements to participate in the study. The participants had no diet intervention and maintained their usual daily activities, dietary habits and social interactions, so they could be considered “free-living population”. Their dietary habits were assessed using a semi-quantitative 136-item Food Frequency Questionnaire (FFQ) previously validated for the Spanish population (De La Fuente-Arrillaga et al., 2010). Participants were asked how often, on average, they had consumed specific foods and beverages with pre-specified portion sizes during the preceding year, also considering seasonal variations and differences between weekday and weekend patterns. Each food included in the questionnaire specified the serving size and offered nine options for frequency of consumption, from “never or almost never” to “more than six times a day”. In particular, the FFQ includes questions related to the frequency of wine intake. Based on the FFQ answers, among the 103 volunteers who made up the cohort, we selected the 24-h urine samples from individuals who consumed wine on a daily basis (1–4 glasses wine/day), excluding individuals with sporadic and weekend consumers. Two groups were stablished: (i) regular consumers (33 participants, 20 males and 13 females) and (ii) non-consumers (43 participants, 8 males and 35 females) who never consumed wine (the control group). In addition, the complete FFQ was used to extract the questions included in the 14-items of the Mediterranean Diet Adherence Score (MEDAS) (Schröder et al., 2011). All the volunteers (wine consumers and non-consumers) in the study adhered closely to the Mediterranean diet ($\geq$ 9). Urine samples were collected from each participant for 24 h. To control risk of bacterial growth, immediately after collection, each 24-h urine samples was coded, divided into aliquots of 10 mL in Falcon tubes and stored at −80 °C until its chromatographic analysis. This ensures that the samples were properly preserved to minimize the risk of bacterial contamination and interference with the metabolomic analysis. This study was framed within the WineGut_BrainUP Project (PID2019-108851RB-C22), and it was approved by the Committee for Ethics in Drug Research in La Rioja, Spain (Reference CEImLAR P.I. 437). The study was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki), and the UNESCO Universal Declaration on Bioethics and Human Rights.

2.3. Targeted metabolomics analysis of 24-h urine and statistical analysis

Before the chromatographic analysis, the urine samples were pretreated by micro-Elution solid-phase extraction (μSPE) using OASIS HLB (2 mg) (Waters, Milford, MA, USA) microcartridges. The methodology used was reported in a previous study (Motilva et al., 2016).

The urine samples were analyzed by ultra-high-performance liquid chromatography with triple-quadrupole mass spectrometry (UHPLC/QqQ-MS/MS) based on the method described by (Royo et al., 2021). Three replicates of each sample were performed and each replicate was analyzed once. The LC analyses were carried out on a Shimadzu Nexera liquid chromatograph (Shimadzu Corporation, Kyoto, Japan), coupled to an AB Sciex 3200QTRAP® mass spectrometer (AB Sciex, Old Connecticut Path Framingham, MA, USA). The analytical column used was a Waters AcQuity BEH C_{18} (100 mm × 2.1 mm i.d., 1.7 μm,) equipped with a VanGuard™ Pre-Column Acquity BEH C_{18} (5 × 2.1 mm, 1.7 μm) from Waters (Milford, MA, USA). The mobile phase solvents and the elution gradients were as described in Royo et al. (2021). Tandem MS analyses were carried out on a 3200QTRAP triple quadrupole mass spectrometer (AB Sciex, USA) equipped with an electrospray ionization source (ESI Turbo V™ Source). Ionization was achieved using the

electrospray (ESI) interface operating in the positive mode $[M-H]^+$ for the analysis of the anthocyanin derivatives, and in the negative mode $[M-H]^-$ for the rest of the phenol metabolites. For each chromatographic peak, two multiple reaction monitoring (MRM) transitions were studied, the most sensitive one being selected for quantification and a second one for confirmation purposes. For each compound, the retention time (RT) and the MRM transitions for quantification and identification, respectively, are shown in Table S1 of Supplementary Material. Data acquisition was carried out with the Analyst ® 1.6.2 software (AB Sciex, USA). Compounds were identified by comparing their chromatographic behavior and mass spectra with those of authentic standards and the literature data. When no standards were available, the phenolic metabolites were identified by using the MS detector system. Subsequently, in order to determine their MRMs, MS analyses of these samples were carried out. These were based on the full-scan mode in the MS mode, and the daughter scan mode in the MS/MS mode according with the procedure described in Royo et al. (2021).

Some of the compounds were quantified using the calibration curves of their corresponding commercial standards. The other compounds were tentatively quantified using the calibration curves of standards with similar chemical structures (Table S1 of Supplementary Material). The results were expressed as nmols (anthocyanin metabolites) and μmols (rest of phenolic metabolites) excreted in 24-h urine, taking into account the total volume of urine collected in 24 h for each volunteer. Statistical comparisons between groups (wine consumers and non-consumers) were performed using Student's *t*-test. Differences were considered significant at a level of $p < 0.05$.

2.4. Untargeted metabolomics analysis of 24-h urine and statistical analysis

Prior to analysis, the urine samples were pre-treated following the protocol described previously by Want et al. (2010) with some modifications. Briefly, each urine sample was diluted 1:10 (*v*/v) with formic acid 0.1 % (RP) or acetonitrile (HILIC) in duplicate. The samples were centrifuged at 20000*g* for 10 min at 4 °C to eliminate particles, and filtered with a 0.22 μm PVDF filter (Scharlab; Barcelona, Spain). Quality control (QC) samples (pool of the 24-h urine samples from all volunteers) were prepared to evaluate the reproducibility of the analysis.

