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Development of a LC−ESI-MS/MS Approach for the Rapid Quantification of Main Wine Organic Acids in Human Urine

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ABSTRACT: The analysis of food components and their metabolome in urine has recently found a growing interest due their potential ability to reflect specific dietary intakes. In the present work, a fast, simple, and environmentally friendly method based on liquid chromatography coupled to electrospray ionization tandem mass spectrometry was developed for the analysis of main wine organic acids in human urine. The proposed method was evaluated in terms of linearity, precision, accuracy, and limits of detection. Quantitative recovery (96−102%) and satisfactory interday precision (RSD <6%) were achieved for all target compounds. To demonstrate the applicability of the method, urine samples from five male volunteers were analyzed before and after consumption of a single moderate dose (200 mL) of red wine. A significant increase ($p < 0.01$) in the urinary concentration of tartaric and malic acids was observed.

KEYWORDS: *biomarker, LC−ESI-MS/MS, organic acids, tartaric acid, urine, wine*

INTRODUCTION

Organic acids play an important role in many foods, as they are responsible for essential sensory properties and may also influence their stability and digestibility.[1,2] In wine they account for a significant fraction and have a marked influence on its overall quality. Tartaric acid is, along with malic acid, one of the two major organic acids found in grape and subsequently wine, whereas succinic acid is produced during alcoholic fermentation at lower concentrations.[3,4]

In the past few years, the analysis of food components and their metabolome in urine is gathering increasing interest due to their potential ability to reflect specific dietary intakes.[5,6] Although there are a variety of methods available to assess diet, such as food frequency questionnaires (FFQs) and dietary recalls, the food intake data can lack enough reliability.[6]

In this regard, there is a need for rapid and reliable methodologies to analyze large batches of urine samples, which are usually essential in clinical and epidemiological studies.

Previous methods for the analysis of organic acids in urine have been based on ion chromatography (IC),[7,8] capillary electrophoresis (CE),[9] and gas chromatography coupled to mass spectrometry (GC/MS).[10,11] Although CE and IC can achieve a good separation, they may lack robustness for routine analysis of biological samples. On the other hand, GC/MS methods involve sample derivatization to increase acid volatility and often require an extensive sample preparation by SPE and/or LLE.[10,12]

The aim of the present work was therefore to develop and validate a robust high throughput method based on LC−ESI-MS/MS, which would allow measuring the main wine organic acids in urine samples within moderate dietary wine intakes. Finally, urine samples from five healthy men were analyzed to demonstrate the applicability of the proposed method.

MATERIALS AND METHODS

Reagents and Standards. L-(−)-Malic acid (≥99%), L-(+)-tartaric acid (≥99.7%), succinic acid (≥99%), and creatinine (≥98%) were purchased from Sigma (Madrid, Spain). The labeled internal standard DL-(±)-tartaric-2,3-d_2 acid was obtained from C/D/N Isotopes (Pointe-Claire, Canada). Formic acid (∼98%), ammonium formate (≥99%), picric acid (98%, moistened ∼33% water), and sodium hydroxide (≥98%) were purchased from Panreac (Barcelona, Spain). Solvents were HPLC grade and all other chemicals were analytical reagent grade. Ultrapure water was obtained from a Milli-Q Gradient water purification system (Millipore, Bedford, MA).

Individual stock solutions of the selected organic acids and a mixture of them were prepared in water. Different working standard solutions were made by appropriate dilution in 0.5% formic acid in water and then stored in amber glass vials at −20 °C.

Wine Samples. A commercial red wine from the Spanish Protected Designation of Origin (PDO) "Penedés" was employed in this study. It was made with grapes of *Vitis vinifera* L. variety

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Table 1. Specific MRM Conditions for Determination of Organic Acids

compd	t_R (min)	parent ion	MRM transition (m/z)	product ion	DP (V)	CE (eV)
tartaric acid	1.09	$[M - H]^-$	149 > 87[a]	$[M - H - CO_2 - H_2O]^-$	−25	−20
			149 > 73	$[M - H - C_2H_4O_3]^-$	−25	−25
tartaric acid-d_2[b]	1.09	$[M - H]^-$	151 > 88[a]	$[M - H - CO_2 - H_2O]^-$	−25	−20
			151 > 74	$[M - H - C_2H_4O_3]^-$	−25	−25
malic acid	1.39	$[M - H]^-$	133 > 71[a]	$[M - H - CO_2 - H_2O]^-$	−25	−22
			133 > 115	$[M - H - H_2O]^-$	−25	−20
succinic acid	2.96	$[M - H]^-$	117 > 73[a]	$[M - H - CO_2]^-$	−25	−25
			117 > 99	$[M - H - H_2O]^-$	−25	−15

[a]Quantifier MS/MS transition. [b]internal standard.

