JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Sulfur Aroma Precursor Present in *S*-glutathione Conjugate Form: Identification of *S*-3-(Hexan-1-ol)-glutathione in Must from *Vitis vinifera* L. cv. Sauvignon Blanc

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When Sauvignon blanc or Gros Manseng grape must was percolated through an immobilized γ-glutamyltranspeptidase column, there was a significant increase in the concentration of *S*-3-(hexan-1-ol)-L-cysteine, the precursor of 3-mercaptohexan-1-ol, a compound that contributes to the varietal aroma of wines made from these grapes. Low- and high-resolution liquid secondary ion mass sepectrometry (LSIMS) analyses established the presence of *S*-3-(hexan-1-ol)-glutathione in Sauvignon blanc must. The identification of this compound suggests that the *S*-3-(hexan-1-ol)-L-cysteine in grapes is produced by the catabolism of *S*-3-(hexan-1-ol)-glutathione. As is the case in other plant or animal organisms, *S*-glutathione conjugates may be involved in certain detoxification systems in vines.

KEYWORDS: ***Vitis vinifera*; Sauvignon blanc; varietal aroma precursors; *S*-cysteine conjugate; *S*-glutathione conjugate; aroma potential; tryptophanase; γ-glutamyltranspeptidase; LSIMS**

INTRODUCTION

Some volatile thiols make a decisive contribution to the varietal aroma of Sauvignon blanc wines. Three of these, 4-mercapto-4-methylpentan-2-one, 4-mercapto-4-methylpentan-2-ol, and 3-mercaptohexan-1-ol, smelling of boxwood, citrus zest, and passion fruit, respectively (*1*, *2*), are released from their odorless precursors in must during alcoholic fermentation. These precursors have been identified as *S*-cysteine conjugates (*3*). This explains why the varietal aroma of Sauvignon blanc becomes more intense during fermentation.

However, the biosynthetic mechanism of formation of *S*-cysteine conjugates in vines had not previously been elucidated. Some *S*-cysteine conjugates in plant and animal organs are intermediate chemicals, produced by the breakdown of the corresponding *S*-glutathione conjugates. These compounds are involved in the detoxification systems of living organisms (*4*−*8*). To investigate the biosynthesis mechanism of *S*-cysteine conjugates in Sauvignon blanc, we analyzed the must for the corresponding *S*-glutathione conjugates and identified *S*-3-(hexan-1-ol)-glutathione in Sauvignon blanc must, using LSIMS.

MATERIAL AND METHODS

Must and Reagents. The Gros Manseng must (1998 vintage) came from the Producteurs de Plaimont cooperative cellars (St. Mont, Gers). The Sauvignon blanc must (1996 and 1999 vintages) was from Clos Floridène (Appellation Graves, Bordeaux). The γ-glutamyltranspeptidase was provided by Sigma, and the chelating Sepharose 4B and activated CH−Sepharose 4B were from Amersham Pharmacia Biotech.

Preparation and Purification of Crude Extract containing Sulfur Aroma Precursors. The crude extract was prepared from 45 L of Sauvignon blanc must by chromatography on a grafted silica C_{18} column, using the method described by Darriet et al. (*9*), except that the aroma precursor was eluted using an ethanol solution at 10% instead of 1%. The eluate was evaporated in a vacuum and diluted in 4.5 mL of ultrapure water. The crude extract was then purified using the method described by Tominaga et al. (*3*), as follows. Two 500-μL samples were prepared by adding 600 μL of a solution containing 100 μL of potassium phosphate buffer (1.0 M, pH 8.0) and percolated through a chelating Sepharose 4B column (2 × 0.5 cm) containing immobilized Cu^{2+} for two-thirds of its height. The column was rinsed with 1.5 mL of potassium phosphate buffer (50 mM, pH 8.0) then eluted using 2 mL of HCl (50 mM). The eluate was evaporated to dryness in a vacuum, the residue was dissolved in 1.5 mL of absolute ethanol, and the insoluble fraction was eliminated by centrifugation for 1 min (13 000 rpm). The supernatant was then evaporated to dryness, and the residue was dissolved in 100 μL of water. The two samples were combined at this stage.

