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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · JANUARY 2014

Impact Factor: 2.91 · DOI: 10.1021/jf402866q · Source: PubMed

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

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 nontargeted metabolomics approaches using CE-MS and FT-ICR-MS analytical platforms. The findings suggest that the addition of <3 kDa permeate isolated from any of the IDYs employed decreases 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 sulfur-containing compounds from yeast origin in g-IDY, and 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 to 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 white wines. The loss of pleasant aromatic notes produced as a consequence of the decrease of important aroma compounds such as polyfunctional thiols, terpenes, and esters[1−3] and the accumulation of other undesirable compounds (hydrogen sulfide, methyl mercaptans)[4] in wines with low exposure to oxygen, which produce the so-called "reduced off flavor", are mainly responsible for the depreciation in the quality of the wines.

Sulfur 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 sulfites, 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 sulfur-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 to 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 sulfur-containing compounds, such as *N*-acetylcysteine, also have a protective effect against wine aroma oxidation.[14,15]

Despite these promising results, the addition of GSH to 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

Received: July 1, 2013
Revised: January 13, 2014
Accepted: January 22, 2014

dx.doi.org/10.1021/jf402866q | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

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. Although, as 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 nontreated 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 the stimulating effect of amino acids and other peptides from the IDY on 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 nontargeted 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 enological IDY preparations were selected for being representative of the current preparations in the enological 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 an 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 evidence 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 was weighed into 50 mL centrifuge tubes. Samples were extracted with 50 mL of water/ethanol solution (87:13, v/v) in an ultrasonic bath (3 cycles, 5 min each) at 4 °C. The mixture was then centrifuged (15 min at 5000*g* and 10 °C), and the supernatant was ultrafiltered using a Centricon device (Amicon Inc., Beverly, MA, USA) with a 10 kDa cutoff membrane. The obtained permeates were submitted to a second ultrafiltration step through a 3 kDa cutoff membrane Centricon (Amicon Inc.). Both ultrafiltration steps were carried out at cold temperature (below 10 °C). The obtained <3 kDa permeates from each IDY preparation were freeze-dried and kept at −20 °C until use. Prior to chemical characterization, permeates were reconstituted with water to 100 mg/mL dry residue. Only for FT-ICR-MS analysis were reconstituted samples dialyzed by using a Float-a-Lyzer G2 device with a 0.1−0.5 kDa cutoff 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-citronellol, α-terpineol, and linalool) from Sigma (Stenheim, Germany) at a final concentration of 25 mg/L each. Finally, 100 μL of the reconstituted <3 kDa fractions isolated from g-IDY or n-IDY at 100 mg/mL was added. In addition, another set of model wines were individually aromatized 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 analyzed at the beginning of the experiment (t = 0 days). The different model wine mixtures were submitted to an accelerated oxidation process during 3 weeks at 25 °C, saturating the headspace of the vials with oxygen (t = 21 daus). All of 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 headspace solid-phase microextraction coupled to gas chromatography−mass spectrometry (HS-SPME-GC-MS). Model wine samples (8 mL), 2.3 g of NaCl, and 40 μL 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 fiber of 2 cm length from Supelco (Bellefonte, PA, USA). Samples were preincubated 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 fiber was cleaned for 20 min to avoid any memory effect. The chromatographic separation was performed in a GC-MS instrument (Agilent 6890GC, 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 increased at 4 °C/min to 240 °C, and held at 240 °C 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, 59, 69, and 69 for linalool, α-terpineol, β-citronellol, and nerol, respectively) was calculated by the data system. 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 of the aroma compounds except for nerol, which 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, USA), a WISP 710B autosampler (Waters), and a HP 104-A fluorescence detector (Hewlett-Packard, Palo Alto, CA, USA) was used following the procedure previously optimized and validated.[18] The mobile phase was composed of methanol (Lab-Scan, Sowinskiego, Poland) and an aqueous solution of phosphate buffer (10 mM $NaH_2PO_4 \cdot 12H_2O$ 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 °C as follows: to a sample vial were added 105 μL from the dithiotreitol (Sigma-Aldrich) solution vial [5 mM and 0.5 mM in

dx.doi.org/10.1021/jf402866q | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

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-naphthalenedialdehyde (NDA) (Sigma-Aldrich) solution (5 mg mL^{-1} in ethanol); then, two mixture cycles of the total content of the insert were carried out, and 100 μL was injected in the chromatographic system. Separation was carried out on a Nova Pack C18 (150 mm × 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 ($\lambda_{excitation}$ = 467 nm, $\lambda_{emission}$ = 525 nm). The derivatization conditions for the determination of γ-glu-cys were the same as previously described. Calibrations were carried out by using pure standard compound solutions of GSH and γ-glu-cys. The analysis of the samples was made in duplicate.

