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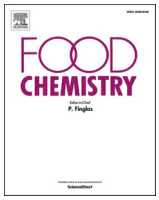

Untargeted metabolomics reveals key biochemical shifts during industrial Muscat of Alexandria alcoholic fermentation

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ABSTRACT

Alcoholic fermentation is a complex biochemical process that profoundly impacts wine quality. Generally, most metabolomics studies focus on controlled laboratory-scale fermentations, and industrial conditions are very rarely investigated. This study addresses this gap by elucidating the metabolomic fingerprint changes of Muscat of Alexandria grape must during industrial-scale alcoholic fermentation, by applying a Mass Spectrometry–based untargeted metabolomics workflow. The results revealed that small peptides exhibited analogous trends to amino acids, since the concentration of some peptides decreased rapidly at the early stages of the fermentation, explaining the increase of the not preferred by yeasts proline, while other peptides accumulated towards fermentation's end. Additionally, terpenes and phenolics glycosidic bonds underwent hydrolysis, and nucleic acid building blocks were released into the must during fermentation. These metabolomic transformations may shape wine's longevity, identity and sensory profile, and therefore the outputs of this work are important in wine science and winemaking.

1. Introduction

The oenological history of the Greek island Limnos started several centuries ago, and still today is an important oenological flagship for Greek winemaking (Robinson, 1986). Nowadays, Limnos wines are distinguished by the volcanic soil and the predominant cultivation of Muscat of Alexandria grapes. This unique combination delivers a diverse array of wines, ranging from dry to fortified styles, including those produced from raisined grapes. Notably, Limnos have two appellations of origin wines: the sweet Protected Designation of Origin (PDO) Muscat of Limnos wine and the dry PDO Limnos wine. These wines are characterized by their floral (roses), fruity (citrus and apricot), and peppermint aromas, stemming from the highly aromatic Muscat of Alexandria cultivar, and exhibit a full-bodied profile (Regulation (EU) No 1308/2013, 2013).

While the metabolomic fingerprint of the grape provides valuable insight, alone is unable to fully explain the organoleptic characteristic of the wine, since during alcoholic fermentation countless biochemical and chemical reactions occur. The yeasts' metabolism is not limited in the production of ethanol from hexoses, but heavily modifies several primary metabolites, including amino acids, organic acids, indoles, amines, sulfur compounds, terpenes, phenols, and carbohydrates, and therefore produces numerous volatile and non-volatile secondary metabolites (Álvarez-Fernández et al., 2019, 2020; Marinaki et al., 2023a; Soufleros et al., 2003; Walker, 2014; Waterhouse et al., 2016). For example, the grape must amino acids are used as nutrients by the yeast during alcoholic fermentation and they are transformed into aroma and other sensory active compounds (Ribéreau-Gayon et al., 2021; Walker, 2014; Waterhouse et al., 2016). Several glycosidic bonds between sugars and volatile (e.g. terpenes, phenols) or non-volatile compounds (e.g.

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phenolics) are hydrolyzed affecting the final organoleptic character of the wine (Caffrey et al., 2021; Garrido & Borges, 2013; Godshaw et al., 2019). The profile of the organic acids is also modified mainly due to the Krebs cycle metabolism and its peripheric metabolic pathways. In addition, at the end of the alcoholic fermentation the yeasts are partially autolyzed, enriching the wine with more metabolites (Ribéreau-Gayon et al., 2021; Waterhouse et al., 2016). Although they are less pronounced, during the winemaking process other chemical reactions occur too, including redox reactions, esterification reactions, acidic cleavages and sulfonations (Nikolantonaki et al., 2022; Álvarez-Fernández et al., 2019, 2020). Monitoring such a complex process with various targeted analysis can give us only a partial image about the metabolomic changes that influence the quality, the longevity and the sensory character of the final product so much.

On the other hand, metabolomics focuses on the complete study of the metabolites of a given sample. Untargeted methods have the ability to measure thousands of features per analysis and have quickly proven to be a potent tool in the discovery of metabolite markers and formulating new hypotheses. This technique has also demonstrated its efficacy in the fields of viticulture and oenology in recent years, since it helped us to understand the metabolomic changes happening during various processes from the vine to the glass, and to discover new metabolites. Several technologies have been used for the analysis of the metabolic profile of wines, grape musts and grape marc beverages including Nuclear magnetic resonance (NMR) (Gougeon et al., 2019; López-Rituerto et al., 2009; Solovyev et al., 2021), and Mass Spectrometry (MS) coupled to Gas Chromatography (GC–MS) (Carlin et al., 2016, 2022; Marinaki et al., 2023b, 2023c) or liquid chromatography (LC–MS) (Arapitsas et al., 2012, 2020; Bordet et al., 2023; Degu et al., 2015; Li et al., 2021; Nikolantonaki et al., 2018; Roullier-Gall et al., 2017, 2022; Sáez et al., 2021; Tzachristas et al., 2021). While numerous studies have explored the volatilome of Muscat of Alexandria wines, there is a significant lack of research focusing on the non-volatile metabolomic profile during industrial-scale alcoholic fermentation. Previous metabolomics studies often rely on controlled laboratory fermentations, which do not fully capture the complexity of real-world production conditions. A recent work, performed the effects of different freezing treatments during the winemaking of a Muscat of Alexandria wine with regard to its phenolic components (Vilar-Bustillo et al., 2023) and an earlier reverse phase LC method with fluorescence detector after derivatization was used to identify the profile of amino acids in Greek white wines, resulting that Muscat of Alexandria sweet and dry white wines of Limnos characterized of high concentration of amino acids (Soufleros et al., 2003).

The aim of this work was to shed light to the changes taking place to the metabolomic fingerprint of Muscat of Alexandria grape must during the alcoholic fermentation by using a state-of-the-art UHPLC–ESI–TIMS–QTOF–MS instrument. To ensure the acquisition of comprehensive and realistic data, an extensive sampling protocol was implemented, encompassing multiple industrial-scale fermentations across various wineries on the island of Limnos over two consecutive vintages.

2. Materials and methods

2.1. Chemicals and reagents

LC-MS grade methanol (MeOH) used as the mobile phase was purchased from CHEM-LAB NV (Zedelgem, Belgium). Formic acid (≥99%), LC-MS additive, was obtained from PANREAC QUIMICA SLU (Barcelona, Spain). A Milli-Q water purification system offered ultrapure water, 18.2 MΩ.cm (Millipore, Bedford, MA, USA). All other used standards were of analytical or higher grade, and for this study obtained from various vendors.

2.2. Sampling and sample preparation

In total, 70 Muscat of Alexandria grape must samples were analyzed in this study. All musts originated from Limnos Island in North Aegean Sea, from 3 different wineries in 2 different vintages (2019 and 2020). Musts were collected on the 0th, 1st, 2nd, 3rd, 4th, 5th, 8th, 11th and 13th day of alcoholic fermentation from 8 different tanks, and more specifically from 2 different tanks from each winery in 2019 (9 samples for every tank), and from 2 different tanks from 1 winery in 2020 (8 samples for every tank). The fermentations were conducted under industrial conditions in stainless steel tanks of 15,000–45,000 L capacity, with inoculation using different commercial *Saccharomyces cerevisiae* yeasts. Sampling was performed from the central valve at mid-height of homogenized tanks following "OIV Guidelines for sampling wines and musts intended for analysis", to ensure representativeness. Table S1 presents additional information on tank volumes and yeast strains. The samples were stored in 2 mL aliquots at −50 °C.

All samples were thawed at room temperature, and they were centrifuged at 14,000 rpm for 10 min. Then, 333 μL of the supernatant were diluted with 667 μL of water, and finally they were filtered by 0.22 μm PTFE syringe filters and placed into 2 mL dark LC-MS glass vials. Quality Control (QC) samples were produced by mixing equal portions of each sample and then were treated like all other samples.

2.3. LC–MS-based metabolomics analysis

Chromatographic separation was performed on an Ultra HPLC Elute system equipped with an ACQUITY HSS T3 (2.1 × 100 mm) (Waters Ltd., Elstree, UK) column and an autosampler, thermostated at 4 °C. The temperature of the column was kept at 40 °C and the flow was 0.35 mL/min for 5 μL of injected sample. The mobile phases were 0.1% (*v*/v) formic acid in water (mobile phase A), and in methanol (mobile phase B), respectively. The gradient was as follows: for 1.5 min 0% of B; then 0–10% of B (1.5–4 min); 10–40% of B (4–8 min); 40–100% of B (8–12 min); 100% of B kept for 2 min (until 14 min of gradient); then 100–0% of B (14–17.01 min) and finally 0% of B kept for 2 min.

