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Pyruvic Acid and Acetaldehyde Production by Different Strains of *Saccharomyces cerevisiae*: Relationship with Vitisin A and B Formation in Red Wines

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The production of pyruvate and acetaldehyde by 10 strains of *Saccharomyces cerevisiae* was monitored during the fermentation of *Vitis vinifera* L. variety Tempranillo grape must to determine how these compounds might influence the formation of the pyroanthocyanins vitisin A and B (malvidin-3-*O*-glucoside-pyruvate acid and malvidin-3-*O*-glucoside-4 vinyl, respectively). Pyruvate and acetaldehyde production patterns were determined for each strain. Pyruvate production reached a maximum on day four of fermentation, while acetaldehyde production was at its peak in the final stages. The correlation between pyruvate production and vitisin A formation was especially strong ($R^2 = 0.80$) on day 4, when the greatest quantity of pyruvate was found in the medium. The correlation between acetaldehyde production and the formation of vitisin B was strongest ($R^2 = 0.81$) at the end of fermentation when the acetaldehyde content of the medium was at its highest. Identification and quantification experiments were performed by HPLC-DAD. The identification of the vitisins was confirmed by LC/ESI-MS.

KEYWORDS: ***Saccharomyces cerevisiae*; vitisin A; vitisin B; acetaldehyde; pyruvate; HPLC-DAD; red wines; anthocyanins**

INTRODUCTION

Anthocyanins are responsible for the color of red wines. Usually, these compounds occur only in the grape skin, different quantities being found in different varieties of vine (*1*−*3*). The ratio of anthocyanidin-3-glucosides to the different acylated derivatives (*4*), or that between the *p*-coumaroyl and acetyl derivatives of anthocyanins, can be used to differentiate varieties (*5*, *6*).

Red wines are fermented in the presence of the grape skins to extract and make use of their anthocyanins and other polyphenol compounds. During fermentation, the yeasts release secondary metabolic products into the medium (*7*), some of which react with the anthocyanins to produce derivatives such as vitisin A and B.

In the same way that the study of the anthocyanidin-3-*O*-glucosides and their acylated derivatives in grapes and wines has been linked to the development of HPLC-DAD (*8*−*10*), the detailed study and characterization of the vitisins has depended on the development of HPLC-MS techniques (*11*−*13*), which have allowed their structures to be confirmed. Some authors had already detected the latter compounds eluting after malvidin-3-*O*-glucoside in certain types of wines (*14*), although they could not be identified at that time.

Vitisin A (**Figure 1a**) and B (**Figure 1b**) were first described in alcoholically strengthened red wines (*11*), and later isolated from grape pressings after fermentation (*12*). These authors confirmed their structures by FABMS, ^{1}H NMR, and ^{13}C NMR. Vitisin A (malvidin-3-*O*-glucoside-pyruvate) is a derivative of malvidin-3-*O*-glucoside with a $C_3H_2O_2$ residue between carbon 4 and the hydroxyl group of carbon 5. C-4/C-5 cycloaddition can also involve other secondary metabolites originating from the activities of yeasts, e.g., acetaldehyde, acetone, acetoine, etc. Vitisin B (malvidin-3-*O*-glucoside-4 vinyl) is also derived from malvidin-3-*O*-glucoside, but has a CH═CH group at the aforementioned position. This is formed through the condensation of anthocyanins with acetaldehyde (vinyl adduct). This adduct of malvidin-3-glucoside and its acetylated ester were first isolated from port (*11*, *15*) and later from red wines (*16*−*20*).

The most interesting properties of these molecules is their partial resistance to discoloration by SO_2, and their greater resistance than malvidin-3-*O*-glucoside to color modification by pH. This is due to the formation of stable quinonoid bases which reduce the formation of noncolored carbinol bases (*21*). In addition, they are more orange than malvidin-3-*O*-glucoside since the new ring in their structures causes a hypsochromic

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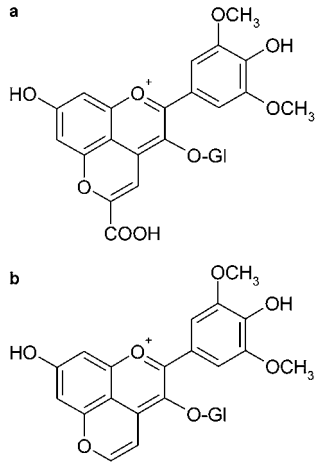

Figure 1. **a**, vitisin A, chemical structure. **b**, vitisin B, chemical structure.

shift in their wavelengths of maximum absorption; in a methanol solution containing 0.01% HCl, the maximum absorption of vitisin A is 518 nm while that of vitisin B is 498 nm (*15*). Though these anthocyanin derivatives have not been detected in grape must (*22*), they are found in significant quantities after fermentation.

Glycolysis involves a series of catabolic reactions that turn glucose into pyruvate. This pathway is operative under both respiratory and fermentatory conditions. In fermentatory metabolism, an organic molecule serves as the terminal acceptor of electrons generated during the conversion of glycolytic metabolites to energy (ATP). In *Saccharomyces*, pyruvate is metabolized into acetaldehyde, which serves as a terminal electron acceptor in the generation of ethanol. Therefore, pyruvate and acetaldehyde are produced in the cytoplasm of *Saccharomyces* during the catabolism of sugars, and although they are metabolized (pyruvate is either decarboxylated to acetaldehyde or used in the formation of acetyl CoA; acetaldehyde is reduced to ethanol), some molecules diffuse out of the cell (*23*).

Several authors have studied the influence of pyruvate and acetaldehyde on the synthesis of vitisin A and B (*17*, *18*, *24*, *25*) taking into account the effect of other molecules such as the organic acids present in wine (*26*) and SO_2. Studies have also been made on the roles of pH, temperature, and aging time on vitisin synthesis and the development of color in wines (*27*).

The aim of the present work was to study the excretion of pyruvate and acetaldehyde by different strains of *Saccharomyces* during the fermentation of Tempranillo grape must, to determine whether there is any correlation between the amounts of pyruvate and acetaldehyde liberated and the quantities of vitisin A and B formed. Strains that produce high levels of pyruvate and acetaldehyde may be useful in providing stable color to aged wine.

