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LC-MS as a Tool to Overcome the Limitations of Self-Reported Dietary Assessments in the Determination of Wine Intake

Patricia Regal *, José J. Porto-Arias, Alexandre Lamas, Lucía Paz, Francisco Barreiro and Alberto Cepeda

Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Veterinary Science, Universidade de Santiago de Compostela, 27002 Lugo, Spain; josejulio.porto@usc.es (J.J.P.-A.); alexandre.lamas@usc.es (A.L.); lucypazlopez@gmail.com (L.P.); francisco.barreiro.buceta@gmail.com (F.B.); alberto.cepeda@usc.es (A.C.)
* Correspondence: patricia.regal@usc.es; Tel.: +34-982-285-900

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Abstract: The nutritional assessment of individuals is usually performed using highly subjective data collecting tools such as food frequency questionnaires, dietary recalls and food records. However, people are not always capable of recalling all foods (and ingredients) consumed, and in some cases, the intake of specific foods is intentionally omitted. Even though wine is considered positive for cardiovascular status, and is an essential part of Mediterranean culture, individuals may not always report its consumption accurately due to the existence of social preconceptions about alcoholic beverages. In this study, the presence of free resveratrol has been determined in human plasma from 25 Spanish volunteers using liquid chromatography coupled to mass spectrometry (LC-MS). This phenolic compound proved to be useful as a dietary biomarker for wine intake in a known population, and the results were compared with those obtained by self-reported dietary assessments. However, certain limitations must also be taken into account such as inter-individual variations and the type of wine consumed. The LC-MS method was validated for *trans*-resveratrol determination in human plasma, with an LOD (limit of detection) of 50 ng·mL^{-1} and an LOQ (limit of quantification) of 150 ng·mL^{-1}, respectively.

Keywords: resveratrol; LC; MS; biomarker; wine; dietary intake; FFQ; self-reported

1. Introduction

Alcoholic beverages are part of the usual diet for many countries and cultures, varying widely over the world in terms of type of beverage, quantity and frequency of consumption. Wine is a traditional beverage obtained from grapes that has been associated with both healthy and harmful effects on regular consumers [1]. In this context, the majority of European countries have included wine among their most consumed beverages since ancient times. This is especially true for those regions considered Mediterranean that follow very similar dietary patterns, such as Spain, Greece and Italy. The well-known Mediterranean Diet is characterized by a moderate consumption of wine (1–2 wine glasses per day), which usually accompanies meals [2]. In Spain, wine consumption has also gained strength as a cultural phenomenon, with tourism being an important factor behind this trend.

Wine has a very complex composition which differs on the basis of multiple factors of its productive process (variety of grape, region, aging, etc.). It contains many natural bioactive compounds with demonstrated beneficial effects in humans, such as resveratrol and flavonoids [1,3]. Resveratrol is a natural polyphenolic and fat-soluble compound, existing in *cis*-, *trans*- and piceid isomeric forms. This compound is found in grapes [4] but also in other plant foods, dried roots, and herbal plants used in

traditional oriental medicine [5], varying in isomerization and percentage of aglycons and glucosylated forms. Resveratrol confers to wine part of its beneficial properties, as it has been reported to have antioxidant effects in cardiovascular, cerebral and metabolic diseases [6,7] and it is also a superagonist of estradiol [8].

The task of collecting wine-intake information faces many challenges, associated with the subjective nature of the common dietary assessment methods. The data obtained from food frequency questionnaires, food records and dietary recalls is highly dependent on individuals and their capability to recall all food consumed as well as specific ingredients. Additionally, some items may not always be reported accurately, on purpose or unintentionally, due to social misconceptions of their consumption. Although wine-drinking is in general well socially accepted, attitudes towards alcoholic beverages vary widely among individuals. These limitations can be overcome by the use of dietary biomarkers; compounds originally present in the food that can be traced in the biological fluids of the consumer (saliva, blood, urine, etc.) [9]. In this study, wine consumption has been assessed in 25 Spanish volunteers through food frequency questionnaires and face-to-face interviews. Plasma samples were collected from each subject and the presence of free resveratrol has been determined using liquid chromatography coupled to mass spectrometry, as a dietary biomarker for wine intake. The only standard commercially available for validation purposes was *trans*-resveratrol, hence, the method was validated only for *trans* isomers. This decision was also based on the faster conjugation of *cis*-isomers in humans and the greater abundance of *trans*-resveratrol in red wine. The limitations of the present approach to determine wine intake are also discussed.

