Measuring the Aromatic Potential of *Vitis vinifera* L. Cv. Sauvignon Blanc Grapes by Assaying *S*-Cysteine Conjugates, Precursors of the Volatile Thiols Responsible for Their Varietal Aroma

Catherine Peyrot des Gachons,* Takatoshi Tominaga, and Denis Dubourdieu

Faculté d'Œnologie, Université Victor Segalen Bordeaux 2, 351 cours de la Libération, F-33405 Talence Cedex, France

The method presented for measuring the aromatic potential of Sauvignon blanc must is based on an assay of the *S*-cysteine conjugate precursors of three volatile thiols involved in the characteristic aroma of wines made from this grape variety: 4-mercapto-4-methylpentan-2-one, 4-mercapto-4-methylpentan-2-ol, and 3-mercaptohexan-1-ol. These compounds were released enzymatically from their precursors by percolating the must through an immobilized tryptophanase column (EC 4.1.99.1), catalyzing an α,*β*-elimination reaction on the *S*-cysteine conjugate. The volatile thiols were analyzed by GC-MS, as were the deuterated analogues that had been released from synthesized deuterated precursors and were added as internal standards. The quantities of volatile thiols released under these conditions were proportional to the *S*-cysteine conjugate content of the must.

Keywords: *Aroma potential; S-cysteine conjugate; flavor precursor; Sauvignon blanc; stable isotope dilution assay; tryptophanase; Vitis vinifera*

INTRODUCTION

Several volatile thiols are involved in the characteristic aroma of Sauvignon blanc wines: 4-mercapto-4-methylpentan-2-one (4MMP), 4-mercapto-4-methylpentan-2-ol (4MMPOH), and 3-mercaptohexan-1-ol (3MH) (Darriet et al., 1995; Tominaga et al., 1998a). These highly odorous compounds are almost totally absent from must and are released into wine from their cysteinylated precursors, *S*-4-(4-methylpentan-2-one)-L-cysteine, *S*-4-(4-methylpentan-2-ol)-L-cysteine, and *S*-3-(hexan-1-ol)-L-cysteine, during alcoholic fermentation (Tominaga et al., 1998c).

Until now it was impossible to estimate by analysis the varietal aroma potential of nonfloral grape varieties such as Sauvignon blanc. This paper describes a method for quantifying this aromatic potential on the basis of an assay of these *S*-cysteine conjugates in must.

MATERIALS AND METHODS

Preparation of the Must. Grape samples (∼4 kg of whole bunches) were taken from a homogeneous Sauvignon blanc vineyard (Clos Floridène, Appellation Graves) in 1998. In the laboratory, the grapes were manually destemmed and any rotten grapes eliminated. Approximately 3 kg of grapes was crushed under inert gas (CO_2) and sulfited at 50 mg/L. The must was left on the skins for one night (∼18 h) at 10 °C. The free-run juice (two pressings at 2 bar) and press-juice (two pressings at 6 bar) were obtained separately, using a pneumatic, laboratory press. The two must samples were sulfited, using 50 mg/L for the free-run juice and 75 mg/L for the press-juice. The two batches of must were blended together, and 2.5 mg/L of a commercial pectolytic preparation (Lafazyme CL) was added. The must was kept at room temperature for 4 h and then chilled to 4 °C overnight to facilitate settling. It was racked, clarified by centrifugation, filtered on a 0.45 μm membrane, and stored at −20 °C in tinted glass bottles.

Enzyme Release of Volatile Thiols from Crude Extract Containing Sulfur Flavor Precursors (CESFPs) in Must. The CESFPs in Sauvignon blanc must (500 mL) was obtained by chromatography on a grafted C_{18} silica column, as previously described by Tominaga et al. (1998c). The enzymatic release of the volatile thiols from the CESFPs was achieved by adding 0.4 mg of apotryptophanase (EC 4.1.99.1) (Sigma, A6007; 90 μg/indole/min/mg) to 250 μL of a reaction medium consisting of a potassium phosphate buffer (100 mM, pH 8.0) containing 1 mM EDTA, 0.1 mM pyridoxal phosphate, and 1 mM glutathion and keeping the solution at 30 °C for 15 min. The volatile thiols released were extracted and analyzed by GC-FPD and GC-MS (see below).