MATERIALS AND METHODS

Must. Musts were obtained from pressing red grapes (*Vitis vinifera* L. cultivar Tempranillo) from the Appellation Contrôlée region of Ribera del Duero. Different authors have identified and quantified the anthocyanins present in this variety (*20*, *22*, *28*). The pH of the must was 3.5; sugar content was 191 g/L. **Table 1** shows the anthocyanin composition of the must.

Yeasts Used in Experimental Fermentations. The yeasts used were nine strains of *Saccharomyces cerevisiae* isolated and selected for the production of Appellation Contrôlée Rioja (4CV, 5CV, and 9CV), Navarra (7EV, 2EV, and 1EV), and Ribera del Duero (3VA, 1VA,

Table 1. Concentration of Anthocyanins in the Must at the Beginning of Fermentation, and in Each of the Wines Made with the Different Yeasts at the End of Fermentation (mg/L)[a]

anthocyanin	D3G	Cy3G	Pt3G	Pn3G	M3G	VIT. B	VIT. A	D3G6Ac	Cy3G6Ac	Pt3G6Ac	Pn3G6Ac	M3G6Ac	M3G6Caf	Pt3G6Cm	Pn3G6Cm	M3G6Cm	total
must	6.10	1.92	8.65	9.03	89.88	nd	nd	0.12	0.16	0.14	0.10	3.54	1.16	0.01	0.01	1.13	121.96
%	5.01	1.58	7.09	7.41	73.70	0.00	0.00	0.10	0.13	0.11	0.08	2.90	0.95	0.01	0.01	0.93	100.00
wines																	
yeast																	
9CV*	0.85 ± 0.022	0.06 ± 0.006	2.28 ± 0.070	0.67 ± 0.042	43.49 ± 1.686	1.38 ± 0.076	1.77 ± 0.055	nd	nd	nd	nd	1.77 ± 0.082	0.66 ± 0.047	nd	nd	0.60 ± 0.039	58.70
2EV*	1.02 ± 0.054	0.02 ± 0.004	2.40 ± 0.093	0.96 ± 0.050	47.56 ± 1.784	0.72 ± 0.126	0.60 ± 0.036	nd	nd	nd	nd	1.96 ± 0.094	0.73 ± 0.053	nd	nd	0.67 ± 0.055	59.49
3VA*	0.93 ± 0.023	0.01 ± 0.021	2.34 ± 0.077	0.73 ± 0.033	42.23 ± 1.387	1.71 ± 0.051	0.97 ± 0.067	nd	nd	nd	nd	1.71 ± 0.054	0.58 ± 0.031	nd	nd	0.58 ± 0.028	53.52
7VA*	0.92 ± 0.035	0.05 ± 0.006	2.21 ± 0.106	0.70 ± 0.034	42.20 ± 2.003	1.45 ± 0.105	1.51 ± 0.064	nd	nd	nd	nd	1.72 ± 0.101	0.56 ± 0.052	nd	nd	0.52 ± 0.044	44.37
S6U*	0.68 ± 0.053	0.03 ± 0.008	2.01 ± 0.144	0.02 ± 0.006	44.81 ± 2.952	0.87 ± 0.204	0.78 ± 0.033	nd	nd	nd	nd	1.91 ± 0.153	0.66 ± 0.091	nd	nd	0.61 ± 0.085	56.63
4CV	1.02	0.00	2.62	0.81	47.60	1.60	1.67	nd	nd	nd	nd	1.99	0.73	nd	nd	0.66	42.13
5CV	1.03	0.07	2.60	0.81	48.33	1.24	1.89	nd	nd	nd	nd	2.01	0.80	nd	nd	0.72	51.79
7EV	0.76	0.01	1.94	0.70	36.06	1.84	0.76	nd	nd	nd	nd	1.41	0.46	nd	nd	0.43	48.20
1EV	0.64	0.00	1.79	0.06	34.98	1.83	0.73	nd	nd	nd	nd	1.35	0.38	nd	nd	0.36	51.85
1VA	0.70	0.01	1.98	0.62	39.79	1.61	0.88	nd	nd	nd	nd	1.61	0.52	nd	nd	0.49	52.37
mean	0.85 ± 0.151	0.03 ± 0.028	2.22 ± 0.283	0.61 ± 0.314	42.70 ± 4.677	1.42 ± 0.384	1.16 ± 0.496					1.74 ± 0.233	0.61 ± 0.132			0.57 ± 0.113	51.91
%	1.64	0.05	4.27	1.17	82.27	2.74	2.23					3.36	1.17			1.09	100.00

[a] Values for the yeasts (*) are means of three fermentations. These values are accompanied by the SD.

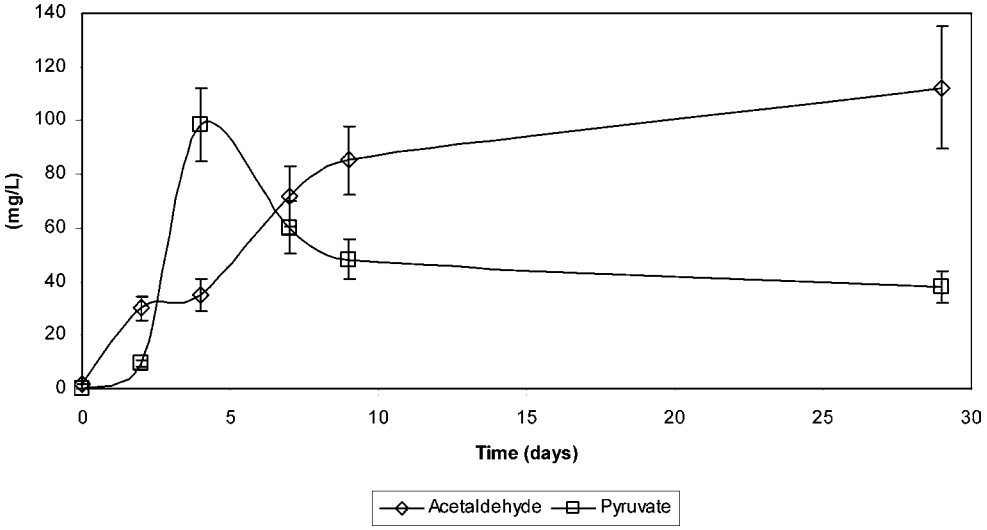

Figure 2. Mean production of pyruvate and acetaldehyde (mg/L) by the 10 yeast strains during fermentation. The SD is given for each point.

and 7VA) red wines. The commercial S6U strain of *S. uvarum* (Lallemand Inc., Canada) was also used.

