Wine metabolomics reveals new sulfonated products in bottled white wines, promoted by small amounts of oxygen

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Abstract

The impact of minute amounts of oxygen in the headspace on the post-bottling development of wine is generally considered very important, since oxygen can either damage or improve the quality of wine. This project aimed to gain new experimental evidence about the chemistry of the interaction between wine and oxygen. The experimental design included 216 bottles of 12 different white wines produced from 6 different cultivars (Inzolia, Muller Thurgau, Chardonnay, Grillo, Traminer and Pinot gris). Half of them were bottled using the standard industrial process with inert headspace and the other half without the inert gas and with extra headspace. After 60 days of storage at room temperature, the wines were analysed using an untargeted LC-MS method. The use of a detailed holistic analysis workflow, with several levels of quality control and marker selection, gave 35 metabolites putatively induced by the different amounts of oxygen. These metabolite markers included ascorbic acid, tartaric acid and various sulfonated compounds observed in wine for the first time, thanks to the untargeted metabolomics approach chosen (e.g. S-sulfonated cysteine, glutathione and pantetheine; and sulfonated indole-3-lactic acid hexoside and tryptophol). The

consumption of SO_2 mediated by these sulfonation reactions was promoted by the presence of higher oxygen at bottling.

Keywords: Untargeted; Non-targeted approach; LC-MS; Sulfonation; Cork; Indole.

1. Introduction

Oxygen is certainly one of the most important players in winemaking, since its effects can be a blessing, benefiting wine quality, or a curse, causing irreversible damage [1–5]. Since oxygen can be introduced into the wine at various stages of winemaking, from grape crushing to wine bottling, oenologists are always very careful and cautious with oxygen management. Oenologists can manage oxygen-wine interactions more easily in their winery, since theoretically the wine is under their complete control. However, when the wine is out of their winery (e.g. during the distribution or the storage in restaurants, supermarkets, wine shops and consumers houses) oxygen-wine interactions are almost impossible to control by the oenologist. For this reason packaging choices during bottling are critical and determinant for the life of wine.

In fact, oxygen contact at bottling is expected to influence the development of the bottled wine. Oxygen management through packaging represents a major challenge in oenology, as different wine varieties or wine styles will behave differently with the same amount of oxygen [1,2,5]. The time between bottling and sale is also fundamental, along with the expected commercial life of the wine. Based on the oenological and commercial parameters of each wine, the winery decides on the type of stopper (cork, synthetic, glass or screw), its oxygen permeability, and the amount of oxygen to insert into the bottle during bottling. For example, the great majority of the white wines are made to be consumed within 12 months from their production, are sensitive to oxidation, and therefore the packaging choices should prevent the entrance of unwanted amount of oxygen [6].

In spite of the recent studies [1,2,5,7,8] about the influence of bottling- and closure-derived oxygen on wine, the management of oxygen at bottling is still largely based on empirical knowledge. This is in part due to the fact that our chemical knowledge is not sufficient to predict the result that a small amount of oxygen may have in the metabolomic space of any wine, probably because so far the oxygen response of a relatively small group of metabolites (e.g. SO_2, volatile sulfur compounds or

anthocyanins) has been investigated [1,5,7–9]. The most common, almost universal experimental designs for studying the post-bottling effects of oxygen in wines – or model wine solutions - are based on targeted analytical methods. Currently, metabolomic fingerprint analysis appears to be one of the most appealing and promising techniques in order to build new hypotheses and better understand the metabolism of various biological systems. In a short time, holistic approaches have been shown to be a powerful tool in metabolite-marker discovery and hypothesis generation, since they can deal with thousand of features per analysis, including a large number of unknown substances, which can be later annotated. In the last few years this technique has also proved its power in the field of oenology and viticulture, by helping to enhance our knowledge of how different oenological practices influence and change the metabolic space of grapes or wine [1,10–14]. However, since metabolomics is a relatively young technique - expanding very rapidly - the workflows are not as robust and well-designed as for targeted analysis and there is still debate about experimental design and method validation, among other things.

From the economical perspective, under the widely accepted assumption that just a few mg of oxygen at bottling could negatively influence the quality especially in the case of white wines, wine industry made huge investments worldwide to install inert bottling lines, which to date represent the standard process. As a praxis, in order to prevent unwanted oxidations, wines are loaded before bottling with standard amounts of exogenous antioxidants (usually SO_2 or a combination of SO_2 and ascorbic acid) so that to protect even the most susceptible wine.

A better understanding of the effects of oxygen at bottling on the consumption of exogenous antioxidants and on the reactivity of other wine metabolites after bottling is expected to shed light on the factors driving the specific consumption of oxygen by different wines.

The general aim of this study, carried out in collaboration with a major Italian winery, a key player in the wine stopper market and an untargeted metabolomics laboratory, was to address some basic questions about the chemistry of the interaction between wine and oxygen, crucial for decisions regarding packaging. In particular, the scope was to compare the metabolic fingerprint of white wines bottled under standard industrial parameters, with the fingerprint of the same wines bottled under sub-optimal but still realistic industrial conditions. To study principally the effect that oxygen can have after two months of storage; wines were bottled without inert gas, with extra headspace, and

with a closure allowing higher oxygen ingress for the sub-optimal conditions. To obtain a high level of variability, the experimental design included 12 white wines made using 6 varieties, with 9 bottles of each wine bottled according to each bottling parameter, using the same industrial bottling line. A parallel aim was to develop and propose a robust and efficient workflow for wine metabolomics.

2. Materials and Methods

2.1. Experimental Design

The sample set included 12 white wines from 6 different grape cultivars (Table 1). All the wines were produced from the MezzaCorona winery (Trentino, Italy) and they were bottled using their industrial bottling system (Bertolaso, Italy). For each wine, 9 samples were bottled using the standard industrial process, with inert headspace and limited exposure to oxygen (low O_2 - LO), along with a further 9 bottles produced using the same bottling line, but without inert gas and with extra headspace (high O_2 - HO). In order to enhance differences in oxygen exposure, LO samples were sealed with a synthetic coextruded stopper allowing lower oxygen ingress (Select 100), whereas HO samples were sealed with a synthetic coextruded stopper allowing higher oxygen ingress (Select 500). Both stoppers were provided by Nomacorc SA (Thimister Clemont, Belgium). After bottling, all the bottles were stored in cardboard boxes at 20^{o}C for two months, and then at 4 ^{o}C until analysis. The volume of all bottles was the typical commercial 750 mL.

Four bottles from each trial/wine were used for untargeted LC-MS analysis to study the effect of the different oxygen levels after two months of storage at 20 ^{o}C (Supplementary Materials: Table S1).

2.2. Oxygen measurement

One bottle from each trial/wine was used to measure oxygen (Supplementary Materials: Table S1). The amounts of gaseous or headspace oxygen and dissolved oxygen were measured by placing a Pst3 oxygen sensor (Nomacorc SA, Thimister-Clemont, Belgium) internally in the head space of the bottle and another dot sensor half-way up of the same bottle, to measure the dissolved oxygen. Since the sensors were placed internally and the measurement was made by a luminescence technology optical fibre externally the bottle glass, the method was totally non-invasive and the bottles remained closed during all the period of analysis. Total package oxygen (TPO) was considered to be the sum of the amounts of headspace and dissolved oxygen. Measurement was

carried out using the NomaSense system (Nomacorc SA, Thimister Clemont, Belgium) weekly during the two months after bottling, in triplicate on each occasion. **Table 1** shows the total package oxygen concentrations in ppm for each wine and bottling condition (HO and LO).

2.2. Sample preparation

Before any treatment, in agreement with the workflow applied in our laboratory [14–16], the sample metadata were uploaded in ISA-Tab format using ISAcreator MetaboLights software [17] and codified according to a randomized sequence, so sample preparation and analysis were completed following this randomized sequence. Wines were uncorked under nitrogen atmosphere and an aliquot was transferred into a 15 mL amber vial (filled to capacity). Then, again under nitrogen atmosphere, quality control (QC) pooled samples were prepared using 0.5 mL of each sample.

