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Research Article

Analysis of Polysulfides in Aged Sparkling Wines From Different Vintages

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Traditional sparkling wines are obtained from a vinification process with an additional 'second' fermentation in the bottle, which has an essential impact on the sensorial characteristics. During this maturation phase, several compounds are released from the yeast in autolysis, including the tripeptide glutathione. This compound has recently gained more interest due to its role in the formation of polysulfides and the subsequent possibility of H_2S release post-bottling. Hence, the release of sulphur-containing compounds like glutathione during an ageing 'sur lies' ought to be considered regarding the accumulation and persistence of polysulfides. In this study, the presence of polysulfides in traditional sparkling wines was validated to gain a preliminary understanding of the role of ageing time and glutathione. UHPLC-HRMS allowed the detection of two glutathionyl trisulfides in a selection of different naturally sparkling wines. Besides, it was found that the wines from vintage 2020 had a relatively high formation of GS_3G and GS_3C compared to vintages 2018 and 2019. Moreover, a trend between the trisulfides and GSSC was observed, possibly related to glutathione condensation and cysteine uptake by the yeast. This study showed the presence of polysulfides in various sparkling wines for the first time, increasing the availability of the current knowledge on this topic.

Keywords: cysteine; glutathione; polysulfides; sparkling wine; wine ageing

1. Introduction

During the last few years, the global wine market size has been expanding, from which the sparkling wine share is expected to follow the fastest compound annual growth rate of 6.6% from 2021 to 2028 [1]. A particularly growing interest in spritz drinks, thanks to the increasing aperitivo culture in both Europe and America, has led to a continuous rising in sales of various sparkling wine brands. These developments resulted in a higher interest in sparkling wine in the research sector as well, as wine producers can benefit from scientific knowledge related to this popular alcoholic beverage. Naturally, winemakers aim to optimise their product with satisfactory sensorial characteristics, considering the entire process of vinification. The occurrence of wine defects is an important topic in wine science in order to gain an understanding of possible factors influencing the formation of undesired scents. Especially sparkling wines produced from the traditional method involve a significant evolution of the chemical composition and organoleptic profile, considering the additional second fermentation on the lees. During this maturation phase, autolysis of yeast takes place, resulting in the release of several cellular compounds influencing the wine composition and sensorial properties [2–5]. Glutathione (GSH) is one of these compounds and has already been studied for its effects on the evolution of traditionally produced sparkling wines. Its slow release in sparkling wine during lees ageing has demonstrated various positive effects on aroma and oxidative stability [6, 7]. However, recent studies have pointed out the role of this tripeptide in the accumulation of polysulfides. The formation of these odourless sulphur compounds is

caused by an oxidation reaction between thiols (like GSH) in the presence of Cu (II) or elemental sulphur (S). Once polysulfides are formed, they represent a potential reservoir of H_2S [8–10], resulting in the appearance of reductive notes during ageing or storage of wine. Hence, the role of polysulfides in wine has become crucial in oenological research during these recent years. The hypothesis that polysulfide accumulation could be significant in traditional sparkling wine has arisen from the substantial contribution of GSH and prolonged contact with yeast. The latter is particularly relevant considering that yeast has an important role in the formation of polysulfides and reductive off-odours from volatile sulphur compounds [9, 11–13]. Yet, performing technological studies on polysulfides in real matrices is challenging due to the low natural concentration of these compounds and their relatively low chemical stability. Besides, the limited sensitivity of the currently accessible instrumentation is an important bottleneck for the detection of polysulfides in wine up until now. Therefore, polysulfide studies in wine have primarily been performed in model or spiked wines in order to increase the concentration of the compounds to exceed the lower detection limit. Only a few studies focussed on the natural presence of polysulfides in wine matrices; the first study that revealed the appearance of GS_3G in real wines was performed by Jastrzembski et al. [14]. More recent work by Van Leeuwen et al. [15] identified six symmetrical and asymmetrical glutathionyl and cysteinyl polysulfides in white wine. However, these studies primarily focus on the identification and characterisation of these compounds, and so far, no comparison studies on the presence of polysulfides in real wines have been carried out.

