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Untargeted metabolomic analysis using liquid chromatography quadrupole time-of-flight mass spectrometry for non-volatile profiling of wines

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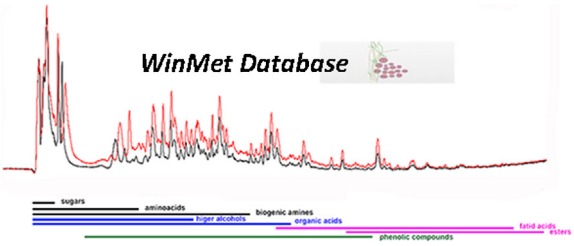
HIGHLIGHTS

- An untargeted metabolomic method for the non-volatile profile of the Graciano wine was developed.
- 411 different metabolites in Graciano *Vitis vinifera* red wine were identified.
- 15 compounds could serve to differentiate Graciano and Tempranillo wines.
- An enological database (WinMet) with 2080 compounds was constructed.

GRAPHICAL ABSTRACT



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ABSTRACT

The current study presents a method for comprehensive untargeted metabolomic fingerprinting of the non-volatile profile of the Graciano *Vitis vinifera* wine variety, using liquid chromatography/electrospray ionization time of flight mass spectrometry (LC–ESI-QTOF). Pre-treatment of samples, chromatographic columns, mobile phases, elution gradients and ionization sources, were evaluated for the extraction of the maximum number of metabolites in red wine. Putative compounds were extracted from the raw data using the extraction algorithm, molecular feature extractor (MFE). For the metabolite identification the WinMet database was designed based on electronic databases and literature research and includes only the putative metabolites reported to be present in oenological matrices. The results from WinMet were compared with those in the METLIN database to evaluate how much the databases overlap for performing identifications. The reproducibility of the analysis was assessed using manual processing following replicate injections of *Vitis vinifera* cv. Graciano wine spiked with external standards. In the present work, 411 different metabolites in Graciano *Vitis vinifera* red wine were identified, including primary wine metabolites such as sugars (4%), amino acids (23%), biogenic amines (4%), fatty acids (2%), and organic acids (32%) and secondary metabolites such as phenols (27%) and esters (8%). Significant differences between varieties Tempranillo and Graciano were related to the presence of fifteen specific compounds.

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1. Introduction

Wine is a widely consumed beverage, and as such has a high commercial value. Therefore, wine authenticity control, mainly in terms of varieties, geographical origin, and/or age, is continuously required to maintain wine quality and to detect any adulteration [1]. Variety, provenance, production year, and quality ratings, which are the most important attributes used for the characterization and description of wines, should be reflected in the total composition of small molecules in wine.

In this sense metabolomic approaches are currently used as methods to ensure the authenticity and traceability of wines. The molecules in wine include a large number of primary metabolites (e.g., sugars, amino acids, organic acids, lipids, etc.) and metabolites (phenolics, alkaloids, sterols, lignans, terpenes, fatty acids, etc.) [2,3]. While hundreds of metabolites (volatile and non-volatile) are detectable in wine, only a fraction of them have been identified. All of these compounds have a strong influence on the quality and character of the wine and are therefore not only important for the characterization and differentiation of wines but also for the detection of frauds [4]. The whole chemical composition of a wine reflects the history of the wine-producing process, including the grape variety, the yeast strain, the containers used for alcoholic and malolactic fermentations, storage, ageing [5], and the enological practice [6,7].

In metabolomic studies of wine tandem mass spectrometry such as hybrid QTOF, coupled to diverse chromatographic platforms is usually employed [1,8,9]. These studies have demonstrated that the restricted target analysis of specific metabolites misses a large part of the molecular information regarding the metabolome of wine and that untargeted metabolomics can be a powerful tool for the molecular fingerprinting of a complex beverage such as wine.

In conjunction with LC–MS systems, the ionization of the compounds may be achieved using different types of sources, such as electrospray ionisation (ESI) [10,11], atmospheric pressure chemical ionisation (APCI) [12,13], or atmospheric pressure photoionisation (APPI) [14]. Currently, new modifications focusing on thermal gradients are carried out in an ESI source design called JetStream technology. This type of ESI source can initially significantly increase the method sensitivity to compounds during the analysis, decreasing sample size requirements, increasing sample throughput, and improving the assay robustness [15].