For the dilution test, the QC sample was diluted 1:1, 1:2, 1:3, 1:4, 1:6, 1:9 with Milli-Q water. Following the dilution experiment results, again under N_2 atmosphere, 1 mL of each wine was diluted with 1 mL Milli-Q water (1:1 dilution), 20 µL of the internal standard was then added (10 mg *o*-coumaric acid in 10 mL of MeOH) and filtered with 0.2 µm PTFE filters into a 2 mL amber vial (MS certificated) prior to LC/MS analysis. The same procedure was followed for the blank, but instead of wine 1 mL of Milli-Q water was used.

2.3. Dilution test – Long term stability test

In order to find the optimum dilution, 20 injections of each QC dilution were carried out, starting with the most diluted QC sample. Between each dilution set, a blank sample was injected. The undiluted wine was also injected, after filtration, in both 5 and 10 µL injection volumes. Following the results of the dilution test, a sequence of 100 injections of the 1:1 (wine:water) QC sample were analysed to evaluate the stability of the method in experimental conditions.

2.4. UHPLC-QTOF MS Analysis

For LC-MS untargeted analysis a Waters Acquity UPLC was used, coupled to a Synapt HDMS QTOF MS (Waters, Manchester, UK) operating in W-mode and controlled by MassLynx 4.1, via an electrospray ionization (ESI) interface. All samples were analysed on a reversed phase (RP) ACQUITY UPLC 1.8 µm 2.1 x 150 mm HSS T3 column (Waters) protected with an Acquity UPLC® BEH HSS T3 1.8 µm, 2.1 x 5 mm precolumn (Waters), at 40 ^{o}C and with a mobile phase flow rate of 0.28 mL/min. Water was used as weak eluting solvent (A) and methanol as strong eluting solvent (B); formic acid 0.1% v/v

was used as additive in both eluents. The multistep linear gradient used was as follows: 0-1 min, 100% A isocratic; 1-3 min, 100-90 % A; 3-18 min, 90-60 % A; 18-21 min, 60-0 % A; 21-25.5 min, 0 % A isocratic; 25.5-25.6 min, 0-100 % A; 25.6-28 min 100% isocratic. Injection volume was 10 µL, unless otherwise stated, and the samples were kept at 4ºC throughout the analysis. Mass spectrometric data were collected in negative ESI mode over a mass range of 50 to 2000 amu, with scan duration of 0.3 s in centroid mode. The transfer collision energy and trap collision energy were set at 6 V and 4 V. The source parameters were set as follows: capillary 2.5 kV for negative scan, sampling cone 25 V, extraction cone 3V, source temperature 150ºC, desolvation temperature 500ºC, desolvation gas flow 1000 L/h and nebulizer gas 50 L/h. External calibration of the instrument was performed at the beginning of each batch of analysis by direct infusion of a sodium formate solution (10% formic acid/0.1 M NaOH/Acetonitrile with a ratio of 1/1/8), controlling the mass accuracy from 40 to 2000 m/z (less than 3 ppm) and mass resolution (over 14000 FWHM). LockMass calibration was applied using a solution of leucine enkephaline (0.5 mg/L, *m/z* 554.2620) at 0.1 mL/min [10]. The injection of samples was carried out following the same randomized sequence used for sample preparation, where at the beginning of the sequence one blank injection and five QC injections were performed to equilibrate the system, and after every six real sample injections one QC injection was inserted to control instrumental stability.

For MS/MS analysis, the Synapt MS was operated in V-mode, scan time was 2 seconds, transfer collision energy was 30 V and trap collision energy 10 V, while all the remaining parameters were as previously described.

2.5. Basic oenological analysis

Alcohol (%v/v) content was measured using an Alcolyzer Wine system (Anton Paar, Graz, Austria); pH, ascorbic acid, free and total SO_2 were measured with PH-Burette 24 1S (Crison, Barcelona, Spain); and all other analysis was performed with a FOSS Wine-Scan (FT-120) rapid-scanning infrared Fourier-transform spectrometer with WineScan software Version 2.2.1 (FOSS, Hillerod, Denmark). Four bottles from each trial/wine (two at the beginning and two at the end of the experiment) were used for the basic oenological parameters (Supplementary Materials: Table S1). All basic oenological analysis were performed in the quality control laboratory of MezzaCorona winery.

2.6. Data processing

The raw LC-MS data were converted to CDF format (Waters Databridge) and then processed using XCMS for feature extraction, grouping and alignment, according to

Franceschi et al. [15]. XCMS data processing was limited to the first 21 minutes of the chromatography to avoid possible carryovers of the last 7 minutes of the chromatography (= column clean up and equilibration before the next injection). The peak table output of XCMS was then used for statistical analysis with the software EZInfo SIMCA-P version 12.0.0 (Umetrics, Umea, Sweden), using Pareto normalization. The PCA (Principal Component Analysis) plots of EZInfo SIMCA-P were used for quality control of the data sets, checking the distribution of the QC injections. The orthogonal partial least-squares discriminant analysis (OPLS-DA) tool of SIMCA-P was used to find features/tentative markers contributing to group/condition discrimination. Tentative markers were considered all features with VIP>2 (variable importance in the projection) and -0.002>CoeffCS>0.002 (CoeffCS: centered and scaled coefficients).

Manual integration of the area of selected features/peaks was performed with the TargetLynx tool of MassLynx on the raw data. SPSS V19 (IBM Statistics) was used for statistical analysis (*p*-values) and visualization (box plots) of the results.

Metabolite identification [18] was performed manually by comparing retention times and accurate mass spectra (mass difference of less than 5 ppm and two ions) to those of the standard, when available. Tentative annotation [18] of the chromatographic peaks, without a standard, was made by using spectral features (mass difference of less than 5 ppm theoretical value, at least one indicative fragment and isotopic pattern), and literature information about chromatographic properties and mass spectra records from an external database such as HMDB, Kegg and MassBank and an internal database for the wine metabolome based on the literature. MS/MS data were also used for further supporting the annotation of a few tentative marker metabolites.

2.7. GSSG and GSH Reactions

Three stock solutions were freshly prepared in model wine solution (10% ethanol, 5 g of tartaric acid and pH 3.4): 39 mg of oxidize glutathione (GSSG) in 10 mL model wine solution (stock solution GSSG); 20 mg of glutathione (GSH) in 10 mL model wine solution (stock solution GSH); and 6 mg of $Na_2S_2O_5$ in 10 mL of model wine solution (stock solution SO_2). The reactions between GSH or GSSH and SO_2 (Supplementary Materials: Table S2) were performed in order to compare the following molar concentration ratios of GSH/SO_2 or GSSG/SO_2: 10/1, 1/1, 1/10; by maintaining constant the concentration of GSH or GSSG. To control the stability of GSH and GSSG in the reaction conditions, the same mixture was prepared without the addition of the stock solution SO_2 (Supplementary Materials: Table S2). All the reactions were preformed

twice, in 2 mL LC-MS vials, which remained in the sample manager of the LC instrument at 4ºC, and were monitored by LC-MS for 24 hours. The instrumental LC-MS analysis were made with the same set up described at section 2.4, apart of the multistep linear gradient which was as follows: 0-1 min, 100% A isocratic; 1-3 min, 100-90 % A; 3-5 min, 90-0 % A; 5-7 min, 0 % A; 7-7.1 min, 100 % A; 7.1-10 min 100% isocratic. The injection volume was 2 µL. Calibration curves were prepared, by using the model wine solution as matrix, for the absolute quantification of GSH and GSSG, while the products were relative quantified as GSH.