The urine samples were analyzed using a Waters Acquity™ Ultra Performance LC system coupled to a Synapt XS HDMS mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray source operating in positive (ESI+) and negative (ESI-) modes. Two chromatographic separations were employed to cover the range of compound polarities. The reverse phase liquid chromatography (RP-LC) was used for the separation of semi-polar and non-polar compounds. For both ionization modes, positive and negative, a (2.1 × 100 mm × 1.8 μm) HSS T3 Acquity premier (Waters) column with the same pre-column composition was used. The injection volume was 0.3 μL and the separation was performed at 0.5 mL/min and 40 °C. The sampler temperature was set at 8 °C. The mobile phase was water (A) and acetonitrile (B), both with 0.1 % formic. The elution gradient was as follows: 1 % B until 1 min, then the gradient increased to 15 % B at 3 min, 50 % B at 6 min, 95 % B at 9 min and it was held there for one minute until 10 min, finally 1 % B at 10.1 min.

In the hydrophilic interaction liquid chromatography (HILIC) (negative ionization mode), an Acquity UPLC® BEH HILIC column (1.7 μm × 2.1 × 100 mm) (Waters) was used with an Acquity UPLC BEH HILIC VanGuard pre-column (1.7 μm × 2.1 mm × 5 mm). The injection volume was 0.3 μL and the flow rate was 0.5 mL/min and 40 °C. The sampler temperature was set at 8 °C. The mobile phase was 10 mM ammonium formiate in water (A) and acetonitrile (B). The gradient started with 95 % B, then it decreased to 82 % B at 9 min, 40 % B at 17 min and it was held there for one minute until 18 min. Then the gradient increased to 95 % B at 19 min and was held there to equilibrate with a total run time of 30 min.

The samples were randomly injected to avoid biases and potential sequence effects during analysis. To generate a random order list for the samples, we used the online tool Random.org (https://www.random.org/). This approach ensured that samples were not introduced in the chromatography sequence according to the volunteer number or experimental group. This randomization minimized the risk of order-based errors and prevented any unintentional patterning in sample injection. Additionally, we included a quality control (QC) sample every 10 samples to monitor and maintain the stability and reproducibility of the instrumentation throughout the analysis. The inclusion of QCs at this frequency allowed us to detect any possible instrumental drift, thereby ensuring the robustness of the results obtained.

Apart from that, prior to the analysis of the urine samples, 10 and 15 QC injections were used for column stabilization in RP-LC and HILIC, respectively. These QC analyses for column stabilization were not considered for retention time alignment. Mass spectrometry data were collected in the continuum format using electrospray ionization (ESI) over an *m/z* range of 50–1200. The capillary and sampling cones were set to 1.75 kV and 40 V respectively for RP-LC and HILIC-neg. The source temperature was set at 120 °C and the desolvation temperature at 500 °C. The gas flow rates were set at 800 L/h for the desolvation gas and 50 L/h for the cone gas, and the nebulizer gas was fixed at 600 KPa. The mass spectrometer was set to acquire in resolution mode with a scan time of 0.2 s. Fragment ion information was acquired using a collision energy ramp from 20 to 40 V. Calibration was performed by infusing leucine enkephalin at 10 μL/min through a lockspray probe and acquired every 30 s. Lockmass correction was $[M + H]^+ = 556.2771$ for the positive mode, and for negative mode, $[M - H]^- = 554.2615$.

The raw data were imported into the Progenesis QI software (Waters, Milford, MA, USA) for baseline filter, peak alignment, retention time alignment and response normalization. The software employed the QC replicates as references to perform retention time alignment automatically. The conditions for peak peaking were set as follows: all runs, limits (automatic), sensitivity (level 4), and chromatographic peak width (minimum of 0.1 min). To perform the deconvolution, the following adduct ion forms were selected for negative ionization analysis: $[M-H]^-$, $[M + Cl]^-$, $[M-2H]^{2-}$, $[2\ M-H]^-$, $[M-H_2O-H]^-$ and $[M + FA-H]^-$. For positive ionization analysis these were $[M + H]^+$, $[M + 2H]^{2+}$, $[M + H-H_2O]^-$, $[M+]^+$, $[M + NH_4]^+$, $[M + Na]^+$, $[M + K]^+$, $[2\ M + H]^+$ and $[2\ M + Na]^+$.

The samples were divided into two groups (non-consumers and consumers of wine) in the "Experimental design setup", following the "Between-subject Design" comparison. Processed data were then exported to EZinfo (V3.03, Umetrics, Sweden). Firstly, Principal Component Analysis (PCA) was used to ensure the correct grouping of the quality control (QC) replicate samples and the absence of outliers. Orthogonal partial least square discriminant analysis (OPLS-DA) was then applied to maximize the separation between the groups and highlight the most robust markers. The separation achieved was acceptable, with coefficients for variance explained (R2Y) ranging from 67 to 99 % and coefficients for variance predicted (Q2) ranging from 43 to 91 % (Table 1). With those selected conditions, the Progenesis QI software performed a one-way ANOVA calculation and a false discovery rate (FDR) optimization approach to test the differences between the two

Table 1
Parameters of OPLS-DA models.

