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Integrating analytical resolutions in non-targeted wine metabolomics

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ABSTRACT

Direct injection Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS), and ultra-high performance liquid chromatography coupled to mass spectrometry (UPLC/MS) were combined for the non-targeted analysis of wine metabolites. The unrivalled resolution on mass measurement allowed by the former and the separation ability of isomeric and isobaric substances by the latter, clearly increases the scope of detectable unknown metabolites in wines. Such methodology is illustrated through the comparison of chemical spaces of a young and an older Pinot noir wine. RP and HILIC chromatography could reveal up to five isomers for a given mass, throughout the explored mass range. CHO, CHOS and CHONS chemical spaces exhibited higher diversities in the older wine, illustrating a molecular oeno-diagenesis process during wine ageing, whereas nitrogen-containing compounds (CHON chemical space) appeared to be fewer in the older wine, likely due to precipitation. This methodological combination is a promising contribution to the wine metabolomics toolkit.

1. Introduction

Most of the current analyses of wine with classical analytical targeted technologies have definitely contributed to a better knowledge of the wine chemistry.[1–3] Alternatively, metabolomics approaches have shown great potential for the study of grape or wine and they have helped to unravel the extremely high yet unknown chemical diversity of wine.[4] Metabolomics is considered here as the non-targeted metabolite analysis through the semi-quantitative description of all low molecular weight metabolites in a specified biological sample (wine).[4–8] Typical analytical techniques used in metabolomic research are FTICR-MS, LC–MS, GC–MS and NMR spectroscopy combined with multivariate statistics, and the advantages and disadvantages of these different analytical tools have been well described.[7,9–12]

Although limited in sensitivity, non-invasive sampling and structural identification of organic compounds are only two of the advantages of NMR spectroscopy, which has allowed the differentiation of wines according to the grape variety,[13] the geographical origin of grapes,[14] or the vintage.[13] In contrast, mass spectrometry allows the identification of a larger number of metabolites and even more when liquid chromatography is used before the MS detection to allow for the determination of different isomers and isobars. Several articles have already been published on wine metabolomics using LC-MS, which enabled the classification and differentiation of wine attributes such as grape variety, vintage and quality,[15] or to visualize the changing metabolic profiles during late berry developments.[16]

Ultra-high resolution mass spectrometry can provide—with an unprecedented sensitivity—a highly resolved description of wine fingerprints, as shown for the first time by Cooper and Marshall in 2001.[17] A few years later, this method allowed the characterization of must and wines of six varieties of grapes,[18] the discrimination of wines according to the geographic origin of oaks used for barrel

Abbreviations: FTICR-MS, Fourier transform ion cyclotron resonance mass spectrometry; HILIC, hydrophilic interaction liquid chromatography; RP, reversed phase; ESI, electrospray ionization; *m/z*, mass/charge; S/N, signal-to-noise ratio; UPLC, ultra-performance liquid chromatography; UHR-ToF-MS, Ultra-high resolution time of flight mass spectrometry.

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ageing,[4] or the identification of distinct chemical fingerprints between a champagne served in a glass and its aerosol above the glass.[19] High resolution FTICR-MS is now able to reveal the diversity of metabolites present in complex biological systems and enables the distinction and characterization of wines according to their terroirs, vintages, or qualities.[15,18,20,21]

Combined analytical methods have already demonstrated the molecular complexity and diversity in various systems including meteorites,[22] *Chlamydia pneumonia* infections[8] or natural organic matter.[23] The possibility of combining analytical tools is vast, but LC–MS and FTICR-MS appear to be very effective combined tools for the study of wine because they combine ultra-high resolution and sensitivity with selectivity and isomer identification. Increasing the range of detectable metabolites in wine or in any other biological samples is a challenge for the better understanding of their physico-chemical and/or organoleptic properties, of their ageing ability or even for authentication purposes.[3] The literature indeed reports various studies where correlations between a specific property of wines and their actual chemical compositions were constrained because of the limited number of targeted compounds,[24] thus illustrating the necessity to integrate a more comprehensive chemical diversity through the research of yet unknown metabolites for the identification of subtle discriminations.[4] To that respect, the combination of DI-FTICR-MS analysis with two orthogonal UHPLC–MS methods increases the scope of detectable metabolites, decreases putative ion suppression effects and achieves a separation between isomeric and isobaric compounds. These analytical platforms combining high resolution mass spectrometry and ultra-high resolution mass spectrometry thus provide an unprecedented synoptic description of the chemical complexity of wines, where results obtained with one platform can directly be validated with data from the other.

2. Experimental

2.1. Materials

Methanol, acetonitrile and water (LC–MS grade) were purchased from Fluka Analytical (Sigma–Aldrich, St. Louis, USA). Standard compounds for UHPLC–MS methods are listed in the Supplementary data Table S1. Low concentration ESI Tuning Mix for Q-ToF calibration was obtained from Agilent (Agilent, Waldbronn, Germany).