Synthesis of *S*-3-(Hexan-1-ol)-glutathione. *S*-3-(Hexan-1-ol)-glutathione was synthesized from *trans*-2-hexenal and glutathione, instead of cysteine, using the method described by Tominaga et al. (*3*) for preparing the corresponding *S*-cysteine conjugate. The quantity of *S*-3-(hexan-1-ol)-glutathione synthesized corresponded to the difference between the total glutathione content (free glutathione + *S*-glutathione conjugate), measured by the ninhydrin method (*10*), and the free glutathione content, measured by the DTNB method (*11*). This synthetic method produced a purity of 98.5%.

Percolating the Must through an Immobilized γ-Glutamyltranspeptidase Column. The γ-glutamyltranspeptidase (20 mg, 25

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10.1021/jf020002y CCC: $22.00
Published on Web 05/29/2002

Table 1. Percentage of the Cystenylated Sulfur Aroma Precursors Released by Percolating the Must through a γ-Glutamyltranspeptidase Column

grape varieties	before percolation	after percolation	% increase
S-3-(hexan-1-ol)-L-cysteine concentration[a]			
Sauvignon blanc			
must 1	2869	4915	71
must 2	21621	32884	52
must 3	10714	16003	49
Gros manseng			
must 1	4199	26740	537
S-4-(4-methylpentan-2-one)-L-cysteine concentration[a]			
Sauvignon blanc			
must 1	589	593	0.5
must 2	1046	1031	
must 3	740	732	
S-4-(4-methylpentan-2-ol)-L-cysteine concentration[a]			
Sauvignon blanc			
must 1	502	492	
must 2	590	603	2.2
must 3	695	703	1.1

[a] ng eq. corresponding thiol.

U/mg) was combined with CH-Sepharose 4B gel under conditions similar to those described for the preparation of the tryptophanase column used to assay the *S*-cysteine conjugate (*12*). The only changes were the coupling buffer (sodium bicarbonate, 0.1 M), with a pH of 8.0, and the fact that no pyridoxal phosphate was added. The activity of the immobilized enzyme was checked by colorimetry, using an l-γ-glutamyl-*p*-nitroanilide solution (1 mM) as substrate. The sample (20 mL of must) was percolated through the column as specified in the protocol described (*12*).

Assay of the *S*-Cysteine Conjugates. The *S*-cysteine conjugates in the must, with or without prior percolation through an immobilized γ-glutamyltranspeptidase column, were assayed using the method described previously (*12*). Deuterated analogues of the cysteinylated precursors were added to the samples as internal standards. The samples were then percolated through an immobilized tryptophanase column and analyzed by GC−MS in SIM mode.

LSIMS Analysis of the Synthetic *S*-3-(Hexan-1-ol)-glutathione and Purified Sauvignon Blanc Must Extract Before and After γ-Glutamyl Transpeptidase Treatment. Synthesized *S*-3-(hexan-1-ol)-glutathione, adjusted to a concentration of 1 mg/mL in water, and 200 μL of purified Sauvignon blanc must extract were analyzed using LSIMS (Micromass Autospec-Q; Cs beam in the positive mode, acceleration voltage 35 keV, calibration with Cs iodine (Mr 200−1500 Da for low resolution, Mr 365−420 Da for high resolution) in a glycerol matrix). The same samples were also analyzed after treatment with γ-glutamyltranspeptidase, using the following protocol: 100 μL of immobilized γ-glutamyltranspeptidase gel was incubated in 500 μL of reaction medium containing 100 μL of potassium phosphate buffer (50 mM, pH 8.0) and 100 μL of synthesized *S*-3-(hexan-1-ol)-glutathione solution (1 mg/mL) or 200 μL of purified Sauvignon blanc must extract. The sample was incubated at 30 °C for 1 h then centrifuged for 1 min (13 000 rpm) to separate the gel from the medium. The supernatant was evaporated to dryness and the residue was dissolved in 1.5 mL of absolute ethanol. The insoluble fraction was eliminated by centrifugation for 1 min (13 000 rpm). The resulting supernatant was evaporated to dryness and the residue was dissolved in 100 μL of water.