Synthesis of the *S*-Cysteine Conjugate. *S*-4-(4-Methylpentan-2-one)-L-cysteine (P-4MMP), *S*-4-(4-methylpentan-2-ol)-L-cysteine (P-4MMPOH), and *S*-3-(hexan-1-ol)-L-cysteine (P-3MH) were synthesized using the method described by Tominaga et al. (1998c).

S-3-(Hexan-1-ol)-L-cysteine-d_1 (P-3MH-d_1) was synthesized by reducing *S*-3-(hexan-1-al)-L-cysteine, using sodium borodeuteride instead of sodium borohydride, under the conditions described by Tominaga et al.(1998a). Similarly, *S*-4-(4-methylpentan-2-ol)-L-cysteine-d_1 (P-4MMPOH-d_1) was prepared by reducing *S*-4-(4-methylpentan-2-one)-L-cysteine under the same conditions. *S*-4-(4-Methylpentan-2-one)-L-cysteine-d_1 (P-4MMP-d_1) was synthesized by an addition reaction of L-cysteine-d_1 [$HOOCCH(NH_2)CH_2SD$] with mesityl oxide, as described by Tominaga et al. (1998c). L-Cysteine-d_1 was obtained by reducing L-cystine with sodium borodeuteride, as follows: The cystine (50 mg) was dissolved in 10 mL of deuterium oxide (D_2O). The pH was adjusted to 9 by adding a few drops of sodium deuteride (NaOD). The reduction reaction was triggered by adding 5 mL of sodium borodeuteride (10 mg/mL) to the cystine solution and maintaining it at 4 °C for 30 min and then at room temperature overnight. After 18 h, the reaction was stopped by adding a few drops of deuterium chloride (DCl).

The quantity of each *S*-cysteine conjugate synthesized was determined according to the Rosen (1957) method. The volatile thiols released enzymatically from 1.5 nmol of cysteinylated precursor were analyzed by GC-MS in SCAN mode.

* Author to whom correspondence should be addressed (telephone +33 5 56 84 64 90; fax +33 5 56 84 64 68; e-mail oenogene@oenologie.u-bordeaux2.fr).

10.1021/jf990979b CCC: $19.00
Published on Web 07/08/2000

Preparation of an Immobilized Tryptophanase Column. A volume of 10 mL of activated CH-Sepharose 4B gel (Pharmacia; 17-0490-01), corresponding to 3.3 g of dry product, was coupled with 30 mg of apotryptophanase in a 0.1 M potassium phosphate buffer at pH 6 containing 0.5 M KCl (Fukui et al., 1975). The gel was then agitated for 1 h at room temperature in a flask placed on an agitation table. It was washed through a fritted glass filter with a 0.1 M potassium phosphate buffer at pH 8 containing 0.1 mM pyridoxal phosphate. The purpose of this last washing was to eliminate any excess enzyme, denature the active *N*-hydroxysuccinimide ester radicals to pH 8.0, and, via its cofactor, stabilize the apotryptophanase that had been fixed. The gel prepared in this way was placed in a column (2.5 × 2 cm).

Standard Curves for *S*-Cysteine Conjugate. Standard samples of the *S*-cysteine conjugates to be assayed were prepared by adding the three *S*-cysteine conjugates in increasing quantities to 50 mL of 0.05 M potassium phosphate buffer at pH 8, containing 0.1 mM pyridoxal phosphate, 0.1 M KCl, 1 mM EDTA, and 0.3 mM chloramphenicol. Quantities used were as follows: 0.04−0.3 nmol (5−40 ng of 4MMP equiv) for P-4MMP, 0.15−1.2 nmol (20−160 ng of 4MMPOH equiv) for P-4MMPOH, and 3−24 nmol (400−3200 ng of 3MH equiv) for P-3MH. The deuterated *S*-cysteine conjugates were added as internal standards in the following quantities: 0.45 nmol of P-4MMP-d_1, 1 nmol of P-4MMPOH-d_1, and 8.7 nmol of P-3MH-d_1.