2. Materials and Methods

2.1. Reagents and Chemicals

Pure standard of *trans*-resveratrol (99% purity) was obtained from Sigma-Aldrich (Madrid, Spain). HPLC grade water, methanol and acetonitrile, and formic acid and acetic acid (≥99% purity) were purchased from Merck (Darmstadt, Germany). A stock solution of resveratrol was prepared at 100 mg·L^{-1} in methanol and stored in the dark at −25 °C.

2.2. LC-MS Method

The HPLC system consisted of a quaternary pump, degasser and autosampler from Agilent Technologies, model 1100 (Santa Clara, CA, USA). A Qtrap 2000 MS with Ion Source Turbo Spray from AB Sciex (Toronto, ON, Canada) was used. Nitrogen was produced by a high-purity nitrogen generator from PEAK Scientific Instruments Ltd. (Chicago, IL, USA).

Sample extracts were separated using a Luna C18 100A (150 × 2 mm, 3 µm) column from Phenomenex (Torrance, CA, USA). The mobile phase was water 0.2% formic acid (A) mixed on a gradient mode with acetonitrile (B), at a flow rate of 300 µL·min^{-1}. After the first 10 min with a very aqueous mobile phase at 85% A, the binary gradient was as follows: (A) from 85% to 20% in 20 min, returned to initial conditions in 5 min, maintained for an additional 5 min for column equilibration between injections. Total run time was 40 min.

Multiple Reaction Monitoring (MRM) data acquisition with electrospray ionization (ESI) in negative mode was used, monitoring the following MRM transitions of resveratrol, in order of abundance: 226.7 > 143.0 and 226.7 > 185.0. Data were collected and processed with Analyst 1.4.1 software package (MDS SCIEX).

2.3. Study Design, Subjects and Samples

Human plasma samples were obtained from 25 healthy volunteers (aged 25–55 years) from Spain, and the collection of fasting blood samples was conducted in the early morning. Plasma samples were stored frozen in the dark at −25 °C until analysis. The study was performed in accordance with the Helsinki Declaration of 1975, as revised in 1996. The corresponding Ethics Committee (*Comité*

Ético de Investigación Clínica de Galicia) approved the study and all participants gave informed consent. The enrolment in this study was voluntary and anonymous.

Information on wine consumption of the participants was obtained using a food-frequency questionnaire (FFQ). This questionnaire was elaborated based on the one used in the PREDIMED study (available online at http://www.predimed.es), including a total of 151 items structured in 12 food categories. Also, all the volunteers were submitted to a face-to-face interview, including some questions about wine. The obtained dietary information about wine-drinking habits was compared with the results obtained for resveratrol from the analysis of the corresponding plasma samples of each subject.

One volunteer agreed on donating two blood samples: one sample at the beginning of the study (type of consumer: occasional wine drinker with 1–3 servings per month) and an additional blood sample after consuming 1 glass/serving of red wine ("Mencía" Spanish grape variety) per day over the course of three consecutive days (blood collection 48 h after the last intake).

2.4. Sample Preparation

Since resveratrol is susceptible to isomerization under light conditions, all samples and standards were handled with no direct exposure to light. Aliquots of plasma samples (1 mL) of each volunteer were treated with glacial acetic acid (30 µL) to precipitate proteins. After vortex-mixing the acidified samples, they were centrifuged at 10,000× *g* at room temperature for 3 min. Samples were then loaded onto Waters Oasis HLB 3cc SPE cartridges, previously preconditioned with 3 mL of methanol and 6 mL of water. After loading sample supernatants, avoiding the protein precipitated pellet, the cartridges were washed with 6 mL of water and 3 mL methanol/water (5:95, *v*/*v*). Resveratrol was eluted from the sorbent with 3 mL of 100% methanol and evaporated to dryness under a nitrogen stream. The residue was reconstituted with 150 µL of initial mobile phase and 25 µL were injected in the LC-MS/MS system.

