Assessing the Aromatic Potential of Cabernet Sauvignon and Merlot Musts Used to Produce Rose Wine by Assaying the Cysteinylated Precursor of 3-Mercaptohexan-1-ol

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The development of a method for assaying *S*-3-(hexan-1-ol)-L-cysteine, the cysteinylated precursor of 3-mercaptohexan-1-ol (P-3MH), in must has made it possible to study its impact on the aromatic potential of Merlot and Cabernet Sauvignon grape varieties used to produce rose wines in Bordeaux. The original feature of this method is the purification of very small volumes of must (500 μL) containing P-3MH by affinity chromatography and gas-phase chromatography coupled with mass spectrometry of the purified precursor in trimethylsilylated derivative form. Assays of the cysteinylated precursor in Merlot and Cabernet Sauvignon grapes showed that it was mainly located in the grape skins (60%). Prolonged juice-skin contact increased the must's P-3MH content, and this phenomenon was more marked at higher temperatures. Assessment of the aromatic potential of must used to produce rose wines by chemical analysis of an S-cysteine conjugate is mentioned for the first time.

Keywords: *Sulfured aroma precursor; S-3-(hexan-1-ol)-L-cysteine; trimethylsilylation; stable isotope dilution assay; Cabernet Sauvignon; Merlot*

INTRODUCTION

Rose wines are usually made by *saignée*, a technique consisting of running off a certain volume of juice from a red grape vat after a variable period of prefermentation skin contact. Many compounds are extracted from the skins during this initial phase in winemaking, similar to the skin contact used in white wine production (*1*, *2*). Skin contact probably also has an impact on the aromatic potential of the must.

However, until now, no research had been done into the concept of aroma precursors in must used to produce rose wines. Studies of aroma precursors in red grape varieties such as Cabernet Sauvignon and Merlot have focused on carotenoids (*3*), monoterpene glycosides, and C-13 norisoprenoids (*4*−*6*). Recent research into the aroma of rose wines made from these grape varieties highlighted the contribution of one odoriferous compound in particular, 3-mercaptohexan-1-ol (3MH), to the fruitiness of these wines (*7*). This volatile thiol is released during alcoholic fermentation from an odorless precursor in the must (*8*). This precursor's chemical structure has now been described (*9*) as an S-cysteine conjugate, an aroma precursor recently identified in passion fruit (*Passiflora edulis*) (*10*).

This article describes the assessment of the aromatic potential of must used to produce rose wines by a direct method for assaying the cysteinylated precursor of 3MH, *S*-3-(hexan-1-ol)-L-cysteine (P-3MH). This work examines the relationship between the P-3MH content of must and the 3MH concentration in rose wines. It identifies the location of the precursor in Merlot and Cabernet sauvignon grapes, as well as assessing the influence of prefermentation skin contact on the aromatic potential of rose wines.

MATERIAL AND METHODS

Must. The must samples were taken at various AOC ("Appellation d'Origine Contrôlée") Bordeaux rose estates during skin contact, between the initial vatting and the final running off. All the grapes were Merlot and Cabernet Sauvignon (1999 vintage), held in stainless steel vats at 20 °C. The first sample was taken from each vat immediately after the juice had been pumped over to homogenize the contents and the last one just before running off. All the 125-mL samples were taken in the same way and kept at − 20 °C until they were analyzed.

Samples for the experiment investigating the effect of temperature were taken from microvinification batches in the same way.

Microvinification and Vinification. Hand-picked grapes from an estate in the AOC Bordeaux appellation (2000) vintage were microvinified. Two 75 kg batches of Merlot and Cabernet Sauvignon grapes were destemmed and crushed under inert gas (CO_2), sulfured at 3 g/hL, and then put into three 30 L vats. The effect of temperature on skin contact was studied at 10, 20, and 25 °C.

The wines made under normal winemaking conditions came from several estates in the AOC Bordeaux appellation, where the grapes were machine-picked.