3. Results

3.1 Oxygen measurement

Measurement of the total amount of oxygen (**Table 1**) present in each bottle at the beginning of the experiment showed the starting difference between the two conditions, which was between 2 and 5.5 mg/L of O_2 (average 4 mg/L). After two months' storage most of the O_2 had been consumed in all the wines for both trials, but not totally since it was still possible to detect a certain amount of oxygen, which proved the mild storage conditions (**Figure 1**). As expected, also after two months of storage the wines bottled under HO condition contained higher amount of oxygen in respect the LO wines. Considering initial and final bottle oxygen content as well as the oxygen ingress of the two different closures, the actual consumed oxygen values was calculated [19], indicating that consumed oxygen ranged between 2.1 mg/L and 5.4 mg/L.

3.2 UPLC-QTOF MS analysis

Metabolomics workflows share many common elements with targeted analysis workflows, but are not as rigid, robust and standardized. Mandatory terms for the concept of method validation in targeted analysis, as limit of detection, limit of quantification, accuracy, precision, specificity, linearity and absolute concentration, are generally missing from the untargeted approach. Indeed, one of the bottlenecks of metabolomics workflows regards the fact that they are generally not well defined and even more frequently are not designed in advance. Based on our previous experience, we developed the workflow presented in **Figure 2**. This describes the methodology followed from experimental design until marker interpretation and hypothesis generation, with specific steps. This workflow includes open source informatics tools about metadata organisation, randomized sequence generation and raw data storage [15].

3.2.1. Method adaptation and evaluation

According to this workflow, an essential step is to adapt the method to the current sample set. Although most of our previous protocols were validated on wine samples, two additional tests were considered important before analysis of the real samples. The first focused on sample preparation, which as noted previously [1,20,21] should be as simple as possible, to avoid bias due to extensive sample manipulation. Since the aim of the project was to study the effect of oxygen on wine, all sample preparation steps took place under N_2 atmosphere to avoid any possible contact with oxygen. Samples were simply diluted with water and filtered, to ensure LC-MS instrument effectiveness during analysis. The results of the dilution test (**Figure 3**) were in line with our previous experience on red wine [10] and the dilution with the largest number of features and good stability was 1:1 (wine:water). In detail, the 20 injections of the diluted pooled sample 1:1 with water gave an average of 8454 features (5.0% RSD), while the second best was the 1:2 dilution, with an average of 7732 features (5.6% RSD). Because of the presence of ~12 % v/v of ethanol in wine, peak broadening and peak doubling was observed in the first part of the chromatogram for the undiluted samples, as expected. For this reason the undiluted wine had the highest variability (11.6% RSD) (**Figure 3**), also caused by signal drop during the sequence, because of the source dirtiness (data not shown).

The second test was designed to check whether the 1:1 dilution was the most suitable choice for our experimental design, compatible with the number of the injections per sequence. This control took place through a 100-injection batch of the same pooled sample. The number of features in this sequence had a variation of 8.5%, while the area of 17 selected compounds had a variation of less than 15% and a retention time of less than 0.5%, so it was decided to use this dilution for the rest of the analysis.

3.2.2 Unsupervised data analysis – Dataset quality control

Sample analysis was organised and performed according to a published pipeline [15], with one of the central tools offering the user the possibility of evaluating the quality of the measurements in parallel with the analysis of the sample, without having to wait for the full dataset at the end of the sequence. To achieve this, during LC-MS analysis, raw files were transformed to CDF format automatically after each injection, and were then moved to the server to perform the XCMS process and finally exported for PCA plotting and other basic statistical analysis [15]. Of course, this brief quality control was not detailed and focused mainly on the distribution of the samples and the QCs clustering, but offered a quick online answer. The QC injections at the beginning of the sequence

helped to equilibrate the LC-MS system, and the QC injections after injection of lots of six real samples helped to evaluate the stability of the system. This quality control through the pipeline took place several times every day during the measurement period, since it was a fast process (~ 25 sec/injection).

The complete sample set included 12 different white wines made from six cultivars (Muller Thurgau, Inzolia, Chardonnay, Traminer, Grillo and Pinot gris), which generated a high amount of statistical noise, because of the chromatographic profile variability (**Figure 4**). Full dataset multivariate unsupervised statistical analysis through PCA of the ~8,000 features gave the plot presented in **Figure 5**. According to this graph it was possible to distinguish the different cultivars and in some cases also different wines belonging to the same variety. Because of the statistical noise generated by cultivar variability, it was not possible to distinguish samples with the two levels of oxygen using unsupervised multivariate analysis on the whole data set. In other terms, we observed that the cultivar is by far the first factor affecting the overall distribution of samples, suggesting that such an untargeted metabolomics dataset should be suitable to support the cultivar identification, while the detection of the small specific effects of minute amounts of oxygen within several different wine lots is a challenging task which must be addressed with a specific strategy of data mining.

This is a common issue for untargeted analysis, caused by the high number and % of features which do not show any significant differences between treated and control samples. In this experiment, because of the strict experimental design, considering the small difference in the amount of oxygen and the short storage time in realistic conditions, it was not expected to find a large number of markers.

The quality of the dataset was controlled before moving on to the next step, namely marker exploration. For this first quality control the distribution of the various wine cultivars and the tight clustering of the QCs was evaluated from the PCA plot in **Figure 5**, as was done during the measurements. A further control was performed by selecting a small number of known metabolites – as compared to the number of features -, whose peaks were integrated, and their area and retention time variations were evaluated for the QC injections. According to this evaluation, the vast majority of the metabolite peak areas had a variation of less than 10% RDS (less than 5.9% for the internal standard) and mainly peaks close to the limit of detection (in term of signal to noise ratio) had higher variability. Average RSD was ca. 0.3% for the retention times and only close to

1% for a few compounds, eluting at the beginning of the chromatography (Supplementary Materials: Table S3).

3.2.3 Marker detection

To solve the problem of the statistical noise discussed above, supervised multivariate methods are often used (e.g. OPLS-DA) although they can generate false positives [22]. On the other hand, statistical noise can also generate false negatives, thus not detecting markers, because the between varieties difference was much larger than the between-trials difference. Such phenomenon (false negatives) could be amplified because each cultivar/wine may behave in a different way because of the different composition. The source of false negatives can also be induced by XCMS misalignment, because of the large variation in cultivar chromatographic profiles. To avoid false negatives the XCMS raw file process followed using OPLS-DA analysis was applied sequentially, once for each cultivar and then once for all the cultivars together. This process was possible because the samples were analysed all together using LC-MS in one randomized sequence. The various features marker lists were merged to give a list of c. 150 markers, out of the ~8K features, thus features with VIP>2 and -0.002>CoeffCS>0.002. This step is described as “markers”, “XCMS” and “OPLC-DA” in the workflow of **Figure 2**.

The next step according to the workflow was “marker validation”, which aimed to filter out false positives (**Figure 2**). This part consisted of: visual inspection of the marker chromatographic peaks and MS spectra; grouping of the features using CAMERA; semi-automatic peak integration of raw files using TargetLynx; and statistical analysis of the TargetLynx results. Visual inspection was an easy way to control whether the marker was a real peak, peak shape, and whether the ion was the principal ion and not one isotope. Semi-automatic integration of raw file data using TargetLynx helped to detect false positives by applying independent evaluation of the markers selected through XCMS and OPLS-DA, since it was applied to the raw files. Semi-automatic integrated areas made it possible to confirm or reject the statistical significance of each marker in distinguishing the two storage conditions and to evaluate the instrumental variability of each specific marker during the analysis by using the QC injections. This step (“marker validation”, “visual control”, “TargetLynx” and “Statistics” - **Figure 2**) allowed us to remove redundancy, decreasing the number of tentative markers to 35 metabolites.

3.2.3 Marker annotation

According to the Metabolomics Society, metabolite annotation is divided into four levels of annotation [18], as follows: 1) identified metabolites (demands 2 or more orthogonal

properties of an authentic chemical standard), 2) putatively annotated compounds (based on public databases and literature data and not requiring matching to data for authentic chemical standards acquired within the same laboratory), 3) putatively characterised compound classes, and 4) unknown compounds.