3. Results and Discussion

3.1. LC-MS/MS Method Development

The high performance liquid chromatography (HPLC) tandem mass spectrometry (MS/MS) method was developed on the basis of the exiting literature on resveratrol determination in various matrices [10–12]. A C_{18} stationary phase is preferred for chromatography because it has proved to be very effective in separating resveratrol and its metabolites in complicated biological samples such as urine and plasma low density lipoproteins [10], and also in wine [12]. The behavior of resveratrol under mass spectrometry was evaluated by infusing a solution of analytes at 1 $\mu g \cdot mL^{-1}$ in acidified methanol (0.1% formic acid), and enabling the selection of its characteristic ion and fragments in negative multiple reaction monitoring. Negative ionization was preferred, as it provided a stronger deprotonated molecule of resveratrol, with a more intense signal than that observed in the positive mode. Acidified (0.1% formic acid) acetonitrile and water, which are common solvents used in chromatography, resulted in good separation and ionization of the analyte.

The clean-up procedure was developed on the basis of the principles of simplicity, quickness and ease of use. Briefly, a version of the protocol used by Urpi-Sarda in 2007 [10] was used with some modifications, including acetic acid precipitation of plasma proteins and a very simple solid phase extraction of the supernatant. The mean recovery of resveratrol in spiked plasma samples was 75% with a relative standard deviation (RSD) of 12%. Recovery was calculated at the lowest fortification level, 100 $ng \cdot mL^{-1}$, and using a total of six replicates analyzed over the course of three consecutive days.

The linearity of the method was verified by analyzing blank plasma (with no resveratrol) and the same sample spiked at three different levels of *trans*-resveratrol, i.e., at 100, 200, and 300 $ng \cdot mL^{-1}$, obtaining a curve for which R^2 was 0.99. The instrumental limits, limit of

detection (LOD) and limit of quantification (LOQ), were based on the standard deviation of the response of the curve and the slope of the calibration curve [13]. They were calculated according to the following formulas: LOD = 3.3 * (standard deviation of the response/slope of the curve) and LOQ = 10 * (standard deviation of the response/slope of the curve). The obtained values for LOD and LOQ were 50 ng·mL^{-1} and 150 ng·mL^{-1}, respectively.

3.2. Resveratrol and Wine Intake

Table 1 shows the data on the wine consumption of 25 Spanish volunteers, obtained through different methods. Information on the frequency of wine consumption of the participants was obtained using a food-frequency questionnaire (FFQ), including many foods and beverages in order to minimize the bias of asking only for one item. Also, a face-to-face interview enabled the collection of some extra information such as moment of consumption (during meals, leisure time/weekends, type of wine consumed, etc.). The main disadvantage of face-to-face interviewing as a method of data collection is that it is costly, requiring more time and personnel, and it is highly dependent on the establishment of trust and rapport between the interviewer and the interviewee in order to obtain accurate data. According to the FFQs, 14 subjects never consumed wine and the rest consumed wine on a monthly (1–3 servings per month) or weekly (1–3 servings per week) basis. Only one volunteer could be classified as an adherent to the Mediterranean pattern of wine consumption, regular but moderate (1–2 wine glasses per day), and usually accompanying meals [2]. During the face-to-face interview the volunteer declared he preferred, on most occasions, "Tempranillo" red wine variety. In contrast, the occasional wine consumers preferred mostly white wines, and the intake was associated mostly with weekends and celebration, special occasions and meetings with friends (social drinking).

Table 1. Wine consumption of 25 Spanish volunteers as declared in food frequency questionnaires (FFQ) and face-to-face interviews. Resveratrol presence in the plasma of each subject was evaluated using HPLC-MS/MS.