In both cases, the must for the rose wine was obtained by running off 10% of the juice (by volume) from a vat of red grapes after a variable period of skin contact. This must was transferred to a vat filled with inert CO_2 gas.

After static cold settling, the turbidity of the must was adjusted to 200 NTU. The must was inoculated with 20 g/hL of a *Saccharomyces cerevisiae* yeast strain (VL3c, CLIB, (Collection de Levure d'intérêt Biotechnologique, France − no. 2016). The alcoholic fermentation for the microvinifications took place in 5 L demijohns.

Fermentation temperatures were between 18 and 20 °C in both cases. When the residual sugar content was below 2 g/L,

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the wines were bottled, stored at 10 °C, sulfured at 4 g/hL, and analyzed rapidly.

Isolating the Different Parts of the Grapes: At harvest time, batches of five grapes were sampled from several different bunches in the same way. The grapes were stored at − 20 °C until they were analyzed. Defrosted grapes were dried with absorbent paper and the skins were removed using a scalpel. The skins were ground in absolute ethanol using an Ultraturrax (IKA, Labortechnick) (13500 TR/min for 5 min). The ground material was centrifuged (7000 rpm, 5 min), the supernatant was evaporated dry under vacuum, and then the residue was diluted in 2 mL of ultrapure water (Milli Q, Millipore). The seeds were removed from the peeled grapes and the remainder (flesh + juice) was ultrasonicated for 5 min and then centrifuged (12 000 rpm, 5 min). The supernatant collected corresponded to the juice fraction.

The skin fraction was percolated on a DEAE Sepharose column (0.5 × 0.5 cm) that had previously been balanced with 2 volumes of potassium phosphate buffer (50 mM, pH 8), to eliminate any phenolic compounds that might distort the precursor assay.

3MH Assay. The 3MH content of the wines was measured one month after the end of alcoholic fermentation, using the method described by Tominaga et al. (*11*) and modified by the same author (*12*).

Anthocyanin Assay. The anthocyanin content of the must was measured using the SO_2 bleaching method described by Ribéreau-Gayon et al. (*13*).

Synthesis of the S-Cysteine Conjugate. The *S*-3-(hexan-1-ol)-L-cysteine was synthesized using the method described by Tominaga et al. (*9*): L-cysteine (Fluka, 30119) was added to trans 3-hexen-1-al (Aldrich, 13, 265-9) then reduced with 50 mg sodium borohydride (SIGMA, S 9125). The *S*-3-(hexan-1-ol)-L-cysteine $^{15}N_2$ was synthesized by the same process, using DL-cysteine $^{15}N_2$ obtained by reducing DL-cystine $^{15}N_2$ (Interchim, N-5270). The quantity of S-cysteine conjugate synthesized was determined by ninhydrin assay (*14*), using *S*-ethylcysteine as a standard.

Purifying P-3MH by Percolation on a Chelating Sepharose 4B Column. Chelating Sepharose 4B gel (Pharmacia, 17-0575-01) and the same gel containing immobilized copper were put into a Pasteur pipet so as to obtain a 3-cm column containing immobilized Cu^{2+} for half its height. The pH of the packing material in the column was adjusted to pH 8.0 by eluting 2 bed-volumes (6 mL) of potassium phosphate buffer (50 mM, pH = 8).

The purification method was as described by Tominaga et al. (*9*), modified in the following way:

A 500 μL volume of clarified must (12000 rpm, 1 min) containing 1000 pmol *S*-3-(hexan-1-ol)-L-cysteine $^{15}N_2$, a stable isotopic analogue of *S*-3-(hexan-1-ol)-L-cysteine, as an internal standard was brought up to pH 8 by adding 200 μL of potassium phosphate buffer (1 M, pH 8). It was then directly percolated through the Chelating Sepharose 4B column prepared as described above. The retained fraction was washed with 3 mL of potassium phosphate buffer (50 mM, pH = 8), eluted by percolating it with 2 mL of hydrochloric acid (50 mM), and evaporated dry under vacuum. The dry residue was diluted in 250 μL of absolute ethanol. The insoluble fraction was eliminated by centrifuging (12000 rpm, 5 min), and the supernatant was evaporated dry under vacuum in a 2-mL vial.