Fermentations. Small scale fermentations were undertaken using 100 mL of the musts inoculated with 2 mL of YEPD medium (*29*) containing 10^8 cfu/mL of yeasts. The inocula were synchronized to obtain homogeneous populations. All fermentations proceeded isothermically at 20 °C.

Fermentations inoculated with 9CV, 2EV, 3VA, 7VA, and S6U were performed in triplicate; all others were performed once.

Determination of Pyruvate and Acetaldehyde. Pyruvate was determined enzymatically using the Sigma-Aldrich Pyruvate kit (procedure 726-UV) (Sigma Diagnostics, Inc. St. Louis, MO 63178). Acetaldehyde was similarly determined using the Acetaldehyde kit No. 668613 (Boehringer Mannheim GmbH, Mannheim, Germany). The enzymatic determination of acetaldehyde (*30*−*32*) is based on the change in absorbance at 340 nm caused by the reduction of NAD^+ to NADH (used as a cofactor by acetaldehyde dehydrogenase during the oxidation of acetaldehyde to acetic acid). The enzymatic determination of pyruvic acid (*33*, *34*) is based on the change in absorbance at 340 nm caused by the oxidation of NADH to NAD^+ (used as cofactor by lactate dehydrogenase during the reduction of pyruvate to lactate).

Pyruvate and acetaldehyde analyses were made on the starting must and at days 2, 4, 7, 9, and 29 of fermentation. Values at day 29, when all fermentations were completely finished, were taken as final values.

Analysis of Anthocyanins by Liquid Chromatography. The anthocyanins contained in the 20 fermentations were analyzed using a Waters (Milford, MA) HPLC chromatograph equipped with a 600-MS controller, a 717 plus autosampler, and a 996 photodiode-array detector. Gradients of solvent A (water/formic acid, 90:10, v/v) and B (methanol) were used in a reverse-phase Nova-pack C_{18} column (300 × 3.9 mm) as follows: 0−20% B linear (0.8 mL/min) from 0 to 5 min, 20−50% B linear (0.8 mL/min) from 5 to 70 min and reequilibration of the column from 70 to 95 min. Detection was performed by scanning from 500 to 600 nm. Quantification was performed against an external standard at 525 nm and expressed as a function of malvidin-3-glucoside (Extrasynthese, France) using a standard calibration curve. Two-hundred microliter samples of previously filtered fermentations were injected into the HPLC. Determinations were made in duplicate.

The following anthocyanins were identified in the fermentations: delphinidin-3-*O*-glucoside (D3G), cyanidin-3-*O*-glucoside (Cy3G), petunidin-3-*O*-glucoside (Pt3G), peonidin-3-*O*-glucoside (Pn3G), malvidin-3-*O*-glucoside (M3G), malvidin-3-*O*-glucoside-pyruvate or vitisin A (VIT. A), malvidin-3-*O*-glucoside-vinyl adduct or vitisin B (VIT. B), delphinidin-3-*O*-(6 acetyl)-glucoside (D3G6Ac), cyanidin-3-*O*-(6 acetyl)-glucoside (Cy3G6Ac), petunidin-3-*O*-(6 acetyl)-glucoside (Pt3G6Ac), peonidin-3-*O*-(6 acetyl)-glucoside (Pn3G6Ac), malvidin-3-*O*-(6 acetyl)-glucoside (M3G6Ac), delphinidin-3-*O*-(6 p-coumaroyl)-glucoside (D3G6Cm), malvidin-3-*O*-(6 caffeoyl)-glucoside (M3G6Caf), cyanidin-3-*O*-(6 p-coumaroyl)-glucoside (Cy3G6Cm), petunidin-3-*O*-(6 p-coumaroyl)-glucoside (Pt3G6Cm), peonidin-3-*O*-(6 p-coumaroyl)-glucoside (Pn3G6Cm), and malvidin-3-*O*-(6 p-coumaroyl)-glucoside (M3G6Cm).

The different anthocyanins were identified by their retention times compared to the majority anthocyanin of *Vitis vinifera* L., malvidin-3-*O*-glucoside; UV−visible absorption spectra were also taken into account (*20*).

The identification of the pyroanthocyanins vitisin A and B was confirmed by liquid chromatography-electrospray mass spectrometry (LC/ESI-MS), using a Hewlett-Packard (Palo Alto, CA) series 1100 chromatography system equipped with a diode array detector (DAD) and a quadrupole mass spectrometer (Hewlett-Packard series 1100 MSD) with an electrospray interface. Separation was performed in a reverse-phase Waters Nova-Pak C_{18} column [150 × 3.9 mm, 4 μm] at room temperature. A gradient consisting of solvent A (water/formic acid, 90/10, v/v) and solvent B (water/methanol/formic acid, 45/45/10, v/v/v) was applied at a flow rate of 0.8 mL/min as follows: 15−80% B linear from 0 to 30 min, 80% B isocratic from 30 to 43 min, followed by washing (methanol) and reequilibration of the column from 43 to 75 min. One-hundred microliters of wine, previously filtered through a 0.45 μm membrane, was injected into the column. DA-detection was performed from 260 to 600 nm. The ESI parameters were: drying gas (N_2) flow, 10 L/min; temperature, 350 °C; nebulizer pressure, 380 Pa (55 psi); and capillary voltage, 4000 V. The ESI was operated in positive scanning mode from *m*/*z* 100 to *m*/*z* 1500 using a fragmenter voltage gradient of 100 V from 0 to 17 min, and 120 V from 17 to 55 min.

Statistics. Means, standard deviations, ANOVAs, and linear regressions were calculated using the PC Statigraphics 5.0 software package (Graphics Software Systems, Rockville, MD).

