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Widely Targeted Metabonomic Analysis to Study Effect of GSH on Metabolites of Chardonnay Wine during Simulated Oxidation

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Abstract: The effects of reduced glutathione (GSH) on non-volatile and volatile metabolites of Chardonnay wine during storage under simulated oxidation were investigated. The metabolites of GSH, which play a key role in the storage of white wine, were identified. In this study, GSHs at 0, 10, and 20 mg/L were added to wine samples and stored at 45 °C for 45 days. Wine samples supplemented with 0 mg/L GSH were used as controls (CK). The samples stored for 45 days were analyzed via ultra-high performance liquid chromatography–tandem mass spectrometry and gas chromatography–tandem mass spectrometry. A total of 1107 non-volatile metabolites were detected, and 617 volatile metabolites were identified. Variable Importance in Projection (VIP) of >1.0 and Fold Change (FC) of ≧2.0 were used to screen differential metabolites. A total of 59 important non-volatile and 39 differential volatile metabolites were screened. Among the non-volatile metabolites, 17 substances were down-regulated, whereas 16 substances were up-regulated. Among the volatile metabolites, 3 substances were down-regulated, while 19 substances were up-regulated. After analysis, some lipids were found to play an important role in the changes to non-volatile substances. This study provides theoretical support for further application of GSH in increasing the oxidation stability of white wine.

Keywords: chardonnay wine; GSH; non-volatile metabolites; volatile metabolites

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1. Introduction

GSH (reduced glutathione) is an active tripeptide and an important antioxidant. As a nuclear substance, it can be directly combined with a reactive electrophilic reagent to maintain the oxidation stability of foods and beverages [1,2]. Oxidation of grape must and wine is one of the main problems that occurs during the production of white wines. Traditionally, sulfur dioxide (SO_2) has been used to prevent oxidation; however, this chemical is toxic and allergenic. Glutathione can inhibit the oxidative browning of wine and prevent the loss of wine aroma, particularly for white wines, as adding glutathione is beneficial to maintaining the wine's characteristics and color stability [3,4]. Studies have been conducted to investigate the use of GSH to replace or reduce SO_2; however, it is still in the experimental stage.

The addition of exogenous food-grade GSH to a wine bottle before storage can prevent the oxidation and aroma loss of wine. Although the levels of GSH can decrease during aging, it is known to remain effective in protecting important aromatic compounds, such as monoterpenes, esters, and volatile thiols [5,6]. The addition of GSH during bottling can not only limit acetaldehyde accumulation, but can also help to preserve aromatic complexity after 12 months of storage [7]. Marchante et al. evaluated the potential of

different antioxidant substances, such as GSH, in white and red wine, and found that they can prevent the formation of acetaldehyde and consume oxygen [8]. Among many antioxidants, GSH has received high attention from the scientific community [9–11]. Yeast derivatives (YDs) applied biotechnology is a new strategy that has been proposed to control wine oxidation through oxygen consumption and antioxidant release during bottle storage. It has been shown that inactivated yeast that is rich in GSH could stabilize wine varietal aromas, such as volatile thiols and terpenes [12]. It has also been shown that under certain conditions, GSH cannot provide the expected protection to wine and might even damage the final color of white wine [13]. Silvia et al. discovered that compared with SO_2, GSH has lower antioxidant efficacy in protecting both the color and aromatic components of wines, and this is probably due to its lower molar concentration. However, the presence of GSH can decrease the oxidative losses of SO_2, especially in wines with higher SO_2 levels [14].

At present, the direct addition of glutathione as a food additive to wine is not allowed in European nations. The effect of GSH on the oxidative stability of wine also depends on the dosage and the way in which GSH is added. Additionally, the effect and mechanism of action of GSH on polyphenols, aromatics, and other substances in wine remain unclear. It is necessary to use a variety of analytical techniques to explore the influence and mechanism of GSH on wine quality. In recent years, metabolomic methods have been used in many research fields to comprehensively analyze non-volatile components. Ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) and gas chromatography–tandem mass spectrometry (GC–MS/MS) have been used to analyze the non-volatile and volatile metabolomics of wine samples supplemented with 20 mg/L GSH after 5 months of storage. The effect of GSH on some phenolic acids, fatty acids, and amino acids has been analyzed, as these substances may play an important role in the changes to non-volatile substances [15]. Ultra-high-resolution mass spectrometry (FTICR-MS) metabolomics has been used to study the oxidation stability of aged Chardonnay wine. One study found that the influence of glutathione on oxidation stability may be related to the antioxidant metabolism of N and S compounds, such as amino acids, aromatic compounds, and peptides, in wine [1].

Widely targeted metabolomics involves the use of UPLC-MS/MS combined with a widely used technique using targeted metabolomic technology to analyze non-volatile metabolites in samples, and volatile metabolomics involves the use of GC-MS/MS to be employed for the detection of volatile metabolites, due to their fast separation, high sensitivity, and wide coverage. To elucidate the effects of GSH addition on metabolites of Chardonnay wine during storage, herein we combined widely targeted metabolomics and volatile metabolomics to study the effects of GSH on non-volatile components and volatile components of Chardonnay wine under simulated oxidation storage conditions at 45 °C, as well as the evolution between these metabolites. This study is expected to provide a theoretical reference and objective basis for the application of GSH in white wine.

2. Materials and Methods

2.1. Materials and Reagents

Chardonnay grapes were obtained from Longhu Agricultural Planting Co., Ltd., Penglai, Shandong Province, China, in October 2021. *Saccharomyces cerevisiae* (LALVIN EC-1118) and pectinase extract (Lafazym) were purchased from Lallemand Inc., Montreal, QC, Canada; 500 g).

Nexera X2 ultra-high-performance liquid chromatography–tandem mass spectrometry UPLC-MS (equipped with Applied Biosystem 4500 qtrap; Shimadsu, Japan) and a temperament combination instrument 8890-7000D GC-MS/MS (Agilent Inc. (Santa Clara, CA, USA)) were used for analysis.

2.2. Sample Preparation

Chardonnay grapes were descaled and pressed to obtain juice. After that, 0.02 g/L pectinase was added, and 60 mg/L potassium metabisulfite ($K_2S_2O_5$) was clarified at

10–12 °C for 24 h. Subsequently, 0.20 g/L yeast EC1118 was added to the supernatant at 18–20 °C, and the fermentation was complete when the reducing sugar content was lower than 2 g/L. Thereafter, the juice was naturally clarified. After 15 days, the juice was transferred to a 100 L airtight jar and stored for 5 months until bottling. The physicochemical indexes of the wine were as follows: alcohol content, 12.67%, and reducing sugar, 1.36 g/L. The experimental arrangement was as follows: 0 mg/L GSH, 10 mg/L GSH, and 20 mg/L GSH were added, respectively, into the wine before bottling. Three replicate samples were prepared for each group of experiments; that is, three wine samples containing GSH at each concentration were prepared. The wine samples containing 0 mg/L GSH were used as controls, and all samples were stored at 45 °C. After 45 days, the wine samples containing 10 mg/L GSH (T), the wine samples containing 20 mg/L GSH (G), and the control wine samples (CK) were subjected to extensive targeted metabolome analysis.