Trimethylsilylation of P-3MH. The vial containing the dry residue thus obtained was capped under a nitrogen stream and 50 μL of trimethylsilylation reagent: *N*,*O*-bis[Trimethylsilyl]trifluoroacetamide (BSTFA) (Pierce 38827): trimethylchlorosilane (TMCS) (Pierce, 88530): pyridine (Pierce, 27530) = 3:1:3, was introduced with a syringe. The reagent mixture was put in an ultrasound bath for a few seconds and then heated to 70 °C for 15 min. A 2 μL sample was analyzed by GCMS.

Calibration and Repeatability of the Assay. The standard curve was prepared by adding increasing quantities of the precursor to a rose must (AOC Bordeaux, Merlot, 1999 vintage): 62.5 to 500 nmol/L (8375 to 67 000 ng equivalent 3MH/L: ng eq 3MH/L), to obtain four different concentrations. The P-3MH content of each of these samples was calculated by measuring the height of the P-3MH peak corresponding to the ion selected (Hs) and establishing the ratio (Hs/Hei) of its height in relation to that of an internal standard (Hei).

Prior analysis of this must showed that it contained some P-3MH so the calibration had to be corrected by subtracting the initial quantity of P-3MH detected (height of P-3MH peak corresponding to the ion selected/height of internal standard peak).

The analysis was repeated five times with the same must to establish the variation coefficient of the assay.

GC-MS. The GC-MS analysis conditions were as described by Tominaga et al. (*9*), except for the initial isotherm (150 °C, 1 min), injector temperature (300 °C), and detector temperature (250 °C).

P-3MH and an internal isotope standard were detected in SIM mode. The selected ions were P-3MH: $m/z = 320$; internal standard: $m/z = 321$.

RESULTS

Fine-Tuning the Assay Method. The method for assaying the cysteinylated precursor, *S*-3-(hexan-1-ol)-L-cysteine, in must from red grape varieties consists of two stages. The first is the purification of this compound by percolating the must on a Chelating Sepharose 4B column. The second stage consists of deriving the precursor by trimethylsilylation and GC-MS analysis in SIM mode.

Figure 1 shows the spectra of P-3MH and the synthesized internal standard P-3MH $^{15}N_2$ in trimethylsilylated derivative form. Natural P-3MH and P-3MH $^{15}N_2$ added as an internal standard were specifically detected in Merlot must by the selected ions (Figure 2). In view of the small volume of must used (500 μL), the quantities of the selected ions detected were remarkably large.

The correlation between the ratio of the height of the P-3MH peak to that of the internal standard and the P-3MH concentrations was linear ($R^2 = 0.9974$). To facilitate interpretation of the analyses, the equation of the standard curve was expressed according to the concentration in ng equivalent of 3MH/L (ng eq 3MH/L): $y = 54819x$. The repeatability of the assay method was assessed over a series of five analyses. The variation coefficient was less than 10% (5.37%) at a risk threshold of $\alpha = 0.01$.

Conversion of P-3MH into 3MH during Alcoholic Fermentation. The quantity of P-3MH in 15 batches of AOC Bordeaux rose must (1999 and 2000 vintages) from several estates, fermented either in large vats or in microvinification, was determined one month after the end of alcoholic fermentation. The 3MH content was also determined in the corresponding wines. A straight-line regression was established between the P-3MH and 3MH content to determine the degree of dependency (Figure 3). The quantity of 3MH released into wine was proportional to the initial P-3MH content in the must. The correlation was statistically significant at the threshold of $\alpha = 0.01$.