Annotation of the features' tentative markers was based on our internal database [23] made up of over 400 metabolites (1st level of annotation), and/or external databases (e.g. HMDB, KEGG, Phenol-explorer, etc) together with oenological references. In all cases, to avoid false positive annotation, all feature marker annotations were explained by organic chemistry, analytical chemistry and oenological knowledge. The usefulness of combining retention time, isotopic distribution, adducts and fragment information with mass accuracy, in order to decrease false positives, was already shown in a previous application on the same sample set [24].

Table 2 presents features with a significant difference between the two bottling conditions, after filtration to remove false positives, together with the results of the annotation process. Of the 35 features, 12 were annotated with the use of their commercial references (1st level annotation), 7 were tentatively annotated based on the literature and public databases, and 16 were unknowns. A substantial majority of these features were found to be higher in the oxygen trial (27 as compared to 8).

4. Discussion

Untargeted metabolomics workflows generally are divided in sample selection, sample treatment, analytical conditions set-up, raw data acquisition, data analysis by chemometrics, marker annotation and database search and hypothesis generation. Often in food metabolomics, raw data are used just to discriminate samples, without any attempt to explain the causes of this discrimination, leaving out the two last steps above described [25]. The proposed minimum reporting standards for chemical analysis in metabolomics doesn't include any guidelines for workflows and validating the analytical part in non-targeted methods [26]. Since metabolomics is a rather new approach with still many problems to overcome and tries to combine multiple analytical techniques (GC, LC, CE, MS, NMR), the proposed workflows are still very generic [27,28]. Subsequently, researchers are using alternative validation methodologies which are commonly applied after the acquisition of the raw data [25,27–29]. False positive and negative biomarkers are difficult to filter and usually this step requires further statistical treatment of the data sets. In this study, based on our previous experience in wine metabolomics, the

406 workflow of **Figure 2** was designed in advance and we propose tools to facilitate the
407 method development/adaptation, the quality control in real time of the raw data with the
408 LC-MS analysis, the false negative marker avoidance and the false positive filtration.
409 The application of this untargeted/holistic approach through a detailed and well-designed
410 workflow allowed quality control with various modes and on various levels during the
411 project, and offered the chance to evaluate the metabolic changes caused by oxygen in
412 wine in an open and wide-ranging manner. The experimental design applied in this work,
413 although very strict and rigorous, was at the same time very realistic [2] and carried out
414 at real industrial scale. The chosen conditions compared were the common values
415 detected in commercial wines [19], avoiding any extreme situation, and the length of the
416 oxygen exposition was short (2 months) but realistic in terms of the expected period
417 between bottling and consumption in relation to the quality of the chosen wines.
418 The metabolite markers known to be influenced by the experimental parameters acted
419 as a control to prove the quality of the analytical method. These markers included
420 ascorbic acid (**Figure 6**), also known as vitamin C, which concentration found in finished
421 wine is mainly a result of addition to increase the protection against oxygen [7]. In our
422 experiment ascorbic acid was higher in the wines bottled and stored with less oxygen, a
423 result later confirmed by targeted analysis, and dehydroxy-ascorbic acid (**Figure 6**) was
424 found to be higher in wines having received more oxygen, although it did not appear
425 among the features with a significant difference according to OPLC-DA analysis (**Table**
426 **2** and **Figure 6C**). **Figure 6C** shows an average ascorbic acid concentration loss of 23%
427 (9.8 mg/L) for wines bottled with low oxygen and a 65% (27.4 mg/L) loss for wines
428 bottled with high oxygen (**Figure 6C**).
429 Glutathione (**Figure 7**) is another antioxidant which is naturally present in grapes and its
430 concentration in wines can also be a result of addition of glutathione enriched products
431 [7]. Like ascorbic acid, glutathione also had a higher concentration in LO wines (**Table 2**
432 and Supplementary Materials: Figure S1). Under oxidative conditions, glutathione can be
433 transformed through different mechanisms, all involving its highly reactive thiol moiety.
434 For example, the oxidation of glutathione is expected to lead to the formation of its
435 disulfide (GSSG – **Figure 7**). However, studies on the oxidation of thiols in real wines
436 reported no formation of disulfides [2,30] indicating the existence of other competitive
437 reaction mechanism(s). Thiols such as glutathione have been reported to react rapidly
438 with the quinones arising from the oxidation of orto-diphenols through nucleophilic
439 addition, resulting in the formation of stable adducts, some of which have been recently

identified [31]. Consistent with these findings, in our conditions we did not detect any GSSG.

On the other hand, the second most significant feature marker in this experiment (VIP = 17.7 in **Table 2**) was tentatively identified as S-sulfonated glutathione ($GSSO_3H$ - **Figure 7**), which was detected at higher concentrations in the HO samples, so it could be assumed that a large amount of glutathione was transformed into its sulfonated analogue (Supplementary Materials: Figure S1), thus simultaneously depleting the concentration of the two major wine antioxidants. Similarly, S-sulfonated derivative of cysteine (**Figure 8)** was also found among the feature markers, with a higher concentration in HO. The S-sulfonate product of pantetheine**,** could also be inserted in the same group of reactions (**Figure 8**).

This is not the first time that sulfonated products have been detected in wine, and lately sulfonated flavonoids were found to be markers of sub-optimal storage [10]. The findings reported here, however, indicate that thiols such as glutathione, cysteine, and pantetheine can also be sulfonated in conditions of wine oxidation, in addition to the known sulfonated adducts of phenolic compounds. Such a reaction would involve two reactants, for example glutathione and SO_2 (or SO_3H), which have been both identified as having a similar nucleophilic capacity against the quinones formed upon wine oxidation [31]. Clarke at 1932 [32] and Waley at 1958 [33], reported the formation of $GSSO_3H$ from GSSG under excess of $Na_2S_2O_5$ at pH 7, and indicated that at lower pH the reaction is too slow (**Figure 7**). To find out if such mechanism could occur also in wine, the behave of GSSG or GSH in the presence of SO_2 (released by $Na_2S_2O_5$) in a model wine solution at pH 3.4 were monitored for 24 hours (Figure 7; Supplementary Materials: Figures S2-S3). In agreement with Clack and Waley [32,33], was found that at the higher concentration of $Na_2S_2O_5$ tested (19.5 mg/L), approximately 30% of GSSG was consumed after 24 hours, producing GSH (2.8 mg/L) and $GSSO_3H$ (12.5 mg/L measured as GSH). Under the analogous conditions, GSH (10.0 mg/L initial concentration) produced small amounts of $GSSO_3H$ (0.3 mg/L measured as GSH), while no GSSG was detected. For the reactions were $Na_2S_2O_5$ was added at minor concentrations, the results were similar but less intense or not detectable (Figure 7; Supplementary Materials: Figures S2-S3). This result indicates that - also in wine - the likely mechanism of this reaction is the sulfitolysis of GSSG produced by the oxidation of GSH, and resulting in the formation of $GSSO_3H$ (**Figure 7**). As GSSG wasn't detected in any of the tested wines, the combination of these new findings indicated that, in the

presence of sufficient SO_2, the GSSG formed through oxidation of GSH in wine should be very fast degraded primary to S-sulfonated glutathione and secondary back to GSH (**Figure 7**).

Another group of markers included three compounds of the metabolism of tryptophan. Tryptophan and its metabolites, especially indole-3-acetic acid, are considered as potential precursors of 2-aminoacetophenone, an aroma compound which causes the "untypical ageing off-flavour" in *Vitis vinifera* white wines [34–36]. The amount of tryptophan metabolites increases significantly during fermentation, although 2-aminoacetophenone is a product of oxidative degradation, whose formation is prompted by sulfonation after fermentation [35]. This group of markers included indole-3-lactic acid hexoside, sulfonated indole-3-lactic acid hexoside, and sulfonated tryptophol (**Table 2**, **Figure 9**, and Supplementary Materials: Figures S4-5). The sulfonated indole-3-lactic acid hexoside and the sulfonated tryptophol have never previously been reported in wine or elsewhere to our knowledge. Lately Fabre et al. [37] isolated and characterised indole-3-lactic acid glucoside in wine and pointed out that its concentration is microclimate dependent and decreases during wine storage/ageing. We believe that we have found the same compound as a marker, because also its MS/MS spectrum (Supplementary Materials: Figure S4) was similar to that of Fabre et al. [37].