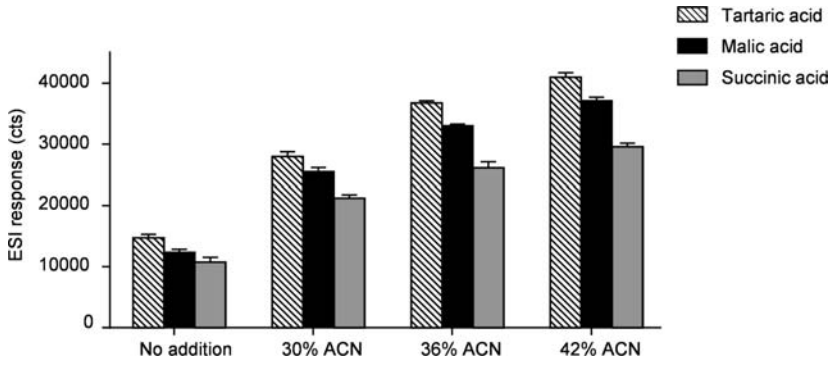

Figure 1. Effect of postcolumn addition of acetonitrile (ACN) on ESI responses of the studied compounds.

Tempranillo from the 2010 vintage. The wine had a pH value of 3.6 and 13.0% alcohol by volume.

For the analysis of the organic acids, 100 μL of wine was filled up to 100 mL in a volumetric flask with 0.5% formic acid in water. An aliquot of the wine dilution was filtered by 0.20 μm and analyzed by LC−ESI-MS/MS as below described.

Subjects and Study Design. Five healthy male volunteers with an age ranging between 20 and 45 years old drank a single moderate dose (200 mL) of red wine at dinner. Participants were asked to collect the first morning urine, 10 h after wine intake. Before the intervention, participants followed a 5-day wash-out period in which they were requested not to consume wine or grape-based products. After the wash-out period, the first morning urine was also collected. All samples were collected in 100 mL randomly coded sterile specimen containers, and immediately stored at 4 °C. Upon receipt of each sample, four aliquots of 1.0 mL were transferred to separate 1.5 mL capped Eppendorf tubes and stored at −80 °C until the analyses.

The participants had no history of cardiovascular, hepatic, or renal disease and had stable alimentary habits. They had not adhered to any special diet for at least 4 weeks prior to the consumption, so the obtained results can be attributed to a normal dietary pattern. The study was explained to subjects through verbal and written instructions, and written informed consent was obtained before participation. The study protocol was approved by the Ethics Committee of Clinical Investigation of the University of Barcelona (Spain) (reference IRB0003099). This trial was registered at controlled-trials.com as ISRCTN63399546.

Sample Preparation. The urine samples were thawed on ice and vortexed for 1 min, and 50 μL was diluted 1:20 (v/v) with 0.5% formic acid in water. Ten microliters of the deuterated internal standard solution in water [DL-(±)-tartaric-2,3-d_2 acid, 60 μg/mL] was then added. The sample dilution was filtered by 0.20 μm and analyzed by LC−ESI-MS/MS. All samples were analyzed in triplicate.

Creatinine adjustment was used to normalize analyte concentrations in spot urine samples.[13] Analyte results were then expressed as micrograms of analyte per milligram of creatinine. Creatinine content was determined by a modification of Jaffés alkaline picrate method.[14] Briefly, 3 μL of urine was mixed with 60 μL of aqueous picric acid solution (1%) and 5 μL of sodium hydroxide (10%) in a 96-well plate. After shaking, the mixture was left for 15 min in darkness at room temperature, 232 μL of Milli-Q water was then added, and absorbance was measured at 500 nm with background subtraction on a Multiskan Spectrum microplate spectrophotometer (Thermo Fischer Scientific, San Jose, CA).