The aim of this work was to use a LC–QTOF system to achieve a detailed, untargeted metabolome profile of the *Vitis vinifera* cv. Graciano wine variety. The interest in the metabolomic study of the Graciano *Vitis vinifera* wine variety resides in the lack of knowledge of the composition of this type of wine and its high market demand due to its unique and appreciated organoleptic characteristics. Graciano *Vitis vinifera* is a singular variety of red grapes that is rarely seen outside its home territory in northern Spain, principally in Rioja and Navarra. It is also grown in Australia under the same name of Graciano and in California as Xeres. This type of variety is highly prised for its intense aromatic properties, red colour, and high acidity but low yielding in the vineyard. *Vitis vinifera* cv. Graciano is used as an integral component of many Rioja wines because it is considered to directly contribute to the quality of the wine. In fact, Graciano *Vitis vinifera* wine is often used as a blending partner for Tempranillo-based wines to increase the quality of these [16].

The current study presents a comprehensive and robust analytical technique for the analysis and identification of the non-volatile/semivolatile metabolome of the Graciano Vitis vinifera wine variety, developing a platform (the WinMet database) suitable for differential QTOF mass spectrometry analyses of wine metabolic profiles that complements the existing tools. The WinMet database contains 2030 putative compounds present in oenological matrices covering 10 different families, such as phenols, organic acids, biogenic amines, sugars, polyols, fatty acids, higher alcohols, aldehydes, lignans and ketones. This database is the first to examine the exact monoisotopic mass of oenological compounds, supporting high throughput MS approaches for the identification and quantification of metabolites present in wine samples.

2. Materials and methods

2.1. Chemicals and reagents

The surrogate standard myristic-d27 acid was purchased from Isotec™ (Sigma–Aldrich, St. Louis, MO, USA). Proline, L-alanine, L-lactic acid, D-fructose, and D-sorbitol obtained from Sigma–Aldrich (Steinheim, Germany) and L-tartaric acid acquired from Riedel de Haën (Seelze, Germany) were used for the alignment of the chromatographic peaks. All standards were of minimum purity of 98%. Optima® LC–MS grade methanol and acetonitrile were supplied by Fisher Scientific (Geel, Belgium), and deionised water was prepared by purifying demineralised water in a Milli-Q water filtration system (Millipore, Milford, MA, USA). Ammonium formate, ammonium acetate, and formic acid, used as mobile phase additives (each ≥99% pure), were supplied by Fluka Analytical (Sigma–Aldrich, St. Louis, MO, USA).

2.2. Wine samples

Young, monovarietal red wines from *Vitis vinifera* cv. Graciano grape varieties, which were guaranteed and provided by wineries from Rioja, Spain, were used to optimise the conditions for the metabolic extraction step as well as the parameters for LC–QTOF.

Metabolomic analysis of *Graciano Vitis vinifera* red wine was performed with the following nine commercial wines from Aldeanueva de Ebro (vintage 2007 and 2011), Villabuena de Alava (vintage 2004 and 2009), La Puebla de la Barca (vintage 2010), Elvillar (vintage 2010), Logroño (2009) and Laguardia (vintage 2008 and 2010). A comparative study was developed with wines elaborated from Tempranillo grapes for the verification of specific compounds identified in Graciano wines. For this part of the work, Tempranillo wines from Villabuena de Alava (vintage 2010 and 2012 (×2)), Elciego (vintage 2013), Baños de Ebro (vintage 2013 (×2)), Laguardia (vintage 2010 and 2011) and Abalos (vintage 2010) were used.

Wine samples employed for sample pretreatment and method optimization consisted of a mixture of the 9 monovarietal Graciano wines or the 9 Tempranillo wines indicated previously (pool). Once optimized the analytical methodology developed was used to analyze all 18 individual wines.

2.3. Sample pre-treatment

Three non-specific pre-treatments were considered: centrifugation, filtration, and direct injection of pooled wine.

Wines were uncorked and pooled before analyses. Five replicates of aliquots of 2 mL samples (pooled an unpooled wines) of *Vitis Vinifera* cv. Graciano wine was spiked with 50 mg L^{-1} deuterated myristic-d27 acid as the internal standard to monitor the retention time and mass drift over the total analytical period.

Centrifugation at 10,000 rpm for 2.5 and 5 min (Allegra™ X-22R Centrifuge, Beckman Coulter, California, USA) was evaluated in the metabolomics analysis. Two millilitres of the supernatant was transferred into a glass vial and injected without any other pre-treatment for LC–QTOF analysis. Methanol/water (50/50) was used as a check to determine whether any carry-over effect was present.