	RP-pos	RP-neg	HILIC-neg
Components	14	12	4
Variance explained R2Y (cum)	99 %	98 %	67 %
Variance predicted Q2 (cum)	88 %	91 %	43 %
Scaling	pareto	pareto	pareto

RP: Reverse Phase liquid chromatography.
HILIC: Hydrophilic Interaction Liquid Chromatography.
pos: positive (ESI+) ionization mode; neg: negative ionization (ESI-) mode.

groups. Statistical significance was set at 95 % (considering significance for the q-value <0.05, adjusted *p*-value using the FDR approach to discard false positives). S-plots were generated to visualize the 24-h urine markers with discrimination power between the two groups, regular wine drinkers and non-consumers (control). Ideally, the most relevant markers are features closer to *p(corr)* 1 or − 1. In this study, a cut-off *p(corr)* $\geq \pm 0.3$ and *p[1] loadings* $\geq \pm 0.1$ were employed. It should be noted that a specific nutritional intervention with wine was not carried out in this work. Hence it was a real-life situation and wine consumption was self-reported through the FFQ. The selected markers with their corresponding tags (wine or no-wine consumption) were imported again into Progenesis QI, and the elucidation process was limited to compounds with a q-value lower than 0.05. The most significant markers highlighted in the OPLS-DA with q-values of less than 0.5 were tentatively identified based on their accurate masses and spectra information. Outliers were identified using Hotelling's ellipse. The Metlin, Metabolic Profiling CCS and ChemSpider (databases: phenol-explorer, KEGG, Human Metabolome Database (HMDB), FooDB, Urine Metabolome Database) libraries allowed the annotation of the urine metabolites.

3. Results and discussion

One of the main strengths of this study is that the volunteers participating in this study are from La Rioja, a wine producing region in the north of Spain. La Rioja is a little and rural region, so the study participants (regular and daily wine consumers and non-consumers) have similar dietary patterns, age and life habits, which makes it a very homogeneous population from a metabolomics point of view. Wine was part of their daily diet and Mediterranean life style in the consumer group, which made the search for specific wine intake biomarkers more complex because the concentration of specific metabolites in biological fluids should be lower than those analyzed in studies with wine diet intervention.

3.1. Wine phenolic metabolites quantified in 24-h urine samples by targeted metabolomic

A wide range of compounds (listed in Supplementary material Table S1) were studied in the 24-h urine samples. Some of these phenolic metabolites are common to other polyphenol-rich foods. That is why more attention was paid in this study to specific phenolic compounds excreted in 24-h urine that could be used as biomarkers for wine intake. A large number of phenolic metabolites are generated from the main phenolic groups (anthocyanins, phenolic acids, flavan-3-ols and stilbenes) present in red wine (Zamora-Ros et al., 2009; Urpi-Sarda et al., 2015; Motilva et al., 2016; Mosele & Motilva, 2021). In the present study, some of the metabolites determined in the urine samples were common to wine non-consumer and consumer groups (Supplementary material Table S2). Nevertheless, Table 2 shows the phenolic metabolites excreted in significantly higher concentrations in the 24-h urine of wine consumers. The main anthocyanin metabolite excreted in urine was the phase-II hepatic metabolite, malvidin-3-glucuronide (Table 2), whose concentration was significantly higher ($p < 0.001$) in the urine of wine consumers. Malvidins are the main anthocyanins in red wine that contribute to its color and antioxidant properties (Royo et al., 2021). Interestingly, some of the native structures of anthocyanin-glucosides (malvidin, peonidin and delphinidin) present in red wine were excreted in higher concentrations in the 24-h urine samples of wine consumers. Note that delphinidin-3-arabinoside and *trans*-delphinidin-3-(6″-coumaroyl)-glucoside were only detected in the 24-h urine of wine consumers, so they could be considered good candidates as specific biomarkers of red wine consumption.

Regarding the phenolic acids excreted in 24-h urine, different compounds were detected in higher concentrations in wine consumers (Table 2). They might arise from phenolic acids present in wine and other small molecules, these being degradation products (colonic fermentation) of other larger polyphenols, such as proanthocyanidins (Motilva et al., 2016). In the group of phenolic acid metabolites, gallic acid sulfate was the most abundant and significant metabolite excreted in 24-h urine of wine consumers (Table 2). The relationship between gallic acid sulfate and wine consumption is indirect but can be traced through the consumption of grapes and grape-derived products, such as wine, after phase-II metabolism. The urine showed a notable increase in the excretion of caffeic acid ethyl ester, protocatechuic acid ethyl ester, ethyl-gallate and syringic acid, among others, in the wine consumption group. Note that ethyl-gallate was only detected in the urine of wine consumers.

Procyanidins are present in grape seeds, skins, and stems, and contribute to the bitterness and astringency of red wine. Among the procyanidin metabolites, catechin sulfate resulting from the phase-II

Table 2
Anthocyanin metabolites (nmols/24 h) and other phenol metabolites (μmols/24 h), determined by targeted metabolomic, excreted in 24-h urine samples with significant differences in the concentration between non-wine consumers and regular wine consumers ($n = 3$). SE: standard error.

