Impact of glutathione enriched Inactive Dry Yeast preparations on the stability of terpenes during model wine aging

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ABSTRACT

The impact of the addition of glutathione enriched Inactive Dry Yeast Preparations (g-IDYs) on the stability of some typical wine terpenes (linalool, α-terpineol, β-citronellol and nerol) stored under accelerated oxidative conditions was evaluated in model wines. Additionally, the effects of a second type of IDY preparation with a different claim (fermentative nutrient) and the sole addition of commercial glutathione into the model wines were also assessed. Model wines were spiked with the low molecular weight fraction (< 3 kDa permeate) isolated from the IDYs, avoiding the interaction of aroma compounds with other yeast components. An exhaustive chemical characterization of both IDY permeates was carried out by using targeted and non-targeted metabolomics approaches using CE-MS and FT-ICR-MS analytical platforms. Our findings suggest that the addition of <3kDa permeate isolated from any of the IDYs employed decrease the loss of typical wine terpenes in model wines submitted to accelerated aging conditions. The g-IDY preparation did indeed release reduced GSH into the model wines, although this compound did not seem exclusively related to the protective effect on some aroma compounds determined in both model wines. The presence of other sulphur-containing compounds from yeast origin in g-IDY, but also the presence of small yeast peptides, such as methionine/tryptophan/tyrosine containing tripeptide in both types of IDYs, seemed to be related to the antioxidant activity determined in the two permeates and in the minor loss of some terpenes in the model wines spiked with them.

Keywords: Inactive Dry Yeast Preparations, Glutathione, Terpenes, Wine oxidation, EC-MS, FT-ICR MS

INTRODUCTION

During wine aging, oxidation can be an undesirable process responsible for important changes in the sensory characteristics of wines, especially in white wines. The loss of pleasant aromatic notes produced as a consequence of the decrease of important aroma compounds such as polyfunctional thiols, terpenes, esters, etc. [1-3] and the accumulation of other undesirable compounds (hydrogen sulphide, methyl mercaptans) [4] in wines with low exposure to oxygen, which produce the so-called "reduced off flavour", are mainly responsible for the depreciation on the quality of the wines.

Sulphur dioxide (SO_2) is the most common preservative used in winemaking, not only because of its antioxidant and antioxidasic properties, but also because of its antimicrobial action. However, due to existing health concerns derived from the consumption of high concentrations of sulphites, there is a current trend to limit its use during winemaking [5]. Therefore, different strategies focused on keeping the original aroma characteristics of young wines while aging in the bottle have been proposed. It has been shown that the addition of some sulphur containing compounds prior to wine bottling might preserve the degradation of certain aromas. Among others, it has been suggested that the addition of gluthatione (γ-L-glutamyl-L-cysteinylglycine, GSH) at 10 mg/L prior bottling might reduce the loss of 3-methylmercaptohexanol in Sauvignon white wines [6]. More recently, Ugliano and collaborators [4] remarked that GSH effectiveness might depend on other wine compositional parameters (e.g. the presence of copper reduces the GSH effect). The protective effect of GSH has also been shown against the loss of some ester and terpene compounds [7-9], which are important contributors to pleasant floral and fruity notes in white wines [3]. This effect has been ascribed to the GSH free sulfhydryl (SH) moiety, which confers unique redox and

nucleophilic properties[10-13]. It has also been found that GSH mixed with some wine polyphenols (caffeic and gallic acids) or other sulphur-containing compounds, such as N-acetyl-cysteine, also have a protective effect against wine aroma oxidation [14, 15].

In spite of these promising results, the addition of GSH to the wine prior to bottling is a winemaking practice under study by the International Organization of Vine and Wine (OIV). However, other alternatives, such as the use of GSH-enriched Inactive Dry Yeast (g-IDY) preparations could be used to increase the levels of GSH in musts and wines [16]. The so-called IDY preparations are yeast derivatives obtained from *Saccharomyces cerevisiae* grown in a highly concentrated sugar medium and subsequently submitted to different inactivation treatments and manufacturing processes to obtain a variety of commercialized products (inactive yeast, yeast autolysates, yeast walls, and yeast extracts) [16]. The use of IDYs is gaining interest within the wine industry because of their large amount of potential applications in winemaking. Among them, as a consequence of its high content in GSH, g-IDYs have been claimed to preserve wine aroma and color during wine storage. However, although, as it has been recently stated, [17] no literature could be found on the industrial preparation of g-IDYs [15], and it is still not clear whether exogenous GSH enrichment is allowed during the manufacturing process, the release of reduced GSH (the form active against oxidation) into the wines has been recently proven [18, 19]. However, the effectiveness of the GSH released by these preparations on wine oxidation inhibition has not yet been investigated. Andujar-Ortíz and collaborators [20] recently revealed significant differences between *rosé* Grenache wines produced by using a g-IDY preparation and non-treated wines in some sensory aroma attributes but only after 9 months of wine aging. This effect could be attributable to the GSH released from IDY or to the stimulating effect of amino acids and other

peptides from the IDY on the GSH synthesis by yeast under winemaking conditions[18, 19].

Considering the current interest of the wine industry in the use of g-IDY preparations to preserve the aroma of wines, and the lack of published literature on this topic, the aim of this work was to evaluate the effect of a g-IDY preparation on some typical and desirable wine aroma compounds (linalool, α-terpineol, β-citronellol and nerol) by using model wines submitted to accelerated oxidative conditions. The effect of a second type of IDY preparation with a different claim (fermentative nutrient) and the effect of commercial GSH were also evaluated. To further understand the role of GSH from the IDY formulations, the wines were spiked with the low molecular weight fraction (< 3 kDa) obtained by cold-ultracentrifugation avoiding the interaction of other yeast components (glycoproteins) with the aroma compounds [21, 22]. To conclude, chemical characterization of both IDY permeates (< 3 kDa fraction) was carried out by using targeted and non-targeted metabolomic approaches using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) and capillary electrophoresis-mass spectrometry (CE-MS) analytical platforms.

MATERIALS AND METHODS

IDY samples

Two types of oenological IDY preparations were selected for being representative of the current preparations in the oenological market and because they are widely used in winemaking: a GSH-enriched IDY (g-IDY) recommended to reduce the oxidation of wine aroma compounds because of the presence of higher amounts of GSH, and a IDY preparation commonly used as fermentation nutrient (n-IDY). The comparison between

both types of IDYs (with and without GSH in their composition) should better provide evidences about the role of GSH released by IDY in wine aroma oxidation.

Isolation of < 3 kDa fraction from the IDY preparations by ultrafiltration

Four grams of each IDY powder were weighed into 50-mL centrifuge tubes. Samples were extracted with 50 mL water-ethanol solution (87:13, v/v) in an ultrasonic bath (3 cycles, 5 min. each) at 4ºC. The mixture was then centrifuged (15 min. at 5000g and 10 ºC) and the supernatant was ultrafiltrated using a Centricon device (Amicon Inc., Beverly, MA, USA) with a 10 kDa cut-off membrane. The obtained permeates were submitted to a second ultrafiltration step through a 3 kDa cut-off membrane Centricon (Amicon Inc.). Both ultrafiltration steps were carried out at cold temperature (below 10 ºC). The obtained <3 kDa permeates from each IDY preparation were freeze-dried and kept at -20ºC until use. Prior to chemical characterization, permeates were reconstituted with water to 100 mg/mL of dry residue. Only for FT-ICR-MS analysis, reconstituted samples were dialysed by using a Float-a-Lyzer G2 device with a 0.1-0.5 kDa cut off membrane from Spectrum (Breda, The Netherlands) to remove salts.

