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Liquid chromatography–quadrupole time of flight tandem mass spectrometry–based targeted metabolomic study for varietal discrimination of grapes according to plant sterols content☆

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ABSTRACT

Grapevine and derived products are rich in a wide range of compounds and its quality mainly depends on its metabolites, as a result of viticulture practices. Plant sterols, also called phytosterols (PS), are secondary metabolites regarded as bioactive substance present in grape berries and other plant-based food. The present study deals with a metabolomic approach focusing on phytosterols family in six varieties of Rioja grapes (*Cabernet Sauvignon, Tempranillo, Graciano, Garnacha, White Garnacha and Viura*), in order to find significant differences among them. Liquid chromatography- mass spectrometry with a quadrupole-time of flight mass analyzer (LC-QTOF) was used to find as many metabolites as possible in the different grape berry fractions, and using statistics to help finding significant clustering of the metabolic profile of pulp, peel and seeds in relation to the variety. The best chromatographic and detection conditions were achieved by gas phase ionization via atmospheric pressure chemical ionization (APCI) in positive mode. Furthermore, analysis with electrospray (ESI) is also needed for phytosterol derivatives confirmation.

Putative compounds of interest in the analyzed samples were found by an automated compound extraction algorithm (Molecular Feature Extraction, MFE) and an initial differential expression from the data was created with the aid of commercial software. Once the data were collected, the results were filtered, aligned and normalized, and evaluating applying one-way analysis of variance (ANOVA) with a 95% significance level. For sample class prediction, partial least square-discriminant analysis (PLS-DA) is used as a supervised pattern recognition method and excellent separation among the grape varieties is shown. An overall accuracy of 93.3% (pulp samples), 100.0% (peel) or 96.7% (seeds) in discriminating between grape varieties was achieved when comparing the different fractions. In general, 7 PS derivatives were identified with ID scores higher than 84%.

1. Introduction

Grapes and wine have been subjected to many metabolomics-based studies providing some very useful information on grapevine chemistry [1–5]. Omics application in wine science is very useful tools to assess wine chemistry. The rapidly developing discipline of metabolomics has made possible high-resolution characterization of hundreds or thousands of metabolites from complex samples in a single measurement [6–8]. Currently, no single analytical technique will provide comprehensive visualization of the metabolome [9,10]. Major advances in analytical tools such as mass spectrometry and the corresponding hyphenated methods, gas chromatography coupled with mass spectrometry (GC–MS) and liquid chromatography coupled to mass spectrometry (LC–MS), have helped separating and confirming a vast number of chemical species in grapevine derived products [11,12]. To date, several analytical techniques based on GC with flame ionization detector (GC–FID) [13–15], GC–MS [16,17] and LC–MS [18–21] have been used to determine phytosterols in grapes tissues. In addition to

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these techniques, and focusing on other lipid compounds with healthy properties (phytosterols and phytostanols, glycerolipids, terpenoids, etc.), nuclear magnetic resonance (NMR) and capillary electrophoresis (CE) have also exerted good results in their identification and/or detection [22,23].

Advances in chemometric and bioinformatic technologies also plaid an important role [24]. When considering the design of a metabolomic study, different sources of variation should be taken into account and must minimized (sampling, sample preparation and metabolite extraction, instrument variation, among others) [25,26]. Metabolomic workflow generally involves several steps, beginning with an appropriate experimental planning that ensures the data is relevant for further biological interpretation. It includes sample preparation (metabolite extraction) before separation and detection. After instrumental analysis, data pre-processing needs to be done by automatic peak detection software. The metabolomics experiment ends with statistical analysis, metabolite identification and result interpretation.

Extraction of metabolites is considered one of the most laborious and rate-limiting step in metabolomics [27]. Minimal sample preparation is usually preferred, especially for untargeted applications. Otherwise, for targeted approaches a relative selective sample-preparation protocol can be used, because there are some preliminary data or knowledge [28,29]. The challenges of sample preparation for metabolomics include efficiency, reproducibility and coverage, among others [30]. As the present research work is regarded as a targeted approach mainly focused on selected predefined group such as PS family, some specific settings should be considered provided it extracts as many PS as possible.

