Analytical and Bioanalytical Chemistry (2022) 414:7805–7812
https://doi.org/10.1007/s00216-022-04314-x

RESEARCH PAPER

Untargeted metabolomic analysis by ultra-high-resolution mass spectrometry for the profiling of new Italian wine varieties

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Received: 21 July 2022 / Revised: 23 August 2022 / Accepted: 31 August 2022 / Published online: 19 September 2022

Abstract

The chemical composition of wine samples comprises numerous bioactive compounds responsible for unique flavor and health-promoting properties. Thus, it's important to have a complete overview of the metabolic profile of new wine products in order to obtain peculiar information in terms of their phytochemical composition, quality, and traceability. To achieve this aim, in this work, a mass spectrometry-based phytochemical screening was performed on seven new wine products from Villa D'Agri in the Basilicata region (Italy), i.e., *Aglianico Bianco*, *Plavina*, *Guisana*, *Giosana*, *Malvasia ad acino piccolo*, *Colata Murro* and *Santa Sofia*. Ultra-high-resolution mass spectrometry data were processed into absorption mode FT-ICR mass spectra, in order to remove artifacts and achieve a higher resolution and lower levels of noise. Accurate mass-to-charge ratio (*m/z*) values were converted into putative elemental formulas. Therefore, 2D van Krevelen diagrams were used as a tool to obtain molecular formula maps useful to perform a rapid and more comprehensive analysis of the wine sample metabolome. The presence of important metabolite classes, i.e., fatty acid derivatives, amino acids and peptides, carbohydrates and phenolic derivatives, was assessed. Moreover, the comparison of obtained metabolomic maps revealed some differences among profiles, suggesting their employment as metabolic fingerprints. This study shed some light on the metabolic composition of seven new Italian wine varieties, improving their value in terms of related bioactive compound content. Moreover, different metabolomic fingerprints were obtained for each of them, suggesting the use of molecular maps as innovative tool to ascertain their unique metabolic profile.

Keywords Wine · Metabolomics · Untargeted · High-resolution mass spectrometry · Van Krevelen

Introduction

Wine has been a part of human culture for 6000 years, being employed for dietary and socio-religious purposes [1, 2]. Its first production goes back to antiquity, as does the discovery of its healthful benefits, now largely attributed to the antimicrobial activity of ethanol [2]. Today, wine is a cultural symbol for many countries, a form of entertainment and a beverage of choice for supporters of its health benefits. Regardless of the region in which the wine is produced or the economic status of the consumer, all wines are expected to be pleasant experiences [3]. In most of wine regions of the world, at least until around the middle of the 1980s, wine was obtained from grapes, following a complex process known as "winemaking," based on the fermentation of grape must with the indigenous yeasts present on the grapes when harvested or introduced from the equipment and cellar during the vinification process [4–7]. Nowadays, the practice of adding selected yeasts to slightly sulfited musts has been widely used to ensure a rapid and complete must fermentation and to produce wines of reproducible characteristics and quality [5, 7, 8]. Winemaking is a biological process characterized

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by a series of biochemical transformations that involve enzymes from different microorganisms, especially yeasts, which are responsible for the principal part of the process, i.e., the alcoholic fermentation [4, 7]. The result of the winemaking process is the production of specific bio-compounds that are considered responsible of the most appreciated properties of this beloved product, such as its taste and its related biological activity [9, 10]. For this reason, a lot of effort has been spent in recent years to improve the knowledge of the metabolic profiles of different wine varieties. This information would be helpful in terms of process optimization, to enhance specific wine properties [8, 10–13]. The use of the modern chromatographic techniques, both gas chromatography (GC) and high-pressure liquid chromatography (HPLC) coupled to mass spectrometry as detection system [14–19], supported by other less sensitive techniques such as thin-layer chromatography (TLC), infrared spectroscopy (IRS), and nuclear magnetic resonance (NMR) [20–28], has allowed the identification of more than 500 compounds in wine, with concentrations ranging between 10^{-1} and 10^{-6} mg/L. Although at low levels, all these compounds greatly contribute to wine aroma and taste [29–31]. In detail, the taste is mainly due to a few compounds that are usually present individually at concentrations higher than 100 mg/L, like water, ethanol, organic acids, sugars, and glycerol [32, 33]. However, also other alcohols, volatile acids, and fatty acid esters are important for wine aroma, and they are produced by yeast during fermentation [34, 35]. Among them, alcohols often cover the higher and more concentrated percentage of all the volatile substances [16, 17]. On the contrary, carbonyls, polyphenols, lactones, terpenes, acetals, hydrocarbons, sulfur, and nitrogen compounds and B vitamins are present in much lower concentrations [36–38]. With regard to B vitamins, among which there's riboflavin (RF), they are released during vinification, and they are strain-dependent. RF wine content is generally up to 0.2 mg/L [39, 40]. Tannins are found in red wine and rarely in significant amounts in white wines [41, 42]. A comprehensive characterization of the wine metabolome could be accomplished using hyphenated techniques by following an untargeted analysis approach [43]. Despite high costs and long analysis times associated to their routine employment for this task, high-resolution mass spectrometry (HRMS) technique is the method of choice for untargeted metabolomic analysis, even if used by following a shotgun, or direct injection, approach [44–47]. Indeed, HRMS has been already used for wine characterization, allowing the identification of thousands of metabolites by using a single direct analysis, with labor times of a few minutes [31, 44, 45]. Moreover, related results provided useful biochemical mechanistic information, highlighting metabolic differences among samples subjected to different winemaking conditions [31, 44, 45].