LC−ESI-MS/MS Analyses. Analyses were carried out in an Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA) consisting of a quaternary pump, a vacuum degasser, an autosampler, and a thermostated column compartment. Chromatographic separation was performed on a reversed-phase column (Atlantis T3 C18, 100 × 2.1 mm, 3 μm) from Waters (Milford, MA) maintained at 25 °C. Mobile phases A and B were, respectively, 0.5% formic acid in water and 0.5% formic acid in acetonitrile. The following linear gradient was used: held at 100% A for 3.5 min, decreased to 10% A over 2 min, held for 2 min, then returned to initial conditions for 1.5 min, and re-equilibrated for 6 min. The flow rate was set at 350 μL/min and the injection volume was 10 μL.

The HPLC system was coupled to a triple quadrupole mass spectrometer API 3000 (Applied Biosystems, Foster City, CA) equipped with a Turbo Ionspray ionization source. For improvement of ionization, postcolumn addition of acetonitrile to the LC eluate was effected using an isocratic pump Agilent 1100 Series at a flow rate of 250 μL/min via a zero-volume mixing T-piece.

The mass spectrometer was operated in negative electrospray ionization (ESI) mode under the following specific conditions: ion spray voltage (IS), −4500 V; source temperature (TEM), 400 °C; curtain gas (CUR), 12 arbitrary units; nebulizer gas (NEB), 10 arbitrary units; entrance potential (EP), −10 V; cell exit potential (CXP), −10 V; focusing potential (FP), −180 V; and collisionally activated dissociation (CAD) gas, 4 arbitrary units. Nitrogen (>99.98%) was employed as curtain, nebulizer, and collision gas.

The detection was accomplished in the multiple reaction monitoring (MRM) mode. Optimized MS/MS ion transitions and the corresponding transition confirmation details for each compound are summarized in Table 1. Analyst v1.4 software (Applied Biosystems) was used for data acquisition and control of all system components except the isocratic pump, which was run independently.

Statistical Analysis. Statistical calculations were made using the software package GraphPad Prism version 5.0 (GraphPad Software,

Table 2. Performance Parameters of the Proposed Method

	% recovery ± SD (n = 5)			intraday precision (% RSD, n = 5)			interday precision (% RSD, 3 days, n = 3)			
compd	0.2 μg/mL	6 μg/mL	200 μg/mL	0.2 μg/mL	6 μg/mL	200 μg/mL	0.2 μg/mL	6 μg/mL	200 μg/mL	LOD (ng/mL)
tartaric acid	97.2 ± 3.7	96.6 ± 2.3	102.0 ± 2.1	1.6	1.3	1.4	2.0	2.2	1.8	25.4
malic acid	101.3 ± 5.1	94.0 ± 3.5	98.3 ± 2.5	2.6	3.4	2.0	5.0	4.3	3.8	28.7
succinic acid	95.5 ± 4.6	92.8 ± 1.5	96.3 ± 4.6	1.1	1.1	1.5	2.3	3.7	1.4	38.3