ORAC-Fluorescein (ORAC-FL) Assay. The antioxidant capacity of IDY permeates and GSH was measured by ORAC-FL assay based on that proposed previously.[24] Briefly, the reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4), and the final assay mixture (200 μL) contained FL (70 nM), AAPH (12 mM), and antioxidant [Trolox (1−8 μM) or sample at different concentration]. The plate was automatically shaken before the first reading, and the fluorescence was recorded every minute for 80 min. 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 Fluostar Galaxy software (version 4.11-0) for fluorescence measurement. Black 96-microwell microplates (96F untreated, Nunc, Denmark) were used. AAPH and Trolox solutions were prepared daily, and FL was diluted from a stock solution (1.17 mM in 75 mM phosphate at pH 7.4). Fluorescence measurements were normalized to the curve of the blank (no antioxidant). From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated. 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 micromoles of Trolox equivalent per milligram of dry permeate (for the <3 kDa permeates from g-IDY and n-IDY) and in micromoles of Trolox equivalent per milligram of 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 milligrams N/L. All of the model wine samples were analyzed in duplicate.

Analysis of Amino Acids by RP-HPLC-FL. Amino acids were analyzed in duplicate by reversed-phase HPLC using a liquid chromatograph as described under Analysis of Reduced Glutathione (GSH), Total Glutathione, and γ-Glutamylcysteine (γ-glu-cys) by RP-HPLC-FL. 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 (150 × 3.9 mm i.d., 60 A, 4 μm) column and the same type of precolumn. Detection was performed by fluorescence ($\lambda_{excitation}$ = 340 nm, $\lambda_{emission}$ = 425 nm).

Analysis of Sulfur-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, UK). 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. Electrophoretic separation was achieved by 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 the ESI chamber temperature was maintained at 250 °C. Spectra were acquired in the m/z 50−700 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: m/z 90.9766, 158.9641, 226.9515, 294.9389, 362.9263, 430.9138, 498.9012, and 566.8886. 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 bond equivalents, as well as a comparison between the theoretical and experimental isotopic pattern (Sigma-Value) for increased confidence in the theoretical molecular formula assignment.

Nontargeted Metabolomic Analysis by FT-ICR MS. FT-ICR MS was used to obtain ultrahigh-resolution (>100,000) mass spectra. Separation and identification of the metabolites were possible without the need of chromatography or derivatization due to the ultrahigh mass accuracy. Commercial beer maltooligosaccharides were used as mass calibrants and tuning 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 as follows: 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 negative mode MS parameters were as follows: −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, defining a mass range from m/z 100 to 1000. The internal detection signal in the cell detector of the FT-ICR was optimized for a mass of m/z 500.

Monoisotopic mass and isotope cluster 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 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

dx.doi.org/10.1021/jf402866q | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

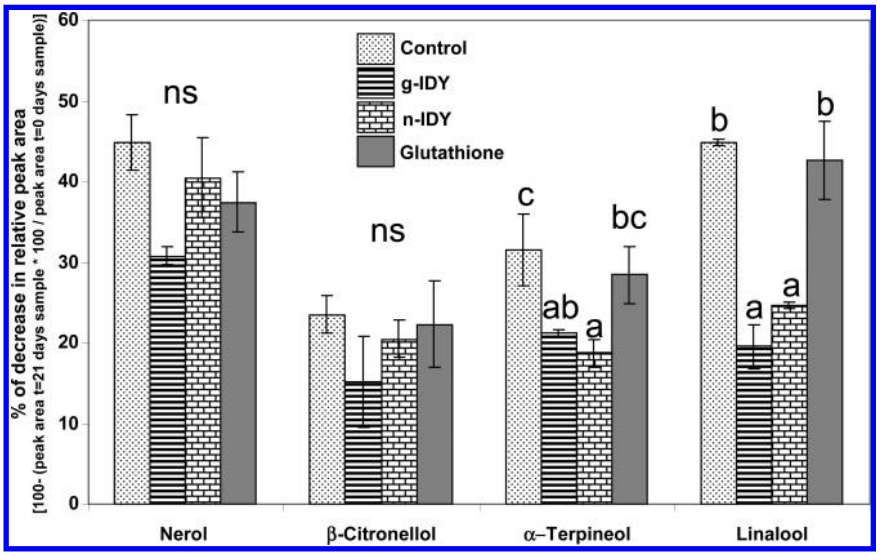

Figure 1. Percentage of decrease in the relative peak area of the aroma compounds in the model wines submitted to accelerated aging conditions (wines supplemented with the <3 kDa permeates isolated from g-IDY and n-IDY, wines with added 10 mg/L of commercial glutathione, and control wines without any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