Model wine solutions under accelerated aging conditions

Model wine solutions (50 mL) were prepared in 100 mL vials by adding ethanol (VWR, Leuven, Belgium) at 120 mL/L and 4 g/L tartaric acid (Panreac, Barcelona, Spain). The pH was adjusted at 3.5 using a 5 M NaOH solution (Panreac). Model wines were spiked with single terpene compounds (nerol, β-citronelol, α-terpineol and linalool) from Sigma (Stenheim, Germany) at a final concentration of 25 mg/L each. Finally, 100 µL of the reconstituted <3kDa fractions isolated from g-IDY or n-IDY at 100 mg/mL, was added. In addition, another set of model wines were individually aromatised with the four aroma compounds and spiked with commercial GSH (Sigma) to a final

concentration of 10 mg/L. Finally, four control model wines, one with each aroma compound, but without addition of the IDY fractions or commercial GSH were also prepared. Two vials of the model wines containing each terpene compound were analysed at the beginning of the experiment (t=0d). The different model wine mixtures were submitted to an accelerated oxidation process during three weeks at 25 ºC saturating the headspace of the vials with oxygen (t=21d). All the preparations were carried out in duplicate.

HS-SPME-GC/MS analysis

Model wine aroma analysis was performed before (t=0 days) and after (t= 21 days) model wine oxidation process. It was carried out by head space solid phase microextraction coupled to gas chromatographymass spectrometry (HS-SPME-GC/MS). Model wine samples (8 mL), 2.3 g of NaCl and 40µ L of an internal standards solution (400 mg/L 3,4-dimethylphenol and 2.5 mg/L methyl nonanoate) were added to a 20 mL SPME vial. The SPME procedure and chromatographic conditions were detailed in a previous work [23]. Briefly, the extraction procedure was automatically performed using a CombiPal system (CTC Analytics AG, Zwingen, Switzerland) with a 50/30 µm DVB/CAR/PDMS fibre of 2 cm length from Supelco (Bellefonte, CA, USA). Samples were pre-incubated for 10 min at 50 °C and extraction was performed in the headspace of each vial for 30 min at 50 °C. Desorption was performed in the injector of the GC system in splitless mode for 1.5 min at 270 °C. After each injection the fibre was cleaned for 20 min to avoid any memory effect. The chromatographic separation was performed in a GC-MS instrument (Agilent6890GC, Agilent 5973 N MS) equipped with a Supra-Wax fused silica capillary column (60 m×0.25 mm i.d.×0.50 µm film thickness) from Konik (Barcelona, Spain). Helium was used as the carrier gas at a flow rate of 1 mL/min. The oven temperature was initially held at 40 °C for 5 min, then, it

increased at 4 °C/min to 240 °C, and held at 240 ºC for 20 min. The acquisitions were performed in scan (from 35 to 350 amu) and electronic impact mode (70 eV). Other MS conditions were 270, 150 and 230 °C for the transfer line, quadrupole and ion source respectively. The signal corresponding to a specific ion of quantification (m/z 93, m/z 59, m/z 69, m/z 69 for linalool, α-terpineol, β-citronellol and nerol, respectively) was calculated by the data system. The compound identification was carried out by comparison of retention times and mass spectra of the reference compounds with those reported in the mass spectrum library NIST 2.0. Data were obtained by calculating the relative peak area (RPA) in relation to that of the corresponding internal standard (3,4-dimethylphenol, for all the aroma compounds except for nerol that was methyl nonanoate).

Analysis of reduced Glutathione (GSH), total Glutathione and γ-glutamylcysteine (γ-glu-cys) by RP-HPLC-FL

Reversed-phase HPLC using a liquid chromatograph consisting of a Waters 600 Controller programmable solvent module (Waters, Milford, MA), a WISP 710B autosampler (Waters) and a HP 104-A fluorescence detector (Hewlett-Packard, Palo Alto, CA, USA) were used following the procedure previously optimised and validated [18]. The mobile phase was composed of methanol (Lab-Scan, Sowinskiego, Poland) and aqueous solution of phosphate buffer (10 mM $NaH_2PO_4 \cdot 12$ H2O at pH 8.5) with a ratio of 15:85 (v/v). The sample (30 µL) was placed in a 1 mL vial (by using an insert) and the precolumn derivatization was automatically made in the autosampler at 12 ºC as following: to a sample vial were added 105 µL from the dithiotreitol (Sigma-Aldrich) solution vial [5 mM and 0.5 mM in borate buffer (0.2 M H_3BO_4, pH 9.2) to determine total GSH or reduced GSH, respectively] and 15 µL of 2,3-naphtalenedialdehyde (NDA) (Sigma-Aldrich) solution (5 mg mL^{-1} in ethanol); then, two mixtures cycles of

the total content of the insert were carried out and 100 µL were injected in the chromatographic system. Separation was carried out on a Nova Pack C18 (150 mm x 3.9 mm i.d., 60 A, 4 µm) column (Waters) in isocratic mode (flow at 1 mL min^{-1}), and detection was performed by fluorescence (λexcitation= 467 nm, λemission= 525 nm). The derivatization conditions for the determination of γ-glu-cys were the same previously described. Calibrations were carried out by using pure standards compounds solutions of GSH and γ-glu-cys. The analysis of the samples was made in duplicate.

ORAC-FL assay

The antioxidant capacity of IDY permeates and GSH was measured by ORAC-fluorescein (ORAC-FL) assay based on that proposed previously [24]. Briefly, the reaction was carried out at 37 ºC in 75mM phosphate buffer (pH=7.4) and the final assay mixture (200uL) contained FL (70 nM), AAPH (12mM), and antioxidant [Trolox (1-8 uM) or sample at different concentration]. The plate was automatically shaken before the first reading and the fluorescence was recorded every minute for 80 minutes. A polestar Galaxy plate reader (BMG Labtechnologies GmbH, Offemburg, Germany) with 485-P excitation and 520-P emission filters was used. The equipment was controlled by the Fluostar Galaxy Software (version 4.11-0) for fluorescence measurement. Black 96-microwell microplates (96F untreated, Nunc, Denamark) were used. AAPH and Trolox solutions were prepared daily and FL was diluted from a stock solution (1.17 mM in 75mM phosphate at pH 7.4. Fluorescence measurements were normalised to the curve of the blank (no antioxidant). From the normalised curves, the area under the fluorescence decay curve (AUC). The regression equation between net AUC and antioxidant concentration was calculated and the slope of the equation was used to calculate the ORAC-FL value by using the Trolox curve obtained for each assay. Final ORAC-FL values were expressed as µmol of Trolox equivalent/mg dry

permeate (for the <3kDa permeates from g-IDY and n-IDY) and in µmol of Trolox equivalent/mg pure compound for commercial GSH.

Total free amino acids and peptides

Free amino acids and peptides in model wine were determined according to the protocols proposed by Doi and co-workers[25]. Free amino acids were determined by the reaction of ninhydrin/Cd with the free amino group by measuring the absorbance at 507 nm (method 5) [25]. On the other hand, free amino acids plus peptides were determined by the reaction of the amino group with ninhydrin/Sn by measuring the absorbance at 570 nm (method 1) [25]. A DU 70 spectrophotometer from Beckman Coulter (Fullerton, CA, USA) was used. Quantification was carried out on the basis of the standard curve of leucine, and results were expressed as mg N/L. All the model wine samples were analysed by duplicate.

Analysis of amino acids by RP-HPLC-FL

Amino acids were analysed in duplicate by reversed-phase HPLC using a liquid chromatograph described in section 2.5. Samples were submitted to automatic precolumn derivatization with o-phthaldehyde (OPA) in the presence of 2-mercaptoethanol following the method described by Moreno-Arribas and collaborators [26]. Separation was carried out on a Waters Nova Pack C18 (150x 3.9 mm i.d., 60 A, 4µm) column and the same type of precolumn. Detection was performed by fluorescence (λexcitation= 340 nm, λemission= 425 nm).