Subject ID [1]	Wine Intake (FFQ) [2]	Face-to-Face Interview	Resveratrol (HPLC-MS/MS)
S1	1 serving per week *	1 bottle per month	Non detected
S2	Never	-	Non detected
S3	Never	-	Non detected
S4	Never	-	Non detected
S5	Never	-	Non detected
S6	Never	-	Non detected
S7	Never	-	Non detected
S8	Never	-	Non detected
S9	Never	-	Non detected
S10	Never	-	Non detected
S11	4–5 servings per week †	Occasional	Non detected
S12	Never	-	Non detected
S13	1 serving per week †	Occasional	Non detected
S14	4–5 servings per week †,*	Occasional: red	Non detected
S15	Never	-	Non detected
S16	2–3 servings per week †	Occasional	Non detected
S17	1 serving per week *	Occasional	Non detected
S18	1–3 servings per month †	Occasional	Non detected
S19	Never	-	Non detected
S20	Never	-	Non detected
S21	1 serving per week †,*	Occasional	Non detected
S22	Never	-	Non detected
S23	1–3 servings per month †	Occasional	Non detected
S24	1–2 servings per day *	Daily, with meals	Non detected
S25	1 serving per day *	3 consecutive days	>100 ng·mL^{-1}

[1] Subject S25 was an occasional wine drinker (1–3 servings per month) who voluntarily agreed on consuming 1 serving/glass of red wine during 3 consecutive days before blood collection (blood collected 48 h after the last glass was consumed). [2] Type of wine consumed: * red wine; † white wine.

Resveratrol (free resveratrol) was determined using a very simple and straightforward analytical method. Resveratrol was extracted from acidified plasma samples using a fast solid-phase extraction protocol, to be analyzed by liquid chromatography coupled to tandem mass spectrometry. Mass spectrometry enabled the unequivocal identification of resveratrol in plasma samples. One of the subjects who declared being an occasional wine consumer (1–3 servings per month of red wine) agreed on donating two blood samples in different time points: one at the beginning of the study and an extra sample 48 h after consuming "Mencía" red wine variety during three consecutive days (1 glass per day, during meals). This subject was included to test the validity and applicability of the analytical method to detect resveratrol in plasma after (red) wine consumption. Figure 1 shows a chromatogram of (**a**) a sample classified as "non-detected" for resveratrol (occasional wine consumer or non-consumer), and (**b**) a plasma sample obtained from the volunteer who donated blood after three days of regular red wine consumption (blood collection 48 h after the last intake) in which resveratrol presence was clearly detected. The chromatographic separation enabled the observation of both aglycon isomers of resveratrol, *cis*- and *trans*-resveratrol, with the latter being more abundant. This finding is explained by the greater abundance of t*rans*-resveratrol in red wine, as well as for the faster conjugation of *cis*-isomers in humans [10]. The plasma of this subject (S25 in Table 1) was tested for resveratrol also at the beginning of the study, and it was not detected.