Mass spectrometer analysis was performed on the trapped ion-mobility time-of-flight (timsTOF) mass spectrometry system (Bruker, Bremen, DE) and the MS data were acquired in positive (ESI+) and negative (ESI−) mode in a mass range of 20–1300 amu. Tuning parameters, for both positive and negative ESI, included capillary at ±4500 V, dry gas 10 L/min, dry temperature at 200 °C, and nebulizer gas 2 bar. For optimum ion transfer, Funnel RF 1 was set to 200 Vpp, Funnel RF 2 to 250 Vpp, Multipole RF at 200 Vpp, deflection delta at 60 V, transfer time at 54 μs, and pre-pulse storage 5 μs. Collision energy and RF were set to 10 eV and 800 Vpp, respectively. A data-dependent acquisition mode was also applied within the MS method with an acquisition frequency of 3 (minimum) and 5 (maximum) Hz. Fragmentation was performed with a collision energy of 30 eV for all precursor ions. During the first segment of the analysis, 10 mM sodium formate was infused with MS with a flow rate of 60,000 μL/h, and clusters were further used for internal calibration.

Before analysis of real samples, we injected blank samples (H_2O) into the system to assess suitability. QCs were analyzed at the beginning, after every 10 samples and at the end of the batch in order to equilibrate the system to the matrix and assess its stability during analysis. Results consisted of 158 full scan raw files (70 in ESI+ and ESI- mode, with 9 QCs in each ionization mode), and 4 MS/MS raw files (one sample at the beginning and one at the end of fermentation in both ionization modes) acquired for the data set. As previously reported, other than full scan mode data dependent acquisition mode was applied in order to enhance identification of possible metabolites related to the initial hypothesis of the study. All raw UPLC–QTOF–MS data generated in this study have been deposited in the MassIVE repository under accession number MSV000099152 (ftp://massive-ftp.ucsd.edu/v10/MSV000099152).

2.4. Data analysis

Data were collected both as profile and centroid spectra. Raw data from each file were recalibrated using clusters of sodium formate (10 mM) with Data Analysis Software (Bruker). MSConvert (ProteoWizard 3.0.11567), an online, free software, was used to convert the files to mzML format. These files were then processed using XCMS (3.2.0) under the R programming language. The XCMS parameters included CentWave algorithm for peak detection (mzdiff 0.01, ppm 30, noise 1000, peak width (6,20)) PeakDensityParam for peak grouping (bw 5, binsize 0.015) resulted in a table with the peak areas of detected features in all samples after peak integration, where these values represent relative, semi-quantitative abundances. Further chemometrics and statistical analysis were performed by MetaboAnalyst, an online free available platform (https://www.metaboanalyst.ca version 5.0, accessed on 6 June 2023). Assessment of correlation and pattern analysis were performed without prior normalization, data transformation, and missing value estimation by applying Pareto scaling. The XCMS workflow (peak detection, alignment, grouping, and peak filling) produced a complete peak table without missing values, since absent peaks were replaced with minimal integrated values. Therefore, no additional missing-value estimation or normalization in MetaboAnalyst was required. Additionally, multivariate statistical analysis including PCA, PLS–DA and OPLS–DA were performed by the SIMCA P software (version 13.0.2).

For metabolite identification MS-DIAL (ver. 4.48), in-house MSMS library and online available databases were used. For MS-Dial raw data files were converted to .abf files by ReifycsAbf Converter (ver. 4.0.0) and annotation was performed using MSMS–Public–Pos–VS15 library. Additionally, the accurate *m/z* values were matched to biomarkers using online available MS databases (Metlin and HMDB). The annotation was performed manually with a mass accuracy of 5 ppm, taking into account the isotopic pattern and in accordance with the four levels annotation (Sumner et al., 2007).

3. Results

3.1. Description and quality control of the datasets

For this experiment, in order to have a biological distribution that covers a big part of Limnos Island wine production, Muscat of Alexandria grapes were harvested and fermented according to the PDO disciplinary and at industrial scale by three different local wineries. The fermentations of eight different tanks (six during the vintage 2019 and two in 2020), were followed by collecting samples during the alcoholic fermentation, from must to wine.

The quality of the LC–MS raw data, acquired in a single batch for each ionization mode, was assessed by a principal component analysis (PCA) as shown in Fig. 1. The number of features registered in the unsupervised analysis was 13,527 in ESI+ and 8396 in ESI-. Both representations, for ESI+ and ESI-, confirmed the good quality of the data set, since the QC sample injections clustered together, created a tight group in the center of each plot, guaranteeing the robustness of the data set and thus for the results and the tentative biomarker discovery (Arapitsas et al., 2020; Sáez et al., 2021; Savoi et al., 2021).

The metabolomic fingerprint of all alcoholic fermentations, irrespective of winery or vintage, exhibited a remarkably consistent trajectory as depicted in Fig. 1A and B. In positive ionization mode, the trajectory commenced from the top right quadrant of the plot (day 0) and concluded in an arc shape at the bottom left quadrant (day 13). Conversely, in negative ionization mode, the fermentation progression mirrored the pattern observed in positive ionization, originating from the top left quadrant (day 0) and culminating at the bottom right quadrant of the plot (day 13). The samples from tanks 8 and 10 grouped together in the lower part of the plots, while the samples from tanks 75_1 and 95_1 were located in the upper part of the plots, possibly because the first two were produced by grapes of organic viticulture, while the second two were of different vintage.

Alcoholic fermentation is a complex procedure, since several metabolomic reactions are carried out simultaneously. The histograms in Fig. 1C and D show the number of detected features in the different days of fermentation, including the standard deviation originating from

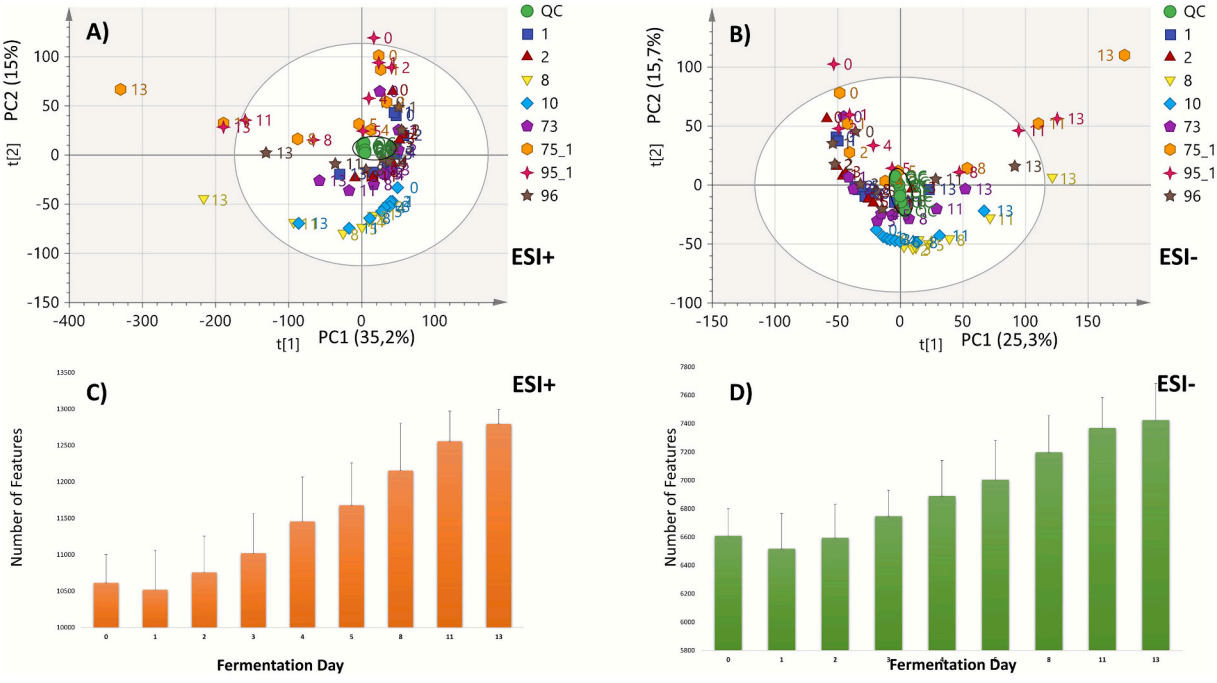

Fig. 1. PCA plots of all features registered in Muscat of Alexandria grape must samples in ESI+ (A) and ESI− − (B). Tanks are represented with different shapes and colors, and numbers indicate the day of alcoholic fermentation. Panels (C) and (D) show the number of features detected at each fermentation day in positive and negative ionization mode, respectively. Error bars represent the standard deviation across the eight industrial tanks.

the eight different tanks. Indicatively, it was observed that in positive ionization an average of about 10,613 features were detected for day 0, 11676 for day 5 and 12,794 for day 13 (end of fermentation), and in negative ionization the number of features for the same time points were 6609, 7004 and 7425, respectively. Generally, in positive ionization were detected more features (almost double), with their number showing a clear increase during fermentation, although an insensible decrease was registered between day 0 and day 1.

