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Metabolite extraction for high-throughput FTICR-MS-based metabolomics of grapevine leaves

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ABSTRACT

In metabolomics there is an ever-growing need for faster and more comprehensive analysis methods to cope with the increase of biological studies. Direct infusion Fourier-transform ion cyclotron-resonance mass spectrometry (DI-FTICR-MS) is used in non-targeted metabolomics to obtain high-resolution snapshots of the metabolic state of a system. In any metabolic profiling study, the establishment of an effective metabolite extraction protocol is paramount. We developed an improved metabolite extraction method, compatible with DI-FTICR-MS-based metabolomics, using grapevine leaves. This extraction protocol allowed the extraction of polar and non-polar compounds, covering all major classes found in plants and increasing metabolome coverage.
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1. Introduction

Grapevine (*Vitis vinifera* L.) is the most widely cultivated and economically important fruit crop in the world, mainly due to the wine industry. Many grapevine varieties are also grown for their use as food products, not only for Table grapes, but also for the consumption of their leaves. Due to their astringent and hemostatic properties and phenolic composition, vine leaves are considered a healthy food and are consumed in several countries, including Saudi Arabia, Turkey and Greece [1]. The biochemical composition of both grapes and leaves is determinant for their nutritional value and taste. Furthermore, some authors believe that the most reliable source of biomarkers for resistance or susceptibility against pathogens is the leaf surface and the polar extracts from defatted leaf tissues [2,3]. Hence, the analysis of the compounds present in leaves is of utmost importance. This is particularly relevant when concerning plants, which are biochemically highly complex and contain a unique metabolome that change with the environment, the development and upon pathogen infections [4].

So far, most of the metabolite studies in grapevine were performed by nuclear magnetic resonance (NMR) spectroscopy and were based on the analysis of a single extract from leaves [5,6]. In these studies by NMR, larger amounts of initial plant material are required (between 25 and 50 mg), the limit of detection is around 10 μM and even using 1D and 2D NMR techniques, and the number of metabolites identified is usually less than 20. More recently, mass spectrometry coupled to liquid chromatography (LC–MS) has been used in the identification and quantification of grapevine metabolites [7]. Although this methodology is more sensitive, only 135 primary metabolites (sugars, amino acids, organic acids and amines) were identified and quantified in a 30-min hydrophilic interaction LC run coupled to a triple quadrupole mass spectrometer [7].

To achieve higher sensitivity and maximum metabolome coverage, we resort to mass spectrometry using high-resolution and high-mass accuracy instruments, based on Fourier transform technology. The sensitivity of this methodology is much higher (typically ρg level) and different fractions can be analyzed (from aqueous to organic extractions) [8]. One of these instruments, the Fourier-transform ion cyclotron-resonance mass spectrometer (FTICR), provides ultra-high-mass accuracy (below 1 ppm) and

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the highest mass resolution (more than 1,000,000) [9]. Moreover, using direct infusion coupled to ultra-high-resolution mass spectrometry, metabolites are analyzed in a high-throughput way, providing a rapid analysis of complex metabolite samples, and eliminating the time-consuming separation by liquid chromatography (LC) [10].

In addition to high mass accuracy instruments, efficient sample extraction methodologies are a priority in metabolomics. These are especially critical when working with plant material, where caution must be taken during harvesting, grinding and metabolite extraction, to avoid consequences in the accuracy of results [11].

Here we present an efficient metabolite extraction protocol for grapevine leaves, suitable for the characterization of the *V. vinifera* metabolome by direct infusion Fourier-transform ion cyclotron-resonance mass spectrometry (DI-FTICR-MS).

2. Materials and methods

2.1. Plant material

V. vinifera cv Pinot noir young leaves were harvested from five different plants (three biological replicates were considered), at the Portuguese Grapevine Germplasm Bank at INIA—Estação Vitivinícola Nacional (Dois Portos), immediately frozen in liquid nitrogen and stored at −80 °C. Leaves were ground in liquid nitrogen and used for metabolite extraction.

2.2. Metabolite extraction

Metabolite extraction from grapevine leaves was performed using different solvent systems coupled to solid phase extraction (SPE) fractionation. We used the mixture 40% methanol (LC–MS grade, Merck)/40% chloroform (Sigma Aldrich)/20% water (v/v/v) as previously described for grapes [12], but the ratio was 0.1 g of grinded leaves to 1 mL of solvent. Samples were vortexed for 1 min and maintained in an orbital shaker for 15 min at room temperature. Samples were centrifuged at 1000*g* for 10 min for phase separation: the lower chloroform fraction and the upper aqueous/methanol fraction. The chloroform fraction (C) was further centrifuged for 5 min at 10,000*g* to remove debris and lyophilized at −40 °C. The aqueous/methanol layer was further processed by SPE using Merck LiChrolut RP-18 columns, pre-equilibrated with methanol. Metabolite fractions were collected by vacuum through sequential elution with 1 mL of water (W), methanol (M) and acetonitrile (A, LC–MS grade, Merck). The water fraction was lyophilized at −40 °C, while both methanol and acetonitrile fractions were evaporated under a nitrogen stream. A workflow of the experimental procedure is shown in Fig. 1.