In this study, data acquired by using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) and processed into absorption mode FT-ICR (aFT) mass spectra were employed to characterize for the first time the general molecular profile of seven new Italian wine varieties, i.e., *Aglianico Bianco*, *Plavina*, *Guisana*, *Giosana*, *Malvasia ad acino piccolo*, *Colata Murro* and *Santa Sofia*, produced in Villa D'Agri in the Basilicata region (Italy). These seven wines (five white wines and two red wines) represent the new main local grape varieties identified in the Basilicata region. Registered species and new species have been classified and reported in the chapter 5 of Basivin_SUD [48], and the general characteristics of grapes and vines have been summarized in Table S1 (see Supplementary information). Since raw data often contain artifacts, ultra-high-resolution mass spectra were processed into absorption mode FT-ICR mass spectra, in order to achieve a higher resolution and lower levels of noise, thus avoiding wrong molecular formula assignment. Considering the huge diversity of organic compounds present in wine varieties and their synergic activity resulting in pleased macroscopic properties, together with the deep correlation between compound levels and winemaking process, it's of great interest to obtain a general metabolomic profile of new wine varieties. Here, the molecular maps of main metabolites were proposed and discussed, with the aim to suggest their use as innovative tool to ascertain wines' unique metabolic profiles.

Materials and methods

Wine samples

Wine samples were obtained from new germoplasms cultivated in the Pollino region, a natural area located in Basilicata (Italy), and were provided by the Agency for Development and Innovation in Agriculture (ALSIA, Agenzia Lucana di Sviluppo e di Innovazione in Agricoltura). The studied samples varied in terms of their type (i.e., red and white). Key features of these new wine varieties, together with a detailed organoleptic description, are described elsewhere [48].

Chemicals

Sodium trifluoroacetate (NaTFA, 98%) and methanol (MeOH, LC–MS grade) were purchased from Sigma-Aldrich

(Milano, Italy). Pure nitrogen (99.996%) was delivered to the MS system as the sheath gas.

Sample preparation

The procedure adopted in this work for the sample preparation was described by Roullier-Gall et al. [45]. In detail, 20 µL of sample were diluted by adding 1 mL of MeOH. The solution was vortexed, passed through a PTFE 0.22-µm syringe filter, and directly injected into the HRMS instrument. For each sample, 3 replicates have been prepared, together with a blank sample obtained by subjecting 20 µL of MeOH to the whole sample preparation step.