RESULTS AND DISCUSSION

Increase in the *S*-3-(Hexan-1-ol)-L-cysteine Content of Must Percolated through an Immobilized γ-Glutamyltranspeptidase Column. The concentration of *S*-cysteine conjugates in three samples of Sauvignon blanc must and one of Gros Manseng was analyzed before and after percolation through an immobilized γ-glutamyltranspeptidase column (**Table 1**). The results indicate quite clearly that percolation through the column produced an increase in the *S*-3-(hexan-1-ol)-L-cysteine content of all the must samples analyzed. The percentage increase in *S*-3-(hexan-1-ol)-L-cysteine was particularly high (537%) in the Gros Manseng must. However, percolation through the γ-glutamyltranspeptidase column had no effect on the concentrations of two other cysteinylated precursors in Sauvignon blanc must, *S*-4-(4-methylpentan-2-one)-L-cysteine and *S*-4-(4-methylpentan-2-ol)-L-cysteine (*3*, *12*).

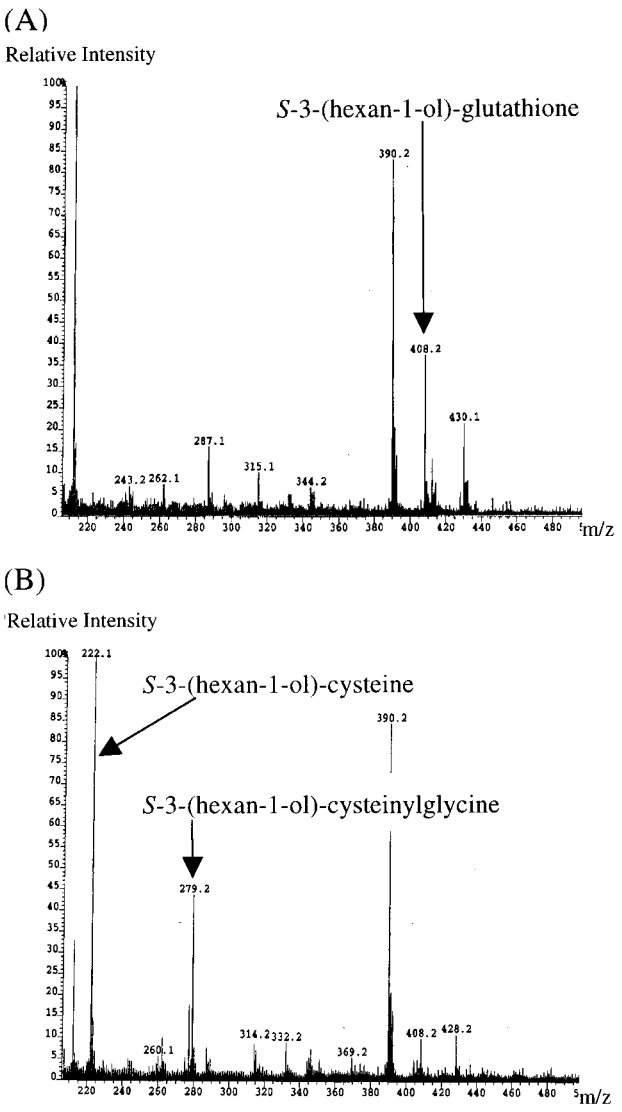

Figure 1. Mass spectra obtained by low-resolution LSIMS analysis of synthesized *S*-3-(hexan-1-ol)-glutathione: untreated (A) and treated (B) with γ-glutamyltranspeptidase.

This result clearly indicates that 3-mercaptohexan-1-ol was present in must as a γ-glutamylcysteinylated conjugate. The possibility of a glutathionylated conjugate could not be excluded, due to the contaminant activity of carboxypeptidase in the commercial preparation of γ-glutamyltranspeptidase used.