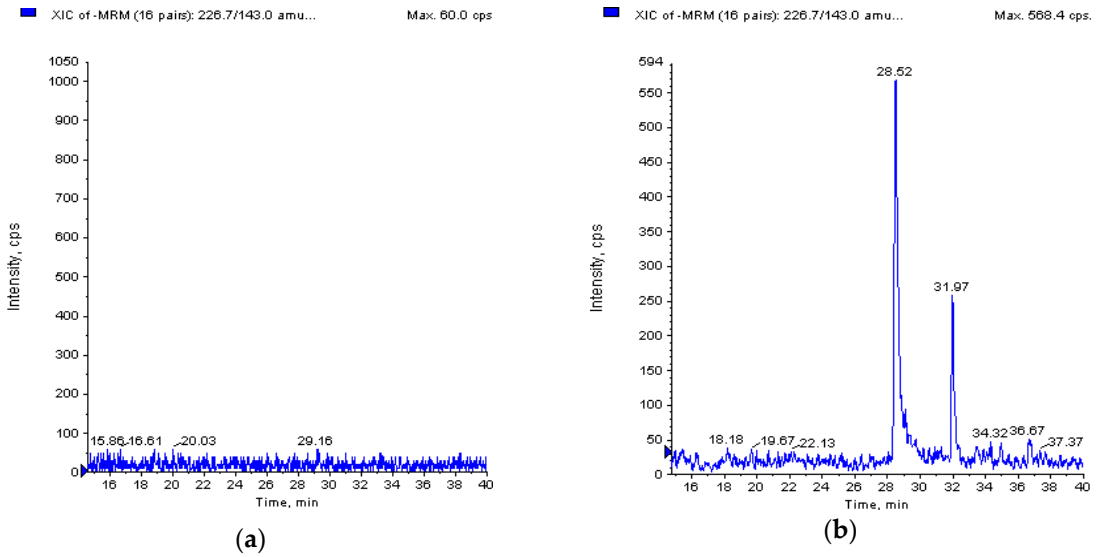

Figure 1. LC-MS chromatogram obtained from the analysis of plasma samples from (**a**) a subject with zero consumption of wine and (**b**) a volunteer who consumed red wine during three consecutive days showing the presence of *trans*- (28.5 min) and *cis*-resveratrol (31.9 min).

The data obtained in this study for wine consumption shows very low adherence to what is considered the Mediterranean pattern. A moderate intake of wine during meals is one of the most representative components of the so-called Mediterranean diet, characteristic of the countries bathed by the waters of the Mediterranean. However, alcoholic beverages consumption in Spain has undergone a slow decline during recent years [14], with wine representing less than 25% of the total alcoholic beverages consumption and undergoing a gradual substitution with beer and non-alcoholic beverages. These facts are in agreement with the data observed in the FFQ obtained from the volunteers of this study, which reflects a higher consumption of beer and/or soft drinks than of wine.

The results of the interview and the FFQs were in agreement with the results obtained for resveratrol in plasma for most subjects. The phenolic compound was not detected in the occasional drinkers, which furthermore mostly preferred white wine varieties, neither in the non-consumers. Red wines are primarily a source of the aglycones *cis*- and *trans*-resveratrol [5]. Piceid hydrolysis

during wine fermentation releases resveratrol, which is contained in considerably higher amounts in red wines than in white wines because it is mainly present in the berry skin. Red wine is known to contain 10-fold more phenolic compounds than white wine [1]. Additionally, resveratrol wine levels can also be affected by wine exposure to light [4].

In previous studies, *trans*-resveratrol bioavailability was shown to be independent from the meal and to pose a high inter-individual variability [11]. This fact supports the idea that the positive health effects that have been associated with wine intake in the Mediterranean regions are related not only to resveratrol but to the whole antioxidant mixture present in (red) wine. Also, the results of this study indicate that not all the grape/wine varieties are important sources of resveratrol and not all wine-consumers can be identified by measuring this biomarker in plasma. The subject who voluntarily ingested 1 glass of wine (150 mL approximately) for three consecutive days showed clear presence of resveratrol in plasma 48 h after the last intake. However, the phenolic compound could not be detected at the same level in the only regular (as self-reported) wine consumer. It is important to note that the latter volunteer consumed "Tempranillo" wines while the three-days experiment was conducted with "Mencía" variety from Galicia (Spain). Previous studies have demonstrated that Galician wines elaborated with "Mencía" are important sources of *trans*-resveratrol [15], while the average resveratrol content in Spanish "Tempranillo" varieties falls far below "Mencía" and other red varieties [12].

It can be concluded that resveratrol is useful as a dietary biomarker for wine intake, but always in combination with self-reported dietary tools. The limitations of this method are inherent to wine and individuals. Since this stilbene is naturally present in several plant species, including some herbs, grapes, berries and peanuts, its presence in human samples cannot be considered exclusively linked to wine intake. In this context, there is still scope for future research to find more specific dietary biomarkers in order to improve the accuracy of self-reported assessment methods.

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Conflicts of Interest: The authors declare no conflict of interest.

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