2.3. Widely Targeted Metabolome Analysis

Sample extraction process: 5 mL of mixed sample was placed in a 10 mL centrifuge tube. The centrifuge tube was then immersed in liquid nitrogen until the sample was completely frozen. After that, the frozen sample was freeze-dried in a freeze-drying machine. After 500 μL of 70% methanol internal standard extract was added, and the mixture was vortexed for 15 min and then centrifuged for 3 min (12,000 r/min, 4 °C). The supernatant was filtered through a 0.22 μm microporous filter membrane before LC-MS/MS analysis.

2.4. LC-MS/MS Analysis

Liquid-phase conditions: The target compounds were separated on an Agilent SB-C18 column (1.8 μm, 2.1 mm × 100 mm) with a mobile phase A consisting of ultra-pure water (0.1% formic acid added) and a mobile phase B consisting of acetonitrile (0.1% formic acid added). The sample size was 2 μL, the column temperature was set at 40 °C, and the flow rate was set to 0.35 mL/min. The elution gradient was set as follows: at 0–9 min, 5%B–95%B and maintained at 95%B for 1 min; at 10–11.1 min, 95%B–5%B and maintained at 5%B for 14 min.

Mass spectroscopy conditions: Ion source, electrospray ion source; temperature, 500 °C; ion spray voltage, 5500 V for positive-ion mode and −4500 V for negative-ion mode; ion source for gas I, gas II, and curtain gas, 50, 60, and 25 psi, respectively; and collision-induced ionization parameters, high. A quadrupole (QQQ) and LIT were applied in quadrupole tuning and mass calibration, respectively, and a polypropylene glycol solution at 10 and 100 μmol/L was used. The declustering potential (DP) and collision energy (CE) scanning and detection of ion pairs were carried out by further optimizing the DP and CE. The metabolites were qualitatively analyzed using the self-established MWDB (Metware database), the second-order spectral information, and the multiple reaction monitoring (MRM) analysis in triple quadrupole mass spectrometry.

2.5. Metabolome Analysis of Volatiles

Sample extraction process: 1 mL of sample was placed in a headspace bottle, and saturated NaCl solution and 10 μL of internal standard solution (benzyl acetate, 50 μg/mL) were added, respectively. The extraction was fully automated using automatic headspace solid-phase microextraction (HS-SPME).

HS-SPME extraction conditions: The samples were incubated at 60 °C for 5 min, extracted with an overhead space at 120 μm DVB/CWR/PDMS for 15 min, and then analyzed at 250 °C for 5 min. The SPME Arrow was used as the extraction head, and its sensitivity could reach 10 times that of the traditional SPME fiber head. The extraction head was incubated for 5 min at 250 °C before sampling, and the new extraction head was incubated for 2 h.

Chromatographic conditions were as follows: Capillary column, DB-5MS (30 m × 0.25 mm × 0.25 μm, Agilent J&W Scientific, Folsom, CA, USA); inert gas, high-

purity helium gas (purity ~99.99%); inlet temperature, 250 °C; and flow rate, 1.2 mL/min. Without shutter injection, the solvent was delayed for 3.5 min, and the temperature was programmed to rise to 40 °C for 3.5 min, to 100 °C at a rate of 10 °C/min, to 180 °C at rate of 7 °C/min, and finally to 280 °C at a rate of 25 °C/min for 5 min.

Mass spectroscopic conditions were as follows: ion source, electron bombardment (EI); ion source temperature, 230 °C; electron energy, 70 eV; mass spectrum interface temperature, 280 °C; quadrupole temperature, 150 °C; and scanning modes, select ion detection mode (SIM) and ion precision scanning (GB 23200.8-2016).

2.6. Statistical Analysis

Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed using R language software (in version 3.3.3, https://www.r-project.org/ (accessed on 15 July 2023)) to analyze the identified metabolites. Variable importance in projection (VIP) of the multivariate analysis OPLS-DA model was determined. Significantly changed metabolites (SCMs) were further screened based on the *p*-value or FC values obtained from univariate analysis. Screening criteria, VIP > 1 and FC $\geq$ 2.0, were adopted to screen metabolites.

3. Results and Discussion

3.1. Effect of GSH on Non-Volatile Metabolites in Chardonnay Wine during Simulated Oxidation

3.1.1. Overall Analysis of Metabolic Components in White Wine

UPLC-MS widely targeted metabolomics technology was used to identify primary and secondary metabolites in wine samples in the CK, T, and G groups stored at 45 °C for 45 days by comparing them with MS/MS spectral information from public databases and standards from MetWare's self-developed metabolite database. A total of 1107 metabolites were identified, including 142 amino acids and their derivatives, 248 phenolic acids, 28 terpenoids, 69 nucleotides and their derivatives, 124 organic acids, 99 lipids, 148 flavonoids, 29 lignin and coumarins, 92 alkaloids, 8 tannins, and 120 other metabolites.

To understand the overall metabolic differences between samples and the variation in samples in the same group, PCA analysis was conducted. The results from PCA are shown in Figure 1a. The PCA of non-volatile compounds indicated that 37.34% of the total variance could be explained by the first two principal components. PC1 and PC2 represented 22.4% and 14.95%, respectively. This reflects the main characteristic information of different samples of white wine. The three groups of samples were clearly grouped and could be better distinguished by the first two principal components. This indicates that the metabolite composition of Chardonnay wine is affected by the addition of GSH at different concentrations. OPLS-DA analysis is a multivariate statistical analysis method with supervised pattern recognition that can effectively eliminate irrelevant effects and screen for differential metabolites. Metabolomic data were analyzed based on the OPLS-DA model and used to draw score maps for each group to further demonstrate the differences between each group. As shown in Figure 1b–d, the three duplicate data points of the CK and G groups, the CK and T groups, and the T and G groups were not superimposed. This indicates that different groups of samples could be clearly distinguished, and the metabolites in these groups were different. The OPLS-DA model was verified. The prediction parameters, the R2Y and Q2 values, of CK and G were 1 and 0.786, respectively, whereas those of CK and T were 1 and 0.603, respectively. The R2Y and Q2 values of T and G were 1 and 0.628, respectively. These parameters were greater than 0.5, indicating that the OPLS-DA models had good prediction ability, and there was no overfitting.