Identification of *S*-3-(Hexan-1-ol)-glutathione in the Crude Extract of Sauvignon Blanc Must using Low-Resolution LSIMS. Synthesized *S*-3-(hexan-1-ol)-glutathione was adjusted to a concentration of 1 mg/mL in water and analyzed using low-resolution LSIMS. The $[M + H]^+$ ion of *S*-glutathione conjugate corresponded to the peak at *m*/*z* 408 in the mass spectrum (**Figure 1A**). The characteristics of γ-glutamyltranspeptidase compared to its substrate made it possible to identify the compound corresponding to this peak. Low-

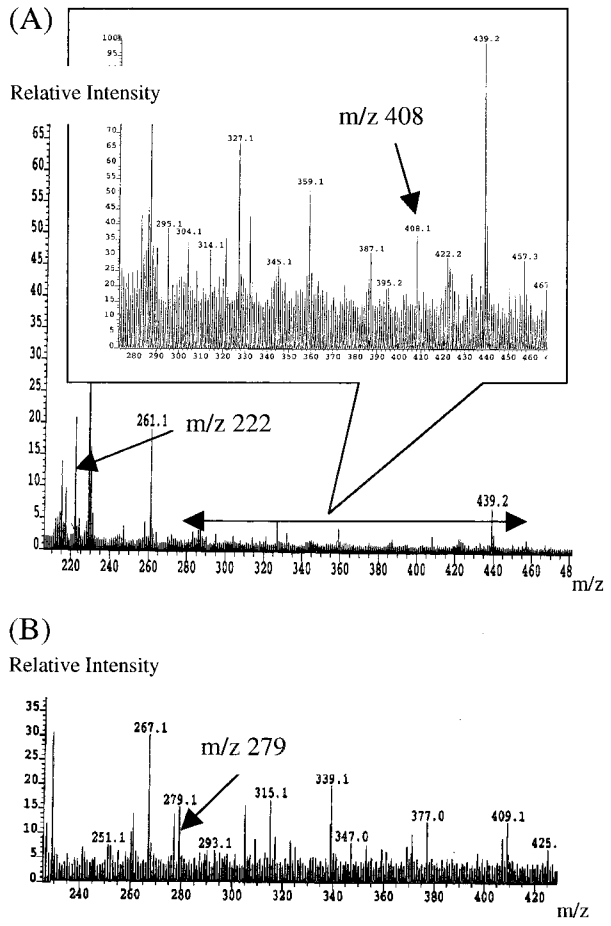

Figure 2. Mass spectra obtained by low-resolution LSIMS analysis of purified Sauvignon blanc crude extract containing sulfur aroma precursor: (A) untreated and (B) treated with γ-glutamyltranspeptidase.

resolution LSIMS analysis of the synthesized *S*-3-(hexan-1-ol)-glutathione (1 mg/mL) incubated at 30 °C for 1 h with 100 μL of the γ-glutamyltranspeptidase gel previously used, showed a decrease in the peak at *m*/*z* 408 and the appearance of a peak at *m*/*z* 279 (**Figure 1B**). This indicated enzymatic conversion of *S*-3-(hexan-1-ol)-glutathione into *S*-3-(hexan-1-ol)-cysteinylglycine (MW = 278) by elimination of the glutamic acid. Furthermore, the appearance of a peak at *m*/*z* 222, corresponding to $[M + H]^+$ of the *S*-3-(hexan-1-ol)-L-cysteine, proved that the commercial γ-glutamyltranspeptidase preparation used contained a carboxypeptidase capable of cleaving the cysteine−glycine bond.

The crude extract prepared from a large quantity of Sauvignon blanc must by percolation through a grafted silica C_{18} column was purified by affinity chromatography on chelating Sepharose 4B gel, then analyzed by LSIMS, as previously described (**Figure 2A**). The fact that the peak at *m*/*z* 408 was present in the crude extract indicated that the must contained *S*-3-(hexan-1-ol)-glutathione. A peak at *m*/*z* 222, corresponding to $[M + H]^+$ of the *S*-3-(hexan-1-ol)-L-cysteine, was also present. Prior incubation of the purified Sauvignon blanc must extract with γ-glutamyltranspeptidase followed by analysis under identical conditions gave the same results as those obtained with synthesized *S*-glutathione conjugate, i.e., the appearance of a peak at *m*/*z* 279 and disappearance of the peak at *m*/*z* 408 (**Figure 2B**). It is, therefore, highly probable that *S*-3-(hexan-1-ol)-glutathione was present in the must. Furthermore, the fact that the Sauvignon blanc must extract did not produce the peak at *m*/*z* 279 unless it was incubated with γ-glutamyltranspeptidase indicated that the must analyzed did not naturally contain *S*-3-(hexan-1-ol)-cysteinylglycine.