The 50 mL of reaction medium was percolated through an immobilized tryptophanase column for 2 h (0.42 mL/min). The volatile thiols in the eluate were combined by adding 10 mL of *p*-hydroxymecuribenzoate solution (1 mM), purified, and assayed by GC-MS in SIM mode (see below), using the method described by Tominaga et al. (1998b).

Repeatability of the Assay. The same Sauvignon blanc must was analyzed five times to establish the variation coefficient of this assay for each cysteinylated precursor.

Influence of "Fatigue" of the Immobilized Tryptophanase Column and Must Composition on the Assay of Cysteinylated Precursors. The same must with added internal standards (deuterated precursors) was percolated three times through 10, 7.5, and 5 mL of gel coupled with tryptophanase. The volatile thiols released from this same must were assayed by GC-MS.

To ensure that the 3MH precursor did not inhibit the tryptophanase activity on the precursor of 4MMP, 0.15 nmol of P-4MMP (20 ng equiv of 4MMP) was percolated in the presence of increasing quantities of P-3MH (20, 80, and 160 times higher than those of P-4MMP).

Assay of the *S*-Cysteine Conjugates in Sauvignon Blanc Must. The Sauvignon blanc must (20 mL) was added to a reaction medium consisting of 0.1 M potassium phosphate buffer at pH 8 containing 0.1 mM pyridoxal phosphate, 0.1 M KCl, and 1 mM EDTA. The final pH was adjusted to 8 using a few drops of KOH (10 N), and the total volume adjusted to 50 mL. The deuterated internal standards were added in the quantities described above. The 50 mL of reaction medium was passed through a DEAE column (1.6 × 1 cm) to eliminate phenolic compounds and coloring matter. The fraction that was not retained was percolated through a tryptophanase column.

GC-FPD and GC-MS. The analysis conditions for GC-FPD and GC-MS in SCAN mode were identical to those described by Tominaga and Dubourdieu (1997). The volatile thiols released and their deuterated analogues were detected by GC-MS in SIM mode, as described by Tominaga et al. (1998b), by ions selected as follows: 4MMP, *m/z* 75; 4MMP-d_1, *m/z* 100; 4MMPOH, *m/z* 100; 4MMPOH-d_1, *m/z* 101; 3MH, *m/z* 134; 3MH-d_1, *m/z* 135.

RESULTS AND DISCUSSION

α,β-Elimination Reaction on an *S*-Cysteine Conjugate Catalyzed by Tryptophanase. The CESFPs of Sauvignon blanc must was incubated with tryptophanase from *Escherichia coli* under the conditions described above. The volatile compounds that formed were extracted using dichloromethane and analyzed by GC-FPD and GC-MS. Three volatile thiols were thus released (Figure 1): 4-mercapto-4-methylpentan-2-one (peak 1), 4-mercapto-4-methylpentan-2-ol (peak 2), and 3-mercaptohexan-1-ol (peak 3). Similarly to *Eubacterium limosum* β-lyase (Tominaga et al., 1998c), *Es. coli* tryptophanase was capable of producing volatile thiols from *S*-cysteine conjugates.