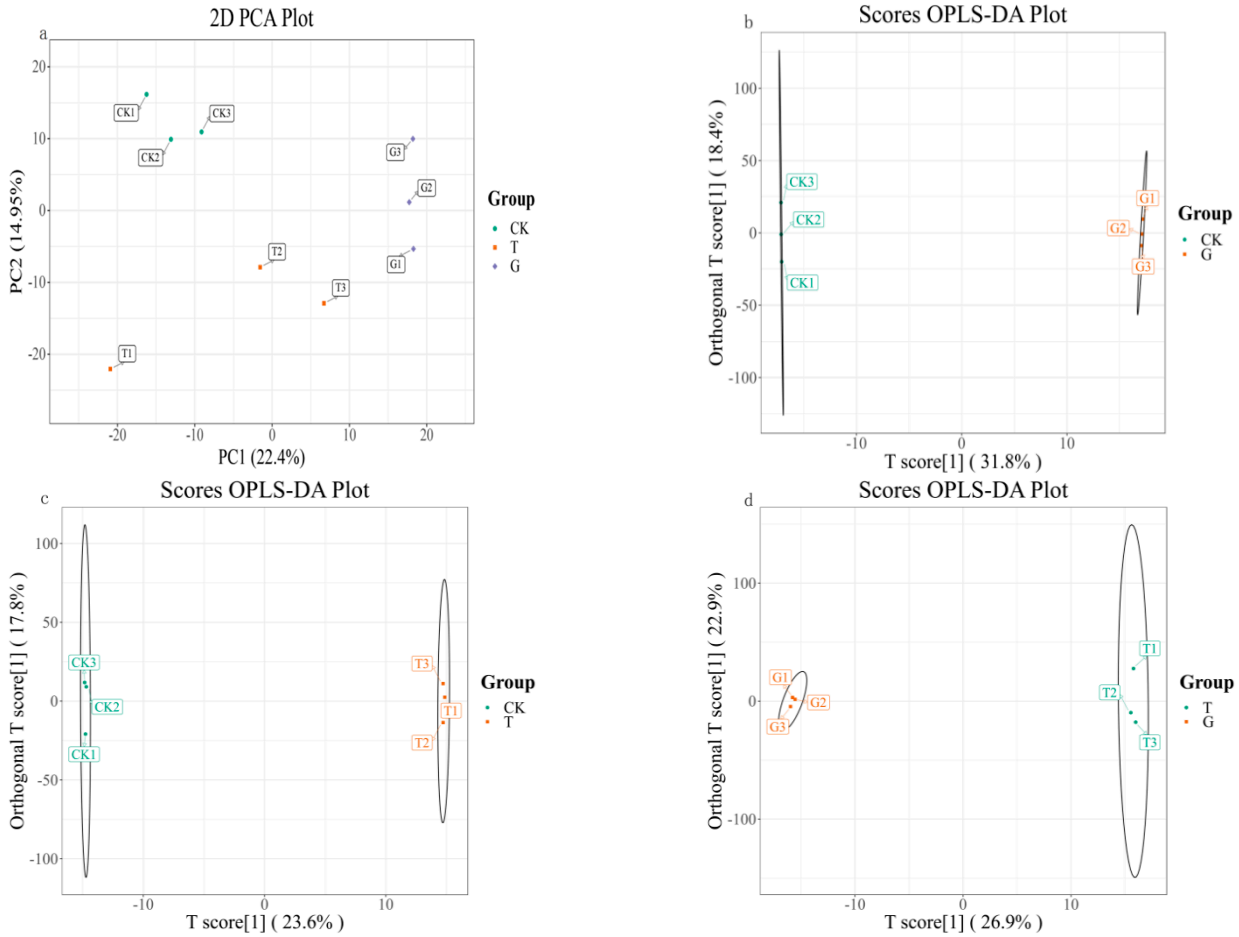

Figure 1. PCA score scatter plot composed of all non-volatile metabolites (**a**) and OPLS-DA of non-volatile metabolites of CK vs. G, CK vs. T, and T vs. G (**b**–**d**).

3.1.2. Screening and Analysis of Differential Metabolites

To elucidate the effect of GSH at different concentrations on non-volatile metabolites during simulated oxidation of Chardonnay wine, different metabolites were screened based on the screening criteria, VIP > 1.0 and FC $\geq$ 2.0. A total of 59 different metabolites were selected, and their percentages are shown in Figure 2. Among all metabolites in the CK and G groups, 38 metabolites were different (Figure 3a: 16 metabolites were up-regulated, while 22 were down-regulated). Additionally, among all metabolites in the CK and T groups, 43 metabolites were different (Figure 3b: 16 metabolites were up-regulated, whereas 27 were down-regulated). A total of 30 metabolites in the T and G groups were different (Figure 3c: 16 metabolites were up-regulated and 14 were down-regulated). According to the Venn diagram (Figure 4), it is apparent that samples in different groups contain both common and unique metabolites.

The CK and G groups and the CK and T groups shared twenty-two differential substances (Table 1), all of which were simultaneously affected by GSH at both low (10 mg/L) and high (20 mg/L) concentrations. These substances included two amino acids and their derivatives, three phenolic acids, three nucleotides and their derivatives, four flavonoids, one alkaloid, four terpenoids, and five lipids. There were a total of twelve metabolic substances with increased relative content, which included two amino acids and their derivatives, three phenolic acids, two nucleotides and their derivatives, two flavonoids, one alkaloid, and two lipids. Among them, L-Leucine and 1-Beta-D-arabinofuranosyluracil showed the largest fold change.

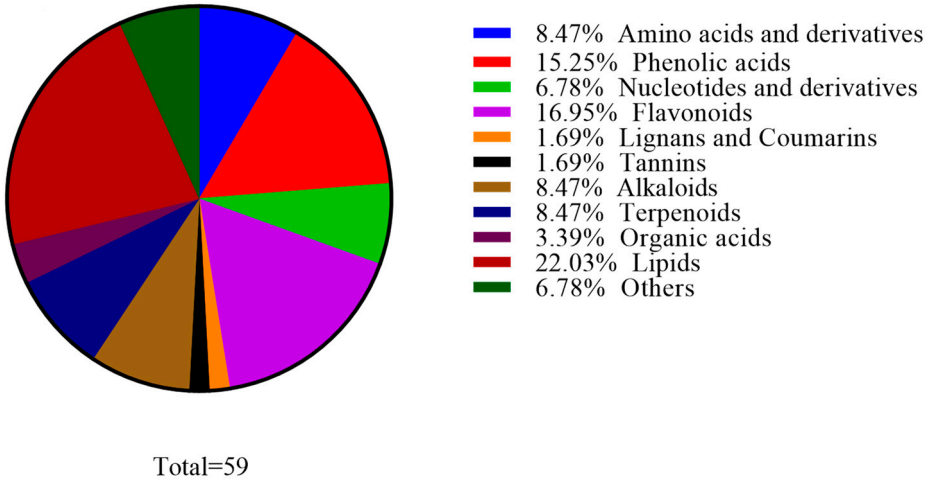

Figure 2. Pie chart of differential metabolites.