Liquid chromatography–mass spectrometry with a quadrupole-time of flight mass analyzer (LC-QTOF) is a powerful tool to determine food authenticity, for instance wine variety [31,32]. Chromatographic separation prior to MS-analysis is particularly important in order to minimize ion suppression as well as to separate isobaric and isomeric compounds. Reverse phase LC provides the most reliable and robust LC stationary phase for separation the majority of the secondary metabolites at low concentration levels [3]. As far as LC–MS interfaces are concerned, electrospray ionization (ESI) is the method of choice in most metabolomics applications. However, in this particular study PS are not ionized under such conditions due to the fact that they are highly lipophilic compounds with few polar functional groups in their structure. Atmospheric pressure chemical ionization (APCI) offers the best choice for sterols detection in positive ionization mode [18,19]. TOF mass analyzer is considered one of the instruments best suited to metabolomics studies since it provides high resolution analysis in a broad m/z range, high sensitivity in full scan mode and high mass accuracy (<5 ppm) [33]. Moreover, the QTOF hybrid instrument provides accurate mass MS/MS spectra facilitating the determination of the molecular formula of unknown metabolites since the number of possible metabolites of a single structure would be reduced.

The complex datasets require visualization software and chemometric and bioinformatic methods for interpretation [34]. Raw data files are transformed into a representation that facilitates easy access to observed ion characteristics, such as m/z, retention time and ion intensity, as well as isotope distribution. A wide variety of software, both commercial and open source, is available to handle metabolomic data [35].

Statistical processing is required to determine whether or not the observed changes in metabolite levels are significant and it relies on careful experimental design including replicate sampling, replicate analyses and application of statistical tests [36].

The identification of the chemical structure of the interesting metabolites, that is the statistical significant entities, comprised the last step in the metabolomic workflow, as well as the study of the metabolic pathway where they are involved. A summary of the most widely used databases for the identification of metabolomic signals by MS has been published by Fukushima [37].

Although the analysis of some phytosterols in grape berries by different analytical techniques have already been published, as mentioned above, to the best of the authorís knowledge, targeted metabolomic studies of phytosterols in wine grape varieties have not been reported in the literature. The main goal of the present study seeks to extend the knowledge of PS family comparing different varieties of grape berries (*Tempranillo*, *Graciano*, *Cabernet Sauvignon*, *Garnacha*, *White Garnacha and Viura*) located in the D.O.Ca. Rioja ("Calificada" Designation of Origin to Rioja). To accomplish this purpose a targeted metabolic study has been carried out with the aim of finding significant clustering of the metabolic profile of pulp, peel and seeds in relation to the variety. Commercially available software was employed for automatic data pre-processing. The compositional information obtained was processed using multivariate statistical analysis. For initial exploration of the data and sample clustering, Principal Component Analysis (PCA) was employed. Afterwards, supervised pattern recognition method such as partial least square-discriminant analysis (PLS-DA) was used so as to predict a model.

2. Experimental

2.1. Chemicals, reagents and reference substances

Isotopically labelled β-sitosterol (5-cholesten-24(RS)-ethyl-3-β-ol-25,26,26,26,17,27,27-d_7, $C_{29}H_{43}D_7O$, MW 421.43, 99 atom% D, purity 98%) and labelled cholesterol (cholesterol-25,26,26,26,27,27,27-d_7,$C_{27}D_7H_{39}O$, MW 393.70, CAS 83199-47-7, 99 atom % D, purity 98%) were purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada) and Sigma-Aldrich, respectively, and were used as internal standards for the subsequent compound alignment and normalization of the obtained peaks.

All organic solvents (acetone, chloroform) of analytical or HPLC grade and acetonitrile and methanol LC–MS grade, were supplied by Scharlab (Barcelona, Spain). Ammonium acetate (≥98%) and formic acid reagent grade (≥95%) were purchased from Sigma-Aldrich. The former was used as additive in the mobile phase and the latter to pH adjustment, when using LC-ESI-QTOF conditions. Ultra-high purity water (UHP), prepared from tap water and pretreated using Elix reverse osmosis cartridges before filtration by a Milli-Q system from Millipore (Bedford, MA, USA), was used throughout the study.

2.2. Instrumental analysis

Metabolomic analysis was carried out on a 1200 Series HPLC system and 6530 QTOF mass spectrometer (Agilent Technologies, PA, USA). The chromatographic separation was performed using an Eclipse Plus C8 column (2.1 × 150 mm, 5 μm) from Agilent Technologies, under gradient mode. The LC system was hyphenated to an accurate-mass QTOF MS with two atmospheric pressure ion sources (API). First, APCI ionization source was used to analyse all grape samples so as to determine as much features as possible. Secondly, ESI interface was only used to confirm the putative conjugates. The injection volume was 2 μL and the analysis was performed in full scan mode.

The QTOF instrument worked in MS mode in extended dynamic range (2 GHz) and mass spectra were acquired by scanning over 100–1000 m/z range at an acquisition rate of 1 spectra/s. The spectrometer working conditions set for the analysis with APCI ion source positive ionization were as follow: capillary voltage was set at 3000 V and corona current at 4000 nA; nebulizer pressure,