2.3. Metabolite analysis by FTICR-MS

W and C fractions were reconstituted in methanol/water (1:1), while M and A fractions were suspended in the respective pure solvent. For the analysis of metabolites, all fractions were diluted 1000-fold in the appropriate solvent: M and A fractions were diluted in the same solvent for positive- (ESI^+) and negative-ion (ESI^-) mode analysis; W and C fractions were diluted in methanol for ESI^+ or in methanol/water (1:1) for ESI^-. The standard leucine enkephalin (YGGFL, Sigma Aldrich) was added to all samples at a concentration of 0.5 μg/mL, and was used as a standard for control and quality assessment of analytical precision ($[M+H]^+$ = 556.276575 Da or $[M\text{-}H]^-$ = 554.260925 Da), through the determination of the relative standard peak deviation and internal calibration. For the analysis in ESI^+, formic acid (final concentration 0.1% (v/v), Sigma Aldrich, MS grade) was added to all samples. Extracted metabolites were analysed by direct infusion in the Apex Qe 7-Tesla Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR-MS, Brüker Daltonics), with a flow rate of 240 $\mu L\,h^{-1}$. Between each sample run, the ESI source was cleaned with methanol or acetonitrile for 10–15 min and the spectrum was collected. Mass spectra were acquired with an acquisition size of 512k, in the mass range between 100 and 1000 Da (with a resolution of 130,000 at 400 *m*/*z*), and 50 scans were accumulated for each sample. In ESI^+, the nebulizer gas flow rate was set to 2.0 L/min and the dry gas flow rate to 4.0 L/min, at a temperature of 180 °C. The capillary voltage was set to 4500 V and the spray shield voltage was 4000 V. In ESI^-, the nebulizer gas flow rate was 2.5 L/min and the dry gas flow rate was set to 4.0 L/min, at a temperature of 220 °C. The capillary voltage was 4300 V and the spray shield voltage was set to 3800 V. In both ionization modes, ions were accumulated in the collision cell for 1.0 s, and a time of flight of 1.0 ms was used prior to their transfer to the ICR cell.

2.4. Data analysis and metabolite identification

Using the Data Analysis 4.1 software package (Brüker Daltonics, Bremen, Germany), the resulting mass spectra were internally calibrated using leucine enkephalin at both ESI modes; external calibration was performed with cyclopamine (*m*/*z* 412.32100, $[M+H]^+$) in all 4 fractions at ESI^+, whereas at ESI^-, hexadecanoic acid (*m*/*z* 255.23295, $[M+H]^-$) was used to calibrate M, A and C fractions, while W fraction calibration was calibrated with glutathione (*m*/*z* 306.07653, $[M+H]^-$). Peak height lists were then exported as ASCII files, setting at a signal-to-noise ratio at 4. The total number of identified ions (peaks) ranged between 1049 and 1346 for ESI^- and 10202 to 11444 for ESI^+ in 3 different biological replicates. The peak lists were combined to a peak matrix with an error of 1.0 ppm, as described by [13], implemented in a Python script based on the Pandas library for data analysis. Peaks with just 1 non-zero intensity (single mass events) were removed from the matrix as well as peaks that were detected in less than 50% of all biological replicates. Overall, 1018 peaks for ESI^- and 6266 peaks for ESI^+ remained after all filtration processes.

For metabolite identification, the mass list was submitted to the MassTrix 3 server (http://masstrix3.helmholtz-muenchen.de/masstrix3/,[14]) server selecting *V. vinifera* as organism, considering possible adducts M + H and M + Na for ESI^+, and M-H and M + Cl for ESI^- data, with a maximum error acceptance of 3 ppm. A total of 221 masses were annotated for ESI^- and 1366 for ESI^+. A manual curation for compounds with biological role was done by searching the annotated metabolites in the public databases PubChem (http://pubchem.ncbi.nlm.nih.gov/, [15]), KNApSAcK (http://kanaya.naist.jp/KNApSAcK/, [16]), KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/kegg2.html, [17]), Lipid Maps (http://www.lipidmaps.org/, [18]) and Metabolomics workbench (http://www.metabolomicsworkbench.org/, [19]).

3. Results and discussion

The analysis of unknown metabolites and the biological interpretation of their relationships represent a very important basis for the profiling of unique metabolic systems and the comparison of such profiles in different phenotypes. To ensure meaningful results and high data quality it is important to have a thorough experimental design and an efficient extraction protocol, specifically designed to be used with an accurate analytical technique.

There is no doubt that mass spectrometry (MS) in metabolomics has facilitated the simultaneous detection and quantification of a large number of metabolites within a large dynamic range. Additionally, it provides structural information through

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