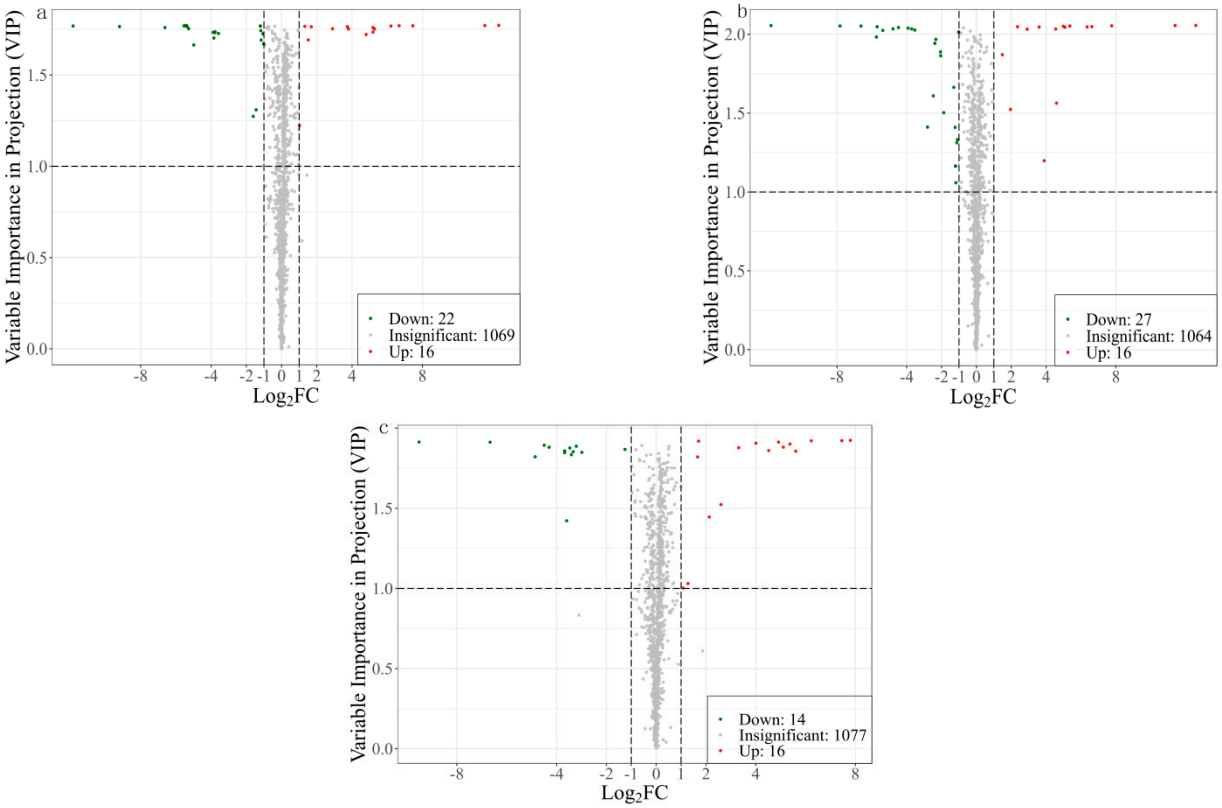

Figure 3. Volcano plot of the differential non-volatile metabolites of CK vs. G, CK vs. T, and T vs. G. (**a**–**c**), red and green dots represent the up-regulated and down-regulated metabolites).

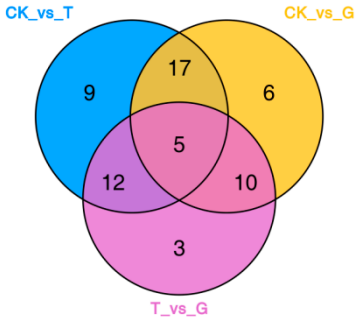

Figure 4. Venn diagram of the differential non-volatile metabolites of CK vs. T, CK vs. G, and T vs. G.

Table 1. Metabolically differential substances between group CK vs. G and group CK vs. T.

Code	Compound	Class I	CK vs. T		CK vs. G	
			Fold Change	Up/Down	Fold Change	Up/Down
1	L-Leucine	Amino acids and derivatives	6157.155	Up	5157.34	Up
2	Oxiglutatione	Amino acids and derivatives	31.665	Up	13.319	Up
3	3,4-Dimethylbenzoic acid	Phenolic acids	24.153	Up	36.448	Up
4	Vnilloyltartaric acid	Phenolic acids	217.094	Up	175.151	Up
5	1-O-caffeoyl-3,4-di-O-galloyl-β-D-glucose	Phenolic acids	41.167	Up	36.542	Up
6	Adenosine 5′-monophosphate	Nucleotides and derivatives	81.771	Up	74.159	Up
7	1-Beta-D-arabinofuranosyluracil	Nucleotides and derivatives	2708.792	Up	2983.773	Up
8	Riboflavin 5′-adenosine diphosphate	Nucleotides and derivatives	0.0759	Down	0.067	Down
9	kaempferol-3-O-(2″-p-coumaroyl)galactoside	Flavonoids	33.753	Up	38.789	Up
10	Diosmetin-7-O-neohesperidoside (Neodiosmin)	Flavonoids	98.80	Up	102.440	Up
11	Quercetin-3-O-sophorotrioside-7-O-arabinoside	Flavonoids	0.429	Down	0.368	Down
12	Quercetin-3-O-(2″,3″-O-digalloyl)-glucoside	Flavonoids	0.458	Down	0.328	Down
13	N′,N″,N‴-p-coumaroyl-cinnamoyl-caffeoyl spermidine	Alkaloids	5.128	Up	3.217	Up
14	3-Epiursolic acid	Terpenoids	0.192	Down	0.024	Down
15	Ursolic acid	Terpenoids	0.241	Down	0.023	Down
16	Mangiferolic acid	Terpenoids	0.199	Down	0.022	Down
17	Isomangiferolic acid	Terpenoids	0.240	Down	0.024	Down
18	Dihydrosphingosine-1-phosphate	Lipids	7.529	Up	7.443	Up
19	13-hydroperoxy-9Z,11E-octadecadienoic acid	Lipids	0.493	Down	0.442	Down
20	7S,8S-DiHODE; (9Z,12Z)-(7S,8S)-dihydroxyoctadeca-9,12-dienoic acid	Lipids	0.493	Down	0.435	Down
21	LysoPE 18:2(2n isomer)	Lipids	14.798	Up	13.934	Up
22	16-Methylheptadecanoic acid	Lipids	0.001	Down	0.001	Down

Note: "Up" and "Down" indicate significant increases and significant decreases in content, respectively.