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 whether the GSH released from IDYs into the wines might have a role in the behavior 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 under IDY Samples. Model wines spiked with this permeate were coded g-IDY-W. Ultrafiltration ensured the removal of glycoproteins (with molecular masses >3 kDa) 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 with added commercial GSH (10 mg/L) referred to as 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 aromatized 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 behavior of the four terpene compounds was evaluated in all of the model wines at the beginning of the experiment (t = 0 days), corresponding to nonoxidized model wines, and after 3 weeks of accelerated aging conditions (t = 21 days). Figure 1 shows the percentage of decrease in relative peak area (RPA) between the initial wine sample (nonoxidized) and the wines after 21 days of aging for each aroma compound and wine type. As can be seen, there was a general decrease in RPAs for all of 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 control rosé wines (without IDY added) and rosé wines produced in cellar conditions with the same type of g-IDY after 9 months of aging.[20]

However, the addition of commercial GSH to the model wine solutions did not have a significant effect under the essayed conditions. Different published works have shown an inhibition of the decline of certain aroma compounds when using GSH at a similar or even lower dosage in wines or model wine systems.[4,6−9] Nonetheless, it is well-known that the main effect of GSH in wines is its ability to react with o-quinones produced by oxidation of caftaric acid (and other polyphenols) to give grape reaction product (GRP) by the action of polyphenol oxidases blocking the subsequent steps in which polyphenols are involved (polymerization) and responsible for browning.[4,6,17] Moreover, o-diphenols can be directly oxidized in the presence of oxygen and some cations (iron, copper) to o-quinones and hydrogen peroxide, which might be involved in subsequent aroma oxidation.[4] In our experimental conditions this action mechanism was limited because of the absence of polyphenols to react with GSH in the model wine, which seems to explain the absence of a noticeable effect of GSH on aroma protection in the model wines supplemented with commercial GSH. However, GSH presents scavenging hydroperoxide and hydroxyl radical properties, which might have allowed it to act as an antioxidant by other mechanisms different from its capacity to interact with o-quinones in polyphenol-free systems.[10,11] Nonetheless, on the basis of our results, this mechanism did not seem as significant in our experimental conditions. Despite this, it is important to highlight that this model system allowed us to uncover the potential role of other

dx.doi.org/10.1021/jf402866q | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

yeast components, different from GSH and contained in the <3 kDa fraction, which seemed to be related to the protection of some terpenes in model 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 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, despite 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-glutamylcysteine were analyzed by using a previously optimized RP-HPL-FL method.[18] These results are shown in Table 1. As 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 enological IDY preparations, only those claimed to be GSH-enriched IDY preparations released reduced GSH into synthetic wines.[18] With the amount of each permeate added into the model wines (100 μL) taken into consideration, the final amounts of reduced and total GSH in g-IDY-W model wines were 2.6 and 6.3 mg/L, respectively, which are 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) was 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 the attempt to elucidate these compounds, a comprehensive chemical characterization of both g-IDY and n-IDY permeates was carried out.

Table 1. Concentration of Total GSH, Reduced GSH, and γ-Glutamyl-cysteine Determined in the <3 kDa Permeates Isolated from g-IDY and n-IDY Preparations

IDY preparation	total GSH (mg/L)	reduced GSH (mg/L)	γ-glutamyl-cysteine (mg/L)
g-IDY	3147 ± 118	1293 ± 76	873 ± 63
n-IDY	nd[a]	nd	nd

[a]nd, not detected.