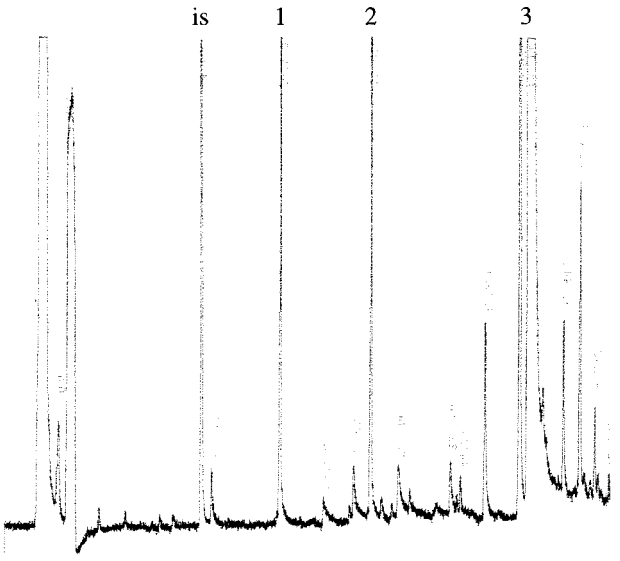

Figure 1. Release of volatile thiols from CESFPs by *E. coli* tryptophanase (is, internal standard; peak 1, 4MMP; peak 2, 4MMPOH; peak 3, 3MH).

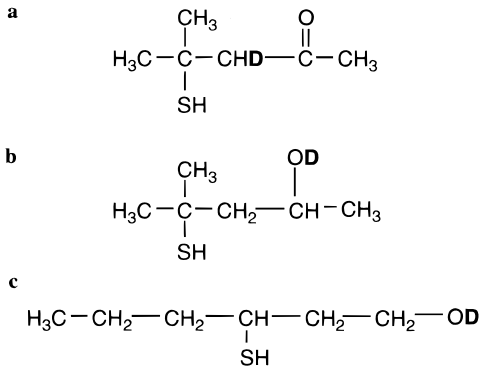

Figure 2. Position of deuterium in (a) 4MMP-d_1, (b) 4MMPOH-d_1, and (c) 3MH-d_1.

The α,β-elimination and β-replacement reaction mechanism catalyzed by tryptophanase (EC 4.1.99.1) has been described in several papers (Morino and Snell, 1967a,b; Newton et al., 1965). This enzyme cleaves the carbon−carbon bond in tryptophan, the carbon−oxygen bond in serine, and the carbon−sulfur bond in *S*-methylcysteine. The effect of tryptophanase on other *S*-cysteine conjugates had not previously been reported.

Release of Natural and Deuterated Volatile Thiols from the Corresponding *S*-Cysteine Conjugates. The mass spectra of the natural and deuterated volatile thiols (Figure 2) released from their precursors in the presence of tryptophanase were compared to check the purity of the synthetic deuterated precursors. P-4MMP-d_1 was 70% pure, as the mass spectrum of the thiol released from it had some ion 132, originating from 4-MMP (Figure 3a,b). On the other hand, P-4MMPOH-d_1 released almost entirely 4MMPOH-