The relative contents of ten metabolic substances decreased, including one nucleotide and its derivatives, two flavonoids, four terpenoids, and three lipids. The decline multiple is less than half, or even lower.

Twenty-one unique non-volatile metabolites of CK and T were only affected by GSH at a low concentration (10 mg/L, Table 2). These volatiles included two amino acids and their derivatives, four phenolic acids, three flavonoids, one lignin and coumarin, one tannin,

three alkaloids, two organic acids, four lipids, and one other substance. There were a total of four metabolic substances with increased relative content, including one amino acid and its derivatives, one flavonoid, and two lipids. The fold change was 2.803~23.43 times that of the control group, and benzoylformic and methyl dihydrojasmonate showed the largest of 23.43 and 14.80, respectively. There were seventeen metabolic substances with decreased relative content, including one amino acid and its derivatives, four phenolic acids, two flavonoids, one lignin and coumarin, one tannin, three alkaloids, two organic acids, two lipids, and one other substance. Among them, 2,4-dihydroxyquinoline and Ellagic acid-4-O-glucoside had the largest decline times.

Table 2. The unique metabolically differential substances of group CK vs. T.

Code	Compound	Class I	Fold Change	CK vs. T
1	N-acetyl-L-tryptophan	Amino acids and derivatives	0.470	Down
2	Glutathione reduced form	Amino acids and derivatives	2.803	Up
3	Isochlorogenic acid B	Phenolic acids	0.436	Down
4	Isochlorogenic acid C	Phenolic acids	0.436	Down
5	1-O-galloyl-6-O-feruloyl-β-D-glucose	Phenolic acids	0.036	Down
6	1-O-galloyl-4-O-feruloyl-β-D-glucose	Phenolic acids	0.045	Down
7	Myricetin	Flavonoids	3.882	Up
8	Quercetin-3-O-rhamnoside (Quercitrin)	Flavonoids	0.407	Down
9	Quercetin-3,7-Di-O-glucoside	Flavonoids	0.180	Down
10	Dihydrodehydrodiconiferyl alcohol	Lignans and coumarins	0.272	Down
11	Ellagic acid-4-O-glucoside	Tannins	0.010	Down
12	4-Hydroxymandelonitrile	Alkaloids	0.143	Down
13	2,4-Dihydroxyquinoline	Alkaloids	0.004	Down
14	Caffeoylcholine-4-O-glucoside	Alkaloids	0.019	Down
15	Benzoylformic acid	Organic acids	0.019	Down
16	Methyl dihydrojasmonate	Organic acids	0.024	Down
17	Docosapentaenoic acid	Lipids	23.43	Up
18	12-Hydroxyoctadecanoic acid	Lipids	14.80	Up
19	LysoPE 18:2	Lipids	0.086	Down
20	LysoPC 17:0	Lipids	0.066	Down
21	Glucarate O-Phosphoric acid	Others	0.442	Down

Note: "Up" and "Down" indicate significant increases and significant decreases in content, respectively.

Sixteen non-volatile substances that were unique to CK and G were only affected by high-concentration GSH (20 mg/L, Table 3). These substances included one amino acid and its derivatives, two phenolic acids, one nucleotide and its derivatives, three flavonoids, one alkaloid, one terpene, four lipids, and two other substances. Overall, there were a total of four metabolic substances with increased relative content, including one amino acid and its derivatives, one terpenoid, and two other types. The fold change was 2.030~27.87 times that of the control group, and S-(5′-Adenosyl)-L-methionine showed the largest fold change. Adding 20 mg/L GSH, all lipids, phenolic acids, and flavonoids decreased significantly.

Fifty-nine differential metabolites mainly included five amino acids and their derivatives, nine phenolic acids, four nucleotides and their derivatives, ten flavonoids, one lignin and coumarin, one tannin, five alkaloids, five terpenoids, two organic acids, thirteen lipids, and four other substances (Figure 2). There were a total of twenty metabolic substances with increased relative content, including four amino acids and their derivatives, three phenolic acids, two nucleotides and their derivatives, three flavonoids, one alkaloid, one terpenoid, four lipids, and two other substances. A total of thirty-nine metabolic substances with decreased relative content included one amino acid and its derivatives, six phenolic acids, two nucleotides and their derivatives, seven flavonoids, one lignin and coumarin, one tannin, four alkaloids, four terpenoids, two organic acids, nine lipids, and two other substances. Among all, twenty-one metabolites were unique to the CK and T groups, accounting for 35.59% of the total metabolites, while sixteen metabolites were unique to the CK and G groups, accounting for 27.12% of the total metabolites.

Table 3. The unique metabolically differential substances of group CK vs. G.

Code	Compound	Class I	Fold Change	CK vs. G
1	S-(5′-Adenosyl)-L-methionine	Amino acids and derivatives	27.87	Up
2	4-Hydroxy-3,5-diisopropylbenzaldehyde	Phenolic acids	0.032	Down
3	2-Phenoxyethanol	Phenolic acids	0.448	Down
4	3′-Adenylic Acid	Nucleotides and derivatives	0.072	Down
5	Quercetin-7-O-glucoside	Flavonoids	0.010	Down
6	5-Hydroxy-6,7,3′,4′-tetramethoxyflavone	Flavonoids	0.026	Down
7	5,6,7,4′-Tetramethoxyflavone	Flavonoids	0.084	Down
8	N-Oleoylethanolamine	Alkaloids	0.480	Down
9	Maslinic acid	Terpenoids	2.494	Up
10	13-Hydroxy-6,9,11-octadecatrienoic acid	Lipids	0.442	Down
11	9,10-DHOME; (12Z)-9,10-Dihydroxyoctadec-12-enoic acid	Lipids	0.073	Down
12	Cis-4,7,10,13,16,19-Docosahexaenoic Acid	Lipids	0.070	Down
13	LysoPC 18:0	Lipids	0.493	Down
14	Rhapontigenin	Others	2.863	Up
15	Xylitol	Others	0.002	Down
16	D-Threose	Others	2.030	Up

Note: "Up" and "Down" indicate significant increases and significant decreases in the content, respectively.