3.2. Tentative biomarkers

The preliminary statistical analysis exported a huge number of biomarkers for alcoholic fermentation, thus metabolites that their concentration was modified during the winemaking. The decided strategy was to act according to the following step: a) detect and annotate metabolites known to have characteristic behavior during the fermentation process (e.g., amino acids, organic acids, and sugars.), b) divide them in different groups according to their behavior during the fermentation, c) run correlation analysis to detect other features that their behavior falls in one of these groups, d) annotate these features, and e) make hypothesis about the metabolomic changes that take place during the winemaking. To achieve the most realistic and robust results possible, all fermentations from various vintages and wineries were combined into a single database rather than analyzed separately.

To obtain a significant number of first-level annotated metabolites (Table S2), reference standards were initially analyzed under the same instrumental conditions. Some of these metabolites are known to exhibit specific and characteristic behavior during alcoholic fermentation. The patterns of selected metabolites were visually inspected individually and subsequently classified into six major behavioral trends, as presented in Fig. 2a. Using the online platform MetaboAnalyst, features that showed a strong correlation (>0.5) with first-level annotated metabolites were identified and grouped into the same six behavioral categories. Group (a): Metabolites whose concentration rapidly decreased in the first few

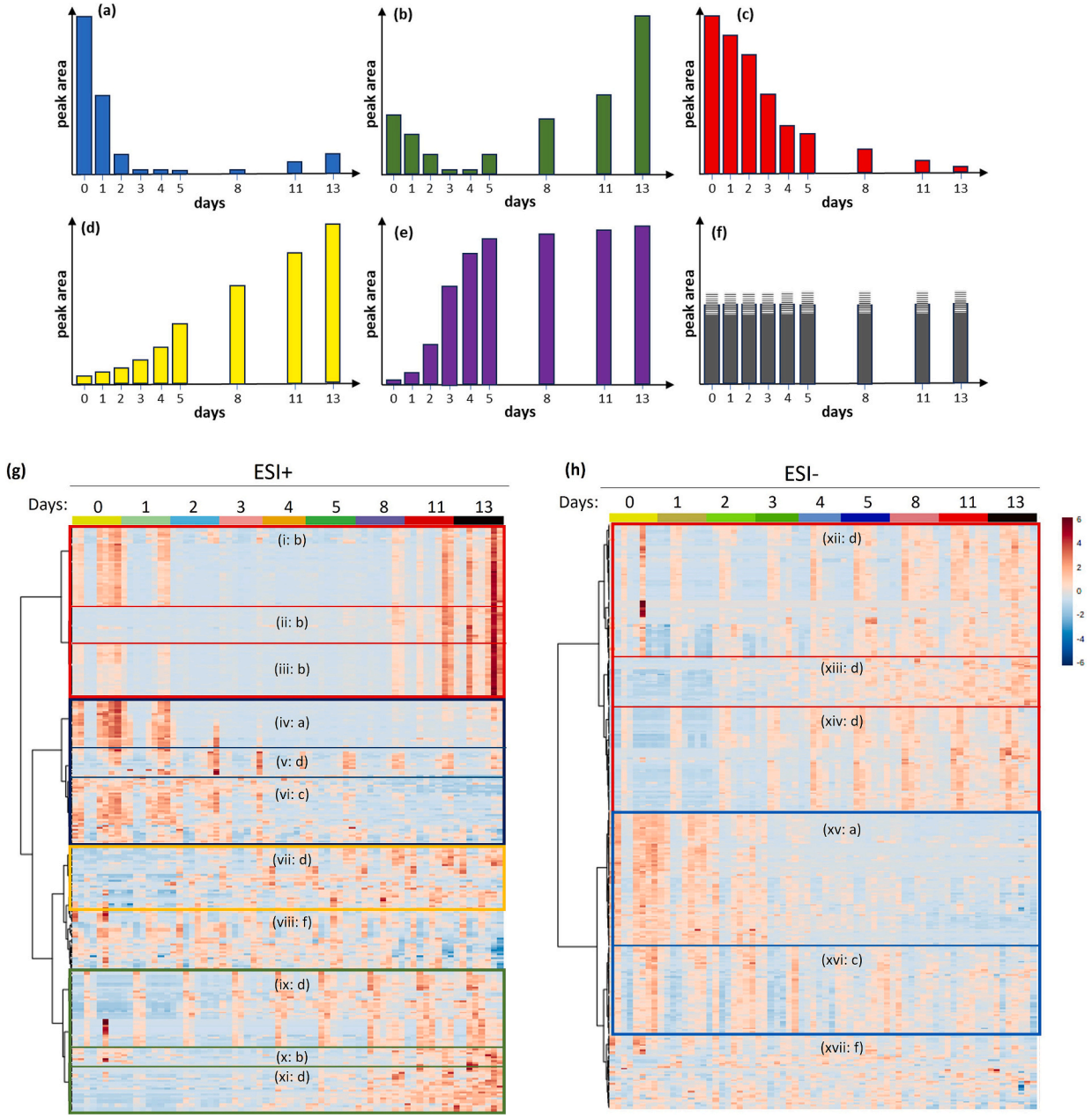

Fig. 2. Groups of temporal patterns for the annotated tentative biomarkers (a-f), and Heatmaps of the selected biomarkers in ESI+ (g) and ESI− (h).

days of fermentation, with a slight increase towards the end (e.g., amino acids tryptophan, phenylalanine, histidine, valine, and arginine). Group (b): Metabolites that decreased during the initial days but showed a substantial increase in the later stages of fermentation (e.g., amino acids isoleucine, threonine, and glutamic acid). Group (c): Metabolites that gradually decreased throughout the entire fermentation process (e.g., nicotinamide, nicotinic acid, and hexoses). Group (d): Metabolites that gradually increased over the course of fermentation (e.g., leucine, citrulline, histidine, tyrosine ethyl ester, choline, cytosine, glutathione, guanine, 2-ketogluconic acid, gluconic acid, and malonic acid). Group (e): Metabolites that increased rapidly in the early stages and then remained relatively stable (e.g., riboflavin, indole-3-acetaldehyde, and kaempferol). Group (f): Metabolites that remained generally stable with minor variations throughout fermentation. The selected features were annotated following the four-level annotation system (Sumner et al., 2007), with most falling into the third level. A comprehensive list of annotated features—including their chemical formula, retention time, *m/z*, theoretical *m/z*, *p*-value, fragments, annotation level, and patterns provided in Table S3 (positive ionization) and Table S4 (negative ionization). Additionally, the peak areas of annotated features are presented in Tables S5 and S6 for ESI+ and ESI-, respectively.

The majority of annotated potential biomarkers from both ionization modes, responsible for differentiating samples during alcoholic fermentation, were nitrogen-containing compounds, primarily amino acids and short-chain peptides. Chitinases and thaumatin-like proteins are known to be the major proteins in grape must, with Muscat of Alexandria being one of the richest grape cultivars in these proteins. This could explain the high concentration of peptides observed after the protein denaturation and degradation during winemaking (Flamini, 2008), but other processes (e.g. yeast metabolism) could be responsible too. Other tentative biomarkers belonged to various chemical classes, including flavonoids, non-flavonoids, terpenoid glycosides, nucleosides, vitamins, sugars, and organic acids. The heatmaps in Fig. 2g–h were based on these selected tentative biomarkers in both ESI+ and ESI-, highlighting 17 distinct subgroups based on their behavioral patterns.