Mass spectrometry analysis

ESI (±) Fourier transform ion cyclotron resonance mass spectrometry (ESI-FT-ICR MS) technique was used for the untargeted analysis of the sample. High-resolution mass spectra were acquired on a Bruker (Bruker Daltonik GmbH, Bremen, Germany) solariX XR Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) equipped with a 7 T superconducting magnet and an ESI source. The capillary voltage was set to 3.9 and − 4.5 kV for negative and positive ionization modes, respectively, with a nebulizer gas pressure of 1.2 bar and dry gas flow rate of 4 L/min at 200 °C. Samples were analyzed by direct infusion, with syringe flow rate of 5.0 µL/h. Spectra were acquired with a time domain size of 16 mega-word, an accumulation time of 0.1 s, and a mass range of 100–2000 m/z. For each sample, the number of scans was set to 50. Before the analysis, the mass spectrometer was externally calibrated with NaTFA. High accuracies were reached, with a root mean square (RMS) error lower than 0.1 ppm. FT-ICR mass spectra were subjected to several data pre-treatment steps. In detail, recorded free induction decays (FIDs) were subjected to apodization, and related absorption mode mass spectra were obtained. Phase correction, mass recalibration, and baseline correction have been performed, together with blank subtraction [49, 50] and noise filtering by following the N-Sigma methodology approach [51, 52]. More specifically, noise level has been estimated, and peaks showing a signal-to-noise ratio (S/N) higher than 2 were retained. Thus, the obtained FT-ICR mass spectra were exported to peak lists. From these, possible elemental formulas were calculated for each MS signal. To obtain unequivocal formulas, several constraints were applied, such as atoms number limitations, i.e., $C \leq 100$, $H \leq 200$, $O \leq 80$, $N \leq 5$, and $S \leq 1$ [44–47], restrictions on atoms to carbon number ratios, i.e., $0.2 \leq H/C \leq 3.1$, $O/C \leq 2$, $N/C \leq 1.3$, and $S/C \leq 0.8$, $RDBE > 0$, nitrogen rule (for m/z ratio values lower or equal to 500), and isotopic pattern filtering. Moreover, Kendrick mass defect (KMD) was performed to help formula assignment [44, 45]. In detail, building blocks with a higher number of occurrences were identified and chosen for the analysis. For this step, experimental mass differences values were examined, and only those in the range ± 1 mDa of the building block exact mass were considered [53, 54]. To further improve the reliability of results, building blocks with occurrences lower than a threshold value (properly chosen to remove all the noisy data) were excluded, being higher the probability for these to have occurred randomly [55]. The absorption mode FT-ICR MS data obtained from both positive and negative mode analysis of all wine samples were processed by using AutoVectis Pro (v.8.9, Spectroswiss, Lausanne, Switzerland). R software (v3.6.3, www.r-project.org) was used for FT-ICR MS data analysis, in order to plot the van Krevelen 2D diagrams.

Results and discussion

Ultra-high-resolution ESI(±)-FT-ICR MS data acquired by direct injection were used to obtain a general description of metabolome of new Italian wine variety samples. In detail, five types of white wines, i.e., *Aglianico Bianco*, *Guisana*, *Giosana*, *Malvasia ad acino piccolo*, and *Santa Sofia*, and two types of red ones, i.e., *Colata Murro* and *Plavina*, were analyzed. A huge number of peaks (data not shown) were detected from our wine samples in both positive and negative mode. Through the analysis, nearly 500 signals were selected and included in our data (a maximum of 421 signals for the *Malvasia ad acino piccolo* wine), thus revealing the wide diversity of metabolites present in wine samples. However, it should be pointed out that possible artifacts can occur during FT-ICR data acquisition [56, 57] and, at the same time, background/noisy signal can interfere with hamper the identification of low intensity ionic species, thus making overall data elaboration more complex. Working with absorption mode mass spectra turned out to be the best solution to overcome these issues. Indeed, peak resolution and signal-to-noise ratios (S/N) were markedly improved, leading to the identification of a higher number of ionic species. The use of specific tools to accomplish this task, i.e., the AutoVectis Pro software, was crucial to perform a quick phase correction step efficiently and, thus, to obtain a readable absorption mode mass spectrum, task that couldn't be achieved for almost 40 years [49, 52, 56, 58]. Indeed, the use of absorption mode in FT-ICR mass spectrometry has been required for a long time either specially adapted instrumentation or a manually intensive process of phase correction. Instead, AutoVectis pro software allows to automatically do phase correction and baseline deviation remotion, thus greatly reducing user interaction. In detail, AutoVectis Pro software performs the phase correction step in few milliseconds to obtain aFT mass spectra, by employing