Compound	Non-wine consumers		Wine consumers		
	Average	SE	Average	SE	Sig. level
Anthocyanin metabolites (nmols excreted in urine/24 h)					
Malvidin-3-glucoside	0.012	0.012	2.390	0.892	<0.001
Malvidin-3-glucuronide	0.004	0.001	8.807	1.727	<0.001
Cyanidin-3-glucuronide	0.227	0.138	0.816	0.362	<0.001
Peonidin-3-glucoside	0.096	0.048	0.463	0.158	<0.001
Peonidin-3-(6″-acetyl)-glucoside	0.809	0.225	2.130	0.482	0.003
Peonidin-3-glucuronide	0.515	0.364	1.843	0.802	0.005
Peonidin-diglucuronide	0.062	0.028	0.182	0.082	0.001
Methyl-peonidin-3-glucuronide-sulfate	0.036	0.022	0.160	0.070	<0.001
Delphinidin-3-arabinoside	nd	nd	0.341	0.238	<0.001
trans-Delphinidin-3-(6″-coumaroyl)-glucoside	nd	nd	0.288	0.161	<0.001
Phenolic acid metabolites (μmols excreted in urine/24 h)					
Caffeic acid ethyl ester	0.134	0.201	1.300	1.668	<0.001
cis-Coumaric acid	0.189	0.145	0.284	0.207	0.047
trans-Fertaric acid	0.046	0.110	0.152	0.276	0.038
4-Hydroxybenzoic acid	2.089	0.185	3.032	0.322	0.032
4-Hydroxybenzoic acid sulfate	2.917	0.299	3.661	0.485	0.015
Protocatechuic acid ethyl ester	0.016	0.010	0.220	0.054	<0.001
Ethyl-gallate	nd	nd	0.488	0.203	<0.001
Gallic acid	0.029	0.020	0.397	0.125	<0.001
Gallic acid sulfate	1.302	0.354	6.917	1.247	<0.001
Syringic acid	0.771	0.188	1.854	0.346	<0.001
Syringic acid glucuronide	2.086	0.277	3.479	0.551	0.005
5-(3′.4′-dihydroxyphenyl)-valeric acid	0.265	0.049	0.789	0.274	0.008
Procyanidin metabolites (μmols excreted in urine/24 h)					
Catechin sulfate	6.155	1.735	15.440	4.283	0.015
Methylcatechin sulfate	2.955	0.572	6.804	1.598	0.003
Methylepicatechin glucuronide	0.096	0.053	0.269	0.095	0.011
Methylepicatechin sulfate	3.121	0.537	5.919	1.334	0.001
Stilbene metabolites (μmols excreted in urine/24 h)					
trans-Resveratrol glucoside	nd	nd	0.006	0.006	0.021
trans-Resveratrol glucuronide	0.013	0.013	0.324	0.169	<0.001
trans-Resveratrol sulfate	0.314	0.131	3.671	0.877	<0.001
Dihydroresveratrol	0.407	0.064	10.192	6.7629	0.021
Dihydroresveratrol sulfate	0.964	0.144	24.545	17.185	0.023
Dihydroresveratrol sulfate-glucuronide	0.005	0.004	0.351	0.317	0.023
Elagitannin metabolites (μmols excreted in urine/24 h)					
Urolithin A	1.035	0.304	1.523	0.599	0.05

nd: not-detected.

metabolism of catechin, and various conjugated forms of methyl-catechin were found in greater abundance in the 24-h urine of wine consumers. On the other hand, stilbenes are phenolic compounds derived from the phenylalanine pathway in the grapevine metabolism. Red wine is one of the major dietary sources of stilbenes for human nutrition, especially in European countries (Benbouguerra et al., 2021). Among the commonly identified stilbenes, resveratrol is the most popular compound and a recognized biomarker for wine consumption (Trius-Soler et al., 2023; Zamora-Ros et al., 2009). In this work, the concentration of *trans*-resveratrol conjugated after phase-II hepatic metabolism, predominantly in its sulfate form, was significantly higher ($p < 0.001$) in the group of wine consumers. Note the high concentration of dihydroresveratrol and dihydroresveratrol sulfate, its conjugated phase-II metabolite, detected in the 24-h urine of the wine consumer group. Finally, urolithin A, a metabolite of ellagitannins resulted from the colonic microbiota metabolism, was detected in higher concentrations in the wine consumer group. The connection between urolithin A and wine consumption lies in the fact that ellagic acid is present in red wine. It is important to note that the capacity to produce urolithin A from ellagic acid and the extent of this production can vary between individuals and depends on variations in gut microbiota composition (Espín et al., 2013). However, the differences in the urine concentration of urolithin A between wine consumers and non-consumers were lower than for other metabolites detected in this study (Table 2), so it might not be proposed as an exclusive biomarker for wine consumption.

3.2. Study of wine intake biomarkers in 24-h urine samples by untargeted metabolomics

The RP-positive ionization (RP-pos), RP-negative ionization (RP-neg) and HILIC-negative ionization (HILIC-neg) chromatographic analyses were performed independently and the annotation of markers was done for each chromatographic approach. Fig. 1A shows the PCA score plots of wine consumers and non-wine consumers and Fig. 1B shows the OPLS-DA score plots of wine consumers vs. non consumers. Outliers were identified using Hotelling's ellipse. According to what is described in the Material and Methods (*Section 2.4*), the selected markers with their corresponding tags wine or non-wine consumers were imported into Progenesis QI, and the elucidation process was limited to compounds with a q-value lower than 0.05. Based on these criteria, Table 3 shows the selected compounds that have been tentatively annotated based on exact mass, MSMS spectra and databases (phenol-explorer, KEGG, Human Metabolome Database (HMDB), FooDB, Urine Metabolome Database), and that could be proposed as wine-consumption biomarkers (q-value<0.05). Every proposal was supported by the fragments detected in the MSMS spectrum, and the annotation of the metabolites was done with mass accuracy lower than 5 ppm. Additionally, Supplementary Material Table S3 shows features detected in the 24-h urine samples of wine-consumers (q-value<0.05) which could not be annotated based on exact mass, MSMS spectra or databases.