In the current work, the presence of different polysulfides in traditional sparkling wines from different vintages and refinements 'sur lie' was studied for the first time. Moreover, different ageing times and the presence of glutathionyl disulfides from oxidised GSH were evaluated in order to gain more knowledge on the possible role of these two factors during sparkling winemaking. This study is of great relevance considering the gradual polysulfide degradation and consequent H_2S release during prolonged storage, as in the case of wines ageing on the lees. New information on this topic is important for the winemaking industry in order to evaluate the risk of quality alterations and possible wine defects related to different phases of the vinification process.

2. Materials and Methods

2.1. Chemicals and Reagents. LC-MS grade acetonitrile (ACN, LC-MS; 99.9%), methanol (MeOH; LC-MS, 99.9%) and formic acid (FA; 98%) were obtained from Fluka (St. Louis, MO, USA). Copper sulphate ($CuSO_4$; 99.5%) was purchased from Carlo Erba (Milano, Italy), while reduced L-glutathione (GSH; ≤ 98%) and L-cysteine (Cys; 97%) were obtained from Sigma Aldrich. The positive mass calibration solution, containing n-butylamine (characteristic mass: m/z 74.09643), caffeine (m/z 195.08765), methionine-arginine-phenylalanine-alanine peptide (MRFA; m/z 524.26496) and Ultramark 1621 [10, 13, 16] was bought from Pierce® (Rockford, IL, USA). Deionised water was generated using an Arium®Pro Lab Water System (Sartorius AG, Goettingen, Germany).

2.2. Preparation of Standard Solutions of Mixed Polysulfides. Three separate standard solutions of GS_nG, GS_nC and CS_nC were prepared in MilliQ water according to the method described by Dekker et al. [10], with GSH (0.2 mM) and $CuSO_4$ (0.1 mM), GSH (0.2 mM), Cys (0.2 mM) and $CuSO_4$ (0.1 mM) and Cys (0.2 mM) and $CuSO_4$ (0.1 mM), respectively.

2.3. Preparation of Sparkling Wine Samples. The 57 sparkling wines from different vintages (2018, $n = 21$; 2019, $n = 17$; 2020, $n = 19$) were obtained from the micro-winery of Fondazione Edmund Mach (San Michele all'Adige, TN, Italy). The wines had been obtained from different grape juices from Trentino and subsequently subjected to the same vinification conditions, fermented with the same yeast. All wines were stored under the same conditions (18°C, dark environment, typical winery storage ambient) but for a different time depending on the production year; vintages 2018, 2019 and 2020 were subjected to maturation for 4, 3 and 2 years, respectively. After ageing, all bottles were opened at the same moment, added separately in 50 mL centrifuge tubes and manually degassed by shaking for 10 min each. Subsequently, the wines were centrifuged at 5000 rpm for 10 min and transferred to 2 mL HPLC vials. After the sample preparation, the samples were directly analysed by UHPLC-HRMS.

2.4. UHPLC-HRMS Analysis for Polysulfide Detection. UHPLC (Ultimate 3000, Dionex, Thermo Fisher Scientific) coupled to a Q-Exactive™ mass spectrometer (Thermo Fisher Scientific, Sunnyvale, CA, USA) with a heated ESI source (HESI-II, Thermo Fisher Scientific) was used for the analysis of the wines. An online sample clean-up was carried out before chromatographic separation, adapting the method according to Dekker et al. [17]: 2 times 100 μL was loaded on the solid-phase extraction (SPE) cartridge (Strata® C18-E 20 mm × 2.0 mm, 20 μm, MercuryMS™, Phenomenex®, Danaher, Torrance, CA, USA). Subsequently, the samples were chromatographically separated using an IonPac CS12A-MS column (2 mm × 100 mm, 8.5 μm; Thermo Fisher Scientific), according to Dekker et al. [17]. The mass analysis was operated in positive ion mode according to the conditions described by Van Leeuwen et al. [15]. For the compound and peak analyses, instrument software Chromeleon™ 7.3 (Dionex, Thermo Fisher Scientific) was used. Peak areas were normalised for the TIC signals of each sample, and statistical analysis of the data was performed using XLSTAT.