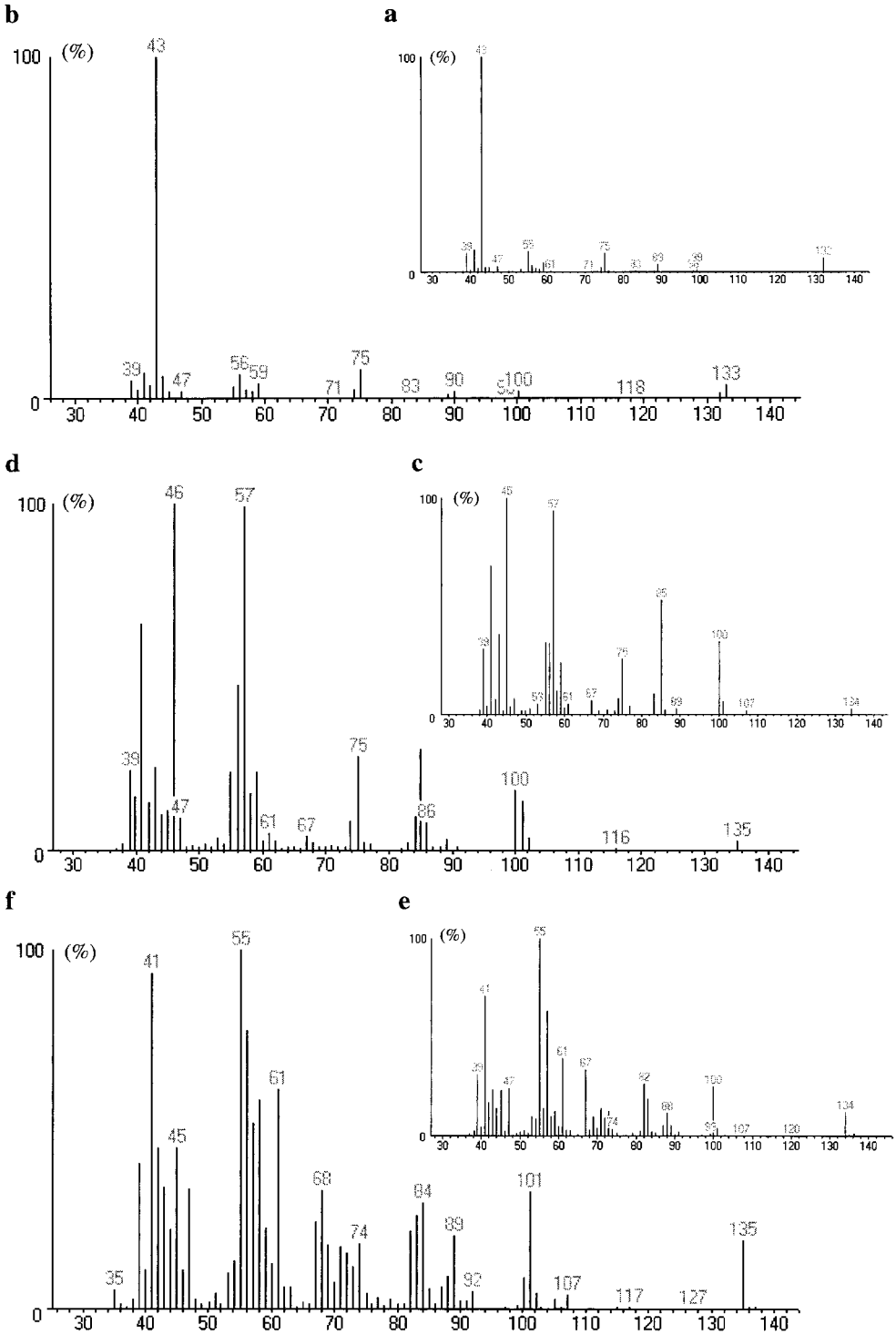

Figure 3. Mass spectra of 4MMP, 4MMPOH, and 3MH (a, c, and e), and their deuterated analogues (b, d, and f).

d_1, and the mass spectrum did not include any ion 134 (Figure 3c,d). P-3MH-d_1 was also almost completely pure (no ion 134) (Figure 3e,f).

Standard Curves for *S*-Cysteine Conjugate. The volatile thiols released enzymatically from increasing concentrations of natural, cysteinylated precursors were assayed using GC-MS. The ratio of the height of each peak for the selected ion to that of its deuterated internal standard was represented graphically in relation to the concentrations of natural precursors added. The correlation between the two variables was linear for the three precursors: P-4MMP, $y = 6.173x - 27.840$ ($r^2 = 1.000$); P-4MMPOH, $y = 43.451x - 43.728$ ($r^2 = 0.999$); and P-3MH, $y = 341.081x - 44.946$ ($r^2 = 1.000$).

Repeatability of the Assay. Table 1 shows the variation coefficient of the assays of the three cysteinylated precursors. Repeatability of the assay was satisfactory as none of the variation coefficients exceeded 6%.