To provide a framework for interpreting the metabolomic changes observed, we prepared a schematic representation of the main pathways highlighted in this study (Fig. 7). The diagram integrates peptide and amino acid metabolism, glycolysis and the TCA cycle (tricarboxylic acid or Krebs cycle), and transformations related to aroma-active compounds, glycosidase activity, and nucleotide turnover. In the following subsections, the observed metabolite trends are described within this pathway context.

3.2.1. *Amino acids and peptides*

Nitrogen, a crucial element, profoundly influences yeast cell growth and fermentation kinetics during wine alcoholic fermentation. While inorganic nitrogen is preferred by yeasts, they can also utilize amino acids, peptides, and oligopeptides as secondary nitrogen sources. Nitrogen supplementation at the beginning and/or during fermentation can effectively prevent sluggish or stuck fermentation. Additionally, nitrogen availability impacts the formation of volatile compounds, crucial for shaping wine aroma profiles (Becerra-Rodríguez et al., 2020; Ribéreau-Gayon et al., 2021).

Amino acids are typically classified based on *Saccharomyces cerevisiae's* nitrogen preference into three groups: prematurely consumed (lysine), early consumed (Aspartate, threonine, glutamate, leucine, histidine, methionine, isoleucine, serine, glutamine and phenylalanine), and later consumed (valine, arginine, alanine, tryptophan, and tyrosine). Proline is considered the only amino acids that *Saccharomyces cerevisiae* is incapable of being utilized under anaerobic condition (Crépin et al., 2012; Ribéreau-Gayon et al., 2021) and that its concentration remains stable during the winemaking.

In this study, most amino acids fell into the first two behavioral groups, (a) and (b) (Fig. 2). The free aromatic amino acids (tryptophan, tyrosine and phenylalanine), the sulfur–containing amino acids (methionine and histidine), valine, ornithine and arginine exhibited a rapid decline in concentration during the early stages of fermentation, with some showing a slight increase towards the end. A similar pattern was observed for various oligopeptides, particularly those containing leucine/isoleucine and valine, as well as other branched amino acids like proline, alanine, threonine, tyrosine, phenylalanine, and glutamate residues. In contrast, a distinct group of free amino acids — including leucine, isoleucine, threonine, glutamate and aspartate — showed a significant increase in concentration at the end of fermentation, surpassing their initial levels in the must. Their behavior correlated well with oligopeptides containing valine, alanine, and leucine/isoleucine, with less frequent occurrences of threonine, tyrosine, and proline residues. The remaining annotated amino acids and N-containing metabolites showed a constant increase during the fermentation, correlating with the third group of behavior (Fig. 2d), and included proline, citrulline, histidine and several peptides containing mainly proline but also leucine, valine, and phenylalanine residues. As shown in Fig. 3, peptide hydrolysis and amino acid transamination feed into higher alcohol and ester formation, explaining the observed changes in free amino acids and oligopeptides.

However, some studies have reported an increase in proline during alcoholic fermentation (Son et al., 2009a, 2009b). The increase of several amino acids at the end of the fermentation, due to cell autolysis and/or de novo biosynthesis, is also a known phenomenon (Álvarez-Fernández et al., 2020; Braus, 1991). However, it is worth noting that the differences in the kinetics of amino acid assimilation by different strains prevent a strict comparison of the transporters' regulation, and this is the reason why in other studies, some of these amino acids are characterized as non-preferable, or delayed (Crépin et al., 2017; Kessi-Pérez et al., 2023; Marsit et al., 2016).

The intriguing aspect of this study was the behavior of the peptides. As written above, several peptides, whose concentration decreased at the beginning of the fermentation and/or increased at the end, were annotated. This finding suggests that yeast used the peptides as N–source and that at the end they released peptides in the wine. The utilization of oligopeptides as a nitrogen source by yeast has been known for decades (Damlé & Thorne, 1949) while the increase in oligopeptides at the end of fermentation may result from de novo biosynthesis by yeast or breakdown of larger peptides or proteins (Fig. 3) (Martínez-Rodríguez et al., 2002; Martínez-Rodríguez & Polo, 2000; Perrot et al., 2002).

The development of multiple transport systems among fungi and yeasts underscores the importance of oligopeptide transport to fulfil the microorganisms' nitrogen source requirements for building essential macromolecules such as proteins and nucleic acids. The various mechanisms that enable the entrance of peptides in the cell via membrane peptide transporters, the variability and the specificity of such transporters have been previously discussed in the literature (Becerra-Rodríguez et al., 2020; Berg et al., 2023). Generally, these transporters are divided based on the peptide length they allow to internalize in the cell, and/or their affinity to specific amino acids. Studies carried out with $^{15}NH_4Cl$ and ^{15}N–labelled yeast hydrolysate, demonstrated that approximately 40% of the total nitrogen in the yeast protein fraction originated from yeast hydrolysate and that ammonia contributed only 20%. According to the same authors, their results indicate that several amino acids are more readily obtained from peptides (Kevvai et al., 2016). These observations can be interpreted in the context of the nitrogen catabolite repression network, which regulates amino acid and peptide uptake in yeast. The consumption of oligopeptides rich in leucine/isoleucine and valine is consistent with the activation of PTR and OPT transporters. From a winemaking perspective, this reinforces the importance of managing nitrogen supplementation strategies to avoid sluggish fermentations, particularly in musts naturally poor in preferred amino acids.

Therefore, literature provides us with several tools to explain the findings of this study regarding oligopeptides. The slight increase of proline and the increase of proline containing oligopeptides could be

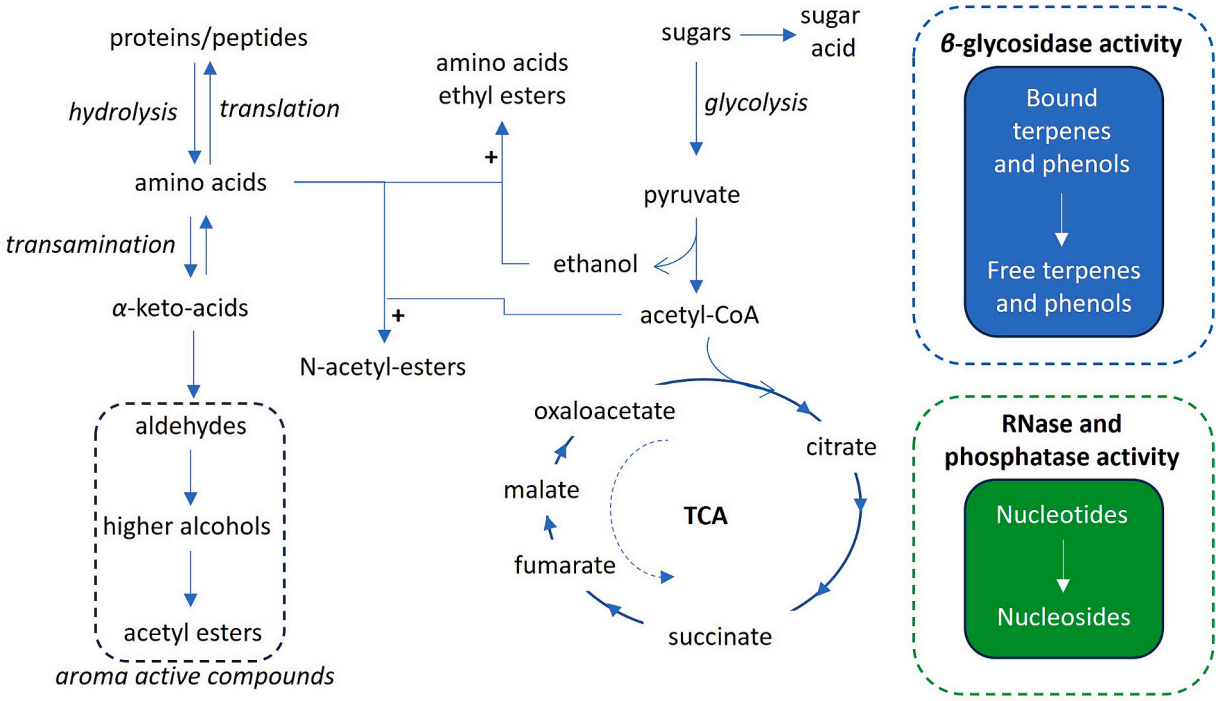

Fig. 3. Schematic overview of the main biochemical pathways highlighted during industrial alcoholic fermentation of Muscat of Alexandria. The diagram integrates peptide and amino acid metabolism, glycolysis and the TCA cycle, and links to the formation of aroma-active compounds, together with *β*-glycosidase and RNase/phosphatase activities.

