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# 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 to be 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 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 (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 levels of oxygen on bottling.



## 1. Introduction

Oxygen is certainly one of the most important players in wine-making, 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 own wineries, since theoretically the wine is under their complete control. However, during wine distribution or storage in restaurants, supermarkets, wine shops and consumers' homes, it is almost impossible for the oenologist to control oxygen–wine interaction. For this reason packaging choices during bottling are critical and decisive for the life of wine.

Indeed, oxygen management through packaging represents a major challenge in oenology, as different wine varieties or wine styles will behave differently under 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 during bottling. For example, the vast majority of white wines are designed to be consumed within 12 months of production and are sensitive to oxidation, so packaging choices should therefore prevent the entrance of unwanted amounts of oxygen [6].

In spite of recent studies [1,2,5,7,8] about the influence of bottling- and closure-derived oxygen on wine, the management of oxygen at bottling time 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 effect that a small amount of oxygen may have in the metabolomic space of any wine, probably because to date the oxygen response of only a relatively small group of

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metabolites (e.g. $SO_2$, volatile sulfur compounds or anthocyanins) has been investigated [1,2,5,7,8]. 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 thousands 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 shown its effectiveness 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 [1,9–13]. 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 economic perspective, given the widely accepted assumption that just a few mg of oxygen at bottling can negatively influence quality, especially in the case of white wines, the wine industry has made huge investment worldwide in order to install inert bottling lines, which to date have represented the standard process. In order to prevent unwanted oxidation, it is the customary practice for wines to be loaded with standard amounts of exogenous antioxidants (usually $SO_2$ or a combination of $SO_2$ and ascorbic acid) before bottling, in order to protect even the most susceptible wine. A better understanding of the effects of oxygen inserted at bottling time on the consumption of exogenous antioxidants and 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 ultimate goal is to contribute towards promoting a knowledge-based reduction in the amount of exogenous antioxidants. 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 using standard industrial parameters with the same wines bottled in sub-optimal – but still realistic – industrial conditions. To study the effects that oxygen can have after two months of storage, for the sub-optimal conditions wines were bottled without inert gas, with extra headspace, and with a closure allowing higher oxygen ingress. To obtain a high level of variability, the experimental design included 12 white wines made using 6 varieties, with 9 bottles 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 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 °C for two months, and then at 4 °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 °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 measurement was carried out using luminescence technology optical fibre outside the glass bottle, the method was totally non-invasive and the bottles remained closed throughout 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 [14]. Table 1 shows the total package oxygen concentrations in ppm for each wine and bottling condition (HO and LO).

### 2.3. Sample preparation

Before any treatment, in agreement with the workflow applied in our laboratory [13,15,16], the sample metadata were uploaded in ISA-Tab format using ISAcreator MetaboLights software [17] and codified according to a randomised sequence, so sample preparation and analysis were completed following this randomised 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.

### 2.4. 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.