Influence of "Fatigue" of the Immobilized Tryptophanase Column and Must Composition on the Assay of Cysteinylated Precursors. Because of the use of deuterated internal standards, the assay of the precursors did not depend on variations in the activity of the immobilized tryptophanase due to various causes

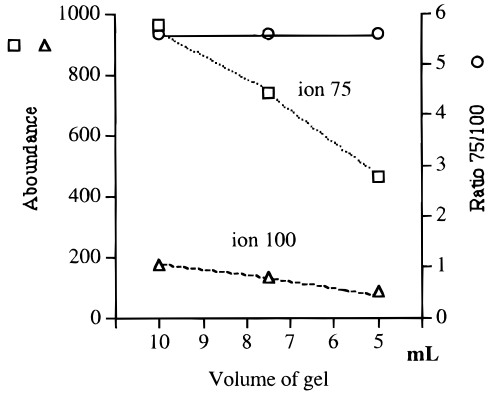

Figure 4. Constant release of 4MMP by percolation on three decreasing volumes of gel coupled with tryptophanase (10, 7.5, and 5 mL).

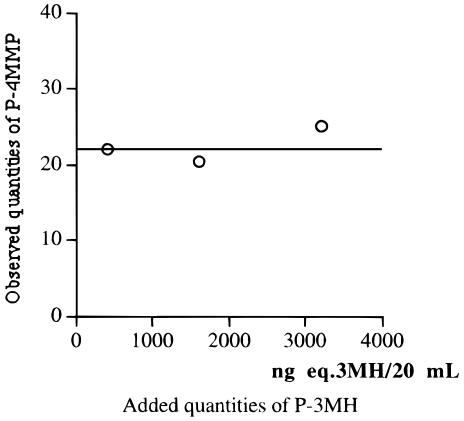

Figure 5. Constant release of 4MMP in the presence of an excess of P-3MH (20, 80, and 160 times more than P-4MMP).

Table 1. Repeatability and Statistical Values of Assays of Cysteinylated Precursors (Nanogram Equivalents of Thiols per Liter) in Sauvignon Blanc Must

sample	P-4MMP	P-4MMPOH	P-3MH
1	33.5	26.6	3021
2	28.6	23.2	2805
3	31.1	23.2	2923
4	31.8	24.4	3218
5	34.0	24.4	3218
av ($n = 5$)	31.8	24.3	3029
SD	2.15	1.39	173
CV (5%)	5.93	5.01	5.01

(variation in the enzyme fixation rate on the gel, gradual deactivation after several uses, inhibition by certain components in the must, etc).

Thus, percolating the same Sauvignon blanc must through three decreasing volumes of gel containing tryptophanase (10, 7.5, and 5 mL) released decreasing quantities of 4MMP and deuterated 4MMP. However, the ratio (ion 75/ion 100) was constant (Figure 4). Similarly, a decrease in the activity of the tryptophanase column did not affect the assay of the other two cysteinylated precursors (data not shown).

We also examined the release of 4MMP from its precursor, in the presence of an excess of another precursor. Figure 5 shows that the release of 4MMP remained constant, whatever amounts of other cysteinylated precursors were also present.

Assay of the *S*-Cysteine Conjugates in a Sauvignon Blanc Must. We show the evolution of cysteinylated precursors in Sauvignon blanc grapes from the Graves appellation (Bordeaux) during ripening in 1998 and 1999 (Figure 6). For both years, harvest occurred September 16th. The concentrations of P-4MMP, P-4MMPOH, and P-3MH are much higher in 1998 than in 1999. Their evolutions are very different according to the precursor and the vintage. This is the first time the aromatic potential of a must of nonfloral grape varieties has been assayed by analyzing the *S*-cysteine conjugates.

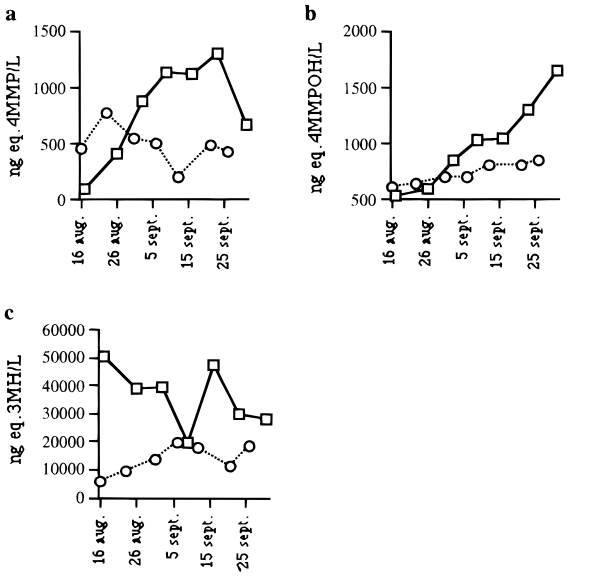

Figure 6. Evolution of the cysteinylated aroma precursors (a, P-4MMP; b, P-4MMPOH; c, P-3MH) in Sauvignon blanc grapes during ripening in 1998 (□) and 1999 (○).