3. Results and Discussion

The presence of symmetric and asymmetric glutathionyl and cysteinyl polysulfides RS_nR was evaluated, with n up to 6.

After chemical analysis using UHPLC-HRMS and subsequent data analysis of the detected peaks, all sparkling wines revealed the presence of trisulfides GS_3G and GS_3C. Only 4 of the 57 samples demonstrated the appearance of CS_3C, and polysulfides with longer S-chains were not identified in any of the wines. Similarly, in the previous study performed by Van Leeuwen et al. [15], both GS_3G and GS_3C were detected in all 15 wines analysed, while CS_3C was only found in 13 of the samples. The low accumulation of symmetric cysteinyl polysulfides is explained by the relatively low concentration of Cys in must and wine as compared to GSH, as the latter is generally from 10 to 100 times higher [18, 19]. Therefore, Cys is also more likely to react with GSH to yield GSSC rather than condensate to CSSC.

Figure 1 shows the boxplots of the detected polysulfides, representing the data from the normalised peak areas of each of the compounds. The boxes indicate the first quartile, median and third quartile and the whiskers the minimum and maximum. The basic physicochemical parameters of the wines are provided in Table S1 in the electronic supplementary material (ESM).

Figure 1 demonstrates that the sparkling wines from the vintage 2020 generally presented a higher polysulfide intensity compared to the two older vintages. This was expected considering results from previous work about the polysulfide degradation in time [10], suggesting that older wines would be more depleted of these compounds. A Kruskal–Wallis two-tailed test ($\alpha = 0.05$) was performed to confirm the effect of the vintage and effectively showed a significantly higher abundance of the glutathionyl trisulfides in the wines from 2020 compared to the other years. Despite the expectation that younger wines would present an increased presence of polysulfides, a relatively higher abundance of both types of trisulfides was observed in the wines from 2018 compared to 2019. In fact, Figure 1 shows that polysulfides were not found in detectable numbers in the wines from 2019. This finding indicates the possible influence of intrinsic (for example, grape variety) and extrinsic effects (climatic and agronomical factors) potentially modifying the composition of the must and consequently the wine after fermentation. A principal component analysis (PCA) was performed using the basic physicochemical characteristics specified in Table S1. However, the PCA, observed from Figure 2, did not allow for speculation with regard to one of the variables, as the samples from the different years were homogeneously distributed. In fact, Figure 2 does not show any grouping of the samples of the three different vintages by means of the tested variables (indicated by the vectors in red). Principal component 1 (F1) explained 38.02% of the variance and principal component 2 (F2) explained 21.26% of the variance, representing 59.3% of the total variance. Correlations were found between the different variables—such as dry extract with density and glucose + fructose—but no sample clusters were observed. Nonetheless, external factors related to the vintage year could have an influence on compositional variables other than the ones included in the PCA.

The results shown in Figure 1 also showed an augmented presence of symmetric glutathionyl trisulfides as compared to the asymmetric trisulfides composed of both glutathione and cysteine. In fact, for the vintage 2020, a Wilcoxon signed-rank test ($\alpha = 0.05$) revealed a significantly higher intensity of GS_3G compared to GS_3C. Similar observations were found in previous studies performed on spiked Chardonnay wines, revealing increased formations of symmetric glutathionyl trisulfides. However, in that study, the wines were spiked with both GSH and Cys in excess amounts. In the present study, the relatively high formation of glutathionyl polysulfides is more likely attributed to a higher concentration of GSH compared to Cys naturally present in must and wine [18, 19]. Moreover, GSH is released during fermentation and cell autolysis, resulting in increasing concentration during the maturation phase. In contrast, Cys can possibly be taken up by the yeast when present in traditional sparkling wine during the ageing process. The presence of Cys could be the result of cell wall degradation compounds (among which mannoproteins) from autolysis and the release of this amino acid upon the proteolytic activity of living yeast. The extent of proteolysis could depend on the yeast strain and the time of ageing [20–23] and subsequently influencing amino acid release and the content of Cys. However, very few studies have investigated autolysis during bottle storage of sparkling wine, and no reports are available on the Cys uptake during maturation.