The Maillard reaction is the reaction between amino compounds (amino acids or proteins) and reducing sugars that forms brown or black macromolecular substances. This reaction can lead to the non-enzymatic browning of wine. Therefore, amino acids are linked to wine browning and are responsible for some typical aromas or aging aromas in wine due to the Maillard reaction [16]. After 45 days of simulated oxidation, compared with CK, L-leucine in amino acids was up-regulated in wine samples in the T and G groups, to which 10 and 20 mg/L GSH were added, respectively. S-(5′-adenosyl)-L-methionine was up-regulated only after the addition of 20 mg/L GSH. The up-regulation of amino acids in wine samples after the addition of GSH at different concentrations was more prominent than the down-regulation. This indicates that GSH had a protective effect on some amino acids in wine, possibly by reducing the non-enzymatic browning caused by amino acids. In addition, amino acids and sulfur-containing peptides can capture quinones formed during oxidation, as well as prevent the browning and loss of varietal aromas in wine [17]. Studies have also shown that nitrogen-containing and sulfur-containing compounds are the main contributors to the antioxidant metabolome of white wine. These compounds mainly consist of amino acids and peptides, which have significant antioxidant capacity (AC) due to their nucleophilicity [1,18]. Therefore, the addition of GSH may increase the antioxidant capacity of white wine by protecting some amino acids.

Phenolic acid compounds can react with sugars, alcohols, organic acids, etc., to form ester compounds. Hydroxycinnamic acids, including caffeic acid, coumaric acid, and ferulic acid, are phenolic acids with a high content in white wine. They are prone to oxidative browning, resulting in a loss of color and aroma, and in turn causing the quality of white wine to reduce [19]. Compared with the CK group of wine samples, 3,4-dimethylbenzoic acid, vanillin tartaric acid, and 1-O-caffeoyl-3,4-di-O-galloyl-β-D-glucose in wine in the G group were found to accumulate after 45 days of simulated oxidation. This indicates that 20 mg/L GSH could protect these phenolic acids against oxidation, and these phenolic acids could undergo changes upon the addition of GSH at 10 and 20 mg/L, respectively. Four phenolic acids that were unique to the T group, including isochlorogenic acid B, isochlorogenic acid C, 1-O-galliyl-6-O-ferucyl-β-D-glucose, and 1-O-galloyl-4-O-ferulic acid-β-D-glucose, were down-regulated. This indicates that 10 mg/L GSH can protect some phenolic acids against oxidation; however, it can also lead to a decrease in the relative content of some phenolic acids. By contrast, 20 mg/L GSH protects some phenolic acids in wine against oxidation without affecting other phenolic acids. Previous studies have shown that adding 20 mg/L GSH can result in the accumulation of phenolic acids, such

as propyl gallate (PG) and benzylsalicylic acid, in white wine after 5 months of storage. GSH can prevent the oxidation of these phenolic acids [15]. The relative contents of caffeic acid, coumaric acid, and ferulic acid in white wines with the addition of GSH were not significantly different from those in the control group, which is consistent with a report by Paneroet and Webber [7,20].

Some studies have shown that the browning that takes place during white wine aging may also be due to some flavanol compounds, such as catechins and epicatechin, in white wine [21]. Additionally, flavanols are important phenolic compounds in wine and are closely related to the bitterness and color of wine [22,23]. After 45 days of simulated oxidation, the flavonoid substances quercetin-3-O-sophora-7-o-arabin and quercetin-3-O-(2″,3″-o-digallic acid) glucoside in the T and G groups were down-regulated compared to the CK group wine. Two unique substances (quercetin-3-O-rhamnoside (quercetin) and quercetin-3,7-di-O-glucoside) in the T group wine were down-regulated. A unique change observed in the G group wine is the down-regulation of quercetin-7-O-glucoside, 5-hydroxy-6,7,3′,4′-tetramethoxyflavones, and 5,6,7,4′-tetramethoxyflavones. This might be due to the hydrothermal hydrolysis of flavonoid glycosides under the temperature of simulated oxidation (45 °C), which can lead to the production of glycosides and glycosomes, such as quercetin [24,25]. However, the down-regulation of flavonoid glycosides caused by the addition of GSH did not cause the up-regulation of quercetin and other glycosides. This indicates that the decomposed aglycone is re-oxidized, causing its content to decrease. However, the down-regulation of flavonoid glycosides caused by the addition of GSH did not lead to the up-regulation of quercetin and other aglycones, which indicates that the decomposed aglycones underwent another cycle of oxidization. GSH was readily depleted by a flavonoid, peroxidase, and H_2O_2 mixture, and the products obtained were dependent on the redox potential of the flavonoid. Flavonoids, such as quercetin and luteolin, contain a catechol B ring or kaempferol and can deplete GSH at a stoichiometric ratio without forming a thiyl radical or GSSG [26]. Since flavonoids belong to colored polyphenols, the decrease in their content may delay the browning caused by GSH [27]; nonetheless, this mechanism needs to be further studied. In this study, the contents of catechins and epicatechin before and after the addition of GSH were not significantly different compared to the control group, which is consistent with a study previously reported by Webber et al. [7].

Compared with those in the CK group, three free fatty acids (13-hydroperoxy-9Z,11E-octadecadienoic acid, 7S,8S-DiHODE; (9Z,12Z)-(7S,8S)-dihydroxyoctadeca-9,12-dienoic acid; and 16-methylheptadecanoic acid) in the T and G groups were significantly down-regulated, while dihydrosphingosine-1-phosphate and LysoPE 18:2(2n isomer) were significantly up-regulated. Two unique free fatty acids (docosapentanoic acid and 12-hydroxystearic acid) in the T group were significantly up-regulated, while lysophosphatidyl ethanolamine 18:2 and lysophosphatidyl choline 17:0 were significantly down-regulated. In the G group, three unique free fatty acids (13-hydroperoxy-9Z,11E-octadecadienoic acid, (12Z)-9,10-dihydroxyoctadec-12-enoic acid, and cis-4,7,10,13,16,19-docosahexaenoic acid) and lysophosphatidylcholine 18:0 were significantly down-regulated. The down-regulation of free fatty acids was more prominent in the wine samples supplemented with 20 mg/L GSH. The decrease in free fatty acids in wine may be due to the formation of some aroma components [28]. Phosphatidyl ethanolamine and phosphatidylcholine can form carbonyl metabolites upon heating, such as hexanal, 2,4-dialdehyde, and 1-octen-3-one [29], and this shows that there is a close correlation between the degradation of phospholipids and the formation of aroma.

3.2. Effect of GSH on Volatile Metabolites in Chardonnay Wine during Simulated Oxidation

3.2.1. Effect of GSH on Volatile Metabolites in Chardonnay Wine

A novel HS-SPME Arrow combined with GC-MS technology was used to identify and analyze volatile metabolites in wine samples in the CK, T, and G groups, which were stored at 45 °C for 45 days. A total of 617 metabolites were detected. The orthogonal

partial least squares discriminant analysis (OPLS-DA) model score graph is shown in Figure 5a–c. As can be seen, certain metabolites were different among samples in different groups. Different metabolites were screened based on the screening criteria (VIP > 1.0 and FC ≧ 2.0), and a total of 39 differential metabolites were selected. These metabolites accounted for 6.32% of the total metabolites, which indicates that the addition of GSH at different concentrations has little effect on the overall volatile substances in Chardonnay white wine after simulated oxidation. There were 34 differential metabolites between the CK and G groups (Figure 6a: 5 metabolites were up-regulated and 29 were down-regulated). That is, the relative content of five volatile substances, including one aldehyde, two esters, one aromatic, and one nitrogen-containing compound, increased after 20 mg/L GSH was added. The relative contents of 29 substances, including 9 esters, 3 aldehydes, 3 terpenoids, 3 alcohols, 2 aromatics, 2 hydrocarbons, 1 amine, 1 phenol, 1 nitrogenous compound, 3 heterocyclic compounds, and 1 other compound, were found to decrease.