RESULTS AND DISCUSSION

Production of Pyruvate and Acetaldehyde. The changes in pyruvate and acetaldehyde contents over fermentation show that their models of production and release into the medium are very different (**Figure 2**). Acetaldehyde production increases over the entire fermentation period (mean value 112 mg/L), reaching its peak concentration on the last day with all the yeast strains studied. The literature reports the production of 50−120 mg/L by wine yeasts (*35*). Pyruvate production grew strongly at the start of fermentation until a peak value of 98 mg/L (mean for all strains) was reached on around day 4 (*36*). After this time, the concentration fell progressively, finishing at below 40 mg/L toward the end of fermentation. This is because pyruvate is a vital metabolic intermediate in the production of acetyl CoA; in the later stages of fermentation,

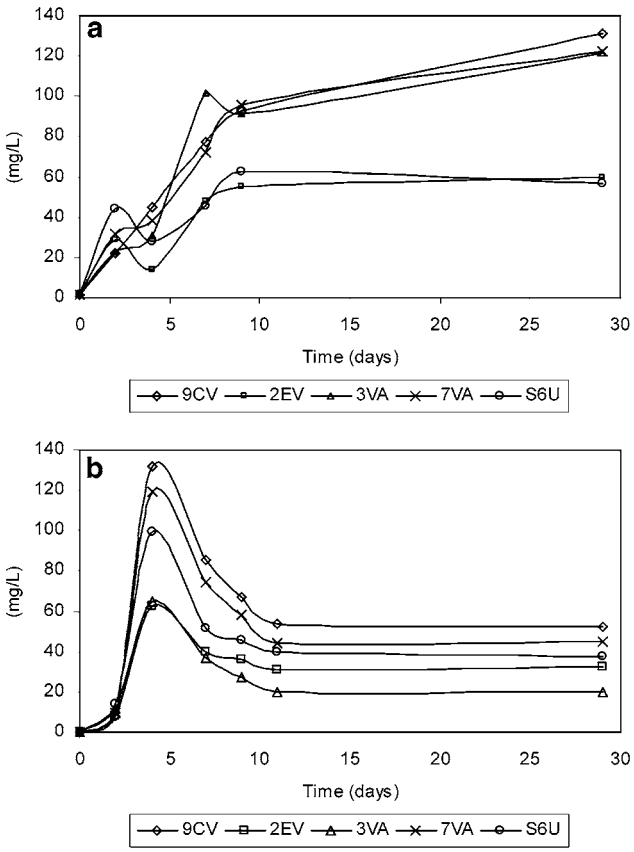

Figure 3. (a) Mean production of acetaldehyde (mg/L) by strains 9CV, 2EV, 3VA, 7VA, S6U during fermentation (each fermentation performed in triplicate). (b) Mean production of pyruvate (mg/L) by strains 9CV, 2EV, 3VA, 7VA, S6U during fermentation (each fermentation performed in triplicate).

when nutrients begin to become scarce, the yeasts consume the pyruvate they previously excreted when the fermentation medium was richer. In wines, pyruvate levels vary between 0 and 500 mg/L (*37*).

Acetaldehyde production differed strongly between yeast strains. **Figure 3**a shows the mean production ($n = 3$) of five of the studied strains, some of which finish fermentation with acetaldehyde contents of over 120 mg/L (9CV, 3VA, and 7VA), and others whose end values are below 60 mg/L (2EV and S6U). Pyruvate production also differed strongly between these five strains ($n = 3$), ranging from 60 to 132 mg/L by the fourth day (**Figure 3b**), and falling to 20−60 mg/L by the end of fermentation. Strains 9CV and 7VA showed the highest levels, and 2EV and 3VA showed the lowest.

Identification and Content of Anthocyanins and Pyroanthocyanins Derivatives. Table 1 shows the anthocyanin content of the starting must and of the wines produced by the 10 yeast strains. Vitisin A, corresponding to the product resulting from the C-4/C-5 cycloaddition of pyruvic acid and malvidin-3-*O*-glucoside (*11*, *12*), was identified through its molecular ion (*m*/*z* 561, $[M^+]$) and by a characteristic fragment (*m*/*z* 399) described in the literature (*13*, *20*). The retention time of vitisin A was 33.0 min in the chromatography conditions used, and its retention time relative to malvidin-3-*O*-glucoside was 1.25 min. Its maximum absorption wavelength was 508.7 nm. Vitisin B, the adduct resulting from the reaction between malvidin-3-*O*-glucoside and acetaldehyde (malvidin-3-*O*-glucoside-4 vinyl or malvidin-vinyl adduct), was identified and confirmed in the same way: by its molecular ion (*m*/*z* 517, $[M^+]$) and by a characteristic fragment (*m*/*z* 355) (*13*, *20*, *25*). The retention time was 30.8 min, while the retention time relative to malvidin-3-*O*-glucoside was 1.17 min. Its maximum absorption wavelength was 500.2 nm.

The total anthocyanin content of the starting must was 121.96 mg/L. Malvidin-3-*O*-glucoside content was 89.88 mg/L (73.7%). At the end of fermentation, mean anthocyanin content was 51.91 mg/L; malvidin-3-*O*-glucoside content was 42.70 mg/L (82.27%). Fermentation therefore led to a loss of 70.1 mg/L of anthocyanins (57.5% of the initial content) and a loss of 47.2 mg/L of malvidin-3-*O*-glucoside (52.51% of the initial content).

No vitisin A or B was detected in must; there are no reports in the literature of these compounds being found in must or grapes (*22*). **Figure 4**a shows a chromatogram of the must at a wavelength of 525 nm. **Figure 4b** shows the chromatogram for the must after fermentation (yeast strain 7VA); a clear reduction in the areas of all the peaks can be appreciated. In fact, some anthocyanins present in the must are detected only in trace amounts in the ferments, especially the acylated derivatives (D3G6Ac, Cy3G6Ac, Pt3G6Ac, and Pn3G6Ac) but also the *p*-coumaroyl derivatives (Pt3G6Cm and Pn3G6Cm), generally the major derivatives of the Tempranillo variety. Two new peaks can also be seen after fermentation: those corresponding to vitisin A and B. The spectra for these molecules show absorption maxima shifted toward lower wavelengths (vitisin A, 508.7 nm; vitisin B, 500.2 nm), clearly different to those of anthocyanins with similar elution times (D3G6Ac, 533 nm; Cy3G6Ac, 518.4 nm) (**Figure 4a**). These hypsochromic shifts with respect to the anthocyanin from which the vitisins are formed, malvidin-3-*O*-glucoside ($\lambda_{max} = 527-528$ nm), occur because of changes in the electron density of the heterocyclic system when a new ring (C-4/C-5) is formed through the union of a pyruvate molecule (to make vitisin A) or a vinyl residue from acetaldehyde (to make vitisin B).