Regarding HILIC-neg analyses, more features were detected compared with the RP, probably related to the high-water content of the urine samples (95 %) (Sarigul et al., 2019). The first compound annotated (*m/z* 291.018) was 3-(3-Hydroxy-4-methoxyphenyl)-2-(sulfooxy) propanoic acid (Table 3) that corresponds with dihydroferulic acid conjugated with a sulfate group to favor its urinary excretion. The presence of ferulic acid 4-*O*-sulfate has been previously reported in plasma and urine samples after cranberry juice consumption and has been described in grape seed (Feliciano et al., 2016). In this study, the dihydroferulic acid sulfate could be derived from the direct absorption and conjugation of hydroxycinnamic acids present in the wine, and also from the colonic metabolism of other flavonoids in the wine, mainly anthocyanins (Motilva et al., 2016). Moreover, other isomers of that compound, such as 3-[3-Hydroxy-5-methoxy-4-(sulfooxy)phenyl]propanoic acid, could also be candidates. On the other hand, the m/z 291.018 was annotated as 2-Hydroxy-6-methoxy-4-vinylphenyl hydrogen sulfate (adduct $[M + FA\text{-}H]^-$) that might be generated during the colonic fermentation of wine procyanidins. However, this annotation could not be explained as properly as the previous one according to the fragments detected in the MS/MS spectrum of the compound.

The compound eluted at 0.86 min was annotated as equol (Table 3). Equol is a colonic metabolite generated from soy isoflavones, daidzein

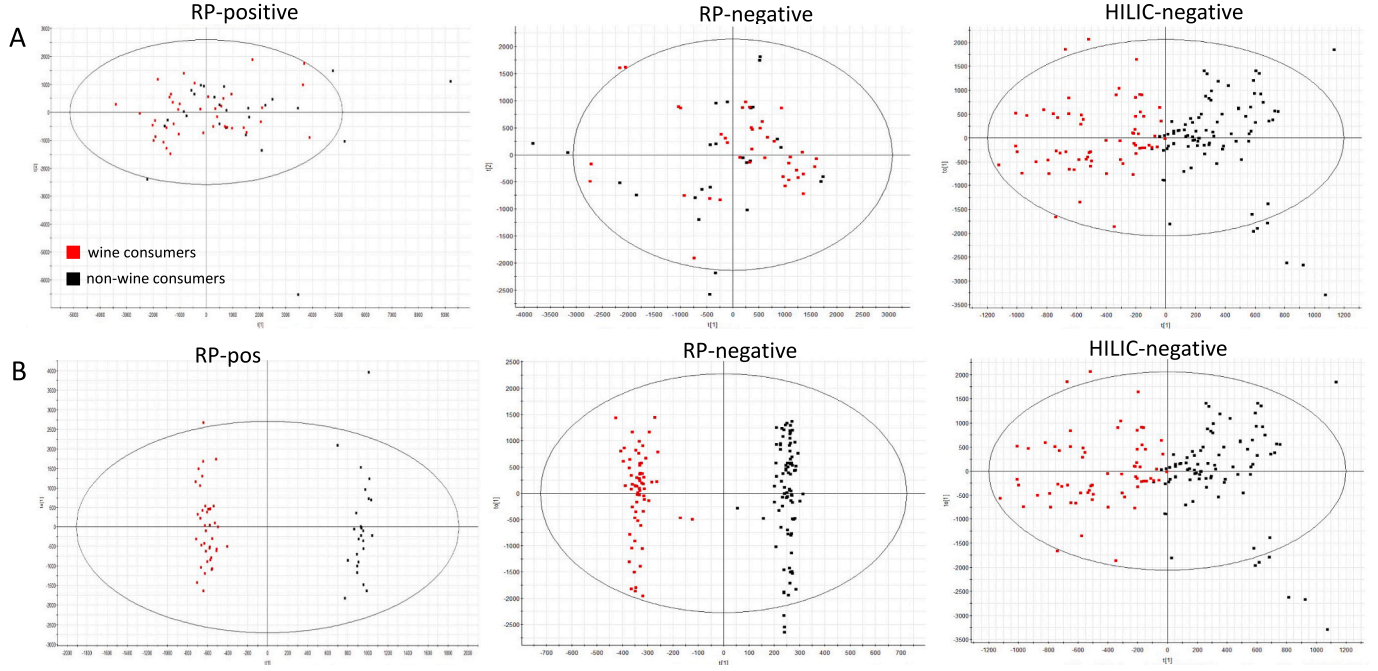

Fig. 1. PCA scores plots and OPLS-DA analysis. (A) The PCA score plots of wine consumers and non-consumers; (B) OPLS-DA score plots of wine consumers vs non-consumers. The samples of wine consumers (red boxplots) and non-wine consumers (black boxplots) are represented in order to evaluate the overall status, distribution, and to maximize inter-group discrimination. Outliers were identified using Hotelling's ellipse. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Compounds detected by untargeted metabolomic approach in 24-h urine samples of wine consumers group that could be proposed as wine-intake biomarkers (q-value<0.05) and tentatively annotated on exact mass, MSMS spectra and databases.