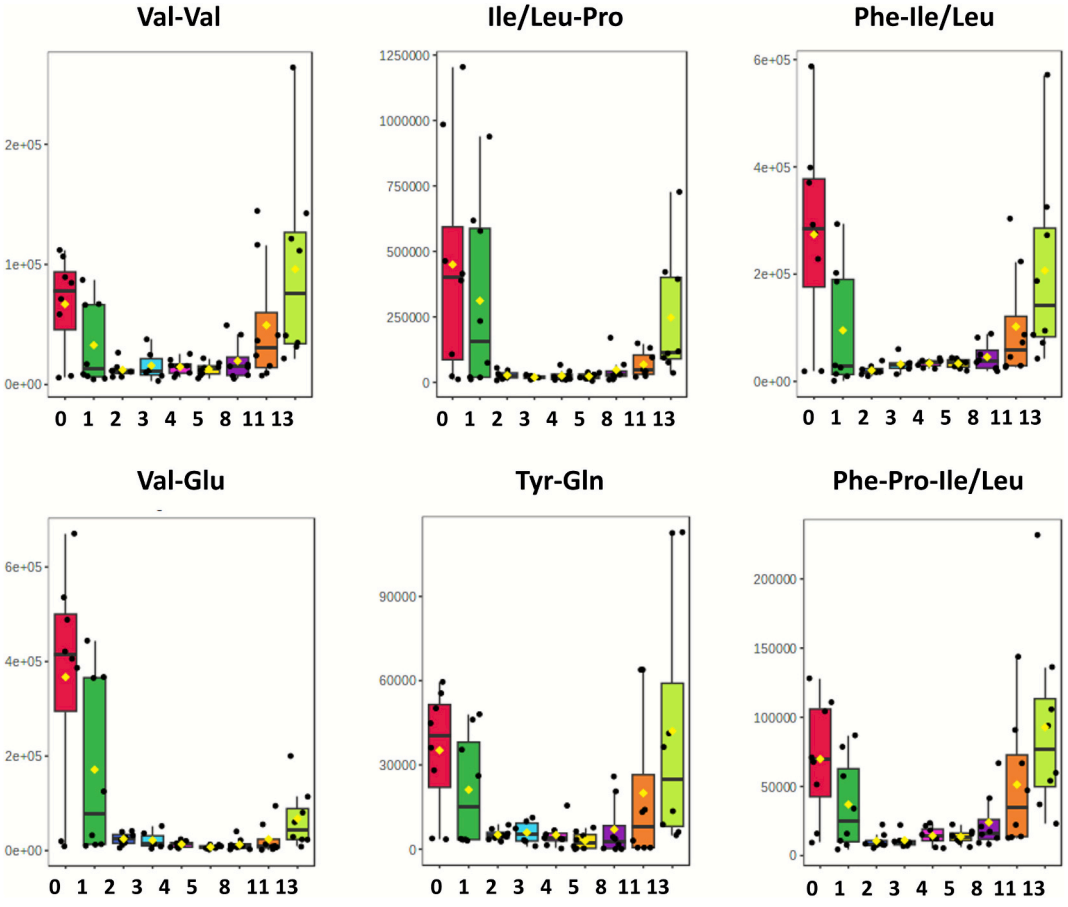

Fig. 4. Behavior of selected oligopeptides during alcoholic fermentation. The y-axis reports the concentrations in chromatographic peak area.

explained by the fact that yeast hydrolysing the oligopeptides to utilize the nitrogen from other amino acids and leaving behind proline residues. As presented in the heatmap of proline-containing compounds in Fig. S1, most peptides containing proline were rapidly consumed, with some being released at the end of fermentation. Some researchers have discovered that proline can enhance the stability of certain proteins, and by taking also into consideration the sweet taste of proline, they used this yeast ability to ensure additional accumulation of proline in sake fermentation (Takagi, 2019).

Most annotated oligopeptides were identified at level 3, with a few at level 2, based on the characteristic fragmentation patterns of their amino acid components (Tables S3 and S4). However, due to the lack of reference standards, it remains uncertain if they are part of larger peptides. Our data revealed that oligopeptides rich in leucine/isoleucine were among the most frequently consumed, aligning with literature findings that dipeptides with isoleucine in the N-terminal position or isoleucine/leucine in the C-terminal position are preferred by yeast peptide transporters (Damon et al., 2011). The same study showed that dipeptides containing acidic amino acids (glutamate or aspartate) were also not efficiently transported, but as discussed above yeast have various oligopeptide transporters with different affinities (Becerra-Rodríguez et al., 2020; Berg et al., 2023) and so they adapt easily at different nutrition conditions (Perrot et al., 2002). In the present study, leucine/isoleucine was a building block in 50 from the about 110 annotated oligopeptides that have a clear decreasing trend the first days of the fermentation, valine was found in 31, tyrosine and alanine in 22,

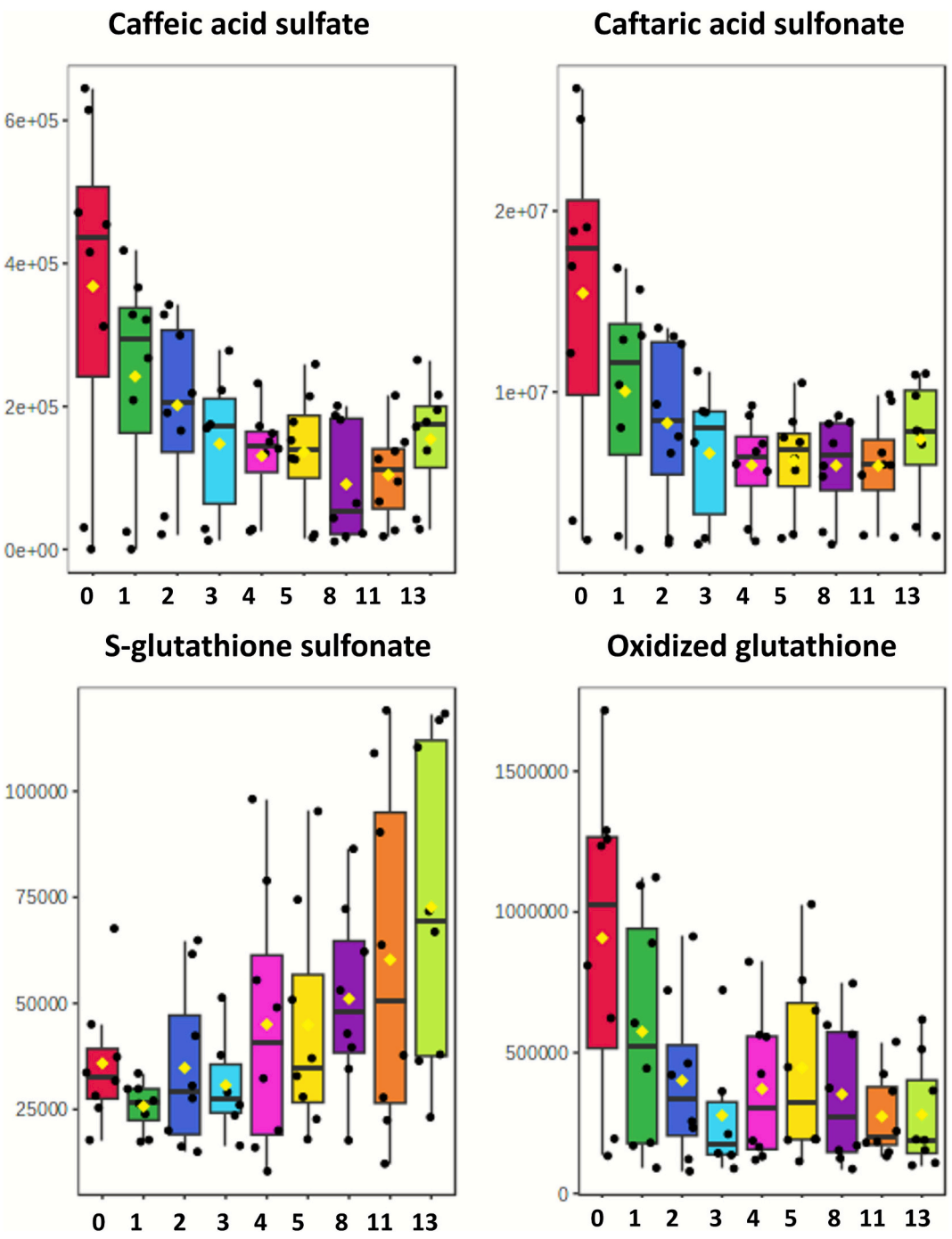

Fig. 5. Behavior of sulfonated products during alcoholic fermentation. The y-axis reports the concentrations in chromatographic peak area.

glutamate in 13, arginine and phenylalanine in 11 and proline in 24 oligopeptides (Tables S3 and S4).