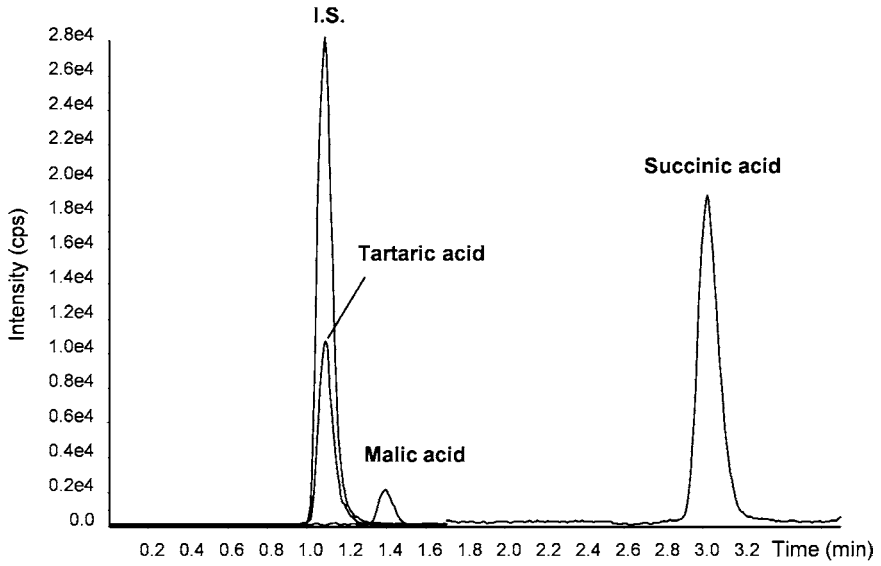

Figure 2. Overlaid MRM chromatograms obtained for a real urine sample after a 5-day wash-out period (tartaric acid at 2.23 ± 0.04 μg/mL, malic acid at 0.463 ± 0.016 μg/mL, and succinic acid at 5.8 ± 0.2 μg/mL).

San Diego, CA). Unless otherwise specified, data are presented as the mean ± SD. Statistical comparisons were performed using paired, two-tailed, Student's t test with the level of significance set at $p < 0.05$.

RESULTS AND DISCUSSION

Method Development. The main objective of the present work was to develop a rapid and simple method based on LC−ESI-MS/MS for the determination of tartaric, malic, and succinic acids in urine samples after wine moderate consumption. Therefore, experiments were conducted to optimize their chromatographic separation as well as their MRM detection.

The polar nature of these acids, i.e., octanol/water partition coefficient (log P) ≤−0.59, requires the use of highly aqueous mobile phases to obtain a suitable retention under reversed-phase conditions. Nevertheless, the main drawback of conventional reversed-phase columns when using 100% aqueous conditions is the loss in retention time that occurred over time as a consequence of pore dewetting.[15] The selected column Atlantis T3 uses a trifunctional C18 phase bonded at an intermediate ligand density, specially designed to be used with 100% aqueous mobile phases.

Since the target compounds are dicarboxylic acids, it was also necessary to use an acidic mobile phase to improve their chromatographic behavior. To this end, formic acid was selected because of its compatibility with ESI sources. Two different proportions were tested (0.1% and 0.5%), and the best results were achieved using 0.5% formic acid in water (pH 2.25).

Under the optimized chromatographic conditions, which are detailed in the experimental section, baseline separation (Rs ≥ 1.5) of all target compounds was achieved in less than 4 min (Table 1). No loss in retention times was observed over the course of the method development, validation, and subsequent application to real urine samples.

Optimization of MRM conditions was carried out by postcolumn infusion of standard solutions. ESI provided negatively single charged $[M - H]^-$ precursor ions for all compounds. Two MS/MS ion transitions were selected for each compound (Table 1); the most intense transition was used for quantification, while the other one was employed for identification. For tartaric and malic acids, the main observed transition corresponded to a neutral loss of 62 u, i.e. CO_2 plus H_2O, which is typical of compounds having a carboxylic acid with a hydroxyl group on the adjacent carbon. In the case of tartaric acid, the selected qualifier transition corresponded to the loss of glycolic acid (76 u), whereas for malic acid the loss of H_2O was used. For succinic acid, the main fragment corresponded to the loss of CO_2 (44 u) from a carboxylic group.

ESI-MS/MS has become a powerful technique for analyzing target metabolites in complex biological samples, although its sensitivity is highly dependent on the mobile-phase composition, as well as the nature of the analyte.[16] Since water possesses high surface tension and viscosity, the use of 100% aqueous mobile phases hampers the desolvation of sample droplets in the ESI interface and reduces compound ionization. Therefore, postcolumn addition of an organic solvent to the eluate may enhance the ionization of analytes. This approach was assessed by adding acetonitrile to the eluate at different proportions (Figure 1). It was found that the addition of 42% acetonitrile improved peak intensities about 3-fold. This higher response may be the result of a more efficient desolvation of analytes