Compound	RT (min)	m/z	Molecular formula	Error (ppm)	Adduct	MSMS fragments	Chromatography/ ESI
3-(3-Hydroxy-4-methoxyphenyl)-2-(sulfooxy) propanoic acid (Dihydroferulic acid sulfate)	0.85	291.0183	$C_{10}H_{12}O_8S$	1.01	$[M-H]^-$	188.0087, 135.0440, 79.9563	HILIC-neg
3-(4-Hydroxyphenyl)chroman-7-ol (Equol)	0.86	241.0881	$C_{15}H_{14}O_3$	4.26	$[M-H]^-$	107.0494, 93.0337	HILIC-neg
3-[1-Hydroxy-2-(2-hydroxyphenyl)ethyl]-5-methoxyphenol			$C_{15}H_{16}O_4$	3.99	$[M-H_2O-H]^-$		
Resveratrol	0.87	227.0718	$C_{14}H_{12}O_3$	1.71	$[M-H]^-$	107.0494, 187.0060, 172.9902, 93.0345	HILIC-neg
6-Hydroxy-4′,5,7-trimethoxyflavone	0.88	271.0988	$C_{18}H_{16}O_6$	1.28	$[M-H_2O-H]^-$	187.0059, 107.0493, 172.9902, 135.0439	HILIC-neg
(4S,4aR,7aR,8R,9aR)-4-Hydroxy-3-(hydroxymethyl)-4a,8-dimethyl-4,4a,7a,8,9,9a-hexahydroazuleno[6,5-*b*]furan-2,5-dione (Isohelenol)	0.92	259.0987	$C_{15}H_{18}O_5$	3.89	$[M-H_2O-H]^-$	183.0111, 112.9845, 173.9857	HILIC-neg
Valine-Histidine (Val-His)	0.93	282.0976	$C_{11}H_{18}N_4O_3$	4.19	$[M + Cl]^-$	183.0110, 150.0553, 173.9857, 230.0116	HILIC-neg
(2Z)-3-[4,5-Dihydroxy-2-(2-hydroxy-2-propanyl)-2,3-dihydro-1-benzofuran-7-yl]acrylic acid (Gravolenic acid)	1.01	261.0777	$C_{14}H_{16}O_6$	3.03	$[M-H_2O-H]^-$	226.0263, 183.0108, 96.9590, 79.9566, 124.9904, 152.0104, 164.0044, 198.0314	HILIC-neg
2-(3,4-Dihydroxyphenyl)-5-hydroxy-4-oxo-4H-chromen-7-yl hydrogen sulfate (Luteolin 7-sulfate)	1.02	411.0031	$C_{15}H_{10}O_9S$	0.96	$[M + FA-H]^-$	277.1844, 150.0311, 263.0219, 121.0284, 399.1825	HILIC-neg
Citramalic acid; Beta-lactic acid; Erythro-3-methylmalic acid; Threo-3-methylmalic acid; other isomers	1.22	129.0186	$C_5H_8O_5$	−4.88	$[M-H_2O-H]^-$	112.9845, 68.9947, 110.9830	HILIC-neg
p-coumaryl alcohol 4-*O*-glucoside	2.05	311.1146	$C_{15}H_{20}O_7$	−3.06	$[M-H]^-$	178.0497, 134.0598, 197.0665, 166.0165, 311.1143, 159.0451	HILIC-neg
2-amino-3-oxoadipic acid; N-formyl-L-glutamic acid	2.42	156.0294	$C_6H_9NO_5$	−4.94	$[M-H_2O-H]^-$	113.0231, 85.0284, 107.0491, 149.0445, 71.0128, 95.0127, 99.0080, 101.0234	HILIC-neg
Galactopinitol A; Galactopinitol B	4.40	337.1134	$C_{13}H_{24}O_{11}$	−1.78	$[M-H_2O-H]^-$	133.0492, 316.9474, 85.0285, 178.9910, 221.1537	HILIC-neg
Methyl (2E)-5-(5-methyl-2-thienyl)-2-penten-4-ynoate	1.62	187.0224	$C_{11}H_{10}O_2S$	0.41	$[M-H_2O-H]^-$	96.9585, 247.8403, 194.9253, 168.8880	RP-neg
Vanillic acid-4-*O*-glucuronide; 1-Caffeoyl-beta-D-glucose; Caffeic acid 3-glucoside	4.38	387.0931	$C_{15}H_{18}O_9$	−0.50	$[M + FA-H]^-$	146.9638, 113.0225, 85.0278, 383.1911, 191.0542, 61.9871, 358.0530, 196.0357	RP-neg
1-*O*-[3-(2,4-Dihydroxy-methoxyphenyl)propanoyl] hexopyranuronic acid; 4-(2-Carboxyethyl)-3-hydroxy-2-methoxyphenyl hexopyranosiduronic acid			$C_{16}H_{20}O_{11}$	−0.39	$[M-H]^-$		
Xanthine	1.45	152.0338	$C_5H_4N_4O_2$	2.22	$[M + Na]^+$, $[M + K]^+$	110.0329, 153.0387, 136.0125, 70.0634, 142.0842, 173.0494, 107.0471, 97.9663	RP-pos
O-adipoylcarnitine; 3-Methylglutarylcarnitine	3.27	290.1585	$C_{13}H_{23}NO_6$	−4.67	$[M + H]^+$	118.0631, 143.0709, 188.0686, 146.0581, 115.0522, 130.0631, 290.1581, 73.0285	RP-pos

and genistein (Vázquez et al., 2020). Nevertheless, the presence of genistein and daidzein has been described in white wine grapes (De Sanctis et al., 2012) and they have been identified in samples of urine after wine consumption (Noh et al., 2017), which could explain the presence of equol in the urine samples of wine-consumers in the present study. Additionally, the metabolite 3-[1-Hydroxy-2-(2-hydroxyphenyl) ethyl]-5-methoxyphenol, corresponding to the adduct $[M-H_2O-H]^-$ with formula $C_{15}H_{16}O_4$, might be an alternative proposal. However, there is nothing in the literature about the relationship between this compound and wine, so equol would be the most feasible proposal.