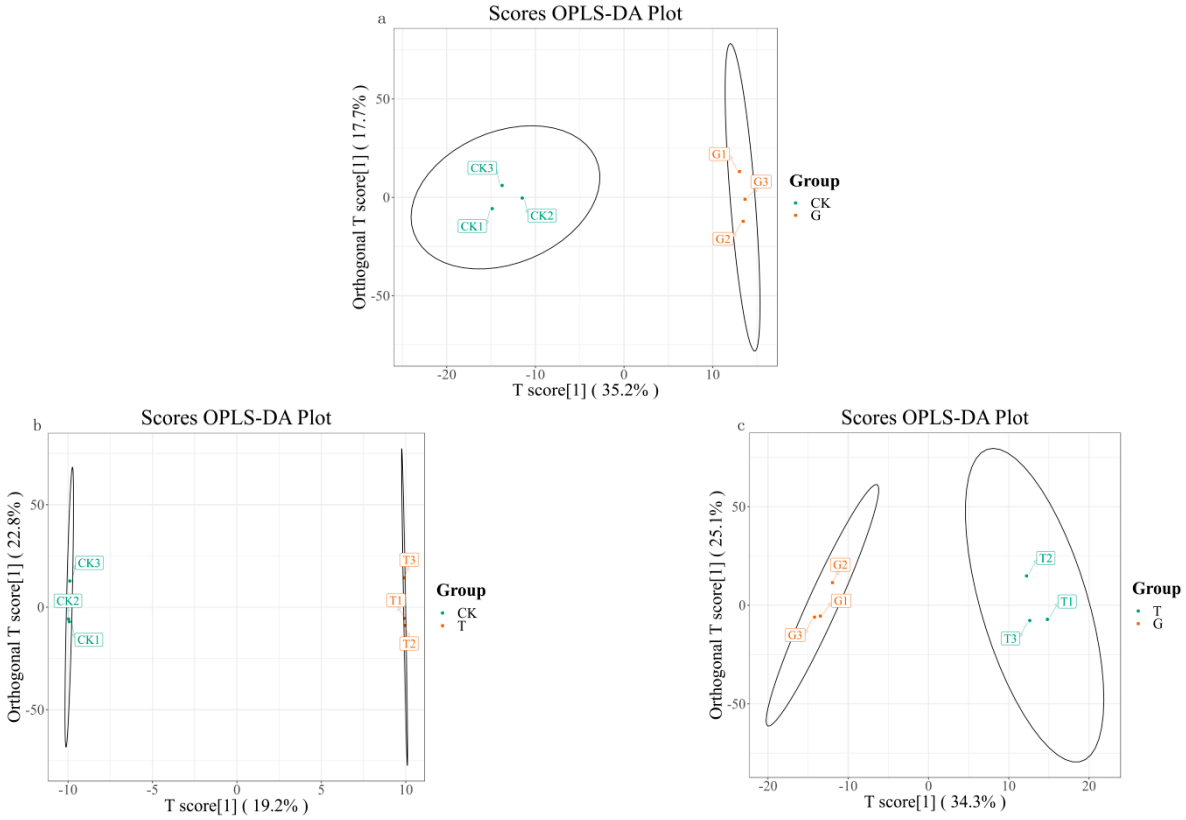

Figure 5. OPLS-DA diagram of differential volatile metabolites of CK vs. G, CK vs. T, and T vs. G (**a**–**c**).

Compared to the CK group, the down-regulation of differential varietal volatiles in group G was more significant than the up-regulation. Among these substances, terpenes, aldehydes, and alcohols were most different. Under the simulated oxidation at 45 °C, some bad volatile substances with high boiling point were formed in the wine. At the same time, compared to those in the wine in the CK group, the relative contents of amines, nitrogen compounds, heterocyclic compounds, and other substances in the wine samples with the addition of 20 mg/L GSH were lower. This shows that 20 mg/L GSH plays a certain protective role in wine. Comparing the CK and T groups, there were seven differential metabolites (Figure 6b: one metabolite was up-regulated and six were down-regulated). That is, the relative content of only one aldehyde ((4Z)-4-heptenal) increased upon the addition of 10 mg/L GSH. Additionally, the relative content of six substances decreased, which were one ester (n-propyl acetate), one amine (acetamide), one hydrocarbon (2,4-dimethyl-decane), one sulfur compound (diallyl sulfur compounds), one aldehyde (3-

methyl-, oxime-butanal), and one alcohol (2-nonen-1-ol). Compared to the CK group, the down-regulation of differential volatile substances was significantly higher than the up-regulation after 10 mg/L GSH was added into white wine. However, (4Z)-4-heptenal has a sweet and milk flavor, which is beneficial to the aroma of wine, while n-propyl acetate, acetamide, diallyl sulfide, and 3-methyl-hydroxamic butyraldehyde are unbeneficial to the aroma. This suggests that the addition of 10 mg/LGSH can promote the decrease in these substances, which is advantageous to wine. There were thirty differences between T and G (Figure 6c, seven metabolite was up-regulated and twenty-three were down-regulated), among which seven substances (including two esters, one aldehyde, two aromatics, one nitrogen compounds, and one amine) have an increase in relative content, while twenty-three substances (including eight esters, three aldehydes, two aromatics, two alcohols, three terpenoids, one phenolic, three heterocyclic, and one amine) have a decrease in relative content, that is, adding 20 mg/L GSH compared to adding 10 mg/LGSH, the down-regulation of differential volatile substances was significantly higher than the up-regulation. Under the simulated oxidation at 45 °C, the addition of GSH might also protect the aroma components of wine. During bottle storage, glutathione prevents the reduction of several aromatic esters and terpenoids, such as isoamyl acetate, ethyl caproate, linalool, and alpha-terpinol, while limiting the accumulation of acetaldehyde [7]. In addition, it inhibits the formation of 2-aminoacetophenone (2-AAP), which is the cause of unpleasant odors in wine [30].