Analysis of sulphur-containing metabolites by CE-MS

CE analyses were carried out in a P/ACE 5500 CE apparatus from Beckman Coulter. The CE system was coupled to a TOF MS instrument from Bruker Daltonics (Bremen, Germany) through an orthogonal ESI interface model G1607A from Agilent

Technologies (Palo Alto, CA, USA). Electrical contact at the ESI needle tip was established via a sheath liquid delivered by a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA). The electrophoretic separation was carried out using an uncoated fused-silica capillary (50 µm internal diameter, 363 µm outside diameter and 80 cm total length) from Composite Metal Services (Worcester, England). Before first use, the separation capillary was conditioned by rinsing with 1 M NaOH for 10 min, followed by 20 min with water, both using pressurized N_2 at 20 psi (1380 mbar). After each run, the capillary was conditioned with water during 2 min, followed by BGE during 4 min. Injections were made at the anodic end using N_2 pressure at 0.5 psi (34.5 mbar) for 80 s. The electrophoretic separation was achieved applying +25 kV at room temperature in a BGE composed of 3 M formic acid. Electrical contact at the ESI needle tip was established via a sheath liquid based on isopropanol-water (50:50, v/v) and delivered at a flow rate of 0.24 mL/min. The mass spectrometer operated in the positive ion mode. The nebulizer and drying gas conditions were 0.4 bar N_2 and 4 L/min N_2, respectively, and maintaining the ESI chamber temperature at 250ºC. Spectra were acquired in the 50-700 m/z range every 90 ms. External and internal calibration of the TOF MS instrument was performed by introducing a 10 mM sodium formate solution through the separation capillary. The ions used for the calibration of the TOF MS instrument were next: 90.9766, 158.9641, 226.9515, 294.9389, 362.9263, 430.9138, 498.9012 and 566.8886 m/z. TOF MS provided a high mass resolution and high mass accuracy with errors usually below 10 ppm. Selected mass spectra were processed through the software DataAnalysis (Bruker Daltonics), which provided a list of possible elemental formulas by using the Generate-Molecular Formula Editor (Bruker Daltonics), which provided standard functionalities such as minimum/maximum elemental range, electron configuration and ring-plus double bonds equivalents, as well

as a comparison between the theoretical and the experimental isotopic pattern (Sigma-Value™) for increased confidence in the theoretical molecular formula assignment.

Non-targeted metabolomic analysis by FT-ICR MS

FT-ICR MS was used to obtain ultra-high resolution (>100,000) mass spectra. Separation and identification of the metabolites was possible without the need of chromatography or derivatisation due to the ultra-high mass accuracy. Commercially beer maltooligosaccharides were used as mass calibrants and tunning standards in both the positive and negative ion modes [27]. The maximum mass error achieved was below 2 ppm.

Experiments were performed on a hybrid triple quadruple-FT-ICR instrument Varian 920 MS provided with a 7.0 T actively shielded superconducting magnet and equipped with an electrospray ionization (ESI) source. The conditions in the electrospray were next: in positive mode the potential in the needle was set at 4.5 kV and 600 V in the shield. The capillary potential to pass the ions from the source to the skimmer was set in a range between 40 and 60 V. Nitrogen was employed as nebulizer gas and its pressure was 50 psi. The pressure for drying gas was set at 18 psi and the temperature at 300 °C. The flow rate of the sample was kept at 15 µL/min and injected by direct infusion. In the negative mode MS parameters were next: -3.5 kV, 600 V, from -70 to -90 V in the capillary, 18 psi and 300 °C. Sample flow rate was 15 µL/min. Air was used instead of nitrogen as nebulizer gas. The spectra were acquired in full scan mode and defining a mass range from 100 to 1000 of m/z. The internal detection signal in the cell detector of the FT-ICR was optimized for a mass of 500 m/z.

Monoisotopic mass and isotope clusters profiles were extracted from the raw data by using Varian MS Peak Hunter software version 4.1.89. These two parameters were used by the same software to get the elemental composition following the next criteria: error was set at 2 ppm and only chemical formulas containing C, H, N, O, P and S were allowed. The spectra were exported to mzXML format and applied against XCMS [28] online METLIN database. When the results from the METLIN database were in good agreement in accordance with the chemical formula found in Varian MS Peak Hunter, the metabolite was given as a good result.

Statistical analysis

Data from the analysis of aroma compounds (RPAs) from the model wine experiments were submitted to one-way ANOVA analysis and LSD to test the effect of wine treatment.

RESULTS AND DISCUSSION

Effect of the addition of GSH and the < 3 kDa permeates isolated from IDYs on specific wine terpenes in model wines under accelerated aging conditions

To determine the effect of GSH-enriched IDY preparations on the evolution of aroma compounds during aging, and wether the GSH released from IDYs into the wines might have a role on the behaviour of aroma compounds during aging, the <3 kDa permeate from a g-IDY preparation was isolated by ultracentrifugation and spiked into model wines, as described in section 2.1. Model wines spiked with this permeate were coded as g-IDY-W. Ultrafiltration ensured the removal of glycoproteins (with higher molecular weights than 3kDa) from the IDY preparations that might interact with volatiles [21, 22], masking the potential action of GSH on the aroma compounds, which was the main objective of this study. In addition, to compare the effect of a different

type of IDY preparation currently commercialized as a fermentation nutrient (without any claim on wine aroma protection), the <3 kDa permeate was also isolated and added to model wines (n-IDY-W). In addition, two other types of model wines added with commercial GSH (10 mg/L) referred to glut-W and control model wines, without any addition, (cont-W)), were also prepared. To avoid chemical transformations due to the high reactivity of terpene compounds [3], each model wine solution was individually aromatised with a single aroma compound (nerol, β-citronellol, α-terpineol and linalool). These aromas were selected because they are characteristic of young wines providing pleasant floral-fruity nuances and are very sensitive to the oxidation phenomena[3, 29, 30]. The behaviour of the four terpene compounds was evaluated in all the model wines at the beginning of the experiment (t=0d), corresponding to non-oxidised model wines and after three weeks of accelerated aging conditions (t=21d). **Figure 1** shows the percentage of decrease in relative peak area (RPA) between the initial wine sample (non-oxidised) and the wines after 21 days of aging for each aroma compound and wine type. As it can be seen, there was a general decrease in RPAs for all the aromas during aging, which ranged from 24 to 45%, therefore, confirming the outstanding effect of aging on the loss produced in these types of aroma compounds [2, 3, 14], which can be attributable to oxidation phenomena [3, 29, 30]. Interestingly, compared to the control wine solution, α-terpineol and linalool showed a lower reduction in RPAs in the model wines supplemented with the < 3k Da permeate isolated from either of both preparations (g-IDY or n-IDY). In the case of nerol, a slightly lower reduction in RPAs was also observed in the g-IDY model wine, although this effect was not statistically significant. β-Citronellol did not show a significant effect either. These results seemed to indicate a protective effect of these preparations on some specific aroma compounds, which is in agreement with the aroma sensory differences recently found between

347 control rosé wines (without IDY added) and rosé wines produced in cellar conditions
348 with the same type of g-IDY after 9 months of aging [20].

349 However, the addition of commercial GSH to the model wine solutions did not have a
350 significant effect under the essayed conditions. Different published works have shown
351 an inhibition of the decline of certain aroma compounds when using GSH at a similar or
352 even lower dosage in wines or model wine systems[4, 6-9]. Nonetheless, it is well known
353 that the main effect of GSH in wines, is its ability to react with orthoquinones produced
354 by oxidation of caftaric acid (and other polyphenols) to give GRP (grape reaction
355 product) by action of polyphenols oxidases blocking the following steps in which
356 polyphenols are involved (polymerization) and responsible for browning [4, 6, 17].
357 Moreover, orthodiphenols can be directly oxidized in the presence of oxygen and some
358 cations (iron, copper) to orthoquinones and hydrogen peroxide, which might be
359 involved in subsequent aroma oxidation [4]. In our experimental conditions this action
360 mechanism was limited because of the absence of polyphenols to react with GSH in the
361 model wine, which seems to explain the absence of a noticeable effect of GSH on aroma
362 protection in the model wines supplemented with commercial GSH. However, GSH
363 presents scavenging hydroperoxyde and hydroxyl radicals properties, which might have
364 allowed it to act as antioxidant by other mechanisms different than its capacity to
365 interact with ortoquinones in polyphenol free systems [10, 11]. Nonetheless, on the basis of
366 our results, this mechanism did not seem as significant in our experimental conditions.
367 In spite of this, it is important highlight that this model system allowed us to uncover
368 the potential role of other yeast components, different to GSH and contained in the
369 <3kDa fraction, which seemed to be related to the protection of some terpenes in model
370 wines submitted to accelerated aging conditions.