Analysis of Other Sulfur-Containing Compounds by CE-MS. In addition to GSH, other biological sulfur-containing compounds have been said to present antioxidant properties.[32] Thus, the analysis of other low molecular weight sulfur-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 sulfur 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 sulfur. After further inspection of n-IDY and g-IDY CE-MS profiles, besides the 2 sulfur-containing amino acids methionine and cysteine, another 14 highly abundant sulfur-containing compounds were found in the permeate from the g-IDY sample. In Figure 2, extracted ion electropherograms (EIEs) from these two amino acids and the most abundant sulfur-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 can be seen in this table, 8 of 14 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 sulfur compounds, including the sulfur-containing amino acids, have been shown to exhibit antioxidant properties in vivo and in vitro,[32,33] and all of them are synthesized from methionine.[29,33] Therefore, the absence of this amino acid in the n-IDY permeate is in agreement with the lack of sulfur-containing compounds in this sample. In synthetic wines, Papadopoulou and Roussis[8] showed that some sulfur-containing compounds, such as *N*-acetylcysteine, are effective at decreasing the rate of reduction of some aroma compounds (including terpenes) during wine aging. On the basis of existent literature and the chemical structure of the sulfur-containing compounds identified in g-IDY, the involvement of these compounds in the antioxidant activity determined in the model

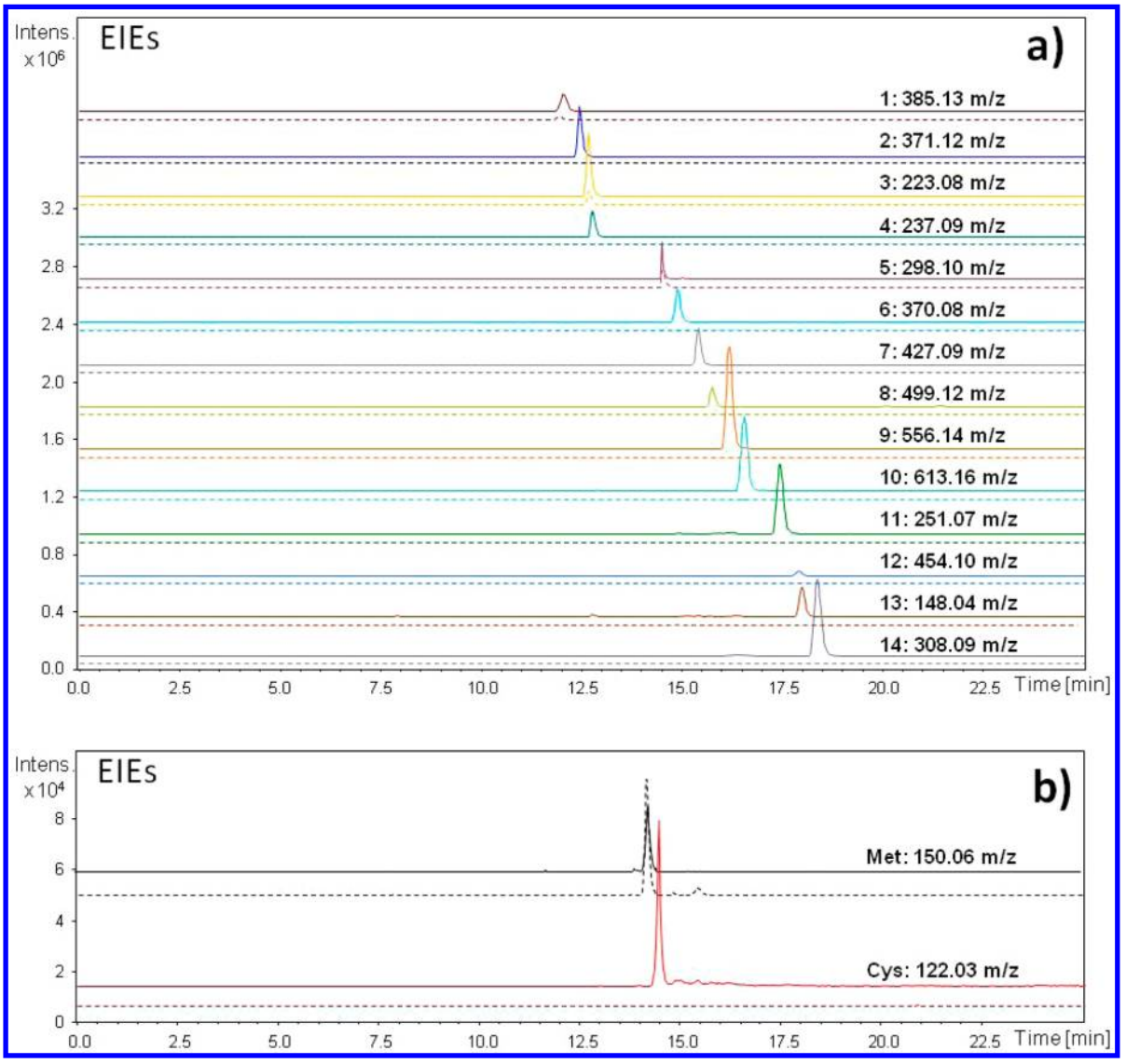

Figure 2. CE-TOF-MS extracted ion electropherograms (EIEs) of (a) the 14 most abundant sulfur-containing compounds and (b) methionine and cysteine from g-IDY and n-IDY samples. A continuous line for g-IDY permeate and a dotted line for n-IDY permeate are used. See Analysis of Sulfur-Containing Metabolites by CE-MS for experimental conditions.