However, as illustrated by the examples of oligopeptides behavior during the fermentation shown in Fig. 4, the annotated oligopeptides were not just consumed during alcoholic fermentation; rather, the concentration of several oligopeptides also increased, particularly during the latter stages of fermentation. Oligopeptides, besides serving as a nutrient source, play crucial and specific roles within living organisms and environments. Yeast may synthesize oligopeptides in response to oxidative stress or as antimicrobial agents to reduce the growth of other microorganisms (Becerra-Rodríguez et al., 2020; Bourbouloux et al., 2000; Williams & Miller, 2001). The annotated oligopeptides whose concentration increased during the last days of fermentation were notably rich in proline residues, as previously discussed, and frequently contained alanine, valine, isoleucine/leucine, and less commonly tryptophan, tyrosine, phenylalanine, serine, and glutamate. Some of these peptides have been annotated in wine metabolomics studies and have shown positive correlation with the wine organoleptic characteristics (Sáez et al., 2021; Sherman et al., 2020). Other oligopeptides, including Ala-Pro, Leu-Ala, Asp-Val and Glu-Glu, have been found imparting in kokumi flavor of Trentodoc sparkling wines, characterized by thickness, mouthfulness, and continuity (Perenzoni et al., 2024). Similarly, they have been observed in sake and have been associated with its sensory attributes (Takahashi et al., 2012). Additionally, oligopeptides containing histidine and cysteine residues are presumed to contribute to the antioxidant properties of wine (Nikolantonaki et al., 2018).

Glutathione, an oligopeptide known for its pivotal functions primarily attributed to cysteine residue, exhibited intriguing patterns alongside its derivatives. With the exception of the oxidized form of glutathione disulfide, which demonstrated a decrease in concentration during the fermentation (pattern c), all the other detected and annotated forms, including glutathione, S–sulfonate glutathione, 2–S–glutathionyl caftaric acid (a.k.a. Grape Reaction Product or GRP) displayed an increase (Fig. 5). In grape musts, glutathione is predominately found in its oxidized form (Fig. S2) and can serve as a source of nitrogen and sulfur for various *Saccharomyces cerevisiae* strains during alcoholic fermentation (Marsit et al., 2015). It can also be assumed that the reductive conditions prevailing during alcoholic fermentation promote the conversion of glutathione to its reductive forms. Alternatively, it can be hypothesized that sulfur dioxide produced by the yeasts during alcoholic fermentation reacts with the glutathione disulfide yielding glutathione, S–sulfonate glutathione. The occurrence of this reaction has been detected recently in wine (Arapitsas et al., 2020) and according to our knowledge this is the first time reported to happen during the alcoholic fermentation.

Next to the oligopeptides, other N-contain annotated biomarkers were several indoles, such as indole 3-lactic acid and its glucoside, indole 3-acetic acids, indole 3-acetaldehyde, kynurenic acid and tryptophol. Although grapes contain several indoles, like the amino acid tryptophan, the hormone auxin, indole lactic acid and its glucosides (Arapitsas et al., 2020; Fabre et al., 2014; Ribéreau-Gayon et al., 2021) during the alcoholic fermentation yeasts metabolized tryptophan and enrich the wine in several other indolic compounds, like the higher alcohol tryptophol, indole 3-acetaldehyde and kynurenic acid (Arapitsas et al., 2018; Ribéreau-Gayon et al., 2021). Lately it was found that some of these indoles have the ability to react with SO_2 and to produce their sulfonated analogs in wine, too (Arapitsas et al., 2016, 2018). In this study, indole–3–lactic acid, its hexoside, acetyl–hexoside and sulphonated analogue had a progressive increase mentor stable trend (behavior groups d and f) during alcoholic fermentation. Tryptophol sulfonated increased during the last stages of the fermentation, having the pattern (d), and kynurenic acid increased during the first days of the fermentation and then reached a plateau, where both behaviors are in agreement with previous finding (Álvarez-Fernández et al., 2019, 2020; Arapitsas et al., 2018; Turska et al., 2019).

3.2.2. Organic acids, sugars and sugar alcohols

Among the annotated biomarkers, several organic acids have been identified as expected, such as succinic acid, tartaric acid, malic acid, gluconic acid etc. The concentrations of certain organic acids such as gluconic acid, keto-gluconic acid, isocitric acid, malonic acid, oxo-adipic acid, phenylacetic acid, and tartaric acid increased constantly during fermentation. All these are known to be part of yeast metabolism during alcoholic fermentation, apart from tartaric acid which yeast are unable to metabolize. Gluconic acid is formed by dehydrogenation and oxidation of glucose and can be metabolized in pyruvate which imparts the TCA cycle (Fig. 3) (Pal et al., 2016). Phenylacetic acid formation can be related to the shikimic acid pathway metabolites (Hayasaka et al., 2017), such as phenylalanine and tyrosine, and cinnamic acids and its esterification with the production of ethyl phenylacetate (Tat et al., 2007). Most probably tartaric acid should derive from the hydrolysis of caftaric acid, coutaric acid and fertaric acid. A second group of organic acid increased the first days, achieved a maximum after 3 to 5 days, and then decreased. In this group belonged metabolites of the TCA cycle, thus citric acid, malic acid, fumaric acid, ketoglutaric acid and succinic acid. These acids are produced through the TCA cycle from acetyl-CoA through the reductive and oxidative branches, depending on the aerobic or anaerobic conditions of fermentation (Fig. 3). In all tanks the oxygenation process took place the 3rd or 5th day of fermentation, a procedure which could activate several enzymes which are inhibited in anaerobic environment or deactivate the enzyme which impart to the formation of these acid, such as the deamination of *a*-ketoglutaric acid (Rankine, 1968), and this could explain the decrease of the organic acids of TCA cycle after 5th day of fermentation (Waterhouse et al., 2016). The decrease of citric, malic, fumaric, and succinic acids after the third to fifth day coincides with oxygenation events and can be explained by the regulatory flexibility of the TCA cycle and peripheral pathways under shifting redox conditions (Fig. 3). This highlights how oxygen management during industrial fermentation not only prevents stuck fermentations but also shapes the organic acid profile, with direct consequences for acidity and sensory balance.

Fermentative hexoses are the principal source of energy for *Saccharomyces cerevisiae*, thus undergoing rapid consumption during alcoholic fermentation. Consistent with this, the majority of the annotated hexoses exhibited decrease during fermentation in this study. However, certain annotated pentoses (thus non–fermentable sugars) and some oligosaccharides demonstrated a stable trend, while other oligosaccharides increased. Additionally, sugar alcohols such as sorbitol and *myo*-inositol, products of yeast activity, displayed an upward trend during the fermentation. This trend can be attributed to the reduction of sugars carbonyl group to alcohol during the fermentation process (Ribéreau-Gayon et al., 2021; Waterhouse et al., 2016).

3.2.3. Phenolics

All the products of the study were white wines, and although the process did not involve skin contact with the must, several phenolic compounds, including both flavonoids and non-flavonoids, were annotated among the biomarkers.

As expected, hydroxycinnamates were the major detected phenolics, since these are present in the pulp of the grapes. Hydroxycinnamates are of paramount importance in wine, participating in various oxidation reactions to produce yellowish colored compounds and enzymatic reaction that yield volatile compounds that influence the wine aroma (Ribéreau-Gayon et al., 2021; Waterhouse et al., 2016). In our study, hydroxycinnamates exhibited diverse behaviors. Their glycosidic forms such as caffeoyl glucoside and feruloyl glucoside decreased, while their corresponding aglycon forms, such as caffeic acid, increased, likely due to *beta*-glycosidase activity (Rodríguez et al., 2004; Zhang et al., 2021). Ethyl caffeate also increased, presumably attributed to esterification with ethanol. The sulfonated products of the caftaric and caffeic acid were also detected, which concentration decreased at the first stages of the fermentation, while their analogues with glutathione increased

(Fig. 5).