Following this rationale, to find out if the observed reduction in peak areas for some of the terpenes employed in our study was effectively related to an antioxidant effect exerted by the IDY permeates, the radical scavenging activity of both of them was calculated by using the ORAC-FL method. The ORAC values were 0.33 and 0.22 µmol TE/ mg dry permeate for g-IDY and n-IDY permeates respectively, showing that the two permeates had a positive and a similar antioxidant capacity. These results are in agreement with the previous experiment, in which g-IDY-W and n-IDY-W wines showed a similar reduction in the corresponding peak areas for the same aroma compounds (linalool and α-terpineol). In addition, to confirm the antioxidant activity of the commercial GSH employed in this experiment, the ORAC value for the pure compound was also calculated, this is 10.7 µmol TE/mg pure compound, thus, corroborating its high antioxidant capacity, comparable to other important wine antioxidants such as polyphenols. As an example, for a representative set of pure polyphenolic compounds, the calculated ORAC values ranged between 2.35 and 18.16 µmol TE/mg pure compound determined for myricetin and caffeic acid respectively [31]. Nevertheless, and as previously stated, in spite of the high antioxidant activity determined for GSH, this compound did not exert a noticeable effect in preventing aroma oxidation in a model wine in the absence of polyphenols, as used in the present work. However, these results confirmed the antioxidant properties of both IDY permeates in agreement with the better preservation of some terpenes observed in the model wines supplemented with them. However, this effect, at least in the g-IDY wines, might have been a consequence of the higher amount of GSH contained in the g-IDY permeate compared to that added into the wines by using commercial GSH (10 mg/L), which on the basis of its radical scavenging properties might be responsible for the

lower aroma loss in g-IDY wines. Therefore, a quantitative determination of GSH, total GSH, and the precursor γ-glutamylcysteine, was carried out.

Determination of GSH, total GSH, and γ-glutamylcysteine

Reduced GSH, total GSH and the precursor γ-glutamyl-cysteine were analysed by using a previously optimised RP-HPL-FL method [18]. These results are shown in **Table 1.** As it can be seen, only the g-IDY permeate presented detectable levels of GSH (1293 mg/L) and γ-glutamyl-cysteine (873 mg/L). The amount of total GSH was higher (3147 mg/L), meaning that only 41% of glutathione was in its reduced form (GSH) and available to act as a potential antioxidant. However, in the n-IDY permeate, there were traces of GSH or GSH related compounds. This is in agreement with some previously published works in which in a screening of commercial oenological IDY preparations, only those claimed to be GSH-enriched IDY preparations released reduced GSH into synthetic wines [18]. Taking into consideration the added amount of each permeate into the model wines (100 µL), the final amount of reduced and total GSH in g-IDY-W model wines was 2.6 mg/L and 6.3 mg/L respectively, which is very close to the amounts determined in model wines when using IDYs at the recommended wine dosage (0.3 g IDYs/L), which has been established to be between 1 and 2.5 mg/L [18, 19]). Considering that no GSH (or other GSH related compounds) were detected in n-IDY-W model wine and that the amount of GSH determined in g-IDY-W was lower than the amount of commercial GSH employed in the GSH-W model wine (10 mg/L), it could be concluded that the observed antioxidant effect of these preparations on the reduction of aroma loss during wine aging did not seem to be linked to the sole antioxidant action of GSH but could be due to other compounds or to the combined action of GSH and other antioxidant compounds from yeast origin present in the permeates (at least in n-

IDY-W model wine). In trying to elucidate these compounds, a comprehensive chemical characterization of both g-IDY and n-IDY permeates was carried out.

Analysis of other sulphur-containing compounds by CE-MS

In addition to GSH, other biological sulphur-containing compounds have been said to present antioxidant properties [32]. Thus, the analysis of other low molecular weight sulphur-containing compounds in the < 3 kDa permeates from both g-IDY and n-IDY samples was performed by using CE-MS. This targeted analysis was carried out on the basis of the presence of at least one S atom in the molecular structure. The existence of sulphur in the molecule requires the presence in the mass spectra of an isotopic peak 2 Da higher than the molecular ion and at least 4% in intensity per sulphur. After further inspection of n-IDY and g-IDY CE-MS profiles, besides the two sulphur containing amino acids methionine and cysteine, another 14 high abundant sulphur-containing compounds were found in the permeate from g-IDY sample. In **Figure 2**, extracted ion electropherograms (EIEs) from these two amino acids and the most abundant sulphur-containing compounds are represented (continuous and dotted lines for g-IDY and n-IDY samples, respectively). The electropherogram from n-IDY showed, however, only three major peaks (compounds 1, 3 and 5) and methionine and cysteine were not detected either. Detailed information about the identity of these compounds is shown in **Table 2**. As it can be seen in this table, eight out of fourteen compounds could be tentatively identified. Most of them corresponded to glutathione derivatives (compounds 7, 8, 10, 11 and 14) and in general, the rest of the identified compounds were compounds related to the amino acids cysteine and methionine. Many sulphur compounds, including the sulphur containing amino acids, have been shown to exhibit antioxidant properties in *vivo* and *in vitro* [32, 33] and all of them are synthesised from methionine [33 29]. Therefore, the absence of this amino acid in the n-IDY permeate is in

444 agreement with the lack of sulphur-containing compounds in this sample. In synthetic
445 wines, Papadopoulou and Roussis [8] showed that some sulphur-containing compounds,
446 such as N-acetylcysteine are effective at decreasing the rate of reduction of some aroma
447 compounds (including terpenes) during wine aging. On the basis of existent literature
448 and on the chemical structure of the sulphur-containing compounds identified in g-IDY,
449 the involvement of these compounds in the antioxidant activity determined in the model
450 wines spiked with the g-IDY permeate seems plausible. However, the absence of
451 sulphur-containing compounds in the n-IDY permeate might indicate that the
452 antioxidant effect determined in n-IDY-W model wines should be due to compounds
453 from a different nature.

454 **Analysis of nitrogen-containing compounds**

455 Previous works have already shown that free amino acids represent the greatest nitrogen
456 fraction released by IDYs into model wines whose specific composition depends on the
457 type of IDYs [22]. In addition, the antioxidant effect exerted by different types of nitrogen
458 compounds such as peptides and amino acids (other than sulphur-containing amino
459 acids) have also been described [34, 35]. Therefore, in order to determine which other
460 chemicals might be responsible for the antioxidant effect found in both permeates, their
461 nitrogen composition was determined. **Table 3** shows the content of total free amino
462 acids, free amino acids and peptides, and individual amino acids determined by RP-
463 HPLC-FL. As it can be seen, both permeates exhibited important qualitative and
464 quantitative differences. Firstly, the content of free amino acids was clearly higher in
465 the g-IDY permeate (2964 mg N/L) than in the n-IDY permeate (1248 mg N/L).
466 However, n-IDY permeate was richer in N from peptides. Besides, the amino acid
467 profile showed important differences between IDYs. For instance, the major amino
468 acids in g-IDY permeate were glutamic acid (62.2 mg/L), threonine (56.27 mg/L) and

469 β-alanine (45.82 mg/L), whilst histidine (56.39 mg/L), glycine (33.15 mg/L), and lysine
470 (21.99 mg/L) were most abundant in the permeate from n-IDY. Some amino acids have
471 been associated to relatively important radical scavenging activities in the order
472 tryptophan > tyrosine > methionine > cysteine > phenylalanine [34]. In this sense, only
473 tyrosine was detected in the free form in both permeates, although in a relatively low
474 concentration (5.6 and 1.05 mg/L for g-IDY and n-IDY permeates, respectively),
475 whereas phenylalanine and tryptophan were not detected in any of the samples.
476 Corroborating the previous results obtained by CE-MS, methionine was only identified
477 in the g-IDY permeate (1.55 mg/L). However, the analytical method employed did not
478 allow the detection of cysteine, although its sole presence in the g-IDY permeate was
479 previously confirmed by CE-MS analysis. Therefore, considering the amino acidic
480 profile, the contribution of free amino acids to the total antioxidant activity of g-IDY
481 and n-IDY permeates did not seem very relevant, meaning that there were still other
482 compounds which should be more related to this activity.