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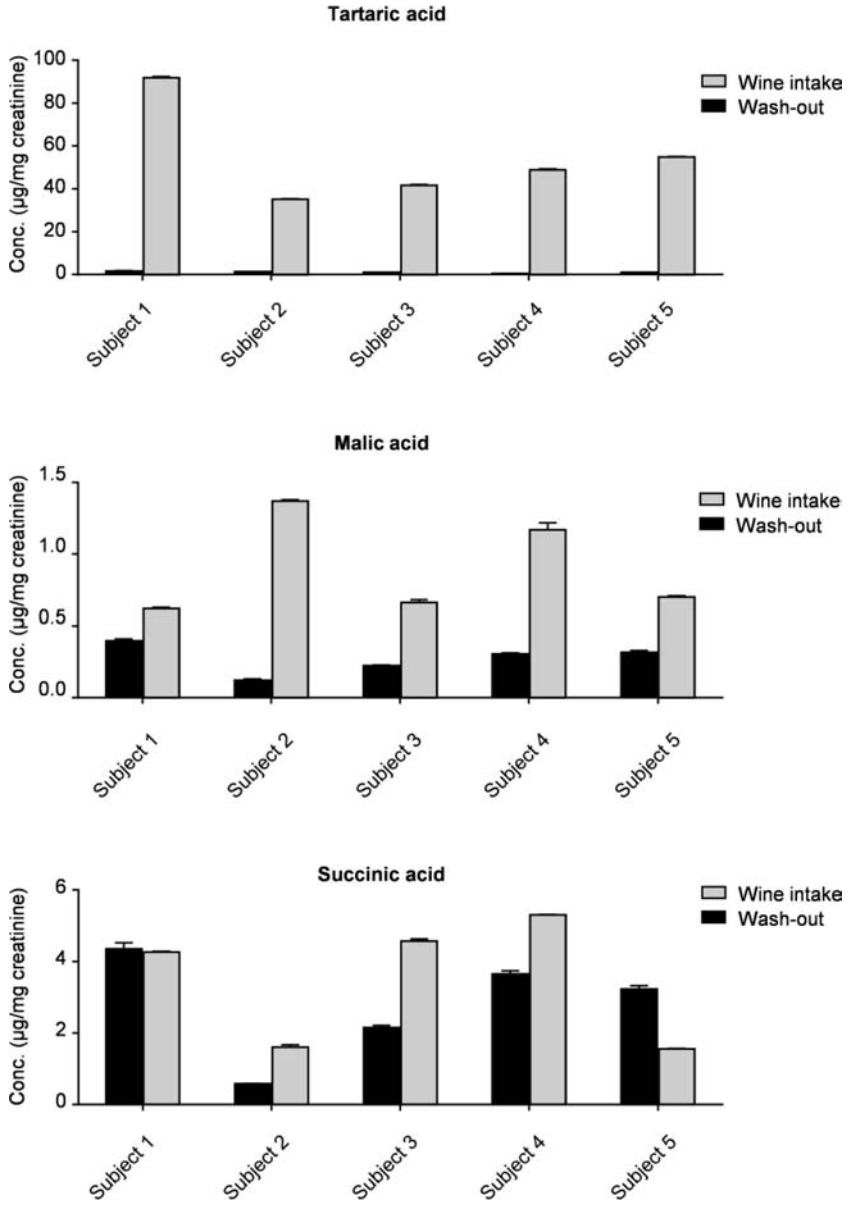

Figure 3. Concentrations of organic acids in urine samples from volunteers before and after consumption of a single moderate dose (200 mL) of red wine.

when a more volatile solvent is used and a higher spray stability due to decreased surface tension.[16] An additional advantage is that the LC eluate, and therefore the sample matrix, is diluted (ca. 2-fold) before reaching the ESI source, thus reducing possible matrix effects. Under these conditions, it was observed that sample preparation could be greatly simplified, so that samples were merely diluted and then directly analyzed by LC–ESI-MS/MS without loss of performance in the analytical system over the course of the analyses.

Method Validation. In order to assess the performance of the proposed method, the main analytical quality parameters were thoroughly evaluated (Table 2). The linearity of the method was tested using standard solutions at nine concentration levels from 0.007 to 15 μg/mL. The weighted $(1/x)$ calibration curves were found to be linear in the studied range with determination coefficients (R^2) $\geq$0.9992 and RSDs $(n = 3)$ $\leq$5%.

Recoveries were evaluated using urine blank samples, previously analyzed to ensure levels of these acids below LODs, that were spiked at three concentration levels (0.2, 6, and 200 μg/mL). Recoveries were calculated by dividing the difference between the measured concentrations for spiked and nonspiked samples by the added concentrations. Quantitative recoveries at all three concentration levels were obtained ranging from 96% to 102% (Table 2).