Acetaldehyde and Pyruvate Contents: Influence on Vitisin Production. Figure 5a compares the vitisin A contents of five of the strains with the levels of pyruvate reached on day four of fermentation (peak concentration). At this time, the greatest proportionality between vitisin A production and pyruvate levels is seen. This suggests that vitisin A production is a consequence of the fermenting activity of the yeasts, and that the amount formed depends, in principle, on the quantity of pyruvate released into the medium.

Figure 5b shows the final pyruvate and vitisin A contents of the wines. The wines from strains 9CV and 7VA maintain the proportionality between the production of both compounds. Differences in the behavior of yeasts 2EV and 3VA can be seen in all the graphs of **Figure 5**. These differences are accentuated with respect to vitisin A by the end of fermentation. 3VA causes more vitisin to be produced than does 2EV. This indicates metabolic differences between these two strains and between these and the remaining strains represented in **Figure 5**.

Similarly, **Figure 6** shows a relationship between the final acetaldehyde content of the wines and the quantity of vitisin B synthesized. However, the relationship between pyruvate and vitisin A, and between acetaldehyde and vitisin B is different. ANOVA of the relationships between the values for vitisin A and pyruvate at the moment of greatest production (day 4) showed significant differences in the fermentations produced by the five yeast strains $p < 0.05$ (calculations not shown). This indicates that the different yeasts have different metabolic behaviors, favoring either greater or lesser production. The difference between the mean values of wines made with strains 7VA and 9CV, however, was not significantly different (**Figure 5**).

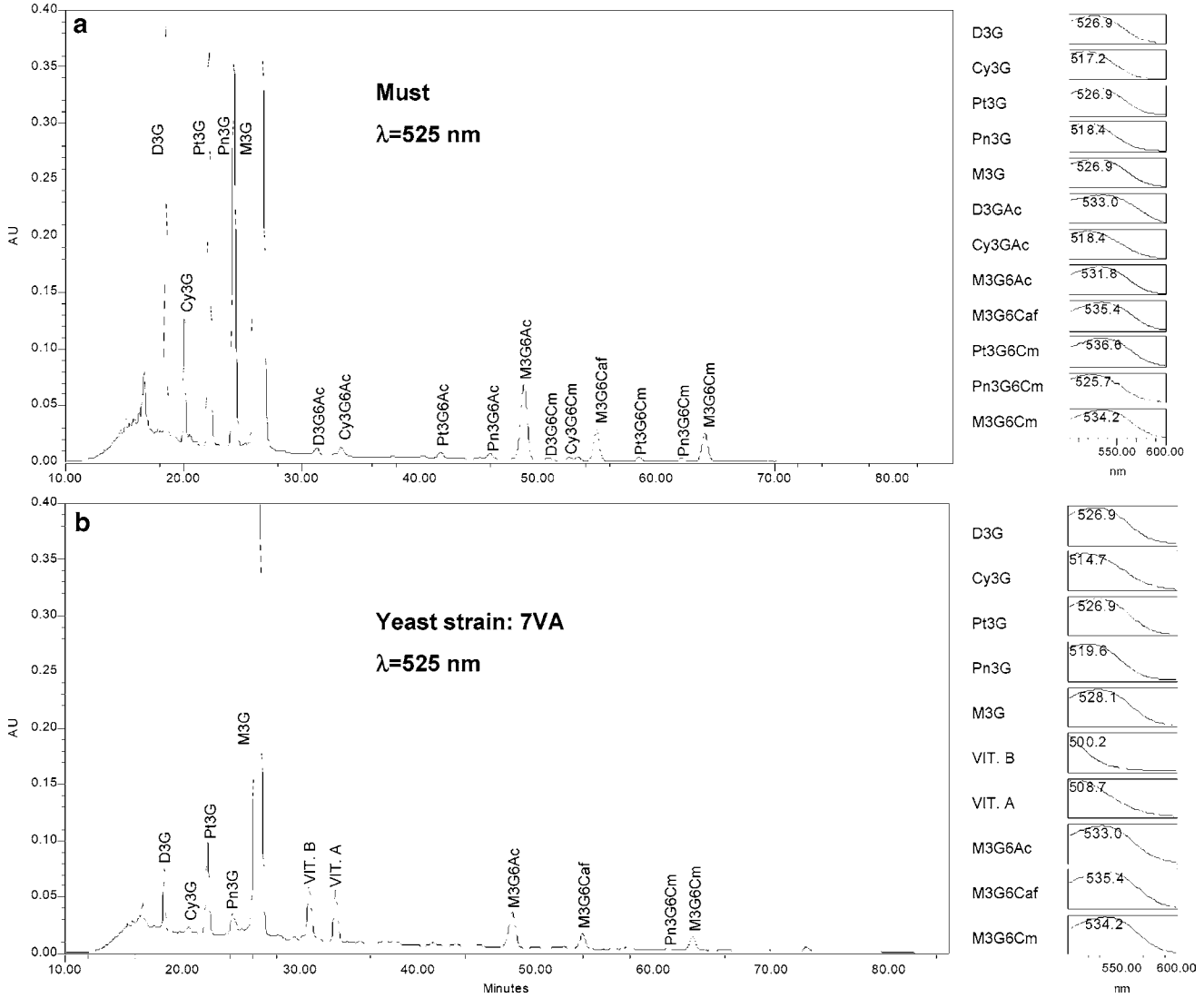

Figure 4. (a) HPLC-DAD chromatogram for the starting must (*Vitis vinifera* L. variety Tempranillo) at 525 nm and absorption spectra of the anthocyanins identified (λ = 500–600 nm). (b) HPLC-DAD chromatogram for the above must after fermentation with strain 7VA at 525 nm plus absorption spectra of the identified anthocyanins (λ = 500–600 nm).

In ANOVA calculations of the relationship between vitisin B and the acetaldehyde present at the end of the fermentation (day 29), no differences were seen between the means (mean = 0.4918; $p < 0.05$). This indicates that either the production of vitisin B from acetaldehyde does not depend on yeast metabolic function (i.e., there is a simple chemical equilibrium between the precursor and the vitisin), or that this metabolic activity is the same in the five yeast strains studied. All this corroborates the deductions made from **Figure 6**.