Similarly, the glycosidic forms of several phenolics decreased fast in the first days of fermentation and their aglycon analogues increased, as shown in Fig. 6. This behavior was observed for galloyl derivatives (monogalloyl glucose and gallic acid), stilbenes (piceid and resveratrol), flavonols (e.g., quercetin hexoside, kaempferol hexoside, quercetin, kaempferol, myricetin), flavones (e.g., apigenin and luteolin), and flavanols (epicatechin hexoside and epicatechin). All these specific modifications showed again a possible action of *beta*–glycosidase activity, which previously have been proved to be active in phenolic too (Fig. 3) (Todaro et al., 2008). Moreover, some researchers demonstrated that flavanols, such as quercetin and kaempferol, also have an activity against *beta*-glycosidase (Chen et al., 2017; Todaro et al., 2008; Watanabe et al., 1997). The systematic hydrolysis of glycosidic conjugates reflects the activity of yeast β-glycosidases, enzymes whose expression is regulated by carbohydrate metabolism. From an applied perspective, this suggests that strain selection or enzyme addition could be exploited to modulate the release of varietal terpenes and phenolics, thereby enhancing aroma expression in Muscat wines.

Finally, interesting behavior was observed for the monomeric and oligomeric flavanols, as most of them exhibited a slight increase during the fermentation. Particularly noteworthy was the behavior of epicatechin–sulfonates, which also increased. The sulfonated epicatechin was found in aged wine (Arapitsas et al., 2014), as a result of the

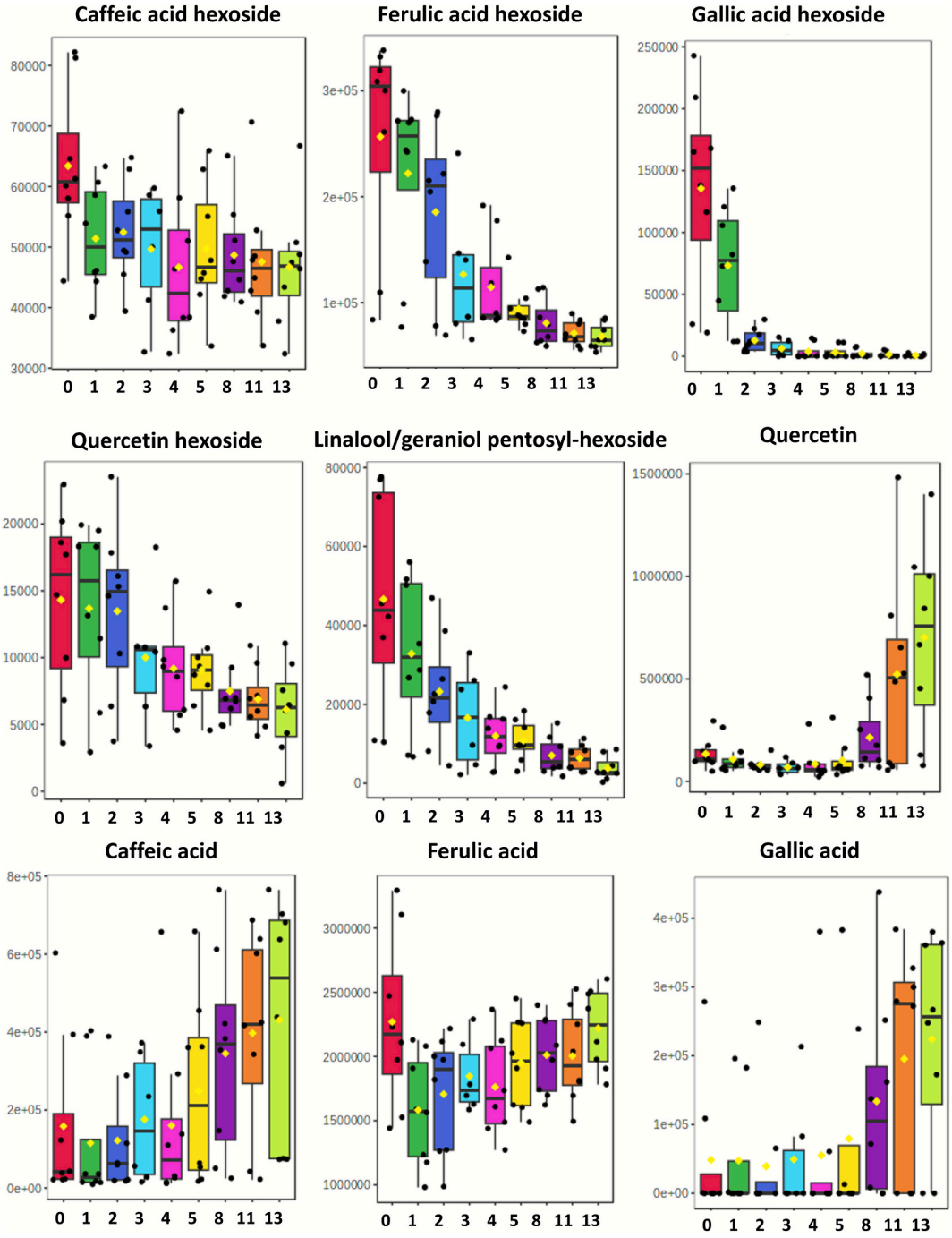

Fig. 6. Boxplots of glycosides of representative phenolic, flavonoid and terpenoid metabolites and their corresponding aglycon forms, presenting their behavior during alcoholic fermentation.

reaction between sulfur dioxide and products of proanthocyanidins acidic cleavage (Bonaldo et al., 2021). Since epicatechin sulfonation can also occur via enzymatic pathway, yielding the O–sulfonation in this case (Borges et al., 2018) we cannot be sure which of the two mechanisms occurred in our experiment.

3.2.4. Terpenoids glycosides

Muscat of Alexandria is a grape cultivar renowned for its abundant aromatic profile, both in chemical and organoleptic terms, since it contains high concentrations in terpenes like linalool, geraniol, *alpha*-terpineol and nerol. In a previous work, based on the same sample set, we measured the aglycon and volatile forms of these terpenes (Marinaki, Mouskeftara, et al., 2023), but the instrument employed in this analysis allowed for the monitoring of their glycosidic forms. The comprehensive study of free and glycosidic terpenes using a combination of GC- and LC-based analyses has been previously described (Barnaba et al., 2018; Caffrey et al., 2020; Ghaste et al., 2015). In this study, MS/MS experiments facilitated the annotation of terpenes attached to sugars. For instance, pentosyl-hexoside derivatives exhibited characteristic fragments at *m/z* 161.0455 and 119.0350, with the former indicative of hexose and the latter of pentose, while methyl-pentosyl-hexoside derivatives displayed the methyl-pentosyl fragment at *m/z* 145.0506. Examples of such MS/MS spectra, both for phenolic and terpene glycosides, are depicted in Fig. S3. With the help of literature data (Flamini et al., 2014; Godshaw et al., 2019; Hou et al., 2023; Rosso et al., 2022), and public databases (e.g., HMDB) was possible to annotate a large number of terpene glycosides (Table S4), and a decreased trend was found for several of them, since they belonged to the behavior group (c). Therefore, it was confirmed that the increase in free terpenes described in our previous work could be attributed to the hydrolysis of their glycosidic forms (Fig. 3).

3.2.5. Others

Nucleotides, although they have little or no flavor or aroma alone, have the ability to enhance the flavor and mouthfeel of other compounds. For this reason, the food and beverage industry uses this characteristic to improve the organoleptic character or specific food products, by enriching them with yeast autolysis extracts (Fuke & Ueda, 1996). However, fermented foods contain naturally nucleotides and are known for their influence in sparkling wines since their production process includes long contact with yeast lees (Charpentier et al., 2005; Vinther Schmidt et al., 2021). In the samples subject of this study where detected several nucleosides (e.g., cytosine, uracil, adenine, guanine, cytidine, uridine, adenosine, guanosine and methyl guanosine) and was found that they increased during the fermentation, falling in the behavior group (d). At the same time, it was found that their corresponding nucleotides (uridine monophosphate, guanosine monophosphate and cytidine–diphosphocholine) were found to decrease (group c) (Fig. 7). Such results could be explained by the activity of *Saccharomyces cerevisiae* RNases for the production of nucleotides, followed by phosphatase activity for the production of nucleosides (Fig. 3) (Charpentier et al., 2005; Stevens, 1985).