wines spiked with the g-IDY permeate seems plausible. However, the absence of sulfur-containing compounds in the n-IDY permeate might indicate that the antioxidant effect determined in n-IDY-W model wines should be due to compounds from a different nature.

Analysis of Nitrogen-Containing Compounds. Previous works have already shown that free amino acids represent the greatest nitrogen fraction released by IDYs into model wines, the specific composition of which depends on the type of IDYs.[22] In addition, the antioxidant effect exerted by different types of nitrogen compounds such as peptides and amino acids (other than sulfur-containing amino acids) has also been described.[34,35] Therefore, to determine which other chemicals might be responsible for the antioxidant effect found in both permeates, their nitrogen composition was determined. Table 3 shows the content of total free amino acids, free amino acids and peptides, and individual amino acids determined by RP-HPLC-FL. As can be seen, both permeates exhibited important qualitative and quantitative differences. First, the content of free amino acids was clearly higher in the g-IDY permeate (2964 mg N/L) than in the n-IDY permeate (1248 mg N/L). However, the n-IDY permeate was richer in N from peptides. Besides, the amino acid profile showed important differences between IDYs. For instance, the major amino acids in g-IDY permeate were glutamic acid (62.2 mg/L), threonine (56.27 mg/L), and β-alanine (45.82 mg/L), whereas histidine (56.39 mg/L), glycine (33.15 mg/L), and lysine (21.99 mg/L) were most abundant in the permeate from n-IDY. Some amino acids have been associated with relatively important radical scavenging activities in the order tryptophan > tyrosine > methionine > cysteine > phenylalanine.[34] In this sense, only tyrosine was detected in the free form in both permeates, although in relatively low concentrations (5.6 and 1.05 mg/L for g-IDY and n-IDY permeates, respectively), whereas phenylalanine and tryptophan were not detected in any of the samples. Corroborating the previous results obtained by CE-MS, methionine was identified only in the g-IDY permeate (1.55 mg/L). However, the analytical method employed did not allow the detection of cysteine, although its sole presence in the g-IDY permeate was previously confirmed by CE-MS analysis. Therefore, consider-

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Table 2. Tentative Identification of Sulfur-Containing Compounds Found in the <3 kDa Permeates Isolated from g-IDY and n-IDY Preparations after CE-MS Analysis

compd	time (min)	peak area (g-IDY)	peak area (n-IDY)	m/z exptl	m/z theor	error (ppm)	formula	tentative ID	HMDB[a] 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[b]	371.1182				NF[c]	
3	12.68	3650062	782426	223.0792				NF	
4	12.75	1427597	ND	237.091	237.09034	−2.7	$C_8H_{16}N_2O_4S$	methionyl-serine, serinyl-methionine, *S*-aminomethyldihydrolipoamide	HMDB29045, HMDB29045, HMDB06239
5	14.49	891150	740927	298.1011	298.0968	−14.3	$C_{11}H_{15}N_5O_3S$	5′-methylthioadenosine	HMDB01173
6	14.87	2142027	ND	370.0768				NF	
7	15.41	2245896	ND	427.0974	427.0952	−5.2	$C_{13}H_{22}N_4O_8S_2$	*S*-glutathionyl-L-cysteine	HMDB00656
8	15.74	3898205	ND	499.1182	499.116311	−3.8	$C_{16}H_{26}N_4O_{10}S_2$	*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	$C_{20}H_{32}N_6O_{12}S_2$	oxidized glutathione	HMDB03337
11	17.43	5710438	ND	251.0716	251.0696	−7.9	$C_8H_{14}N_2O_5S$	γ-glutamyl-cysteine	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	$C_{10}H_{17}N_3O_6S$	glutathione	HMDB00125

[a]HMDB, Human Metabolome Database (http://www.hmdb.ca). [b]ND, not detected. [c]NF, not found.