Conclusion. Until now, appropriate analysis methods were not available for assessing changes in concentrations of flavor precursors of the musts of nonfloral grape varieties. By assaying the *S*-cysteine conjugates in the must using an immobilized tryptophanase column, it is now possible for the first time to assess the aromatic potential of Sauvignon blanc must. It is thus feasible to carry out technologically significant analyses on grapes during ripening that reflect the influence of soil and climate parameters (terroir) and vineyard management techniques (clones, rootstocks, training and pruning methods, fertilization, etc.) on the aromatic potential of grapes. We are conducting such experiments for Bordeaux vineyards.

LITERATURE CITED

Darriet, P.; Tominaga, T.; Lavigne, V.; Boidron, J. N.; Dubourdieu, D.; Identification of a powerful aromatic component of *Vitis vinifera* L. var. Sauvignon wines: 4-Mercapto-4-methylpentan-2-one. *Flavour Fragrance J.* **1995**, *10*, 385−392.

Fukui, S.; Ikeda, S.; Fujimura, M.; Yamada, H.; Kumagai, H. Comparative studies on the properties of tryptophanase and tyrosine phenol-lyase immobilized directly on Sepharose or by use of Sepharose-bound pyridoxal 5′-phosphate. *Eur. J. Biochem.* **1975**, *51*, 155−164.

Morino, Y.; Snell, E. A kinetic study of the reaction mechanism of tryptophanase-catalyzed reactions. *J. Biol. Chem.* **1967a**, *242*, 2793−2799.

Morino, Y.; Snell, E. The relation of spectral changes and tritium exchange reactions to the mechanism of tryptophanase-catalysed reactions. *J. Biol. Chem.* **1967b**, *242*, 2800−2809.

Newton, W. A.; Morino, Y.; Snell, E. Properties of crystalline tryptophanase. *J. Biol. Chem.* **1965**, *240*, 1211−1218.

Rosen, H. A modified ninhydrin colorimetric analysis for amino acids. *Arch. Biochem. Biophys.* **1957**, *67*, 102−115.

Tominaga, T.; Dubourdieu, D. Identification of 4-Mercapto-4-methylpentan-2-one from the Box Tree (*Buxus sempervirens* L.) and Broom (*Sarothamnus scoparius* (L.) Koch.). *Flavour Fragrance J.* **1997**, *12*, 373−376.

Tominaga, T.; Furrer, A.; Henry, R.; Dubourdieu, D. Identification of new volatile thiols in the aroma of *Vitis vinifera* L. var. Sauvignon blanc wines. *Flavour Fragrance J.* **1998a**, *13*, 159−162.

Tominaga, T.; Murat, M. L.; Dubourdieu, D. Development of a method for analyzing the volatile thiols involved in the characteristic aroma of wines made from *Vitis vinifera* L. Cv. Sauvignon blanc. *J. Agric. Food Chem.* **1998b**, *46*, 1044−1048.

Tominaga, T.; Peyrot des Gachons, C.; Dubourdieu, D. A new type of flavor precursors in *Vitis vinifera* L. cv. Sauvignon blanc: *S*-cysteine conjugates. *J. Agric. Food Chem.* **1998c**, *46*, 5215−5219.

Received for review September 1, 1999. Revised manuscript received April 4, 2000. Accepted April 25, 2000. This study received the financial support of Conseil Interprofessionnel des Vins de Bordeaux (CIVB) and Conseil Régional d'Aquitaine.

JF990979B