Xanthine and hypoxanthine were annotated in grape musts of Muscat of Alexandria, presenting an upward trend, mainly at the end of alcoholic fermentation and a very interesting observation is that the tanks of organic wines contained 5 to 10–fold higher concentration of

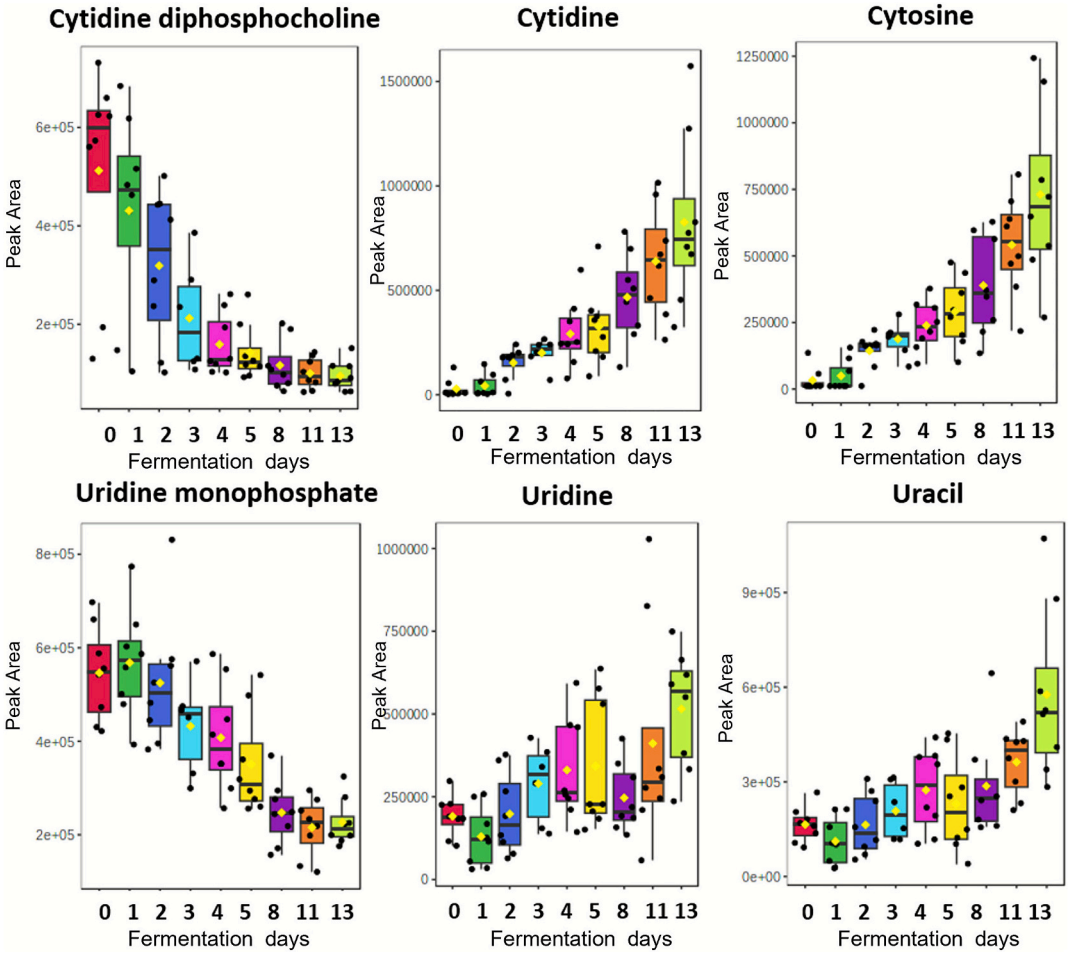

Fig. 7. Boxplots of nucleotides, nucleosides and nucleobases presenting their metabolic behavior during grape must alcoholic fermentation.

xanthine than the tanks of conventional wines.

Other biomarkers, which increased during fermentation, were D-pantothenic acid (vitamin B5), ascorbic acid (vitamin C), riboflavin (vitamin B2), and choline; while pyridoxine (vitamin B6), nicotinic acid (vitamin B3) and nicotinamide (vitamin B3) decreased. Pantothenic acid, the functional group of coenzyme A, is involved in acetylation reactions, nicotinic acid and nicotinamide, are involved in redox reactions and both of them are required for the enzymatic complexes of several intermediates such as organic acids, while riboflavin biosynthesis is performed by *Saccharomyces cerevisiae* during alcoholic fermentation (Chidi et al., 2018).

4. Conclusion

By utilizing a state-of-the-art LC-MS-based untargeted analysis, the metabolomic fingerprint of eight industrial-level fermentations producing commercial Muscat of Alexandria wines in Limnos was successfully monitored. To the best of our knowledge, this is the first study to integrate LC-MS-based metabolomics with industrial-scale winemaking for this significant international and aromatic cultivar. Through the statistical analysis and the annotation processes, the biomarker metabolites were categorized into distinct groups based on their behavior during alcoholic fermentation. In addition to confirming well-known and previously documented fermentation reactions — such as the yeast-driven conversion of sugars and amino acids into organic acids and sugar alcohol — this study also uncovered several previously unreported metabolomic transformations.

Specifically, it was demonstrated that numerous oligopeptides exhibited similar trends to amino acids, suggesting their consumption as nitrogen sources by yeast at the onset of fermentation. Notably, oligopeptides exhibiting this behavior primarily contained isoleucine/leucine, valine, tyrosine, tryptophan, glutamate, and arginine residues.

Our findings revealed metabolomics patterns consistent with heightened activity of various hydrolase enzymes, particularly during the final stages of alcoholic fermentation. Increased levels of diverse oligopeptides, predominantly rich in leucine/isoleucine, proline, alanine, and valine, and secondarily in tryptophan, tyrosine, phenylalanine, serine, and glutamate residues, were detected. This observation potentially explains the elevated levels of proline and proline-containing oligopeptides during fermentation, despite *Saccharomyces cerevisiae*'s preference against proline. Additionally, was assumed that the temporal patterns of several terpene- and phenolic-glycosides is consistent with glycosidase-mediated hydrolysis, which can contribute to the release of aroma-related precursors. Likewise, the behavior of nucleotides, nucleosides, and nucleobases suggests involvement of RNase- and phosphatase-associated pathways, generating N-containing metabolites that are recognized in the literature as possible contributors to flavor modulation in fermented products. While our study did not include sensory analysis, these biochemical shifts may hold relevance for future investigations into wine sensory properties.

Sulfur compounds also appeared to play an important role in the fermentation of Muscat of Alexandria grape must, with antioxidants such as glutathione, cysteine, and histidine increasing concentration during the latter stages of fermentation. The oxidized form of glutathione exhibited rapid depletion in the initial days, possibly due to the reductive conditions of alcoholic fermentation or the production of S-sulfonated glutathione following sulfitolysis. Meanwhile, sulfonated derivatives of caffeic and caftaric acids decreased at the beginning of fermentation, whereas sulfonated derivatives of flavanols and indoles increased towards the end. The enrichment of the wine in such compounds, which have antioxidant properties, could influence wine longevity.

Finally, our untargeted approach demonstrated the potential of metabolomics in comprehensively elucidating the role of fermentation in wine quality, under real industrial conditions too. Future work should include targeted quantitative analyses to validate the tentative biomarkers identified in this study, as well as sensory evaluation under controlled winemaking conditions to better link metabolomic shifts with wine quality attributes.

The insights gained from this study could aid wine scientists and makers in enhancing their understanding of the winemaking process and developing novel tools for improving wine quality. Beyond the specific context of Muscat of Alexandria wines, these results provide reference data of broader relevance for fermentation practice, since nutrient management, aroma release, and product stability are central issues not only in winemaking but also in other fermented beverages. While our study is focused on industrial wine fermentations, we believe these insights may stimulate further comparative research across fermentation industries.

CRediT authorship contribution statement

Panagiotis Arapitsas: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Maria Marinaki:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Christina Virgiliou:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Georgios Theodoridis:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2026.148090.

Data availability

All raw UPLC–QTOF–MS data generated in this study have been deposited in the MassIVE repository under accession number MSV000099152 (ftp://massive-ftp.ucsd.edu/v10/MSV000099152).

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