a genetic algorithm to optimize the phase calibration. Once the user has defined the order of a predefined phase correction function, several others are generated by this software by applying random mutation on related frequency values. An identification of the best phase correction function is done by reiteration, until the complete optimization has been reached. Despite the advantages provided by the absorption mode mass spectrum, it's still difficult to deduce something by simply looking at full MS spectra. To better understand our results, MS signals were assigned to unique elemental formulas (see Materials and methods), and a well-known visualization tool, i.e., the van Krevelen plot, was employed to plot our results on a 2D diagram, by setting the H/C and the O/C ratios as the *y*- and the *x*-axis, respectively [44–46]. Based on H/C and O/C ratio, ionic species obtained from MS spectra will occupy a well-defined area on the van Krevelen 2D plot, and their position is helpful to classify them in one of the major metabolite classes. Briefly, the lipid region is located at the highest H/C ratio ($1.3 < H/C < 2.2$) and lowest O/C ratio ($0 < O/C < 0.3$) (e.g., palmitic acid H/C ratio = 2, O/C = 0.12). The peptide region overlaps with the lipid region but is located at higher O/C ratios ($0.1 < O/C < 0.5$) (e.g., methionine H/C ratio = 2.2, O/C ratio = 0.4). Instead, carbohydrate and polyphenol regions are located in the range $1.5 < H/C < 2.2$, $0.6 < O/C < 1.2$, and $0.4 < H/C < 1.4$ and $0.2 < O/C < 0.7$, respectively (e.g., glucose H/C ratio = 2, O/C ratio = 1 quercetin H/C ratio = 0.67, O/C ratio = 0.47) [59, 60]. Based on the results obtained from van Krevelen plots, the presence of specific types of metabolites in wine samples was proposed, i.e., carbohydrates, polyphenols, amino acids, and peptides and unsaturated fatty acids (Fig. 1, Figs. S1–S6).

From the van Krevelen plots analysis, it is possible to notice differences among metabolic profiles, some of them reflecting what was already found in the literature [45]. By checking the results acquired in negative mode (Fig. 1) (see also Supplementary information Figs. S1A, S2A, S3, S4A, S5A), we can observe that there is a high-density population on the upper right part of the plot (usually assigned to carbohydrates, amino sugars, and peptides [60]), indicating a wider diversity of carbohydrates and glycoconjugates, and this aspect is common for both red and white wines that we analyzed. Furthermore, red wines seem to contain a higher amount of unsaturated glycoconjugate compounds, supporting what was already found regarding the presence of phenolic compounds in wine as glycoconjugates [6, 9, 61–63]. A higher density of points in the middle part of van Krevelen plot of red wines, moreover, indicates the presence of more phenolic derivatives [44, 45]. Polyphenols are important for the characteristics and quality of red wines. Their concentration in white wine is much lower. Polyphenols and related compounds can affect the appearance, taste, mouthfeel, fragrance, and antimicrobial properties of wine [61]. The two primary phenol groups that occur in grapes and wine are the flavonoids and the non-flavonoids. The most common flavonoids in wine are flavonols, catechins, and flavan-3-ols and, in red wines, anthocyanins too [62–64]. Flavonoids come primarily from the skins, seeds, and stems of the fruit [65]. In red wines, they commonly constitute up to 85% of the phenol content, while, in white wines, flavonoids are typically lower than 20% of the total phenolic content. The amount of flavonoids extracted during vinification is influenced by many factors, including temperature, length of skin contact, mixing, type of fermentation vessel, ethanol concentrations, SO_2 yeast strain, and pH [66–70]. The concentration of phenolics in wine increases during skin fermentation and subsequently begins to fall as phenols aggregate and precipitate with proteins and yeast cell remnants [37, 38]. As regard to white wines, they showed a little cluster in the middle left part of the plot (Fig. 1B),