Identification of *S*-3-(Hexan-1-ol)-glutathione in the Sauvignon Blanc Must Extract by High-Resolution LSIMS. *S*-3-(Hexan-1-ol)-glutathione was finally identified in the crude extract of Sauvignon blanc must by high-resolution LSIMS. In this case, the molecular weight of the compound corresponding to the *m*/*z* 408 ion was measured very accurately in the purified Sauvignon blanc must extract, then compared to the molecular weights calculated from various empirical formulas. Poly(ethylene glycol) was used as the standard for calibration. LSIMS (*m*/*z*): $[M + H]^+$ calculated for $C_{16}H_{30}N_3O_7S$, 408.180448; found, 408.180239 (+0.5 ppm).

LSIMS was used to identify an *S*-glutathione conjugate in Sauvignon blanc must. This technique is effective for analyzing nonvolatile compounds, provided samples are properly purified to obtain satisfactory resolution. For this purpose, a Sauvignon blanc crude extract containing sulfur aroma precursor was purified by affinity chromatography on chelating Sepharose 4B gel. Certain amino acids (cysteine, tryptophan, etc.) are known to be retained on this gel via the intermediary of chelated metals (*13*). This property has already been used to identify *S*-cysteine conjugates (*3*). We showed in this study that the *S*-glutathione conjugate is also retained on this gel to a certain extent.

The *S*-glutathione conjugate has only been identified by LSIMS in Sauvignon blanc must. It was, however, highly probable that this *S*-glutathione conjugate was also present in Gros Manseng must, in view of the considerable increase in the concentration of *S*-cysteine conjugate in the must after percolation through the immobilized γ-glutamyltranspeptidase column. Furthermore, the presence of 3-mercaptohexan-1-ol in many red and white wines made from *Vitis vinifera* grapes (*14*, *15*), and of its cysteinylated precursor in Cabernet Sauvignon, Cabernet Franc, and Merlot must (*16*) may indicate that these grapes also contain an *S*-glutathione conjugate.

In both the plant and animal kingdoms, *S*-cysteine conjugates are frequently found alongside the corresponding *S*-glutathione conjugates. *S*-glutathione conjugates are usually involved in the detoxication systems of living organisms. The endogenous or exogenous toxic compound to be eliminated is conjugated with glutathione by *S*-glutathione transferase (EC 2.5.1.18), then the product is broken down by γ-glutamyltranspeptidase (EC 2.3.2.2), which eliminates glutamic acid, and a carboxypeptidase, which eliminates glycine, thus forming an *S*-cysteine conjugate (*17*). Marrs et al. (*7*) suggested that certain anthocyanins are recognized, conjugated with glutathione, then transported and metabolized in vacuoles, in response to the toxicity of quercitin, an intermediary in the biosynthesis of these pigments. The same is true of many other *S*-glutathione conjugates (*18*). The fact that *S*-3-(hexan-1-ol)-L-cysteine is preferentially located in Sauvignon blanc grape skin cells (*16, 19*), which have large vacuoles, and the presence in must of both *S*-3-(hexan-1-ol)-L-cysteine and *S*-3-(hexan-1-ol)-glutathione may indicate that *S*-3-(hexan-1-ol)-L-cysteine is involved in the catabolism of the *S*-glutathione conjugates responsible for cell detoxification.

ACKNOWLEDGMENT

We thank C. Vitry (CESAMO, Université Bordeaux1) for LSIMS analyses.

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Received for review January 2, 2002. Revised manuscript received April 9, 2002. Accepted April 10, 2002. The Conseil Interprofessionnel des Vins de Bordeaux (C.I.V.B.) made a financial contribution to this work.

JF020002Y