The precision of the method was also assessed by calculating the relative standard deviation (RSD). The intraday precision was determined from the analysis of five replicates on the same day, whereas the interday precision was calculated from analyses performed over five consecutive days, with three replicates per day. Both intra- and interday precision studies presented satisfactory results for all compounds, with RSDs below 4% and 6%, respectively (Table 2). Limits of detection (LODs) were calculated as the average concentration of compound producing a signal-to-noise ratio (S/N) of 3 using

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the less sensitive MS/MS transition, i.e. the one permitting the unambiguous identification of the analytes. LODs ranged from 25.4 ng/mL for tartaric acid to 38.3 ng/mL for succinic acid, which enable their determination in urine samples from subjects within a realistic range of dietary wine intakes.

One area of concern in biological sample analysis is the introduction of relatively large amounts of salts into the ion source, which may degrade the performance of the instrument.[17] However, no deterioration in absolute sensitivity was observed over the course of each batch or between batches, and other assays run on the same instrument were unaffected. Figure 2 shows the overlaid chromatograms for the quantifier MRM transitions in a real urine sample (tartaric acid at 2.23 ± 0.04 μg/mL, malic acid at 0.463 ± 0.016 μg/mL, and succinic acid at 5.8 ± 0.2 μg/mL).

Application to Urine Samples. The applicability of the proposed method was demonstrated by analyzing urine samples from five volunteers after the consumption at dinner of a single moderate dose of red wine (200 mL) (Figure 3). The concentration of acids in the wine used in this study was as follows: tartaric acid, 1943 ± 21 mg/L; malic acid, 21.81 ± 0.13 mg/L; and succinic acid, 624 ± 18 mg/L. Therefore, the corresponding intake was of 387 ± 4 mg for tartaric acid, 4.36 ± 0.03 mg for malic acid, and 125 ± 4 mg for succinic acid. The subjects were requested not to consume wine or grape-based products during the previous 5 days. First morning urines were collected the day before the wine intake and in the morning following the intervention. Urine samples were corrected for their creatinine concentration, which was in all cases within normal limits (1.1−2.8 mg/mL urine).

A significant increase in urinary tartaric acid ($p < 0.01$) was observed 10 h after the wine consumption. Its urinary concentration ranged from 35.11 to 91.84 μg/mg creatinine. These results are similar to those reported by Lord et al.,[18] who also showed an increase in urinary tartaric acid after the intake of grape juice. Only small amounts (≤1.7 μg tartaric/mg creatinine) could be measured after the 5-day wash-out period, probably due to the previous intake of some food containing low levels of tartaric acid. Although the main source of this acid in diet are grapes and grape-based products, it can be also found in other fruits, such as mango, bananas, or blackberries, at concentration levels that can be considered negligible as compared to grapes or wine.[1]

Urinary concentration of malic acid was also significantly increased ($p < 0.05$) after the wine intake, although at much lower concentrations (0.62−1.37 μg malic acid/mg creatinine). Malic acid is present in grape musts at levels between 1 and 6.5 g/L,[3] but in red wines and some whites, its concentration highly decreases (0−30 mg/L) during the malolactic fermentation.[1] Unlike tartaric acid, malic acid is one the most widespread organic acids in fruits, such as apples, plums, cherries, and apricots, and many vegetables,[1] so its presence in urine might be due to many dietary sources.

Succinic acid significantly increased after wine consumption, showing concentrations in the range 1.56−5.3 μg/mg creatinine. Although succinic acid is usually present in wine at concentrations up to 2 g/L,[19] it is also involved in lipid metabolism and the Krebs cycle,[3] so being also an endogenous metabolite, its urinary concentration may be more influenced by the human metabolism than the dietary intake.

In this work, a simple, inexpensive, and environmentally friendly LC−ESI-MS/MS approach was proposed for the analysis of main wine organic acids in urine samples. The developed method allowed increasing sample throughput, while sample preparation and solvent consumption were minimized. The method was completely validated, providing a sensitive analysis for organic acids detection and showing satisfactory data for all parameters tested. Good results were obtained with respect to linearity and recovery as well as an excellent level of precision. The applicability of the proposed method was assessed through the analysis of real urine samples from five volunteers after consumption of single moderate dose of red wine. Further research is still needed to evaluate the possible physiological effects of these metabolites in urines and their use as potential urinary biomarkers of wine consumption in clinical and epidemiological trials.

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Notes

The authors declare no competing financial interest.

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