A first evaluation of the possible relationship between GSH and glutathionyl polysulfide formation was performed, comparing the presence of disulfides and trisulfide. In general, free GSH rapidly condensates to form GSSG due to its very low standard redox potential ($E^\circ = -240\,mV$ for thiol/disulfide exchange); hence, in the presence of oxygen, the free form is often present in relatively low amounts [9, 24, 25]. Therefore, the disulfide was considered more representative regarding the total amount of GSH compared to free GSH. In Figure 3, the relative abundance of both glutathionyl trisulfides is plotted against both the corresponding disulfides, separated in four different graphs.

From Figure 3, it can be observed that the accumulation of trisulfides did not reveal a significant correlation with GSSG, with an R^2 of 0.108 and 0.099 for GS_3G and GS_3C, respectively. This was in contrast to our expectations, as it was presumed that an increased GSH content would subsequently lead to more GSSG and more trisulfides. GSH could also have reacted with matrix compounds like phenols [26]; however, in white wines, the phenolic content is relatively low.

Surprisingly, when the trisulfide formation was compared with the presence of GSSC, a considerably better correlation was found with an R^2 of 0.347 and 0.368 for GS_3G and GS_3C, respectively. A reason for this increased correlation as compared to GSSG could possibly be explained by the role of Cys in intracellular polysulfide accumulation. In a recent study by Huang et al. [27], the addition of Cys to a model grape juice led to an increased formation of some polysulfides, in particular glutathionyl hydropersulfides and two oxidised forms after analysis of the yeast cells. The detected $CysS_3H$, $CysS_4H$ and GS_4H can possibly react through their free thiolate group when

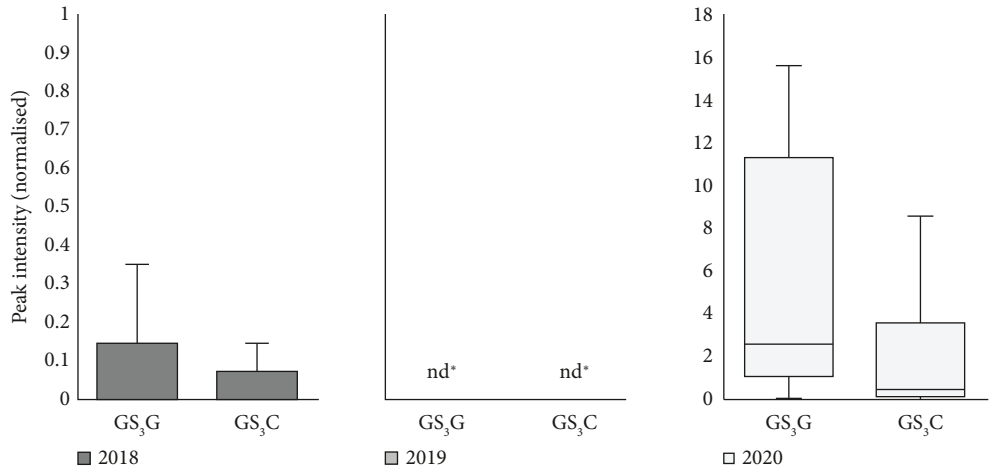

FIGURE 1: Boxplots of the formation of two identified glutathionyl trisulfides in sparkling wines from 3 different vintages (2018, $n = 21$; 2019, $n = 17$; 2020, $n = 19$). The boxes represent the first quartile, median and third quartile, the X the mean value and the whiskers the minimum and maximum. *nd: not detected.