483 **Non-targeted metabolomic ESI FT-ICR MS analysis**

484 Direct infusion ESI-FT-ICR-MS was further employed to gain insight on the chemical
485 metabolites responsible for the antioxidant effect exerted by the two permeates. This
486 technique has been proposed as one of the best techniques to directly investigate
487 complex natural mixtures [36] due to the high mass resolving power and mass accuracy. It
488 has also been recently applied to food materials such as coffee [37] and other metabolomic
489 studies of natural products [38].

490 **Figures 3a** and **3b** show the ESI-FT-ICR-MS spectra from g-IDY and n-IDY permeates
491 respectively. Although they were acquired in the positive and negative ion mode, figure
492 3 only depicts the MS from the positive mode. Visually, it is possible to see, that both

493 MS profiles were substantial different. This is in good agreement with the more through
494 ion identification study that was performed and summarized in **Table 4.** Using positive
495 and negative ionization modes it was possible to tentatively identify a total of 10
496 compounds, in which eight of them were detected in the g-IDY permeate and only four,
497 in the n-IDY permeate. Some of the identified compounds were sulphur containing
498 compounds, such as S-glutathionyl-L-cyteine, γ-glutamyl-cystine and oxidized
499 glutathione which were already identified by CE-MS in the g-IDY sample. In addition,
500 the ion 556.1379 m/z was identified as a biotinil-5-AMP, an intermediary in the
501 synthesis of biotine [39]. This compound was already detected but not identified by CE-
502 MS in the g-IDY permeate. In any case, in agreement with the results obtained from
503 other analytical techniques (HPLC-FL, CE-MS), there were non sulphur-containing
504 compounds in n-IDY permeate. However, a very interesting finding was the detection in
505 both samples of some small peptides, specifically tripeptides. Two of them, were found
506 in both permeates and were tentatively identified as Histidine/Cysteine/Lysine and a
507 Methionine/Lysine/Histidine containing peptides. Their MS and chemical structures are
508 shown in **figure 4.** Because of their low concentration in the sample it was not possible
509 to confirm their sequence. Even more interestingly, was the finding of another two
510 peptides only in n-IDY permeate containing Methionine/Aspartic/Triptophane and
511 Tyrosine/Histidine/Methionine (**Figure 4**). It is worth mentioning that the antioxidant
512 properties of small peptides, mainly contained in fermented food, have been extensively
513 documented [35]. As it was previously commented, small peptides containing tryptophan,
514 tyrosine, methionine, cysteine and phenylalanine have been described to exhibit a high
515 antioxidant activity [34]. In the present study, from the two peptides detected in the
516 permeates from g-IDY and n-IDY, only one (histidine/cysteine/lysine) had an amino
517 acid (cysteine) which could be involved in the antioxidant properties. However, the two

518 peptides exclusively identified in the n-IDY permeate (methionine/aspartic
519 acid/tryptophane and tyrosine/histidine/methionine), contained two of these amino acids
520 each. Even more, both peptides contained tryptophan and tyrosine, the two highest
521 antioxidant amino acids. Previously published works have already described the
522 biological activities (antioxidant, antihypertensive) of peptides from yeast origin found
523 in synthetic wines submitted to autolytic conditions [40] and in red wines [41, 42], although
524 their chemical structure remained unresolved. Moreover, considering these results, the
525 preservation of aroma and reduction of aroma loss in wines aged on lees that has been
526 linked to the GSH released by yeast autolysis [6] might also be attributable to other types
527 of small peptides, which could have even higher antioxidant properties than GSH.

528 In conclusion, it has been proven that the use of IDY preparations (with or without
529 GSH) reduce the loss of certain terpenes during the accelerated aging of model wines. It
530 has also been shown that g-IDY preparations do in fact contain GSH in its reduced state
531 which can contribute to the aroma preservation in model wines, but they also contain
532 other sulphur compounds of yeast origin that might also act as antioxidants. In addition,
533 both g-IDY and n-IDY contained small peptides (tripeptides) with methionine,
534 tryptophan and tyrosine, which seem to be involved in the antioxidant properties
535 determined in the permeates isolated from both IDYs also being effective in the
536 preservation of some terpenes during model wine aging. Taking into consideration the
537 instability of GSH in wines (easily oxidized, fast combination with polyphenols, etc),
538 this finding could be of technological interest assuming the higher stability of these
539 antioxidant peptides when used for example, after wine bottling. The oncoming work
540 will be directed to unequivocally identify the sequence of these compounds and further
541 studies are needed to confirm the antioxidant effect of these peptides in closer
542 winemaking conditions. Undoubtedly, this will be interesting for the wine and

biotechnological industry in order to redirect the formulation of IDY preparations to achieve specific and effective winemaking applications.

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727 **FIGURE CAPTIONS:**

728 **Figure 1.** Percentage of decrease in the relative peak area of the aroma compounds in
729 the model wines submitted to accelerated aging conditions (wines supplemented with
730 the <3kDa permeates isolated from g-IDY and n-IDY, wines added with 10 mg/L of
731 commercial glutathione and control wines without any treatment) compared to the
732 original model wines (0 days). Results of ANOVA and LSD test are indicated with
733 different letters (a-c). ns: no significant differences

734 **Figure 2.** CE-TOF-MS extracted ion electropherograms (EIEs) of (A) the 14 most
735 abundant sulphur-containing compounds, and (B) methionine and cysteine from g-IDY
736 and n-IDY samples. Continuous line for g-IDY permeate and dotted line for n-IDY
737 permeate, are used. See Section 2.9 for experimental conditions.

738 **Figure 3.** ESI (+) FT-ICR MS of the <3kDa permeate from g-IDY (a) and n-IDY (b)
739 samples.

740 **Figure 4**. Chemical structures and EI-FT-ICR MS corresponding to the peptides
741 identified in the <3kDa permeates from g-IDY and n-IDY. (Met/Asp/Trp and Tyr His
742 Met) were only identified in the permeate from n-IDY

743

744 **TABLES:**

745 **Table 1.** Concentration of total GSH, reduced GSH and γ-Glutamyl-cysteine
746 determined in the <3kDa permeates isolated from g-IDY and n-IDY preparations

IDY preparation	Total GSH (mg/L)	Reduce GSH (mg/L)	γ-Glutamyl-cysteine (mg/L)
g-IDY	3147 ± 118	1293 ± 76	873 ± 63
n-IDY	n.d.	n.d.	n.d.

747

748

Table 2. Tentative identification of sulphur-containing compounds found in the <3kDa permeates isolated from g-IDY and n-IDY preparations after CE-MS analysis.

Compound	Time (min)	Peak area (g-IDY)	Peak area (n-IDY)	m/z (exp)	m/z (thr)	Error (ppm)	Formula	Tentative ID	HMDB* code
1	12.03	1489462	204104	385.1318	385.1289	7.6	$C_{14}H_{20}N_6O_5S$	S-Adenosylhomocysteine	HMDB00939
2	12.45	2807046	ND*	371.1182				NF*	
3	12.68	3650062	782426	223.0792				NF	
4	12.75	1427597	ND	237.091	237.09034	-2.7	C8H16N2O4S	Methionyl-Serine, Serinyl-Methionine, S-aminomethyldihydrolipoamide	HMDB29045, HMDB29045, HMDB06239
5	14.49	891150	740927	298.1011	298.0968	-14.3	C11H15N5O3S	5'-Methylthioadenosine	HMDB01173
6	14.87	2142027	ND	370.0768				NF	
7	15.41	2245896	ND	427.0974	427.0952	-5.2	C13H22N4O8S2	S-Glutathionyl-L-Cysteine	HMDB00656
8	15.74	3898205	ND	499.1182	499.116311	-3.8	C16H26N4O10S2	N,N'-Bis (γ-glutamyl)cystine	HMDB38458
9	16.18	7992966	ND	556.1404	278.5817			NF	
10	16.55	6240395	ND	613.16, 307.0921	613.1592	-2.5	C20H32N6O12S2	Oxidized glutathione	HMDB03337
11	17.43	5710438	ND	251.0716	251.0696	-7.9	C8H14N2O5S	γ-Glutamylcysteine	HMDB01049
12	17.9	369996	ND	454.096				NF	
13	17.98	2125131	ND	148.0446				NF	
14	18.37	6861257	ND	308.0952	308.0911	-13.4	C10H17N3O6S	Glutathione	HMDB00125