In contrast to wine research, the aromatic sulfonation of indoles under aerobic oxidation is a known phenomenon in organic chemistry. In 1984 Yang [38] proposed a possible mechanism of (2-sulfoindole)-3-acetic acid formation by indole-3-acetic acid. Hoeniche et al. [36] suggested that the formation of 2-aminoacetophenone acid could be triggered by oxidative degradation of indole-3-acetic after sulfonation with potassium bisulfite. In view of the fact that 2-aminoacetophenone is responsible for the “untypical ageing off-flavour” in wine, study of the possible effects of indole-3-lactic acid derivatives on wine quality is of great importance for the wine industry.

All the above-described reactions (S-sulfonates and indole sulfonates) require the presence of SO_2. The main reason for which SO_2 is added to wine is to protect from oxidations, thus slowing down the development of the bottled wine, while free SO_2 decreases over time. Since SO_2 is an antioxidant, it was expected to measure a much lower concentration in wine bottled with a higher amount of oxygen (**Figure 6A-B**). Indeed, after two months of storage, free SO_2 decreased by ~20% (average value considering all 12 wines) in the LO wine samples, and ~40% in the HO wine samples. Nevertheless, it was expected to find the total SO_2 concentration stable over time. In this

experiment total SO_2 decreased by ~5% in wines bottled with a low amount of oxygen and ~14% in wines bottled with a high amount of oxygen (**Figure 6A-B**). This finding suggest that the sulfonation reactions described in **Figures 7-9** could explain a substantial part of the loss of sulfites during wine development, and is in agreement with our previous work about wine storage [10]. Given that this is the first time these reactions have been experimentally observed and reported in the oenological literature, this project shows the importance of untargeted analysis in exploring new potential markers.

In view of such reactions (**Figure 7**) occurring between the antioxidants added to the wine, their possible additive interaction effect should be revaluated, since this could also turn out to be antagonistic and finally their coaddition/copresence could provide less effective protection. In addition, the information that the indoles are able to trap the added SO_2 (**Figure 9**), even if stored for a short time and in mild conditions, could help in terms of a smarter use of SO_2 in wines. Wines containing high amounts of indoles might need the addition of higher amounts of SO_2 or should be bottled under low oxygen conditions.

Other observed metabolites (**Table 2**), also known to be markers of wine aging, were tartaric acid, caffeic acid and ethyl caffeic acid, probably as products of the hydrolysis of caftaric acid. Caftaric acid, the ester of caffeic acid with tartaric acid, had a higher (but not significant) concentration in the LO trial. Quercetin, higher in the LO trial, should be the result of hydrolysis of quercetin 3-glucoside. The monomeric (catechin and epicatechin) and dimeric (procyanin B2) flavanols, and the flavonol quercetin, are all known to be influenced by the oxygen level in wine, and they also appear in the short list in **Table 2**. Procyanidin B2 had a higher concentration in the LO trial for Inzolia, Muller, two Grillo and one Pinot gris. In Chardonnay and Muller Thurgau catechin and epicatechin were higher in the LO trial, but for the two Pinot gris wines the effect was the opposite. So it is hard to claim that flavonoids had a similar trend for all wines. This finding support the hypothesis that different varieties (and even different lots) behave differently with the same amount of oxygen and so specific packaging strategies matching the reactivity of each wine are required (e.g. stoppers with different permeability to oxygen).

5. Conclusions

In conclusion, this work proposes a clear and functional step-by-step workflow for wine LC-MS metabolomics, with several levels of quality control and the possibility of filtering both false negatives and positives. The application of this workflow to a project exploring how small amounts of oxygen introduced during bottling can influence the metabolic fingerprint of white wines, showed that the key player in the first crucial months of wine storage is sulfur. The antioxidant SO_2, added to protect wine from unwanted reactions, takes part in various reactions, several of which were unknown in wine to date. Specifically, the sulfonated derivatives of indole-3-lactic hexoside, tryptophol, glutathione, cysteine and pantetheine were detected in wine for the first time, thanks to the untargeted metabolomics approach chosen. These findings could explain the phenomenon that GSSG is not detectable in wines. Further studies of the mechanism(s) of such reactions could help to decrease SO_2 addition in wine, and make smarter use of the various oenological antioxidants in correlation with varietal information, the amount of total package oxygen and the choice of stopper.

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Figure Captions

Figure 1. Concentrations of total package oxygen (TPO) for each wine in the two bottling conditions - low (LO) versus high (HO) amount of TPO - time of bottling (Bottling HO and Bottling LO) and after two months (HO and LO).

Figure 2. The workflow used in this study

Figure 3. The number of features of the QC sample injected with different dilutions, and two injection volumes for undiluted wine. The injection volume was 10 µL except for "no-dil 5", which was 5 µL. % RDS, based on 20 consecutive injections, was 15.9% (blank), 2.3% (1:9 wine:water dilution), 1.7% (1:6), 4.9% (1:4), 5.7% (1:3), 5.6% (1:2), 5.0% (1:1), 11.6% (no-dil 10), and 2.2% (no-dil 5).

Figure 4. Typical BPI (basic peak intensity) LC-MS chromatograms of Pinot gris, Inzolia, Muller Thurgau, Chardonnay, Traminer and Grillo. Is visible that each variety has a very different chromatographic profile, in respect the other varieties of our experimental design. This variability explains the clustering of the PCA plot (Figure 5).

Figure 5. PCA plot of untargeted LC-MS analysis for wine. Each point plotted in the PC1-PC4 space corresponds to a different bottle (96 bottles), except the QC points, which are instrumental replicates of the same pooled sample. Different colours indicate different varieties and samples belonging to the same wine are indicated by freeform lines.

Figure 6. The concentration of total (**A**) and free (**B**) SO_2 in the wines on the day of bottling and after two months for the two bottling conditions, high (HO) and low (LO) oxygen concentration. **C**: In the presence of oxygen the antioxidant ascorbic acid (**1**) was oxidized in dehydro-ascorbic acid (**2**). The concentration of ascorbic acid decreased in all the wines after two months of storage, but for wines bottled with a larger amount of oxygen (HO) ascorbic acid loss was more dramatic as compared to wines bottled with less oxygen (LO).

Figure 7. Glutathione (GSH) in the presence of oxygen gives glutathione disulfide (GSSG), and GSSG reacts with SO_3H^- to provide S-sulfonated glutathione ($GSSO_3H$). Such mechanism was favoured in wine stored with a higher amount of oxygen. The kinetic reactions of GSSG (A and C) or GSH (B) under three molar concentration ratio with SO_2 (1/10, 1/1 and 10/1) in model wine solution, shown that the formation of $GSSO_3H$ was favoured when GSSG was the starting material and that the reaction depended from the concentration of SO2. The GSSG/SO_2 reactions produced both $GSSO_3H$ (A) and GSH (C), but no GSSG was detected between the products of the GSH/SO_2 reactions.

Figure 8. The white wines stored with a higher amount of oxygen favored the formation of S-sulfonation (**3** → **4**) of cystein and (**5** → **6**) pantetheine.

Figure 9. The sulfonation of tryptophol (**7**) and indole-3-lactic acid hexoside (**8**) to their corresponding derivatives **9** and **10** was favoured by the higher presence of oxygen in all wines.