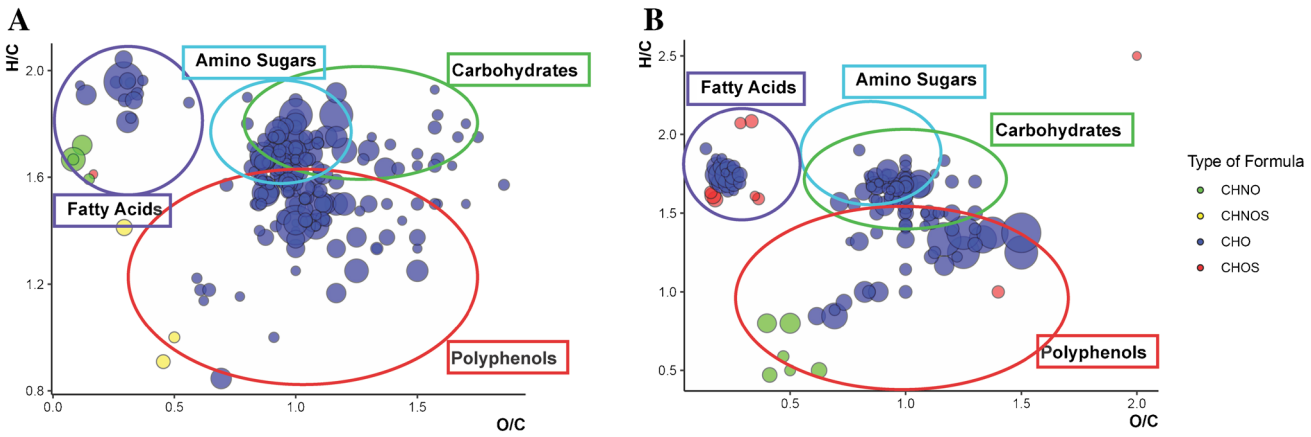

Fig. 1 van Krevelen plots of *Colata Murro* (**A**) and *Aglianico Bianco* (**B**) red and white wine samples, respectively, obtained from related ESI(-)-FT-ICR MS data. The types of formula are distinguished by colors (green for CHNO, yellow for CHNOS, blue for CHO, and red for CHOS)

not observed for red wine varieties, which could be related to the presence of low oxygen content phenolic acids, such as hydroxycinnamic acid derivatives, known to be responsible for the typical yellowish color of white wines [71, 72]. Hydroxycinnamic acid derivatives commonly occur esterified to sugars, organic acids, or various alcohols. The principal grape sugars are glucose and fructose, and they occur in roughly equal proportions at maturity, whereas overripe grapes often have a higher proportion of fructose [73, 74]. As the most important and abundant alcohol in wine is ethanol [75, 76], several ethyl derivatives could be detected. Under standard fermentation conditions, ethanol can accumulate up to ~ 14–15%, but generally ethanol concentrations in wine range between 10 and 13% [75, 76]. Other potentially significant higher alcohols in wine are the straight-chain alcohols: 1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol [61]. The formation of higher alcohols occurs as a by-product of yeast fermentation and is markedly influenced by vinification parameters, such as temperature, presence of oxygen, suspended solids, and yeast strain [5, 6, 10]. With regard to organic acid, tartaric, malic, lactic, succinic, oxalic, fumaric, and citric acids control the pH of wine [73, 77].

Among the same types of wine, differences could be spot related to the absence and the presence of points in specific van Krevelen plots, suggesting the fact that some classes of derivatives are present only in some wine samples, just like the unsaturated CHNO formula type compounds present for the *Giosana* and *Santa Sofia* white wines (see Supplementary information) or the aliphatic amides present in the *Colata Murro* red wine sample only. van Krevelen plots obtained from the analysis of data acquired in positive ionization mode results show a similar profile among all analyzed samples (Fig. 2) (see also Supplementary information Figs. S1B, S2B, S4B, S5B, S6).

In this case, amino acids and peptides, together with their aliphatic derivatives, and aliphatic amides seem to be predominant, as well as other CHO formula type compounds, probably related to unsaturated fatty acid derivatives. Generally, fatty acids as well as their ethyl esters are produced in the first days of the fermentation; after that, their concentration levels decrease. Since a lower amount of external sterols is detectable in the medium during the clarification process of the white wines, these ones show a higher content in fatty acids than red wines [78], as proved by the analyzes made in this work (Fig. 2). It is interesting to notice that, except for the lipidic class, van Krevelen plots do not reveal other remarkable differences between red and white wine metabolic profiles acquired in positive mode. Instead, some differences could be detected by comparing all the results obtained for the white wines. As an example, the *Malvasia ad acino piccolo* van Krevelen plot (Fig. 2B) shows the highest density of points (421 signals), suggesting the presence of several clusters, while in the *Santa Sofia* white wine plot (see Supplementary information Fig. S4B), we observe a uniform distribution with a lower number of points (81 signals). The lower number of compounds reported on in the *Santa Sofia* molecular map as amino acids and fatty acids, compared to those occurring in the *Malvasia ad acino piccolo* one, could be dependent on the winemaking practices and not only on the wine type, i.e., red or white [79]. For the two red wine *Colata Murro* (Fig. 1A) and *Plavina* (Fig. S5A), instead, van Klevelen plots show a similar distribution, suggesting a similar metabolic profile that includes fatty acids, carbohydrates, and polyphenols. For this similarity, a higher number of points could be detected in the