The percentage conversion of precursor into volatile thiol was calculated (Table 1). The percentage did not exceed 10.2% in any of the 15 rose must-wine pairs considered.

Grape Pericarp and Skin. The P-3MH concentrations (ng eq 3MH/g of fresh material) were determined in skin and juice fractions of ripe grapes (Table 2). The precursor content of the seeds was not taken into consideration as it was negligible, and the flesh was included in the juice fraction. Research by Radford et

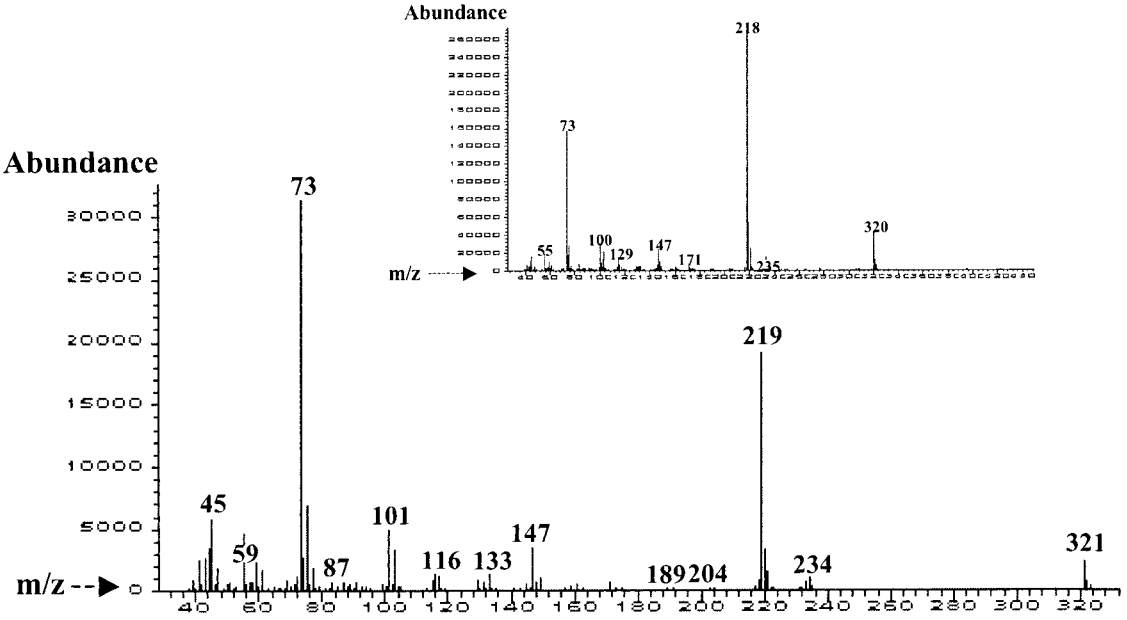

Figure 1. Mass spectra of P-3MH and P-3MH $^{15}N_2$ in trimethylsilylated form.