Linear Correlation between Pyruvic Acid/Acetaldehyde Production by the Different Yeast Strains and the Synthesis of Vitisin A/B. Figure 7a shows the linear regression line between the amount of pyruvic acid in the final wine (day 29) produced by all 10 yeasts and the final content of vitisin A (R^2 = 0.77). The equation for the regression line between these parameters (expressed in mg/L) is

$$[\text{VIT. A}]\ (\text{mg/L}) = -0.16076 + 0.03437[\text{pyruvate}]$$

If linear regression analysis is performed for pyruvate and vitisin A contents at day four (**Figure 7b**), the goodness of fit is better (R^2 = 0.80). The following equation can then be deduced:

$$[\text{VIT. A}]\ (\text{mg/L}) = -0.52894 + 0.01669[\text{pyruvate}]$$

Figure 8 shows a linear regression analysis for the quantities of acetaldehyde and vitisin B. A correlation similar to that seen with pyruvic acid and vitisin A is observed (R^2 = 0.81). The equation for the regression line is

$$[\text{VIT. B}]\ (\text{mg/L}) = 0.52053 + 0.00715[\text{acetaldehyde}]$$

Differences between the Different Strains with Respect to Vitisin Formation. The vitisins produced from the released pyruvate and acetaldehyde amounted to 2.58 mg/L (mean of all strains), i.e., 4.97% of the total anthocyanin content (51.91 mg/L, **Table 1**). They therefore form an important fraction of the coloring material in the wine. Since they are more stable than other anthocyanins, their importance is accentuated during aging: they degrade less easily than others. Further, their reduced discoloration by SO_2 and their stronger coloring effect at higher pHs make their contribution to the color of aged wines more significant. In addition, the formation of the fourth ring makes them more resistant than simple anthocyanins derivatives to oxidation and to discoloration through hydration at C-2.

The importance of the different quantities of pyruvate and acetaldehyde released by the different yeast strains is reflected in differences in the final vitisin contents of the wines. **Table 1** shows that not all the fermentations performed by the yeasts

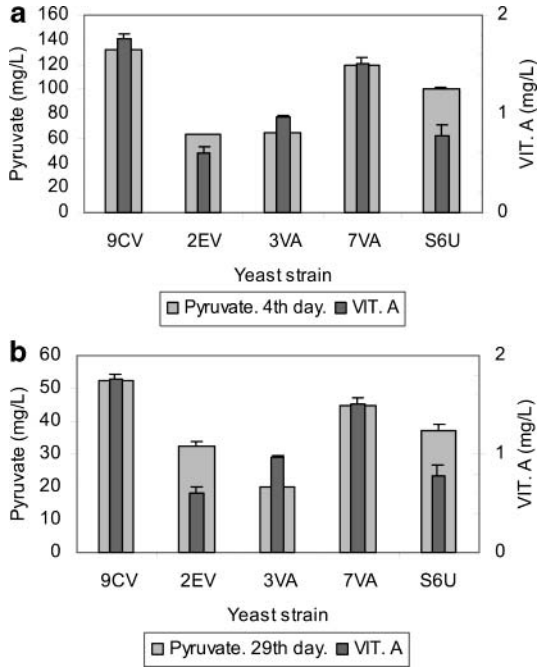

Figure 5. (a) Pyruvic acid content at day 4 for fermentations with strains 9CV, 2EV, 3VA, 7VA, S6U, compared to final content in vitisin A. Error bars are SD ($n = 3$). (b) Mean pyruvic acid content (mg/L) at day 29 for fermentation with 9CV, 2EV, 3VA, 7VA, S6U, compared with final vitisin A contents (mg/L). The SD is given at the top of the bars ($n = 3$).

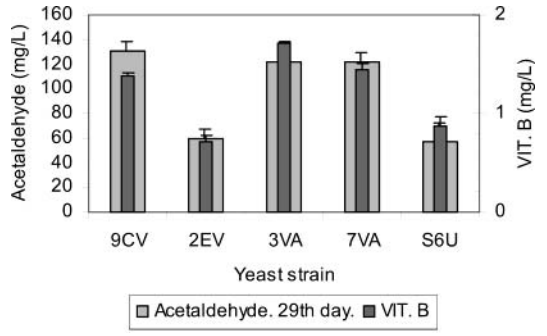

Figure 6. Mean content of acetaldehyde (mg/L) at day 29 of fermentation with strains 9CV, 2EV, 3VA, 7VA, S6U compared to final vitisin B contents (mg/L). The SD is given at the top of the bars ($n = 3$).

contain both vitisins in the same order of concentration (mg/L). The fermentations that contained >78% of one vitisin compared to the other (no matter which is the majority vitisin) were those involving strains 9CV, 2EV, 7VA, S6U, and 4CV. Those that contained 55−60% of one vitisin compared to the other involved strains 3VA, 5CV, and 1VA, i.e, in the presence of any of these yeasts, one vitisin is made at approximately half the concentration of the other. Fermentations involving 7EV and 1EV produced vitisin A at approximately 40% of the concentration of vitisin B.

Of the strains with which triplicate fermentations were undertaken, those with 2EV showed the lowest quantity of vitisin A (0.60 mg/L), while those with 9CV showed greater quantities (1.77 mg/L − nearly three times as much). Fermentation with 2EV led to the formation of 0.72 mg/L vitisin B, while 3VA produced over twice as much (1.71 mg/L). The wines obtained with the strains 9CV and 7VA contained the greatest amounts of both vitisins. Some higher values were recorded in wines obtained with some of the other strains, but only one replicate was available. In tests performed on strains in triplicate, however, the SDs were low, showing these values to be