The next compound (RT 0.87 min) was assigned to a $[M-H]^-$ adduct which corresponds to the molecular formula $C_{14}H_{12}O_3$ that was annotated to resveratrol, one of the most characteristic phenolic compounds in the grapevine and wine. Different studies have detected resveratrol-3-*O*-glucuronide in urine after dietary supplementation with wine (Mosele & Motilva, 2021; Zamora-Ros et al., 2009). It has therefore been proposed as a good biomarker of wine consumption. However, the free form of resveratrol has yet to be described in 24-h urine. Nevertheless, resveratrol in its free form could be generated in the ionization source due to a breakdown of the resveratrol glucuronide. At 0.88 min, the compound 6-Hydroxy-4′,5,7-trimethoxyflavone was annotated with an error of 1.28 ppm. This compound is a flavone derived from scutellarein, which is functionally related to apigenin. Apigenin is present in numerous beverages, including wine, beer and tea. The next compound detected was eluted at 0.92 min. It could be tentatively annotated as isohelenol with a 3.89 ppm error. Isohelenol is a sesquiterpene lactone that has not previously been reported in wine. Nevertheless, wine contains several sesquiterpenes that contribute to its spicy and woody notes (Cincotta et al., 2015). At 0.93 min, a compound with formula $C_{11}H_{18}N_4O_3$ was annotated as the dipeptide Valine-Histidine (Val-His). The next one, gravolenic acid, was proposed for the signal at *m/z* 261.0777 (RT 1.01 min) with an error of 3.03 ppm (Table 3). It belongs to the hydroxycinnamic acid family (coumaric acids), which are abundant in wine. At 1.02 min, the m/z 411.0031 signal was detected, which might correspond to the adduct $[M + FA-H]^-$ and a $C_{15}H_{10}O_9S$ molecular formula with a 0.96 ppm mass error. For this formula, the compound luteolin 7-sulfate was proposed. Luteolin is a polyphenol reported in wine (Fang et al., 2007) and in urinary metabolome after moderate red-wine consumption in an interventional period of 28 days (Esteban-Fernández et al., 2018).

The following compound proposed as wine-intake biomarker (q-value<0.05) was detected at 1.22 min (Table 3). Several compounds

were tentatively annotated for the signal *m/z* 129.0186, these being citramalic acid, beta-lactic acid, erythro-3-methylmalic acid, threo-3-methylmalic acid and other isomers. All of these are supported by the MSMS spectrum and it is plausible for them to be found in wine derived from the malolactic fermentation. The next compound (2.05 min) detected in the 24-h urine samples of wine-consumers could be tentatively annotated as *p*-coumaryl alcohol 4-*O*-glucoside. This is a hydroxycinnamic acid belonging to the coumarin family, a group of compounds known to possess antioxidant and anti-inflammatory properties. *p*-Coumaryl alcohol 4-*O*-glucoside could be generated from coumaric acid (van Summeren-Wesenhagen et al., 2015), which is present in grapes and wine (Salameh et al., 2008) and has been previously reported in urinary metabolome as a biomarker of wine consumption (Noh et al., 2017). At 2.42 min, two compounds, 2-amino-3-oxoadipidic acid and N-formyl-L-glutamic acid, have been annotated for the adduct $[M\text{-}H_2O\text{-}H]^-$ with the molecular formula $C_6H_9NO_5$. The last compound annotated in HILIC-neg eluted at 4.40 min and it may tentatively correspond to galactopinitol A or B (Table 3). This compound has been reported as a biomarker of soy and pulse consumption according to the HMDB and FooDB databases. Nevertheless, to date, this has not been reported in wine or studies related to wine consumption.

In the reverse phase chromatography few significant signals (q-value<0.05) were detected (Table 3), and only two compounds were tentatively annotated in RP-neg, apart from galactopinitol, which was also detected in the HILIC-neg chromatography. The first one, eluted at 1.62 min (*m/z* 187.0224), could be tentatively annotated as Methyl (2E)-5-(5-methyl-2-thienyl)-2-penten-4-ynoate. It belongs to a class of organic compounds known as fatty acid esters. According to Trius-Soler et al. (2023), fatty acid ethyl esters have been proposed as candidate biomarkers for ethanol. The second signal (4.38 min), m/z 387.0931, may correspond to the adducts $[M\text{-}H]^-$ or $[M + FA\text{-}H]^-$ with mass errors of −0.39 and −0.50 ppm, respectively. The deprotonated molecular ion $[M\text{-}H]^-$ was annotated as 1-O-[3-(2,4-Dihydroxy-methoxyphenyl)propanoyl]hexopyranuronic acid and 4-(2-Carboxyethyl)-3-hydroxy-2-methoxyphenyl hexopyranosiduronic acid. For the $[M + FA\text{-}H]^-$ adduct, vanillic acid-4-*O*-glucuronide, caffeic acid 3-glucoside and 1-caffeoyl-beta-D-glucose could be supported by the MSMS data. Vanillic acid-4-*O*-glucuronide is a phase-II metabolite formed through the hepatic metabolism of vanillic acid. In an intervention study with red wine, vanillin 4-sulfate and vanillic acid 4-sulfate were identified in urine samples (Esteban-Fernández et al., 2018) and different derivatives of caffeic acid have been reported as wine-intake biomarkers (Mosele & Motilva, 2021). Regarding the other two candidates for the $C_{15}H_{18}O_9$ molecular formula, 1-caffeoyl-beta-D-glucose and caffeic acid 3-glucoside are hydroxycinnamic acids derived from caffeic acid, a phenolic acid detected in wine. In the study by Esteban-Fernández et al. (2018), 1-caffeoyl-beta-D-glucose and caffeic acid 3-glucoside were not identified, whereas the caffeic acid sulfate and dihydrocaffeic acid 3-sulfate were tentatively identified in the urine samples, being down-regulated due to wine consumption.