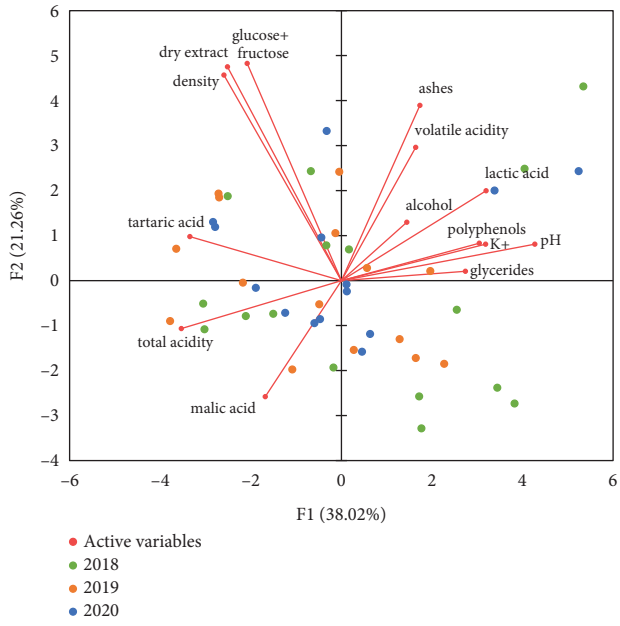

FIGURE 2: Biplot of principal component analysis (PCA), presenting the sample distribution of the wines ($n = 57$) from the different vintages, based on the basic physicochemical parameters (variables in red) and grouped and displayed by colour.

excreted by the yeast to form extracellular polysulfides [8]. Higher Cys concentration in the medium would also result in an increased formation of cysteinyl disulfides upon condensation. It is therefore speculated that when GSSC is more abundant, also hydropersulfide production by yeast is increased, subsequently leading to more GS_3G and GS_3C. This could possibly explain the better correlation with the asymmetric disulfide as compared to the symmetric one, but this finding requires more profound research in order to prove this hypothesis. In fact, the correlations that have been found are considered disputable, considering the possibility of other influencing parameters involved in both the formation of the dimer and the trimers. Nevertheless, the important difference between the two comparisons that have been observed in the present study suggests a high probability of biochemical factors involved in the formation of polysulfides with different sulphur-chain lengths, which ought to be studied in more detail.