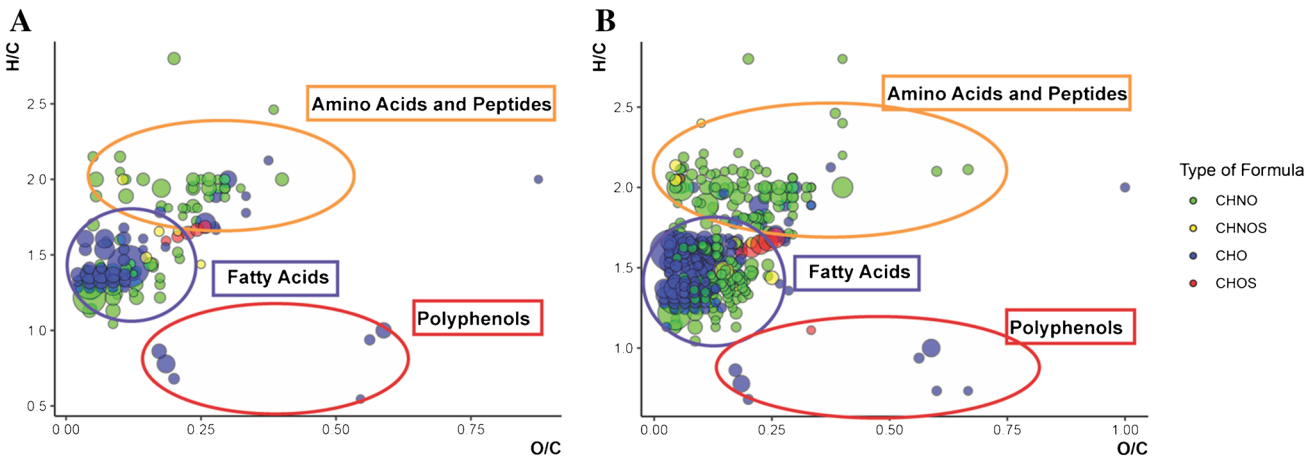

Fig. 2 van Krevelen plots of *Colata Murro* (**A**) and *Malvasia ad acino piccolo* (**B**) red and white wine samples, respectively, obtained from related ESI(+)-FT-ICR MS data. The types of formula are distinguished by colors (green for CHNO, yellow for CHNOS, blue for CHO, and red for CHOS)

carbohydrate and polyphenol regions for the *Colata Murro* red wine, confirming that the polyphenolic composition varies among different wines according to different factors, and the type of grape used is one of the most important ones [80]. Overall, the use of van Krevelen plots provided a comprehensive metabolic fingerprint of the seven wine samples based on data acquired in full-scan mode and allowed to identify the different metabolite classes. This strategy will also provide a hint for further investigation of putative markers by performing MS/MS analysis.

Conclusions

High-resolution mass spectrometry technique was successfully used in this work for the characterization of the metabolome of new Italian type wine varieties, thus confirming its suitability for quick and efficient untargeted metabolic analysis of natural samples. Useful information about types of metabolites present in wine samples were obtained, since a classification of identified species has been made possible by the utilization of the van Krevelen plot, a well-known visualization tool useful for HRMS data interpretation. Results helped to identify principal classes of metabolites and to spot principal differences among related metabolic profiles. Thus, they are very promising as innovative tool to ascertain their unique metabolic profile.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00216-022-04314-x.

Funding The authors would like to thank ALSIA (Agenzia Lucana di Sviluppo e di Innovazione in Agricoltura) for providing the samples analyzed within this work and Yury O. Tsybin and Konstantin O. Nagornov for their support in the processing of recorded time domain ion signals into absorption mode FT-ICR mass spectra.

Declarations

Competing interests The authors declare no competing interests.

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Part of the results of this research has been shared as oral communication in occasion of the 4°MS Italian Wine Day Conference (June 22–24th, Carlentini, Sicily, Italy).