Tables

Table 1. Wines sample information and basic oenological parameters recorded on the day of bottling

code	Variety	SO_2 free (mg/L)	SO_2 total (mg/L)	Ascorbic acid (mg/L)	pH	alcohol vol (%)	TPO HO (ppm)	TPO LO (ppm)
INZ	Inzolia	42	123	41	3.40	13.01	6.09	1.08
MLR	Muller Thurgau	38	101	36	3.28	12.37	8.38	2.95
CHR	Chardonnay	37	118	39	3.35	13.14	6.52	2.33
TRM	Traminer	36	109	33	3.50	13.31	8.09	2.5
GRL-1	Grillo	38	116	47	3.32	13.33	6.95	2.3
GRL-2	Grillo	38	116	44	3.31	13.18	7.03	2.91
GRL-3	Grillo	37	111	44	3.32	13.35	5.93	3.46
PNT-1	Pinot gris	36	111	39	3.33	12.65	7.85	2.79
PNT-2	Pinot gris	42	113	47	3.29	12.42	6.65	3.21
PNT-3	Pinot gris	40	106	39	3.30	12.64	6.22	2.74
PNT-4	Pinot gris	37	111	47	3.31	12.58	6.3	2.56
PNT-5	Pinot gris	35	109	47	3.30	13.31	6.21	4.25

TPO: total package O_2; HO: high O_2; LO: Low O_2.

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Table 2. Feature tentative marker data and annotations

rt (min)	m/z	VIP	Higher in	Annotatation	Theoretical mass	m/z error (ppm)
1.30	199.9693	6.03	HO	S-sulfonate cysteine[b]	199.9692	-0.50
1.33	193.0350	4.21	HO	glucuronic[a], keto-glucuronic[a]	193.0354	1.98
1.35	348.0069	5.72	HO	Unknown 1		
1.39	195.0504	2.30	HO	gluconic acid[a]	195.0510	3.25
1.42	149.0084	2.01	HO	tartaric acid[a]	149.0092	5.15
1.44	302.0019	5.55	HO	Unknown 2		
1.63	208.9765	6.26	LO	Unknown 3		
1.70	256.9910	8.03	HO	Unknown 4		
1.80	386.0328	17.71	HO	S-sulfonate glutathione[b]	386.0333	1.41
1.96	115.0027	10.05	LO	malic acid[a] - H_2O	115.0031	3.77
2.16	175.0243	15.77	LO	ascorbic acid[a]	175.0248	2.96
2.37	271.9902	5.18	HO	Unknown 5		
2.41	306.0767	5.88	LO	glutathione[a]	306.0765	-0.56
2.56	447.9594	6.35	HO	Unknown 6		
4.05	387.0179	4.30	HO	Unknown 7		
4.81	380.0657	3.90	LO	Unknown 8		
5.03	313.0536	6.00	HO	Unknown 9		
5.31	414.0670	7.73	HO	Unknown 10		
6.29	351.0057	4.38	HO	Unknown 11		
6.36	331.9944	4.95	HO	Unknown 12		
6.58	293.9745	4.63	HO	Unknown 13		
6.78	357.0797	7.12	HO	S-sulfonate pantetheine[b]	357.0795	-0.56
7.40	412.1199	4.14	HO	Unknown 14		
8.78	577.1356	4.75	LO	procyanidin B2[a]	577.1352	-0.77
10.15	289.0707	11.43	HO	catechin[a]	289.0718	3.69
10.23	446.0770	10.34	HO	indole lactic acid hexoside sulfonate[b]		
10.74	240.0333	12.85	HO	tryptophol sulfonate[b]		
12.33	179.0346	8.65	HO	caffeic acid[a]	179.0350	2.17
13.14	289.0712	5.41	HO	epicatechin[a]	289.0718	1.96
14.41	366.1193	8.52	LO	indole lactic acid hexoside[b]		
14.75	197.0449	10.71	HO	ethyl gallate[a]	197.0456	3.31
15.58	619.1288	5.47	HO	Unknown 15		
16.47	483.1523	6.05	HO	Unknown 16		
20.78	207.0653	18.85	HO	ethyl caffeic acid[b]	207.0663	4.83
20.88	301.0356	9.53	LO	quercetin[a]	301.0354	-0.72

[a]1st level annotation; HO: [b]2nd level annotation; High O_2; LO: Low O_2

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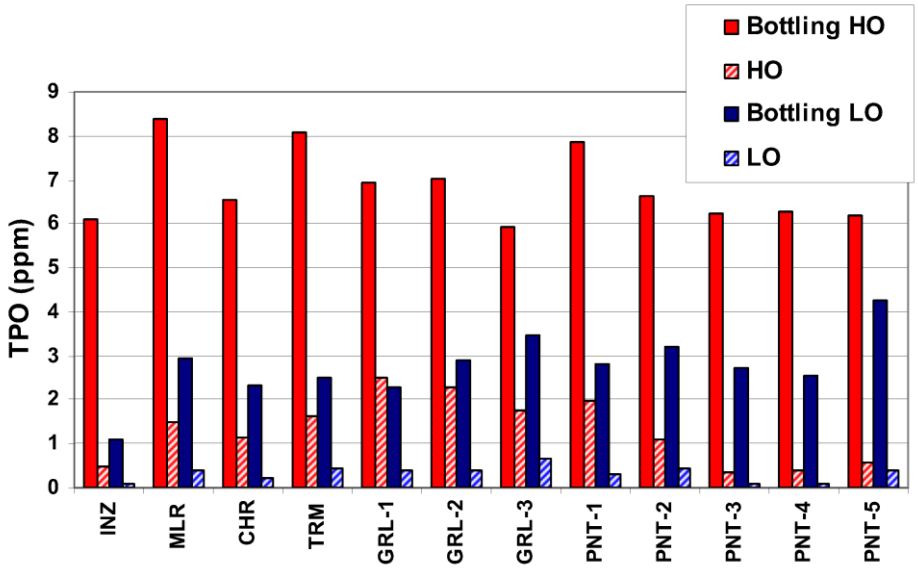

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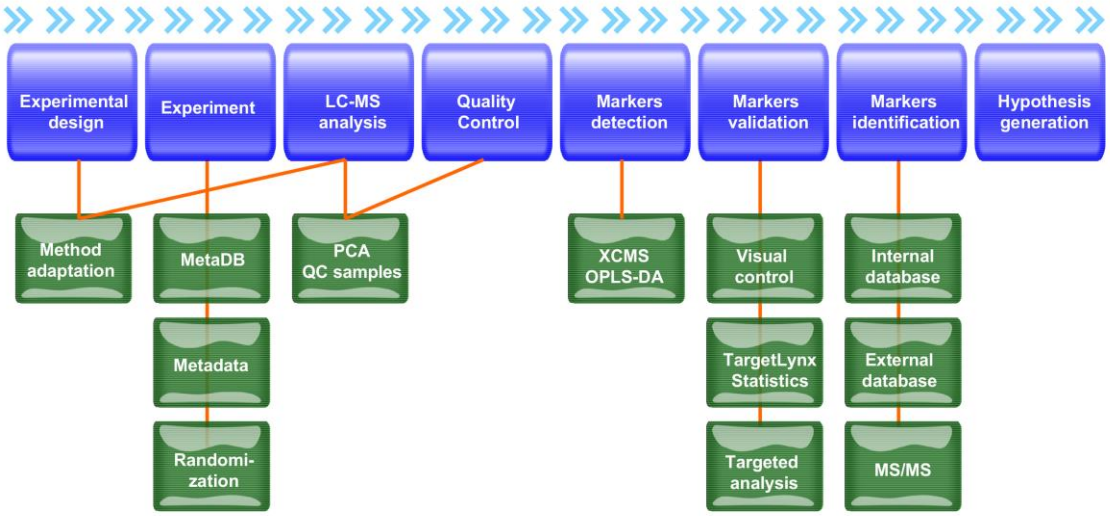
774 FIGURE 2