*ND, not detected
*NF, not found
*HMDB, Human Metabolome Database (http://www.hmdb.ca)

Table 3. Amino acidic composition of the <3 kDa permeates isolated from g-IDY and n-IDY preparations

	g-IDY		n-IDY	
Amino acids (mg/L)	Mean	±SD	Mean	±SD
Free amino acids	2964	342	1248	22
Free amino acids and peptides	2865	29	1604	74
Aspartic acid	19.56	0.51	18.66	0.48
Glutamic acid	62.21	2.31	n.d.	
Asparragine	7.88	0.17	5.17	0.11
Serine	15.75	0.53	6.60	0.22
Glutamine	21.57	0.37	10.62	0.18
Histidine	24.53	0.65	56.40	1.50
Glycine	n.d.		33.15	1.08
Threonine	56.43	1.51	12.77	0.34
Arginine	8.79	0.22	6.95	0.17
β-Alanine	45.82	0.32	4.43	0.03
α-Alanine	n.d.		3.41	0.10
γ-Aminobutitic acid	4.01	0.14	4.05	0.14
Tyrosine	5.70	0.08	1.05	0.01
α-Aminobutiric acid	2.61	0.02	2.51	0.02
Methionine	1.59	0.01	n.d.	
Valine	2.32	0.05	1.76	0.04
Phenylalanine	n.d.		n.d.	
Tryptophan	n.d.		n.d.	
Isoleucine	4.38	0.11	4.67	0.11
Leucine	n.d.		n.d.	
Ornithine	27.75	0.48	5.43	0.09
Lysine	31.47	0.54	22.00	0.37

Table 4. Tentative identification of the compounds found in the <3kDa permeates isolated from g-IDY and n-IDY preparations after FT-ICR-MS analysis.

m/z (exp)	m/z (thr)	Error (ppm)	Formula	g-IDY	n-IDY	Tentative ID	METLIN ID	HMDB code
399.1447	399.1451	1.0	$C_{15}H_{22}N_6O_5S + H$			S-Adenosylmethionine	6064	HMDB01185
425.1137	425.1131	1.4	$C_{17}H_{22}N_4O_8S + H - H_2O$	*		S-(4-Nitrobenzyl)glutathione	4098	
425.1364	425.1368	0.9	$C_{15}H_{26}N_6O_4S + K$	*	*	His Cys Lys	21589	
427.0949	427.0952	0.7	$C_{13}H_{22}N_4O_8S_2 + H$	*		S-Glutathionyl-L-cysteine	63433	
431.1416	431.1394	5.1	$C_{20}H_{26}N_4O_6S - H_2O - H$		*	Met Asp Trp	18421	
453.1675	453.1681	1.3	$C_{17}H_{30}N_6O_4S + K$	*	*	Met Lys His	21819	
470.1526	470.1479	10.0	$C_{20}H_{27}N_5O_5S + Na - 2H$		*	Tyr His Met	18106	
499.1173	499.1163	2.0	$C_{16}H_{26}N_4O_{10}S_2 + H$	*		N,N'-Bis-γ-glutamylcystine	63634	
556.1371	556.1379	1.4	$C_{20}H_{28}N_7O_9PS + H - H_2O$	*		Biotinyl-5'-AMP	58228	HMDB04220
561.3273	561.3235	6.8	$C_{28}H_{50}N_4O_3S + K$	*		Oleic Acid-biotin	45287	
613.1599	613.1598	0.2	$C_{20}H_{32}N_6O_{12}S_2 + H$	*		Oxidized Glutathione	45	

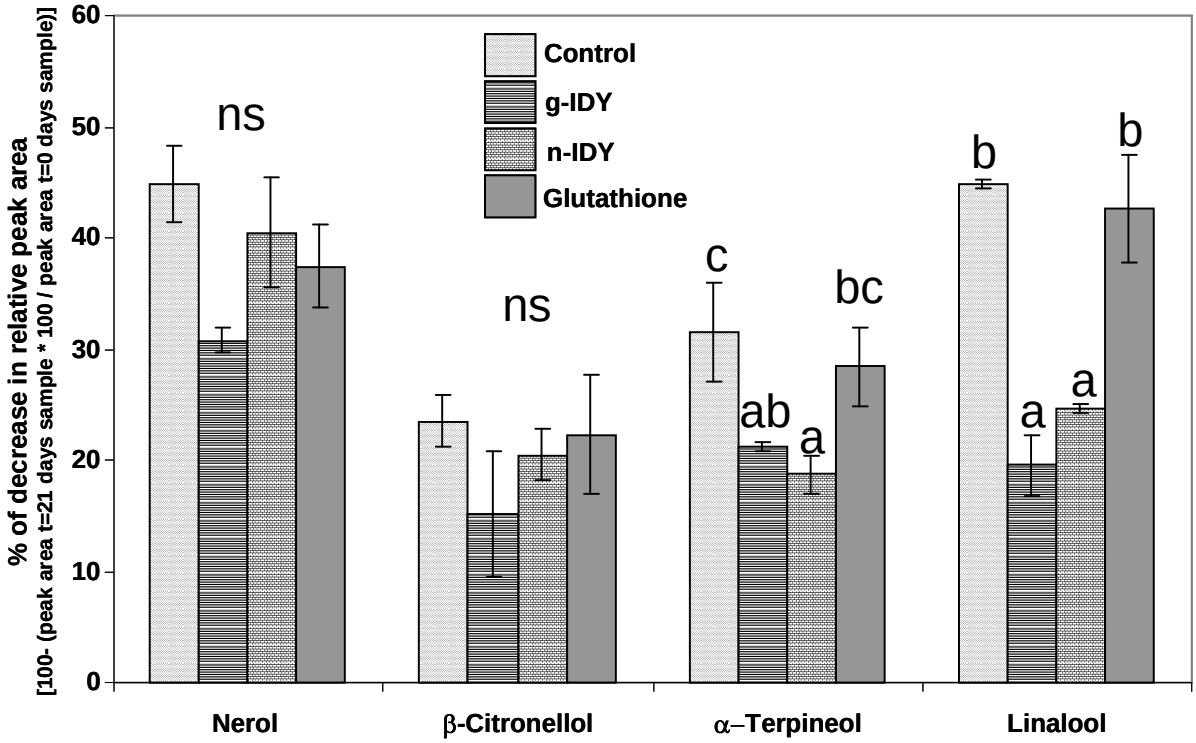

% of decrease in relative peak area
[100- (peak area t=21 days sample * 100 / peak area t=0 days sample)]
60
50
40
30
20
10
0
Control
g-IDY
n-IDY
Glutathione
ns
ns
c
ab
a
bc
b
a
a
b
Nerol
β-Citronellol
α–Terpineol
Linalool