Table 3. Amino Acid Composition (Milligrams per Liter) of the <3 kDa Permeates Isolated from g-IDY and n-IDY Preparations

amino acid	g-IDY mean	g-IDY ±SD	n-IDY mean	n-IDY ±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	nd[a]	
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	nd		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	nd		3.41	0.10
γ-aminobutyric acid	4.01	0.14	4.05	0.14
tyrosine	5.70	0.08	1.05	0.01
α-aminobutyric acid	2.61	0.02	2.51	0.02
methionine	1.59	0.01	nd	
valine	2.32	0.05	1.76	0.04
phenylalanine	nd		nd	
tryptophan	nd		nd	
isoleucine	4.38	0.11	4.67	0.11
leucine	nd		nd	
ornithine	27.75	0.48	5.43	0.09
lysine	31.47	0.54	22.00	0.37

[a]nd, not detected.

ing the amino acidic profile, the contribution of free amino acids to the total antioxidant activity of g-IDY and n-IDY permeates did not seem very relevant, meaning that there were still other compounds which should be more related to this activity.

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

Panels a and b of Figure 3 show the ESI-FT-ICR-MS spectra from g-IDY and n-IDY permeates, respectively. Although they were acquired in the positive and negative ion mode, Figure 3 depicts only the MS from the positive mode. Visually, it is possible to see that both MS profiles were substantially different. This is in good agreement with the more thorough ion identification study that was performed and summarized in Table 4. Using positive and negative ionization modes, it was possible to tentatively identify a total of 10 compounds, 8 of which were detected in the g-IDY permeate and only 4 in the n-IDY permeate. Some of the identified compounds were sulfur-containing compounds, such as *S*-glutathionyl-L-cyteine, γ-glutamyl-cystine, and oxidized glutathione, which were already identified by CE-MS in the g-IDY sample. In addition, the ion *m*/*z* 556.1379 was identified as a biotinil-5-AMP, an intermediary in the synthesis of biotin.[39] This compound was already detected but not identified by CE-MS in the g-IDY permeate. In any case, in agreement with the results obtained from other analytical techniques (HPLC-FL, CE-MS), there were non-sulfur-containing compounds in the n-IDY permeate. However, a very interesting finding was the detection in both samples of some small peptides, specifically tripeptides. Two of them were found in both permeates and were tentatively identified as histidine/cysteine/lysine- and methionine/lysine/histidine-containing peptides. Their MS and chemical structures are shown in Figure 4. Because of their low concentration in the sample, it was not possible to confirm their sequence. Even more interestingly was the finding of another two peptides only in the n-IDY permeate containing methionine/aspartic/tryptophane and tyrosine/histidine/methionine (Figure 4). It is worth mentioning that the antioxidant properties of small peptides, mainly contained in fermented food, have been