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778 FIGURE 3

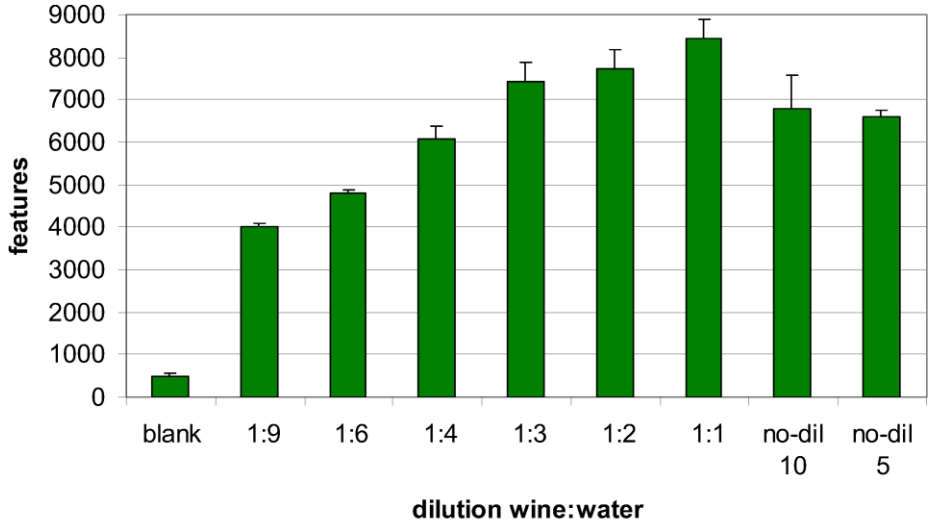

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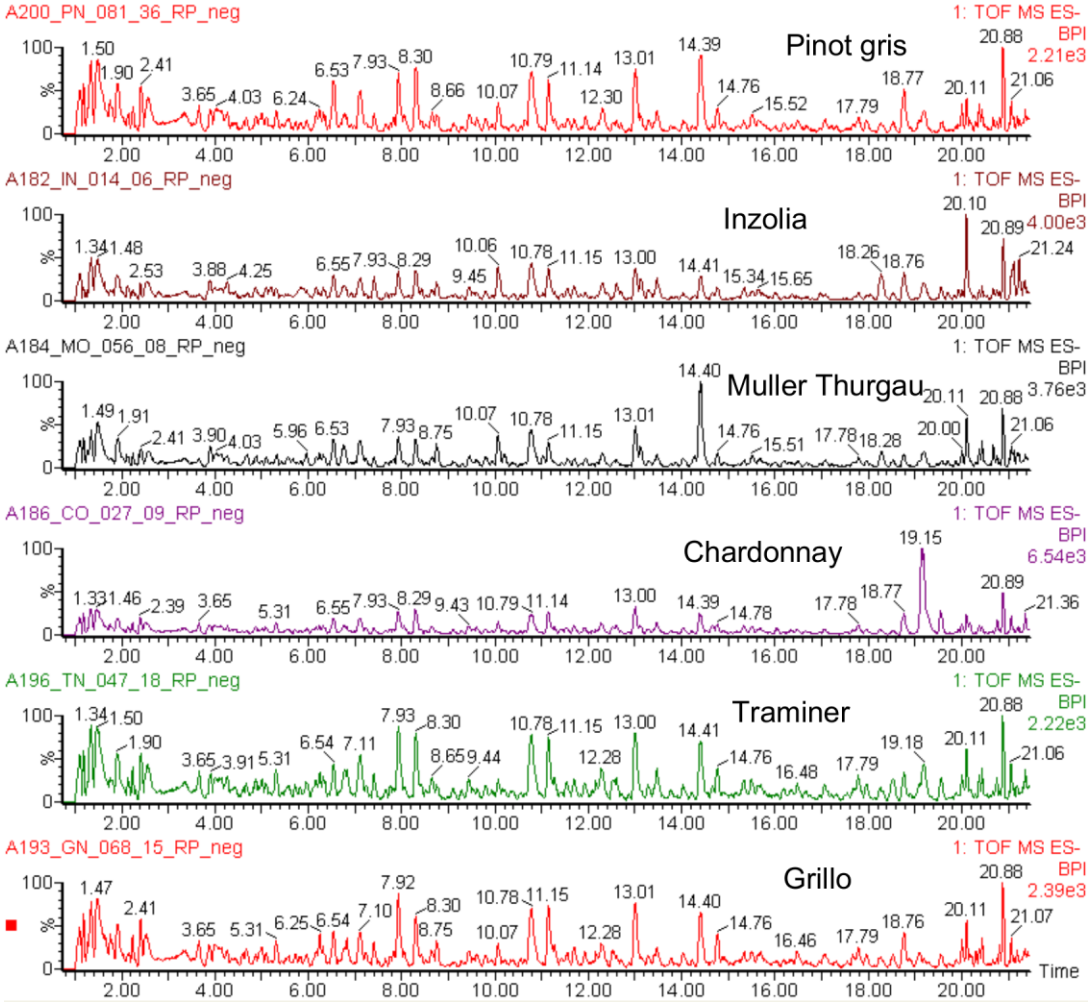
FIGURE 4


A200_PN_081_36_RP_neg
1: TOF MS ES-
BPI
2.21e3
Pinot gris
A182_IN_014_06_RP_neg
1: TOF MS ES-
BPI
4.00e3
Inzolia
A184_MO_056_08_RP_neg
1: TOF MS ES-
BPI
3.76e3
Muller Thurgau
A186_CO_027_09_RP_neg
1: TOF MS ES-
BPI
6.54e3
Chardonnay
A196_TN_047_18_RP_neg
1: TOF MS ES-
BPI
2.22e3
Traminer
A193_GN_068_15_RP_neg
1: TOF MS ES-
BPI
2.39e3
Grillo
%
Time