Figure 1

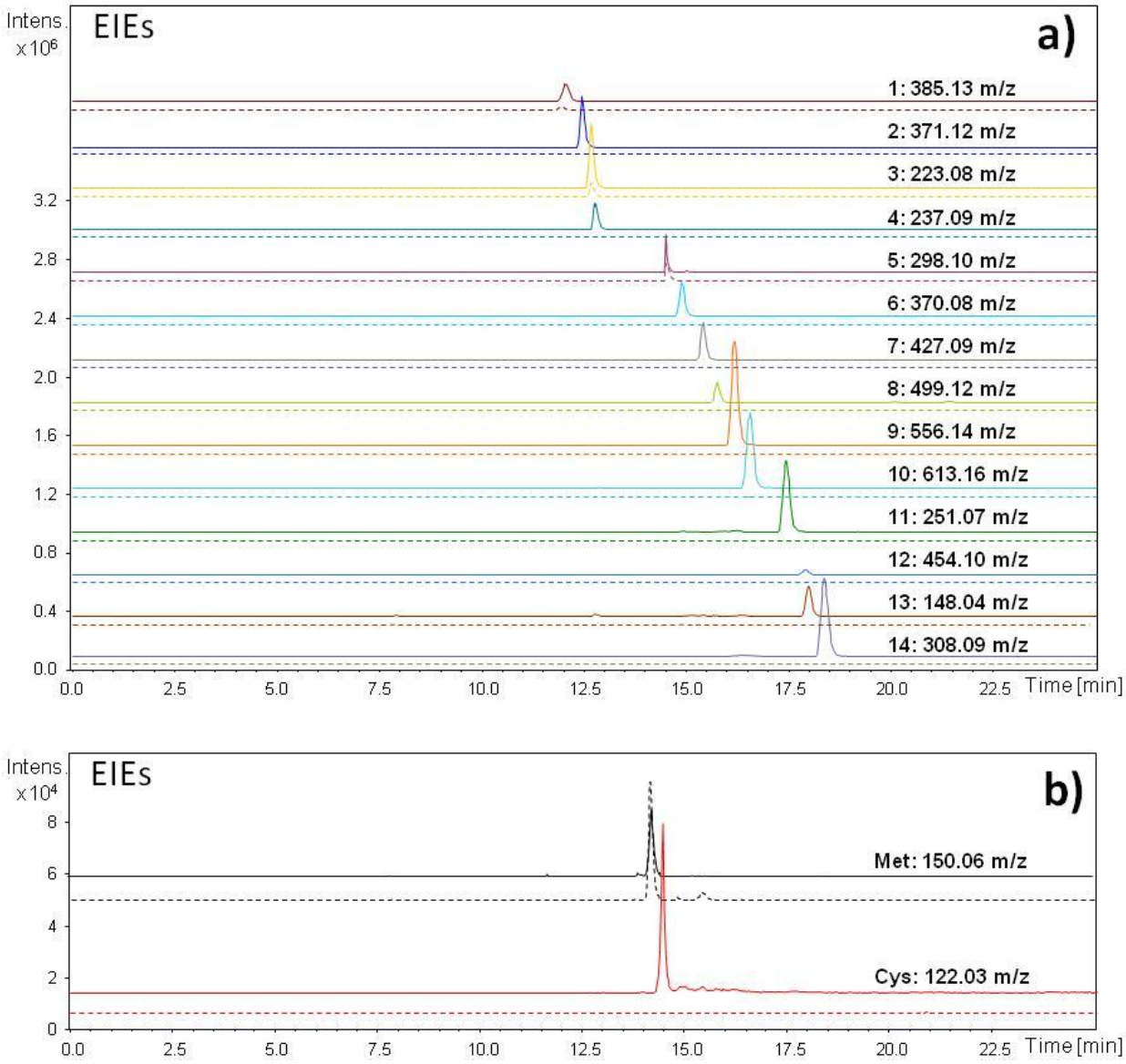

Intens.
x10^6
EIEs
a)
1: 385.13 m/z
2: 371.12 m/z
3: 223.08 m/z
4: 237.09 m/z
5: 298.10 m/z
6: 370.08 m/z
7: 427.09 m/z
8: 499.12 m/z
9: 556.14 m/z
10: 613.16 m/z
11: 251.07 m/z
12: 454.10 m/z
13: 148.04 m/z
14: 308.09 m/z
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
0.0 2.5 5.0 7.5 10.0 12.5 15.0 17.5 20.0 22.5 Time [min]
Intens.
x10^4
EIEs
b)
Met: 150.06 m/z
Cys: 122.03 m/z
8
6
4
2
0
0.0 2.5 5.0 7.5 10.0 12.5 15.0 17.5 20.0 22.5 Time [min]

Figure 2

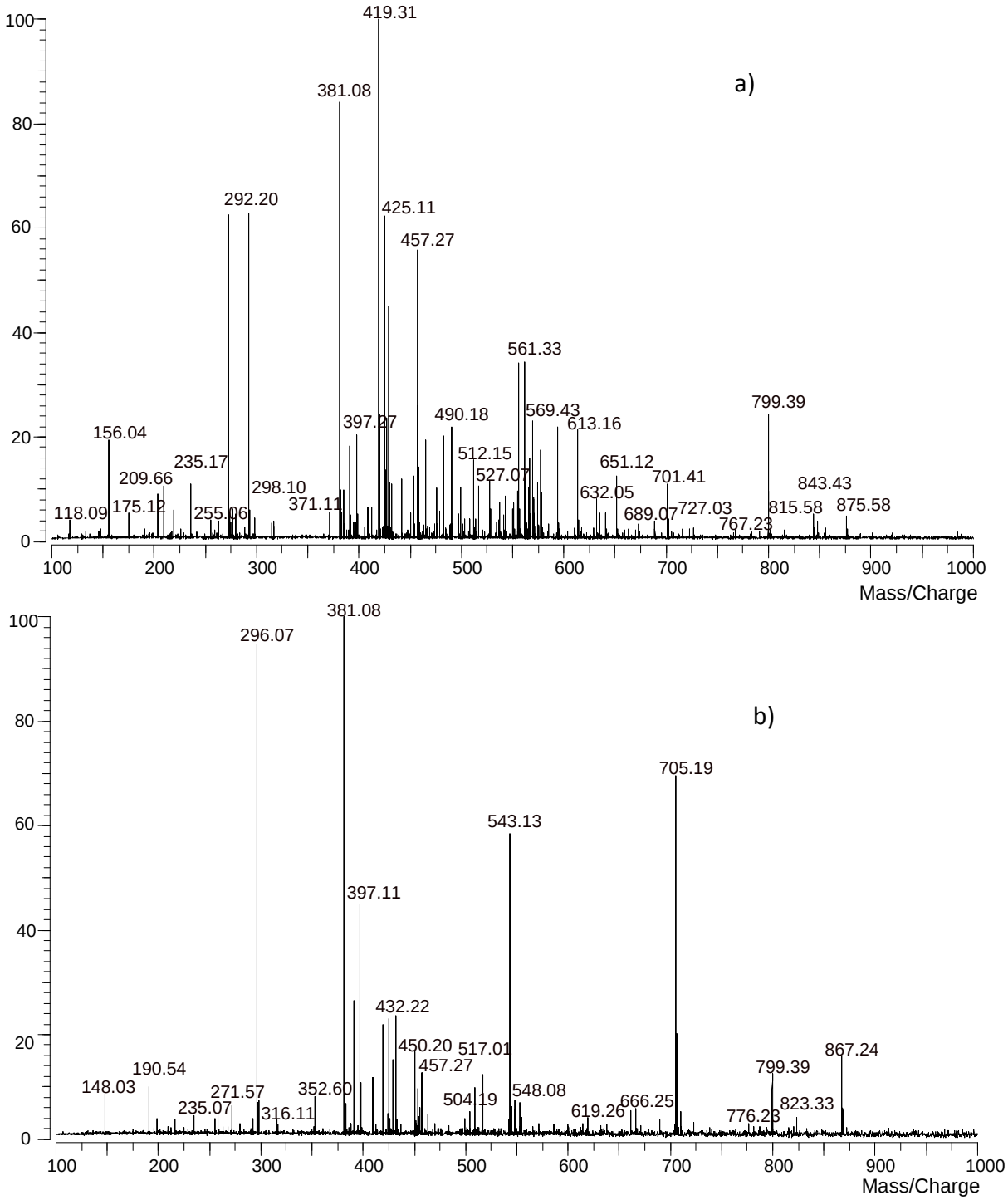

a)
419.31
381.08
292.20
425.11
457.27
561.33
799.39
490.18
569.43
613.16
397.27
156.04
512.15
651.12
235.17
527.07
701.41
209.66
298.10
632.05
843.43
118.09
175.12
255.06
371.11
689.07
727.03
815.58
875.58
767.23
Mass/Charge
b)
381.08
296.07
705.19
543.13
397.11
432.22
450.20
517.01
867.24
190.54
457.27
799.39
148.03
271.57
352.60
548.08
504.19
666.25
235.07
316.11
619.26
823.33
776.23
Mass/Charge

Figure 3.