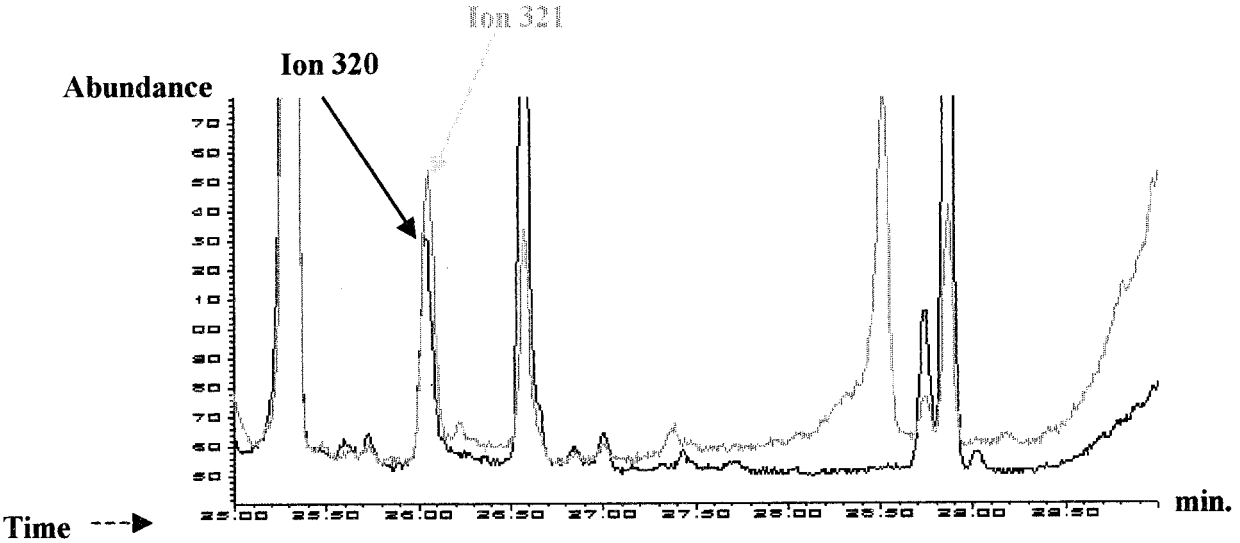

Figure 2. Chromatogram obtained by trimethylsilylation of purified must extract with detection of 320 and 321 ions.

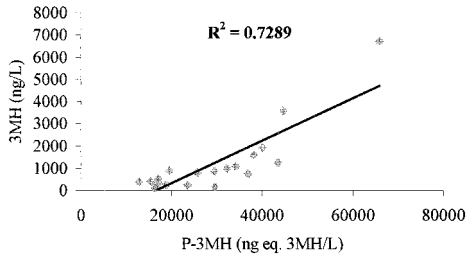

Figure 3. Correlation between the P-3MH content in must and the 3MH content in the corresponding rose wines.

Table 1. Percentage Conversion of P-3MH into 3MH (*n* = 15)

minimum %	maximum %	ave %	SD	variation coefficient (5%)
0.6	10.2	3.2	2.2	33%

al. (*15*) on various fruit juices and Wilson et al. (*16*) on grapes demonstrated that the presence of aromatic compounds (free or bound) in the flesh was due to a simple phenomenon of adsorption on flesh cell debris.

Table 2. P-3MH Content (ng eq de 3MH/g Fresh Material) in the Skin and Juice of Ripe Cabernet Sauvignon (CS) and Merlot (M) Grapes

	CS 1	CS 2	M 1	M 2	M 3
skin	14.53	15.25	16.3	17.93	19.7
juice	1.31	1.27	1.006	1.72	2.18

The P-3MH content was, on average, 11 times higher in the skin than the juice fraction (Table 2).

Considering the respective proportions (g of fresh material) of juice and skin in grapes, it was possible to estimate the distribution of precursor between these two fractions. As shown in Figure 4, and in agreement with the results obtained by Peyrot des Gachons et al. on Sauvignon Blanc grapes (*17*), the P-3MH was mainly located in the skin (60% on average).

Processing Grape to Wine. The first factor studied was the influence of skin contact time. Figure 5 shows the increase in the P-3MH and anthocyanin content of the must in two separate vats of Merlot, from the time

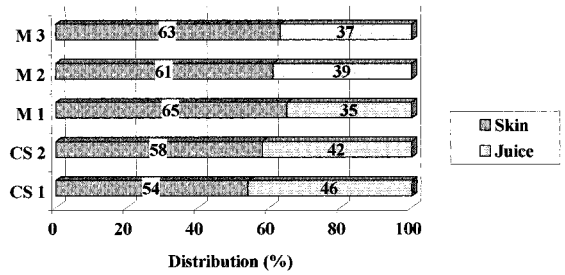

Figure 4. Distribution of P-3MH in ripe Merlot (M1, M2, M3) and Cabernet Sauvignon (CS1, CS2) grapes.