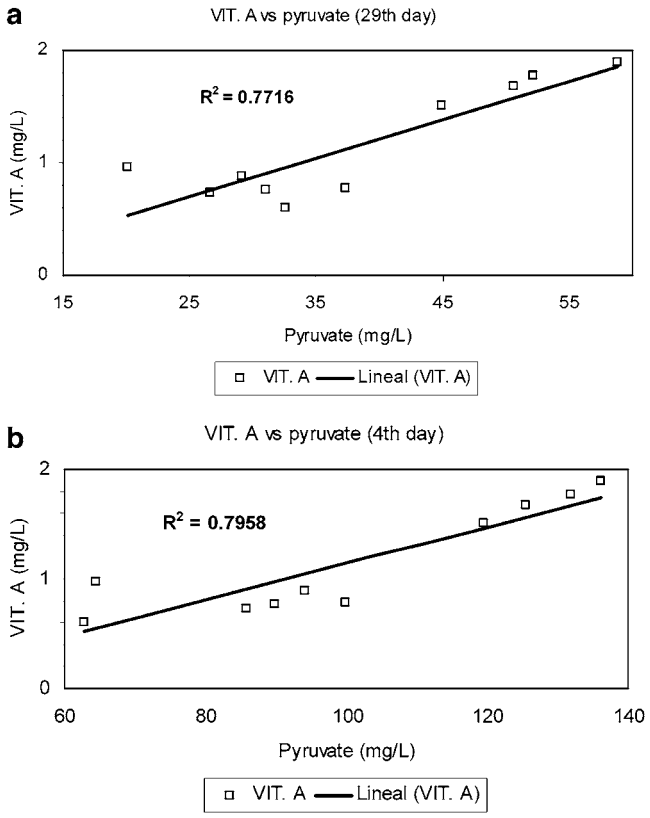

Figure 7. (a) Least squares linear regression between pyruvate content (mg/L) on day 29 of fermentation and final vitisin A contents (mg/L) for the 10 yeast strains. (b) Least squares linear regression between pyruvate (mg/L) at day 4 of fermentation and final vitisin A contents (mg/L) for the 10 yeast strains.

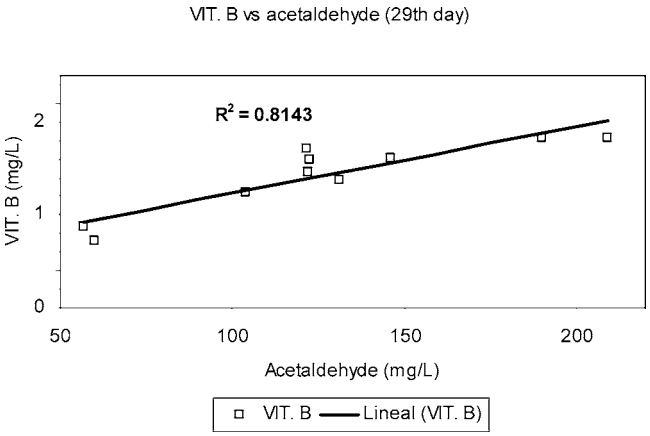

Figure 8. Least squares linear regression between acetaldehyde content (mg/L) on day 29 of fermentation and final vitisin B contents (mg/L) for the 10 yeast strains.

trustworthy. The wine of strain 5CV had 1.89 mg/L of vitisin A, while 7EV had 1.84 mg/L of vitisin B.

The differences between the influence of the strains with respect to vitisin production is shown by the high coefficient of variation of their concentrations: 42.7% for vitisin A and 27% for vitisin B. This indicates that the production rate of pyruvate and acetaldehyde is not the only influencing factor: there must also be metabolic differences between the strains which affect vitisin formation.

In practice, the selection of the *Saccharomyces* strains that produce and excrete more pyruvate and acetaldehyde and will lead to greater vitisin A and B concentrations; this is important

in red wine fermentation. The amounts of vitisin A and B are proportional to the release of these metabolites, although the formation of vitisin A does not depend on this alone. In the present work, undertaken with yeast strains that produce large amounts of acetaldehyde and pyruvate, the total vitisin content oscillated between 2.2% (2EV) and 7.8% (4CV) of total anthocyanins. This may be especially important for red wines destined to be aged (especially if they are aged in the barrel) or are to undergo a second fermentation (e.g., sparkling wines). In young wines, the anthocyanins turn into less colored forms or become polymerized with other flavonoids to produce pigments with a maximum absorption of around 500 nm. The vitisins therefore play an important role since they are proportionally more stable than other pigments. The color of these compounds is now less affected by changes in the pH (*15*, *24*) or sulfur content, and they are more stable against oxidation. This protects the wine from the brown tones produced by oxidation. Although these vitisins absorb short wavelengths, the wines maintain their red tones.

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LITERATURE CITED

(1) Mazza, G.; Miniati, E. Grapes. In *Anthocyanins in Fruits, Vegetables and Grains*; CRC Press, Inc.: Boca Raton, FL, 1993; pp 152−153.

(2) Ribéreau-Gayon, P. The anthocyanins of grapes and wines. In *Anthocyanins as Food Colors*; Markakis, P., Ed. Academic Press: New York, 1982; Vol. 6, pp 214−215.

(3) Bakker, J.; Timberlake, C. F. The distribution of anthocyanins in grape skin extract of Port wines wine cultivars as determined by high performance liquid chromatography. *J. Sci. Food Agric.* **1985**, *36*, 1315−1324.

(4) Mattivi, F.; Scienza, A.; Failla, O.; Villa, P.; Anzani, R.; Tedesco, G.; Gianazza, E.; Righetti P. *Vitis vinifera*: A chemotaxonomic approach. Anthocyanins in the skin. *Vitis* (special issue) **1990**, 119−133.

(5) Gómez-Cordovés, C. Influencia de la variedad, el clón y la producción de la vid sobre el perfil antociánico de los vinos. *II Oenologie* **1998** OIV, II, 213−220.

(6) Marx, R., Holbach, B., Otteneder, H. Determination of nine characteristic anthocyanins in wine by HPLC. F. V. N*o*. 1104. OIV.

(7) Boulton, R. B.; Singleton, V. L.; Bisson, L. F.; Kunkee, R. E. Yeast and biochemistry of ethanol fermentation. In *Principles and Practices of Winemaking*; Chapman and Hall: New York, 1996; pp 127−137.

(8) Wulf, L. W.; Nagel, C. W. High-pressure liquid chromatographic separation of anthocyanins in *Vitis vinifera*. *Am. J. Enol. Vitic.* **1978**, *29*, 42−49.

(9) Williams, M.; Hrazdina, G.; Wilkinson, M. M.; Sweeney, J. G.; Iacobucci, G. A. High-pressure liquid chromatographic separation of 3-glucosides, 3,5-diglucosides, 3-(6-*O*-p-coumaroyl) glucosides and 3-(6-*O*-p-coumaroyl glucoside) 5-glucosides of anthocyanins. *J. Chomatogr.* **1978**, *155*, 389−398.

(10) Mazza, G.; Fukumoto, L.; Delaquis, P.; Girard, B.; Ewert, B. Anthocyanins, Phenolics, and Color of Cabernet Franc, Merlot, and Pinot Noir Wines from British Columbia. *J. Agric. Food Chem.* **1999**, *47*, 4009−4017.