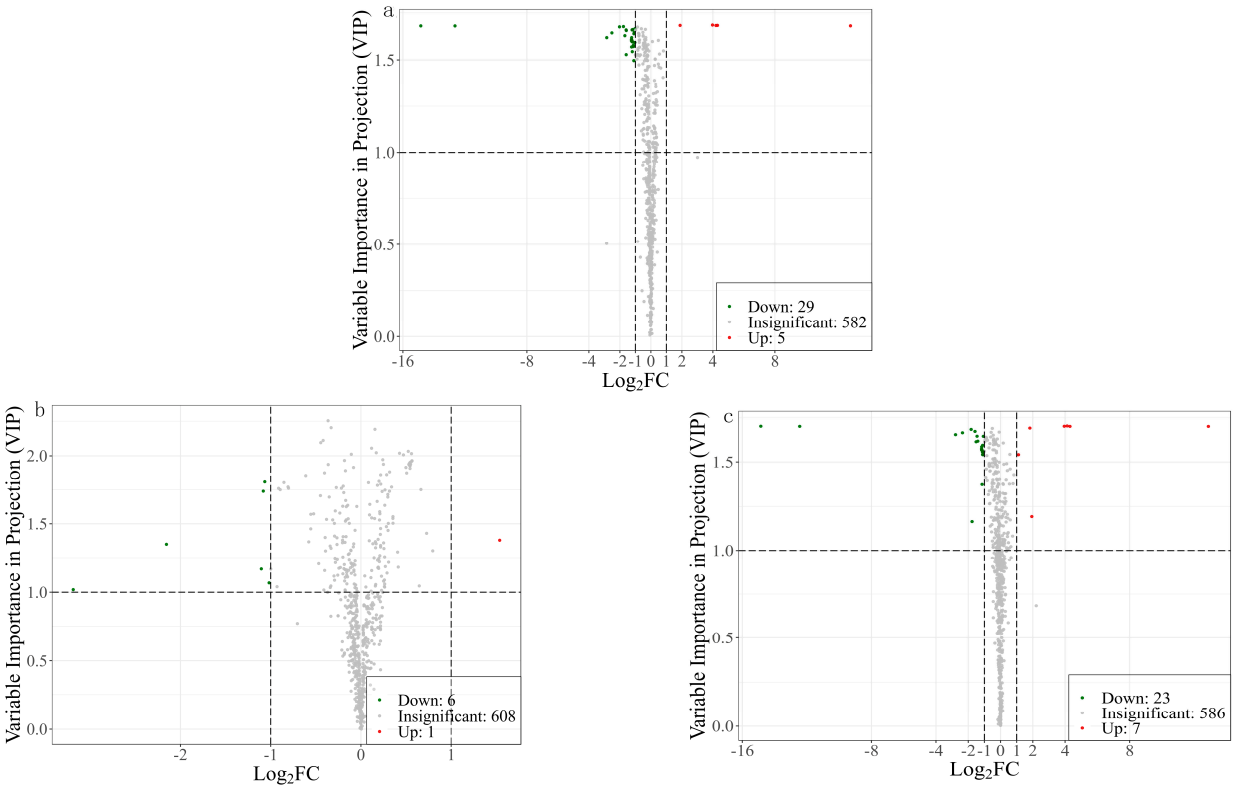

Figure 6. Volcano plot of the differential volatile metabolites of CK vs. G, CK vs. T, and T vs. G. (**a**–**c**), red and green dots represent the up-regulated and down-regulated metabolites).

3.2.2. Effect of Key Non-Volatile Metabolites on Volatile Metabolites in Chardonnay Wine

Non-volatile and volatile components in wine play an important role in its flavor and aroma. The interaction between non-volatile metabolites (amino acids, polyphenols, fatty acids, sugars, ethanol, etc.) and volatile metabolites (esters, terpenes, alcohols, etc.) can affect the sensory and chemical properties of the wine. Compared to those in the CK group, four free fatty acids (13-hydroxy-9z peroxide, 11e-octadecadienoic acid, 7,8-dihydroxy-9,12-octadecadienoic acid, and 16-methylheptadecanoic acid) in the T and G groups were

significantly down-regulated. Lysophosphatidyl ethanolamine 18:2 and lysophosphatidyl choline 17:0 were found to be significantly down-regulated in the T group. Three unique free fatty acids (13-hydroxy-6,9,11-octadecyl trienoic acid, 9,10-dihydroxy-octadecyl-12-enoic acid, and cis-4,7,10,13,16,19-docosahexaenoic acid) and lysophosphatidylcholine 18:0 in group G were significantly down-regulated. The catalysis of fatty acids by lipoxygenase (LOX), hydroperoxide lyase (HPL), and alcohol dehydrogenase (ADH) generates aldehydes and alcohols that are further transformed into the corresponding esters [28]. Upon heating, phosphatidyl ethanolamine and phosphatidylcholine can generate carbonyl metabolites, such as hexanal, 2,4-dialdehyde and 1-octene 3-one [29]. After the addition of 10 and 20 mg/L GSH into white wine, some phenolic acid substances such as 3,4-dimethylbenzoic acid, vanillin tartaric acid, and 1-O-caffeoyl-3,4-bis-O-galtoyl-β-D-glucose were accumulated. Overall, further research is required in order to verify the effect of these phenolic acids on aroma. Thus far, studies have shown that phenolic acids can inhibit the volatilization of esters in wine, retaining aroma compounds in the wine matrix, which is beneficial to the retention of esters in wine liquid and the improvement in the overall aroma intensity and quality of wine [31,32].

4. Conclusions

During the simulated oxidation of Chardonnay wine, extensive targeted metabolomics and volatile metabolomics were employed to analyze the change in the non-volatile and volatile metabolites of the wine before and after the addition of 10 and 20 mg/L GSH. A total of 1107 non-volatile metabolites and 617 volatile metabolites were identified, and 59 important differential non-volatile metabolites and 39 differential volatile metabolites were screened. Compared to the control group, the addition of GSH at different concentrations up-regulated the generation of amino acids in the wine samples, and the up-regulation by 20 mg/L GSH was more significant than that by 10 mg/L GSH. GSH could up-regulate the generation of some phenolic acids in wine; however, compared to 20 mg/L GSH, 10 mg/L GSH could also down-regulate the production of some phenolic acids. The production of some flavonoids and free fatty acids in wine samples containing different concentrations of GSH was down-regulated. Under simulated oxidation, the up-regulation of volatile substances in the wine samples with 20 mg/L GSH was more prominent than that in the wine samples with 10 mg/L GSH. Further analysis indicated that fatty acids, phenolic acids, and amino acids played an important role in the variation in non-volatile substances. It appears that GSH at different concentrations exerts its certain protective effect on the aroma of Chardonnay wine by reducing the relative content of harmful volatile substances. Nonetheless, further research is required to study the specific protective mechanism. This study provides a new theoretical basis for the application of GSH in white wine and the formation mechanism of metabolites.

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