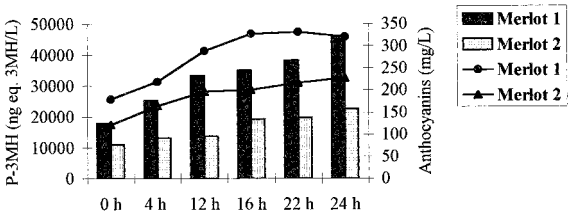

Figure 5. Impact of skin contact time on the P-3MH (histograms) and anthocyanin (curves) content of Merlot must used to make rose wines.

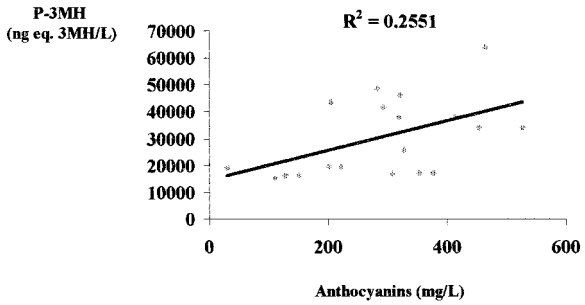

Figure 6. Correlation between the P-3MH content and the anthocyanin concentration of rose must at the end of skin contact ($n = 20$).

the grapes were put into the vat ($t = 0$) until the must was run off ($t = 24$ h). In every case, the must already contained some precursor at the beginning of skin contact and the concentration increased continuously until running off. Identical results were obtained in other vats of Cabernet Sauvignon and Cabernet franc (results not shown): 24 hours' skin-contact increased the P-3MH concentration in the must by an average of 62% as compared to the initial concentration. Furthermore, the grape solids were apparently still capable of releasing more of this compound into the liquid fraction after 24 hours' skin contact. The anthocyanin content of all the must samples submitted to the precursor assay was also determined. The diffusion of coloring matter in the must had similar kinetics to that of P-3MH at the beginning of skin contact (Figure 5). However, the must with the highest anthocyanin content did not necessarily contain the most P-3MH (Figure 6).

At a later stage, we studied the influence of temperature during skin contact. Higher temperatures during skin contact considerably increased the quantity of P-3MH in the must (Figure 7, Table 3): after 36 h at 20 °C, the two batches of must contained an average of 59% more precursor than that of the vats kept at 10 °C. An increase in skin contact temperature also resulted in greater extraction of anthocyanins (Table 3). Little or no difference was observed, however, between batches where skin contact took place at 20 and 25 °C.

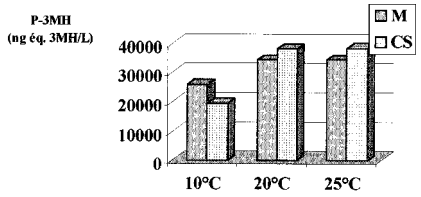

Figure 7. Impact of skin contact temperature on the P-3MH content of must (M: Merlot, CS: Cabernet Sauvignon).

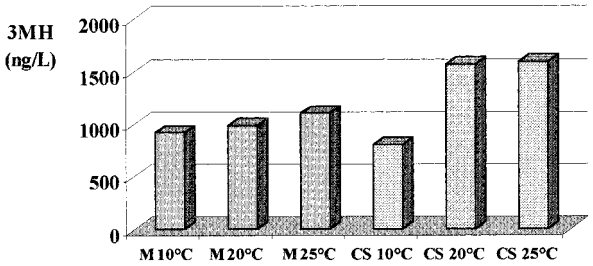

Figure 8. Impact of skin contact temperature on the 3MH content of rose wine made from Merlot (M) and Cabernet Sauvignon (CS).