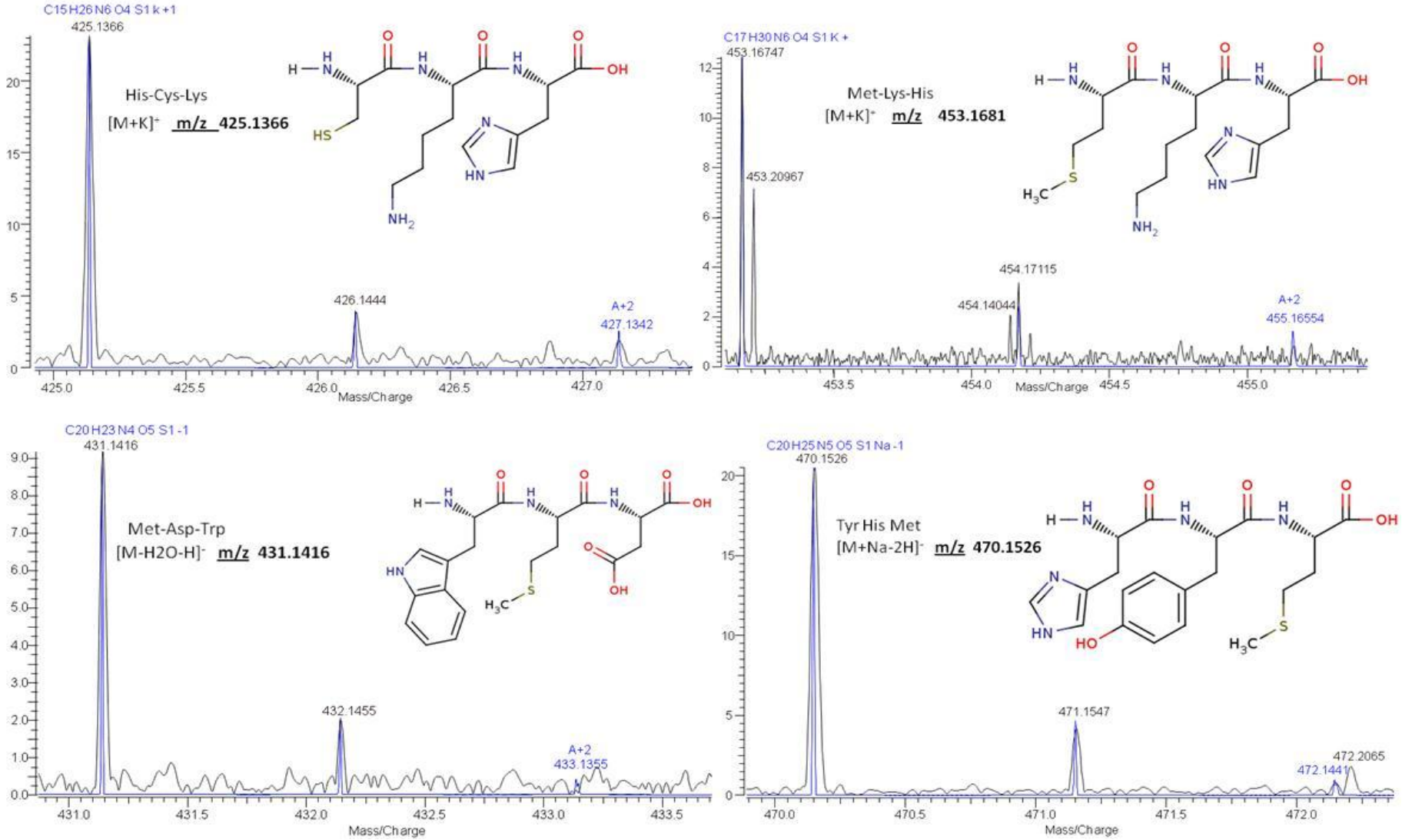
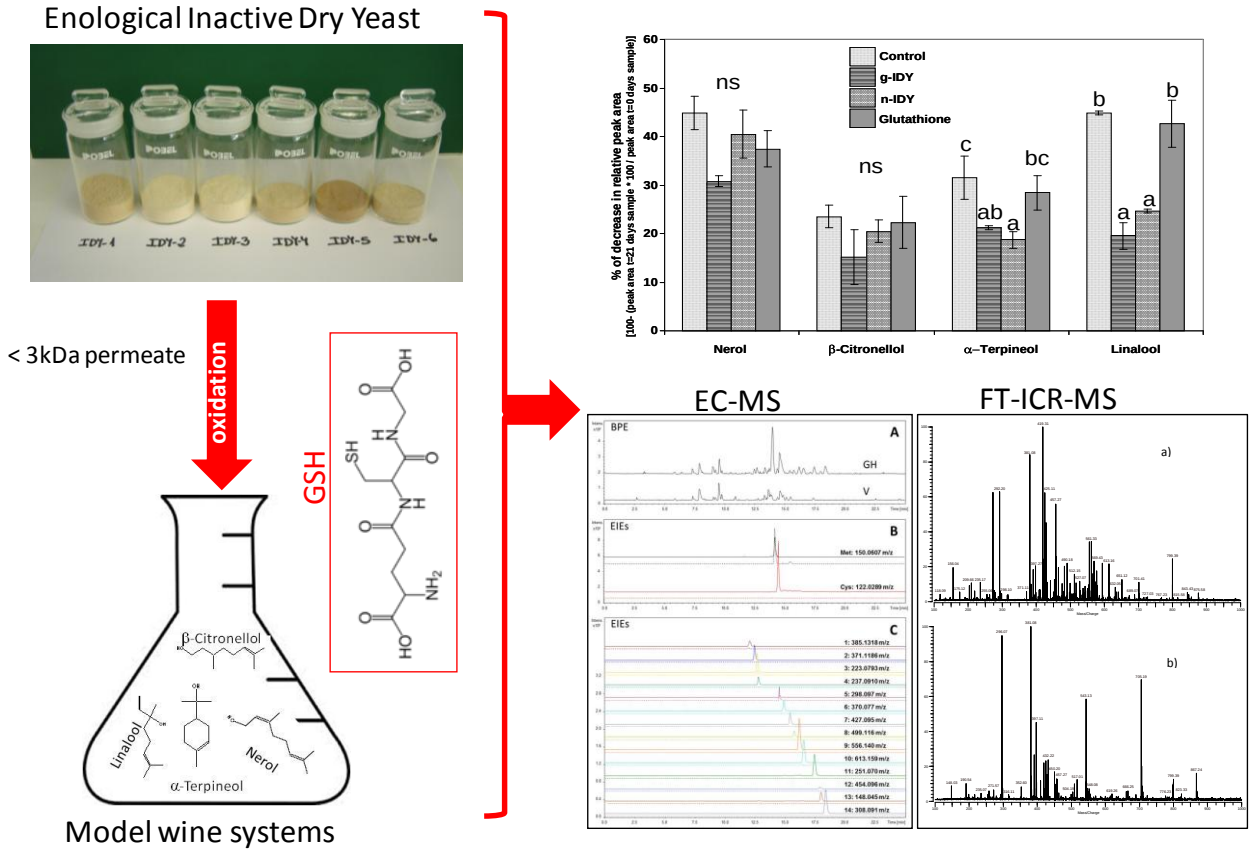

C15 H26 N6 O4 S1 k +1
425.1366
His-Cys-Lys
[M+K]+ m/z 425.1366
426.1444
A+2
427.1342
Mass/Charge
C17 H30 N6 O4 S1 K +
453.16747
Met-Lys-His
[M+K]+ m/z 453.1681
453.20967
454.17115
454.14044
A+2
455.16554
Mass/Charge
C20 H23 N4 O5 S1 -1
431.1416
Met-Asp-Trp
[M-H2O-H]- m/z 431.1416
432.1455
A+2
433.1355
Mass/Charge
C20 H25 N5 O5 S1 Na -1
470.1526
Tyr His Met
[M+Na-2H]- m/z 470.1526
471.1547
472.2065
472.1441
Mass/Charge

Figure 4

TOC graphic


Enological Inactive Dry Yeast
< 3kDa permeate
oxidation
GSH
β-Citronellol
Linalool
Nerol
α-Terpineol
Model wine systems
Control
g-IDY
n-IDY
Glutathione
% of decrease in relative peak area
[100- (peak area t=21 days sample * 100) / peak area t=0 days sample]
ns
ns
c
ab
a
bc
b
a
a
b
Nerol
β-Citronellol
α-Terpineol
Linalool
EC-MS
FT-ICR-MS