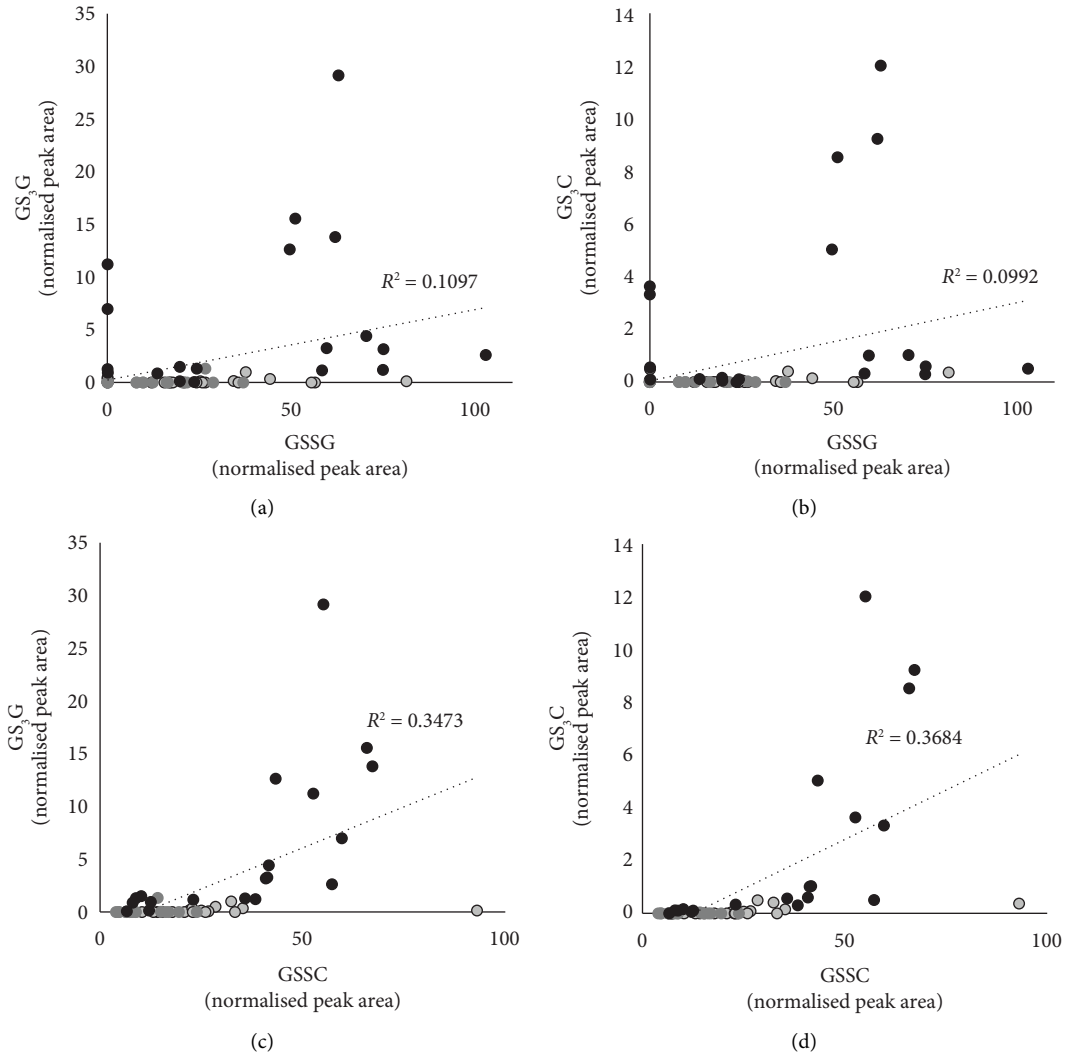

FIGURE 3: Scatter plots of glutathionyl trisulfide against glutathionyl disulfide (GS_3G vs GSSG (a); GS_3C vs GSSG (b); GS_3G vs GSSC (c); GS_3C vs GSSC (d)) accumulation in sparkling wines from different vintages: 2018 ($n = 21$), light grey; 2019 ($n = 17$), dark grey; 2020 ($n = 19$), black.

4. Conclusions

This study shows the first evidence of the presence of polysulfides in traditional sparkling wines. The accumulation of two glutathionyl trisulfides was successfully analysed in wines from different vintages with different periods of ageing, which allowed us to obtain a first result on the possible effect of the year and the maturation time. It was speculated that the release of GSH during autolysis of the yeast could play a role in the formation of polysulfides during the second fermentation in the bottle. No clear trend was found between vintage year and detected polysulfides, but a slight correlation was found between each of the trisulfides and GSSC, which was suggested to be related to the Cys uptake and subsequent polysulfide synthesis by the yeast. To the best of our knowledge, this is the first time that a comparison study on accumulated polysulfides in real sparkling wines has been performed. It is suggested to perform further profound studies on the kinetics of polysulfide formation and degradation during wine ageing on the lees and the parallel monitoring of yeast metabolites that play a potential role, in particular thiolate compounds. Moreover, an extended study on the compositional differences of grape must after harvest, caused by vintage-related factors, could be a stepping stone for finding linkages between the presence of polysulfides in wine and their chemical origin.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. *(Supporting Information)*

The basic physicochemical parameters of the wines used in the present research were determined in order to chemically characterise the wines. The data obtained are attached as Supporting Information and are presented in Table S1. Table S1: minimum (min), median (med) and maximum (max) of basic physicochemical parameters of sparkling wines, divided for each vintage.

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