787 FIGURE 5
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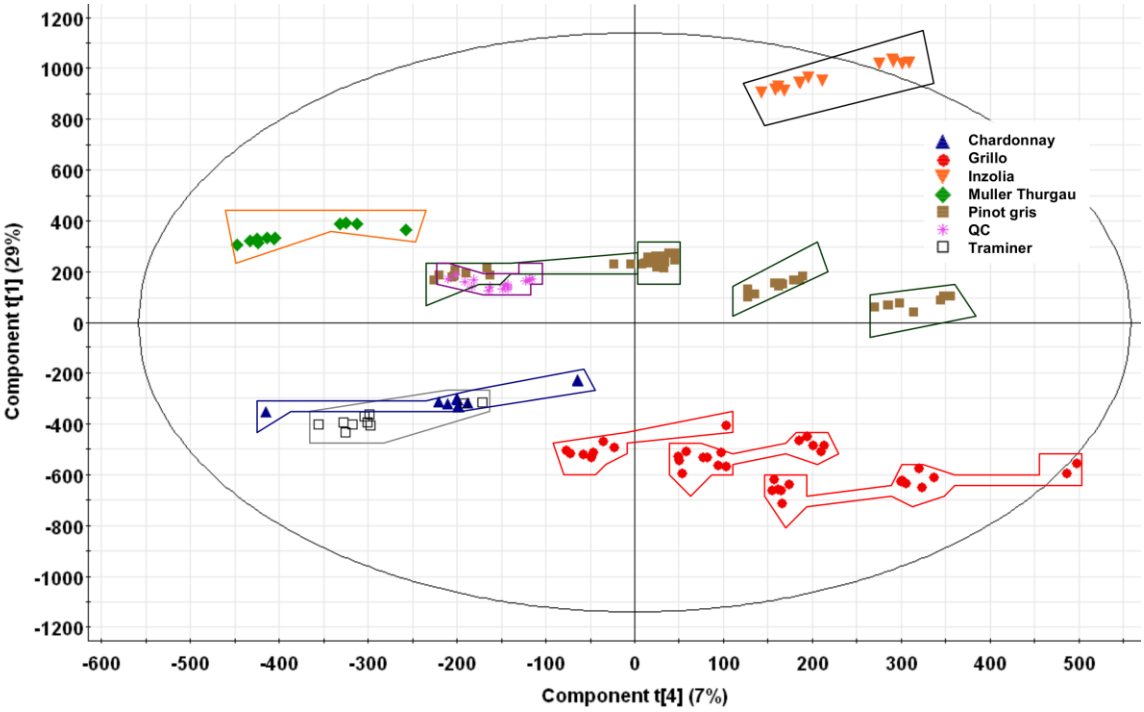

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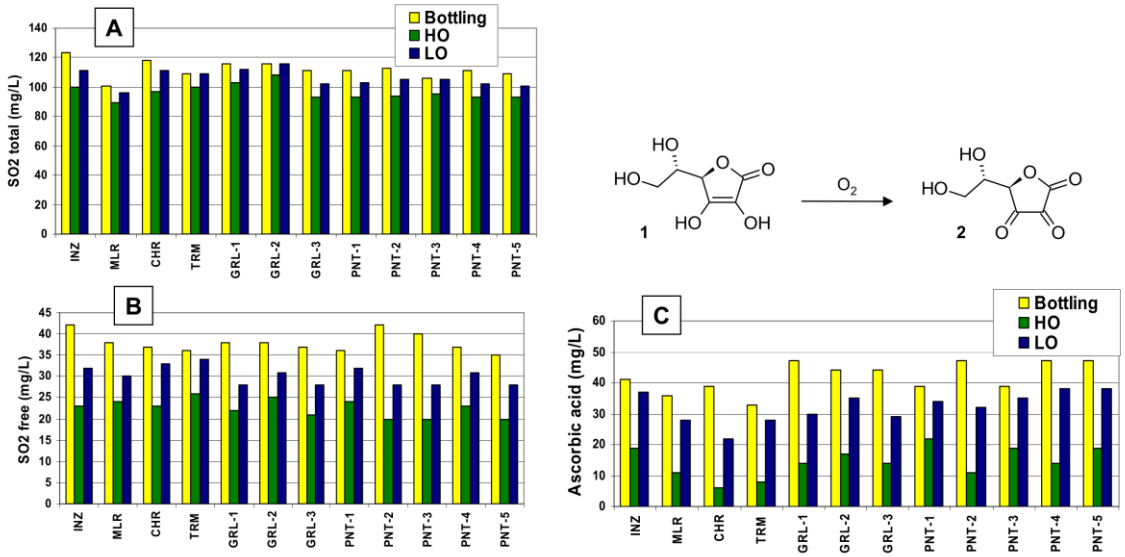
791 FIGURE 6



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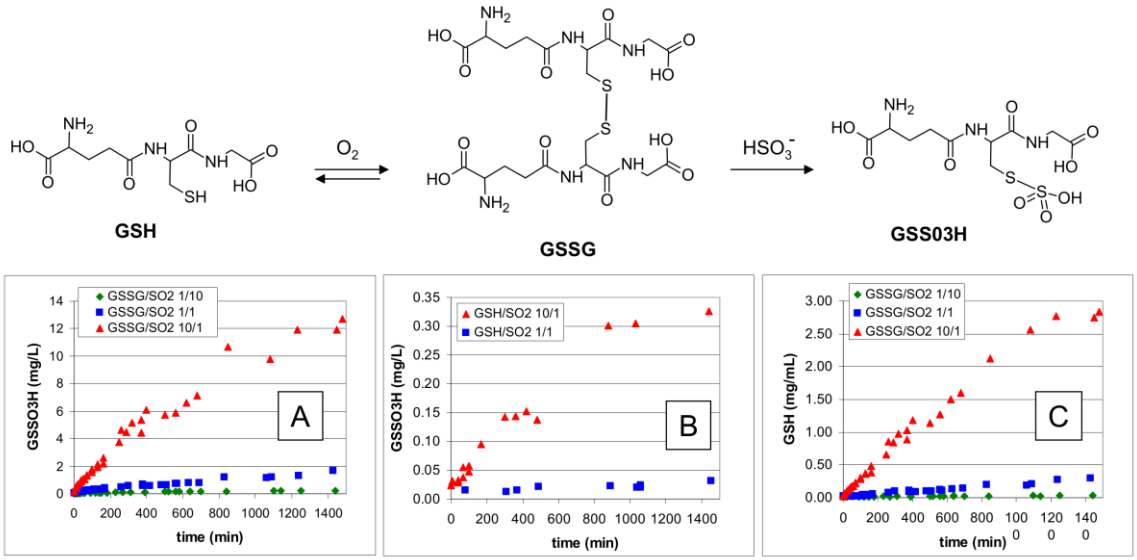
795 FIGURE 7



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798 FIGURE 8
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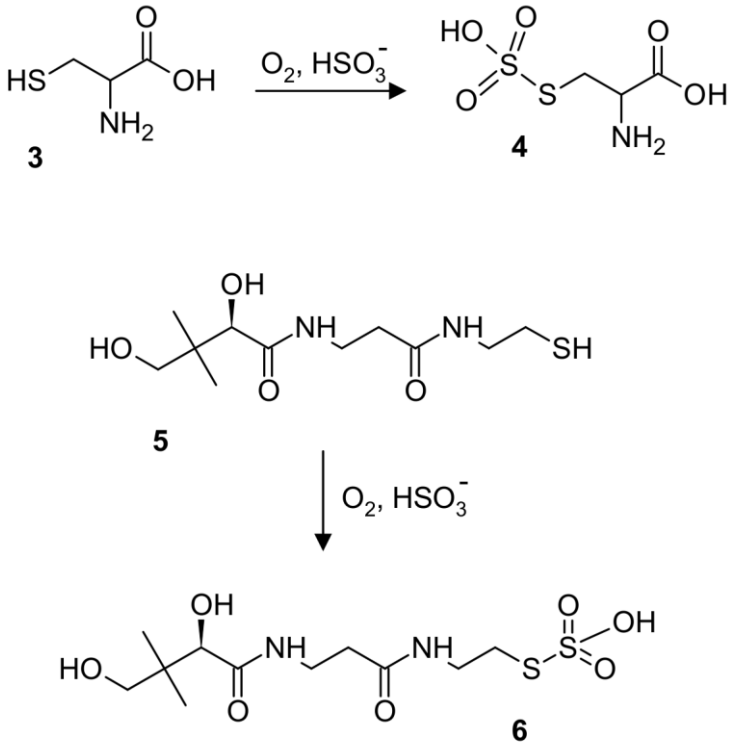

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803 FIGURE 9
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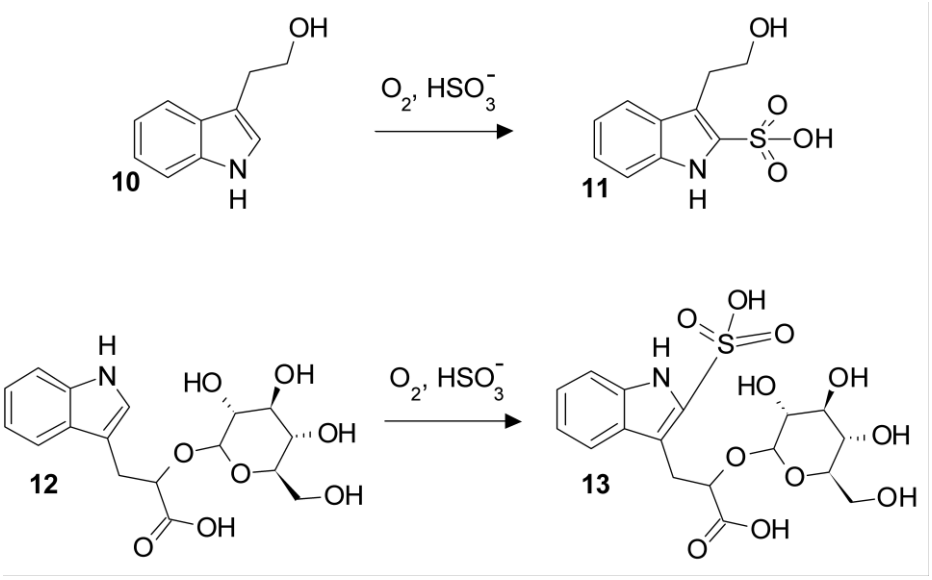

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