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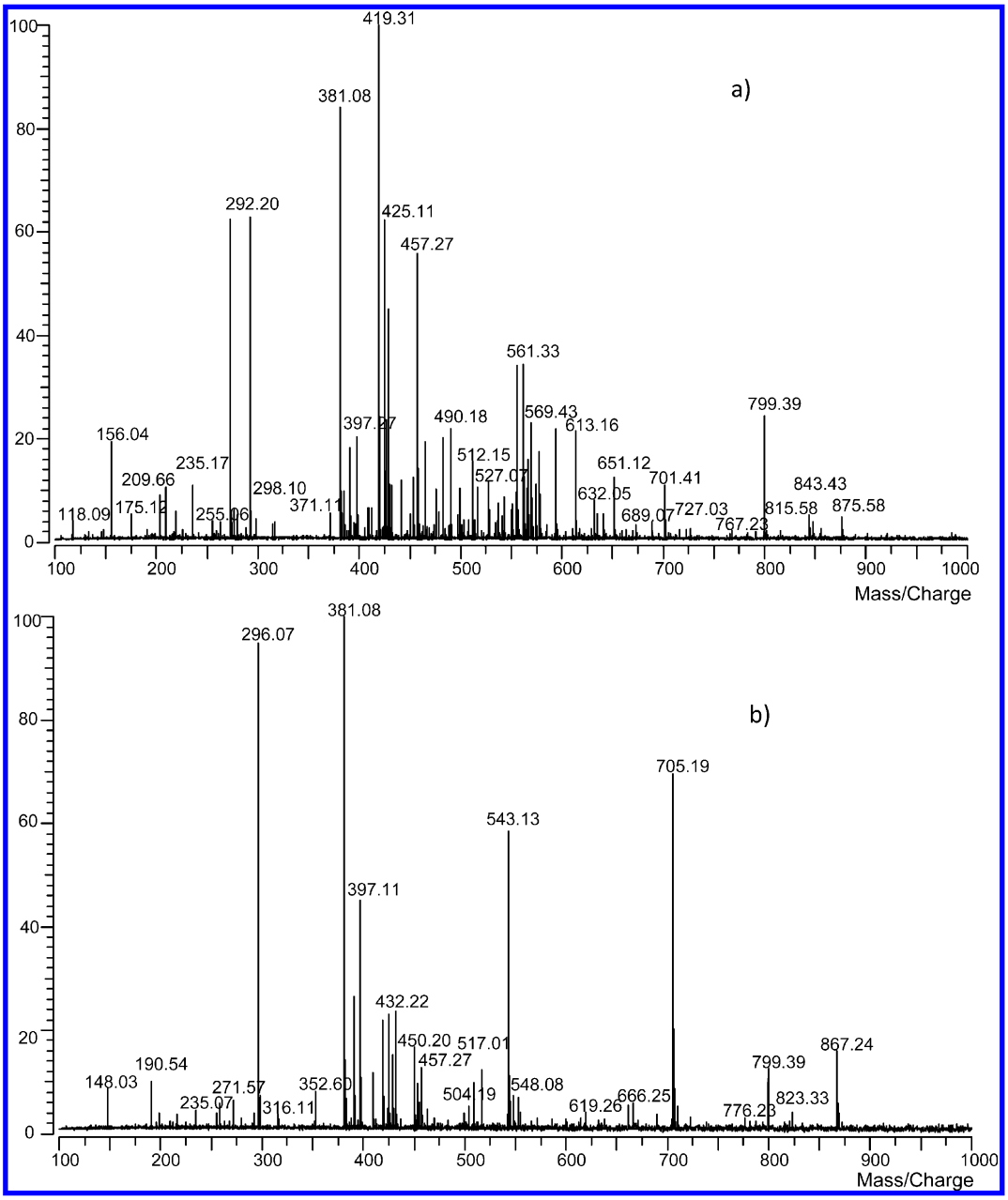

Figure 3. ESI (+) FT-ICR MS of the <3 kDa permeate from g-IDY (a) and n-IDY (b) samples.

Table 4. Tentative Identification of the Compounds Found in the <3 kDa Permeates Isolated from g-IDY and n-IDY Preparations after FT-ICR-MS Analysis

m/z								
exptl	theor	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	

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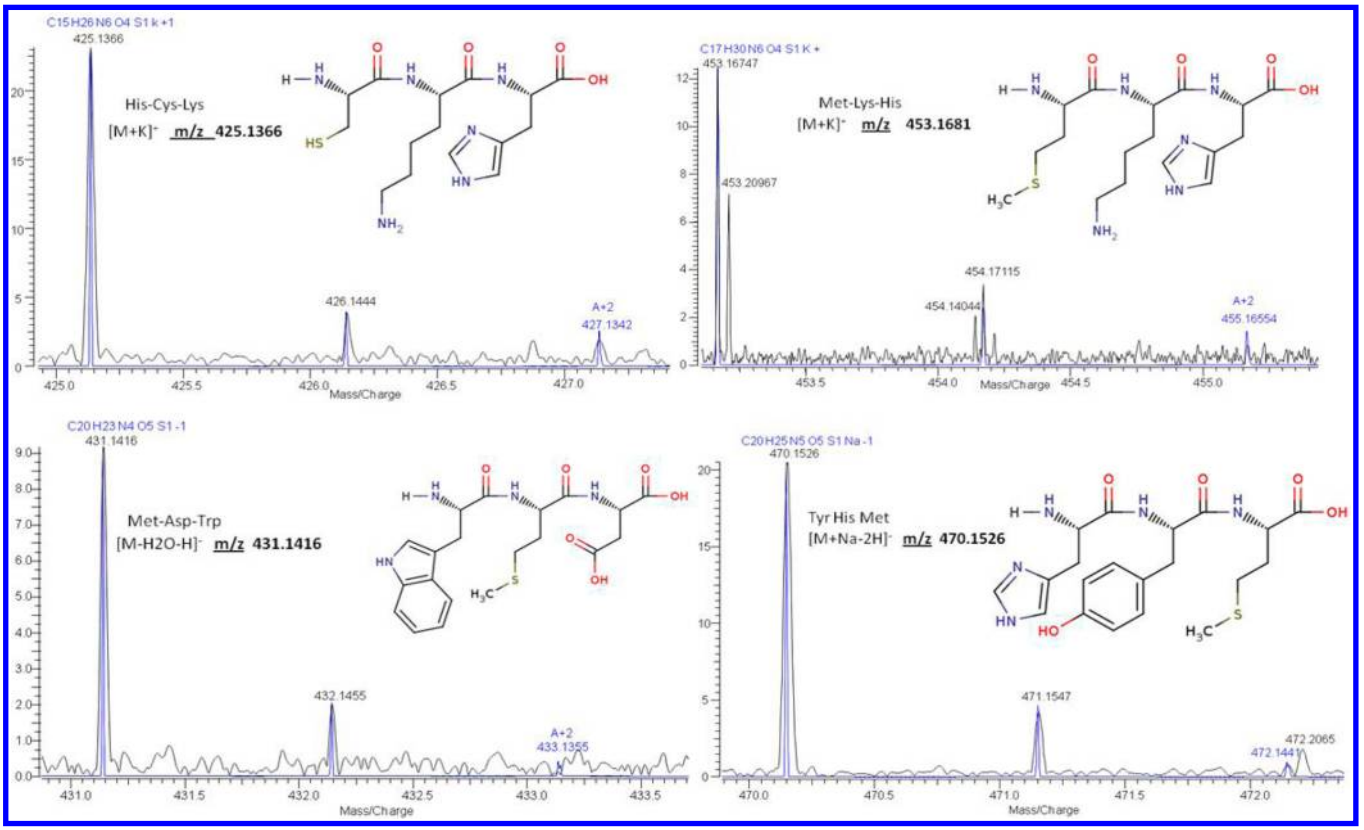