2.2. Sampling and sample preparation

All of the measurements were done on two Pinot noir wines from Burgundy with a young 2009 vintage and an older wine from the 19th century (exact age unknown), sampled directly from the bottles in 2 ml vials under argon to protect them from oxygen. Wine of 50 μl was diluted into 950 ml methanol for FTICR-MS analysis and 40 μl of acetonitrile was added to 960 μl of wine with for both RP and HILIC LC–MS analysis.

2.3. Fourier transform ion cyclotron resonance mass spectrometry analysis (FTICR-MS)

Ultra-high resolution mass spectra were acquired using an FTICR-MS instrument (solariX, Bruker Daltonik, Bremen, Germany) equipped with a 12 T superconducting magnet and an Apollo II electrospray ionization source operated in the negative ionization mode. Samples were introduced at a flow rate of 120 μL h^{-1} using a syringe pump. The MS was externally calibrated on clusters of arginine (10 mg L^{-1}) in methanol. Spectra were acquired with a time domain of 4 mega-words per second with a mass range from m/z 100 to 1000 to guarantee a high accuracy in elemental formula assignments in this proof-of-principle study. Up to 500 scans per sample were accumulated. Fourier Transform Ion Cyclotron Resonance (FTICR) spectra were internally recalibrated on list composed by fatty acids and recurrent compounds in wine, linear until m/z 600, with mass errors below 50 ppb and peak with a signal-to-noise ratio (S/N) of 4 and higher were exported to peak lists. In conjunction with an automated theoretical isotope pattern comparison, the generated formulae were validated by setting sensible chemical constraints (n rule; O/C ratio $\leq$1; H/C ratio $\leq 2n+2$; element counts: C $\leq$100, H $\leq$200, O $\leq$80, N $\leq$3, S $\leq$3 and P $\leq$1).

2.4. Ultra-performance liquid chromatography coupled to quadrupole time of flight mass spectrometry analysis (UPLC/Q-ToF-MS)

Analyses were performed on a Waters Acquity UPLC (Waters, Milford, USA) coupled to a maXis™ UHR-ToF-MS (Bruker Daltonik, Bremen, Germany) system using reversed phase (RP) and hydrophilic interaction liquid chromatography (HILIC) separation. Sample analyses were carried out in (−)ESI.

RP separation was performed using a Waters ACQUITY UPLC BEH C18 column (1.7 μm; 1.0×150 mm) using gradient elution with an initial isocratic hold of 100% A for 0.5 min, followed by a linear increase to 100% solvent B in 4.9 min, isocratic conditions for 3 min and return to initial conditions in 1.6 min (solvent A: 10% ACN, 90% water, 1 mM ammonium formate; solvent B: 100% ACN).

HILIC separations were performed on a Waters ACQUITY UPLC BEH Amide column (1.7 μm; 2.1×150 mm) using a two-step gradient elution programme from 100% A to 100% solvent B (solvent A: 95% ACN, 5% water, 1 mM ammonium formate; solvent B: 50% ACN, 50% water, 1 mM ammonium formate).

The flow rate for both separation modes was set to 0.25 ml/min with a column temperature of 40 °C and a full loop injection of 10 μl.

UHR-ToF-MS acquisitions were carried out in profile spectra mode with 1 Hz accumulation time. Instrument tuning focused on detection and resolution of molecular weight compounds in the mass range of 50–2000 Da. Mass calibration was carried with low concentration ESI Tuning Mix (Agilent, Waldbronn, Germany). Data was exported to mzXML format using Bruker Data Analysis 4.0 (Bruker Daltonik, Bremen, Germany). MZmine 2.6 was applied for peak alignment and extraction (http://mzmine.sourceforge.net/).

2.5. Network analysis

To obtain chemical formulae for subsequent use in querying chemical databases, exact masses were subjected to mass difference network analysis using the Netcalc algorithm and in-house software tool.[25] In a mass difference network, nodes represent m/z values (metabolite candidates) and edges represent chemical reactions. Netcalc enables network reconstruction by comparing the mass differences of all experimental masses of a mass spectrum to a list of user-defined theoretical mass differences (selected according to atomic units, e.g., C, H, N, O, S, P, or common functional groups, e.g., homologous series of CH_2, H_2, or OH (Table S1)). The mass difference list used in this example was optimized to detect chemical differences between all annotatable nodes in the experimental data in order to reveal patterns in compositional or functional chemical spaces. The purpose of mass difference network analysis is the visualization of the sample's compositional structure and the calculation of elemental formulae of experimental masses. In such a scale-free network, highly inter-connected nodes tend to cluster together while sparsely connected ones are peripherally dispersed. Netcalc increases the percentage of m/z peaks, which can be assigned to a preliminary formula up to a 40–60% per dataset including isotope peaks ^{13}C.[6] The goal of this model is the visual