Table 3: Percentage Increase in P-3MH and Anthocyanins in Rose Must (Merlot (M) and Cabernet Sauvignon (CS), 1999 Vintage) Following 36 Hours' Skin Contact at 10, 20, and 25 °C

	M 10	M 20	M 25	CS 10	CS 20	CS 25
P-3MH (%)	55	66	66	31	52	52
anthocyanins (%)	76	82	85	56	70	77

The wine's aroma was enhanced proportionately to the increase in precursor in the must (Figure 8).

DISCUSSION

The selectivity of Chelating Sepharose 4B gel in relation to S-cysteine conjugates in must has been clearly demonstrated and used to identify this chemical family in Sauvignon Blanc must (*9*). This gel is capable of fixing certain amino acids, particularly tryptophane and cysteine, via the intermediary of chelated metals (*18*). The S-cysteine conjugate selectively retained on the gel was then eluted using an HCl solution. The purity was quite satisfactory for preparing a trimethylsilylated derivative, which made it easy to detect this compound by GC-MS in SIM mode without any interference due to contamination by derivative osides.

The advantage of this method was that it was quick and easy to assay P-3MH in must made from red grape varieties containing large quantities of phenolic compounds. The enzymatic assay method developed by Peyrot des Gachons et al. (*19*) was not suitable for assaying cysteinylated precursors in these batches of must, due to the deactivation of tryptophanase in media with high concentrations of phenolic compounds. However, the method presented here was not capable of assaying the other two known cysteinylated precursors in Sauvignon Blanc must, *S*-4-(methylpentan-2-one)-L-cysteine and *S*-4-(4-methylpentan-2-ol)-L-cysteine, as the concentrations are approximately 50 times lower than that of P-3MH (*19*). The proposed method can only be used to assay P-3MH in all types of must, as well as in grapes and wine.

A high concentration of precursor in the must produces a high 3MH content in the wine (Figure 3). However, the rate of conversion into aroma of small quantities of precursor is apparently relatively variable (Table 1). Furthermore, the P-3MH content of the must

was extremely high as compared to the 3MH content in the corresponding rose wines. There are three hypotheses to explain the low percentage conversion of precursor into aroma (3.2% on average): either the 3MH released was partially oxidized, or a certain quantity of P-3MH, or 3MH was metabolized by yeast.

The P-3MH, mainly located in the skins of red grapes, was extracted during skin contact prior to running off (Figure 5). The increase in anthocyanin content during skin contact in rose wine making is a phenomenon well-known to winemakers and was previously described by Roson (*1*). This research showed for the first time that the P-3MH content in the must also increased.

Many studies have shown that higher temperatures during prefermentation skin contact lead to an increase in the diffusion of various components of the skin in white (*20*−*22*) as well as rose and red must (*1*, *23*). A higher skin contact temperature promotes the release of P-3MH in rose must by increasing its extractability from the solid parts of the grapes (Table 3, Figure 7).

CONCLUSION

The assay method presented in this article is a new tool for studying the aromatic potential of Cabernet Sauvignon and Merlot grapes used to produce rose wines. The method's speed and the fact that it requires only a small volume of must makes it perfectly suited to daily assays of large numbers of samples.

This method has made it possible to demonstrate the correlation between the P-3MH content of must and the 3MH content of the corresponding rose wine. In view of the decisive impact of this compound on the fruity aroma of Bordeaux rose wines (*7*), it is obviously useful to assay its precursor in must.

We identified the location of this compound in the skins of Merlot and Cabernet Sauvignon grapes and provided the first analytical demonstration of the increase in precursor concentrations in must during prefermentation skin contact. Skin contact before running off in rose wine making is therefore justified for enhancing the wine's aromatic potential.

When rose wines are produced by running off, the skin-contact phase not only promotes diffusion of coloring matter, but also improves the must's aromatic potential. Higher temperatures during juice−skin contact improve extraction of both aroma precursor and coloring matter.

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