(11) Bakker, J.; Bridle, P.; Honda, T.; Kuwano, H.; Saito, N.; Terehara, N.; Timberlake, C. F. Identification of an anthocyanin occurring in some red wines. *Phytochemistry* **1997**, *7*, 1375−1382.

(12) Fulcrand, H.; Benabdeljalil, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. A. A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. *Phytochemistry* **1998**, *47*, 7, 1401−1407.

(13) Heier, A.; Blaas, W.; Drob, A.; Wittkowski, R. Anthocyanin analysis by HPLC/ESI-MS. *Am. J. Enol. Vitic.* **2002**, *53*, 1, 78−86.

(14) Bakker, J. Studies on the colors and pigments of port wines. Ph.D. Dissertation, The University of Bristol, 1985.

(15) Bakker, J.; Timberlake, C. F. Isolation, identification and characterization of new color-stable anthocyanins occurring in some red wines. *J. Agric. Food Chem.* **1997**, *45*, 35−43.

(16) Revilla, I.; Pérez-Magariño, S.; González-San José, M. L.; Beltrán, S. Identification of anthocyanin derivatives in grape skin extracts and red wines by liquid chromatography with diode array and mass spectrometric detection. *J. Chrom. A.* **1999**, 847, 83−90.

(17) Vivar-Quintana, A. M.; Santos-Buelga, C.; Francia-Aricha, E.; Rivas-Gonzalo, J. C. Formation of anthocyanin-derived pigments in experimental red wines. *Food Sci. Technol. Int.* **1999**, *5*, 4, 347−352.

(18) Hayasaka, Y.; Asenstorfer, R. E. Screening for potential pigments derived from anthocyanins in red wine using nanoelectrospray tandem mass spectrometry. *J. Agric. Food Chem.* **2002**, *50*, 756−761.

(19) Atanasova, V., Fulcrand, H., Cheynier, V. and Moutounet, M. Effect of oxygenation on polyphenol changes occurring in the course of wine-making. *Anal. Chim. Acta* **2002**, *458*, 15−27.

(20) Monagas, M.; Nuñez, V.; Bartolomé, B.; Gómez-Cordovés, C. Anthocyanin-derived pigments in Graciano, Tempranillo and Cabernet-Sauvignon wines produced in Spain. *Am. J. Enol. Vitic.* **2003**, *54*, 163−169.

(21) Romero, C.; Bakker, J. Interactions between grape anthocyanins and pyruvic acid, with effect of pH and acid concentration on anthocyanin composition and color in model solutions. *J. Agric. Food Chem.* **1999**, *47*, 3130−3139.

(22) Nuñez, V.; Monagas, M.; Gómez-Cordovés, C.; Bartolomé, B. *Vitis vinifera* L. cv. Graciano grapes characterized by its Anthocyanin profile. *Postharvest Biol. Tec.*, in press.

(23) Pretorius., I. S. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* **2000**, *16*, 675−729.

(24) Romero, C.; Bakker, J. Anthocyanin and colour evolution during maturation of four port wines: effect of pyruvic acid addition. *J. Sci. Food Agric.* **2000**, *81*, 252−260.

(25) Benabdeljalil, C.; Cheynier, V.; Fulcrand, H.; Hakiki, A.; Mosaddak, M.; Moutounet, M. Mise en evidence de nouveaux pigments formés par reaction des anthocyanes avec des metabolites de levure. *Sci. Aliments* **2000**, *20*, 203−220.

(26) Romero, C.; Bakker, J. Effect of acetaldehyde and several acids on the formation of vitisin A in model wine anthocyanin and colour evolution. *Int. J. Food Sci. Technol.* **2000**, *35*, 129−140.

(27) Romero, C.; Bakker, J. Effect of storage temperature and pyruvate on kinetics of anthocyanin degradation, vitisin A derivative formation, and color characteristics of model solutions. *J. Agric. Food Chem.* **2000**, *48*, 2135−2141.

(28) Hebrero, E.; Santos-Buelga, C.; Rivas-Gonzalo, J. C. High performance liquid chromatography-diode array spectroscopy identification of anthocyanins of *Vitis vinifera* variety Tempranillo. *Am. J. Enol. Vitic.* **1988**, *39*, 227−233.

(29) Yarrow, D. Methods for the isolation, maintenance and identification of yeasts. In *The Yeast, A Taxonomic Study*; Kurtzman, C. P., J. W. Fell, Eds.; Elsevier: Amsterdam, 1998; Vol 11, pp 79−80.

(30) Beutler, H. O. *Methods of Enzymatic Analysis*, 3rd ed.; Bergmeyer, H. U., Ed.; Verlag Chemie: Weinheim, Deerfield Beach/Florida, 1984; Vol VI, pp 606−613.

(31) Jojeux, A.; Lafon-Lafourcade, S. Dosage de l'acétaldéhyde dans les vins par méthode enzymatique, comparación avec les méthodes chimiques. *Ann. Falsif. Expert. Chim.* **1979**, *72*, 321−324.

(32) Delcour, J. A.; Caers, J. M.; Dondeyne, P.; Delvaux, F.; Robberechts, E. An enzymatic assay for the determination of acetaldehyde in beers. *J. Inst. Brew*. **1982**, *88*, 384−386.
(33) Bucher, T.; Czok, R.; Lamprecht, W.; Latzko, E. *Methods of Enzymatic Analyses*; Bergmeyer, H. U., Ed.; Academic Press: New York, 1963; pp 253−259.
(34) Fleischer W. R. *Enzymatic Methods for Lactic and Pyruvic Acids. In Standard Methods in Clinical Chemistry*; McDonald, R. P., Ed.; Academic Press, New York, 1970; Vol. VI, pp 245−259.
(35) Fleet, G. H.; Heard, G. M. Yeast. Growth during fermentation. In *Wine Microbiology and Biotechnology*; Fleet, G. H., Ed.; Harwood Academic Publishers: Camberwell, Victoria, Australia, 1992; pp 165−182.
(36) Whiting, G. C.; Coggings, P. A. Organic acid metabolism in cider and perry fermentations. III. Keto-acids in cider-apple juices and ciders. *J. Sci. Food Agric*. **1960**, *11*, 705−709.
(37) Radler, F. Yeast. Metabolism of organic acids. In *Wine Microbiology and Biotechnology*; Fleet, G. H. Ed.; Harwood Academic Publishers: Camberwell, Victoria, Australia, 1992; pp 165−182.

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