Figure 4. Chemical structures and EI-FT-ICR MS corresponding to the peptides identified in the <3 kDa permeates from g-IDY and n-IDY. Met/Asp/Trp and Tyr/His/Met were identified only in the permeate from n-IDY.

extensively documented.[35] As was previously commented, small peptides containing tryptophan, tyrosine, methionine, cysteine, and phenylalanine have been described to exhibit a high antioxidant activity.[34] In the present study, from the two peptides detected in the permeates from g-IDY and n-IDY, only one (histidine/cysteine/lysine) had an amino acid (cysteine) that could be involved in the antioxidant properties. However, the two peptides exclusively identified in the n-IDY permeate (methionine/aspartic acid/tryptophan and tyrosine/histidine/methionine) contained two of these amino acids each. In addition, both peptides contained tryptophan and tyrosine, the two most highly antioxidant amino acids. Previously published works have already described the biological activities (antioxidant, antihypertensive) of peptides from yeast origin found in synthetic wines submitted to autolytic conditions[40] and in red wines,[41,42] although their chemical structures remained unresolved. Moreover, considering these results, the preservation of aroma and reduction of aroma loss in wines aged on lees that has been linked to the GSH released by yeast autolysis[6] might also be attributable to other types of small peptides, which could have even greater antioxidant properties than GSH.

In conclusion, it has been proven that the use of IDY preparations (with or without GSH) reduces the loss of certain terpenes during the accelerated aging of model wines. It has also been shown that g-IDY preparations do in fact contain GSH in their reduced state, which can contribute to the aroma preservation in model wines, but they also contain other sulfur compounds of yeast origin that might also act as antioxidants. In addition, both g-IDY and n-IDY contained small peptides (tripeptides) with methionine, tryptophan, and tyrosine, which seem to be involved in the antioxidant properties determined in the permeates isolated from both IDYs, also being effective in the preservation of some terpenes during model wine aging. Because of the instability of GSH in wines (easily oxidized, fast combination with polyphenols, etc.), this finding could be of technological interest, assuming the higher stability of these antioxidant peptides when used, for example, after wine bottling. Subsequent work will be directed to unequivocally identify the sequence of these compounds, and further studies are needed to confirm the antioxidant effect of these peptides in closer winemaking conditions. Undoubtedly, this will be interesting for the wine and biotechnological industry to redirect the formulation of IDY preparations to achieve specific and effective winemaking applications.

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Funding

This work was funded by the MINECO (AGL2012-04172-C02-01) and CONSOLIDER INGENIO 2010 (FUN-C-FOOD, CSD2007-063, Projects) and by Agrovín, S.A. (I+D 20132446 Contract). J.J.R.B. thanks CSIC for the JAE-doc contract.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Hernández-Ledesma for her valuable assistance during the ORAC-FL analysis.

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