Finally, in the reverse phase with the positive ionization mode (RP-pos), two compounds were annotated (Table 3). The first compound eluted at 1.45 min, with a main signal at m/z 152.0338 with a formula of $C_5H_4N_4O_2$ (2.2 ppm mass error), was annotated as Xanthine or 7H-Purine-6,8-diol. Xanthines are purine bases found in most human body tissues and fluids, as well as in other organisms. The second compound was detected at 3.27 min (m/z 290.1585) with the molecular formula $C_{13}H_{23}NO_6$ and was annotated as O-adipoylcarnitine or 3-Methylglutarylcarnitine. According to HMDB, both are acylcarnitine and it is believed that there are more than 1000 types of acylcarnitine in the human body and they are involved in fatty acid metabolism.

3.3. Comparison between targeted and untargeted metabolomics

Different metabolites, related with regular wine consumption, were proposed based on the comparative results of the 24-h urine analysis of wine consumers vs non-consumers resulting of the both targeted (Table 2) and untargeted metabolomics (Table 3) approaches. It stands out that some of them, or at least related compounds, were detected in both metabolomics approaches. However, it should be noted that the sensitivity of the QqQ-MS/MS detector used in the targeted metabolomics is higher than the TOF (qToF) detector, which may explain the lower number of compounds detected in the untargeted approach. In addition, to prevent saturation of the TOF detector due to the presence of major compounds in urine differ from the low molecular weight metabolites studied, sample dilution (1:10, *v*/v) was necessary to minimize the saturation of the detector.

To start with, in the 24-h urine of wine consumers, different hepatic phase-II metabolites of resveratrol were tentatively identified with targeted metabolomics (Table 2) and their free form was also detected with untargeted metabolomics (Table 3). Similarly, caffeic acid ethyl ester was detected in higher concentrations in the 24-h urine samples of the wine consumers (Table 2) by the targeted metabolomics approach and two caffeic acid derivatives were detected in the wine consumer group by untargeted metabolomics (Table 3). Finding the metabolites in both metabolomics approaches reinforces their tentative identification.

Urolithin A and a phase-II metabolite of gallic acid (gallic acid sulfate) were detected in higher concentrations in the wine consumer group (Table 2) when urine was analyzed by targeted metabolomics. Both were tentatively annotated in the urine of wine consumers by untargeted metabolomics (q-value<0.05). Nevertheless, their MSMS spectra were not very informative and it was not possible to draw conclusions about the accurate annotation of these two compounds. For this reason, these two compounds were not included in Table 3. The fact that this study was carried out without diet intervention implies greater inter-individual variability between samples and lower concentrations of metabolites derived from wine intake than if diet intervention with wine had been carried out in a controlled way.

Generally, dietary and health biomarkers can be classified into 3 groups: exposure/intake, effect and susceptibility/host factors (Odriozola & Corrales, 2015; Gao et al., 2017). Intake biomarkers encompass the compounds present in wine (such as resveratrol), and these reflect the level of wine consumption. Function or effect biomarkers refer to the functional response of the human body to an exposure. And susceptibility/host factors represent the individual susceptibility to an exposure, predicting the intensity of its effect on the individual. Some of the biomarkers reported in this study might not be directly related to wine, but rather to the effect of the wine components on the human body. In this sense, some proposals in Table 3, such as *O*-adipoylcarnitine/3-Methylglutarylcarnitine or Methyl (2E)-5-(5-methyl-2-thienyl)-2-penten-4-ynoate (metabolites involved in fatty acid metabolism) are not present in wine but rather could derive from the functional response of the human body to sustained wine consumption.

4. Conclusions

The present study demonstrated that the combination of targeted and untargeted metabolomics approaches could be a useful tool for exploring biomarkers of regular and moderate wine-consumption using 24-h urine samples from a free-living population without dietary intervention, allowing them to maintain their usual daily activities, dietary habits and social interactions. The great potential of LC-HRMS for detecting biomarkers of wine consumption in the context of untargeted metabolomic studies was illustrated by the annotation of sixteen compounds associated with regular wine-consumption. This approach enables the detection of a broader range of metabolites compared to more classical techniques, which are often limited in sensitivity and scope. Unlike targeted methods, LC-HRMS allows for the identification of unexpected or novel metabolites that may serve as more accurate biomarkers of wine intake, providing a comprehensive metabolic profile and offering deeper insights into the biochemical impact of wine consumption. Note that none of the proposed biomarkers were detected in

the urine samples of non-wine consumers (control group), or at least, if they were detected in some of the urine samples of the control group, their relative abundance was not significant (q-value>0.05) between the two groups. Additionally, the results of the targeted metabolomics approach have shown a wide range of biological phenol metabolites, mainly hepatic phase-II conjugates, whose concentration was significantly higher in the urine samples of regular wine consumers. Anthocyanin and stilbene derivatives were highlighted as the most selective wine regular consumption biomarkers.

Hence, this study was performed as a starting point for the optimization and subsequent evaluation of the usability of the metabolomic strategies categorized into two approaches, untargeted and targeted metabolomics, applied to 24-h human urine samples from a specific group from La Rioja, for the selection of regular wine consumption biomarkers. Future studies should focus on the validation of these biomarkers in the broader populations.

CRediT authorship contribution statement

Marta Jiménez-Salcedo: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **José Ignacio Manzano:** Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Silvia Yuste:** Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **María Iñiguez:** Investigation, Formal analysis, Data curation. **Patricia Pérez-Matute:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Maria-Jose Motilva:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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