Synergistic Effects of Branched-chain Amino Acids and Phenylalanine Addition on Major Volatile Compounds in Wine during Alcoholic Fermentation

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The effects of adding branched-chain amino acids (BCAAs, including L-valine, L-leucine and L-isoleucine), L-phenylalanine and a mixture of them (BCAAs + Phe) on the fermentation profiles of wine yeast EC1118 and the production of volatile compounds were investigated in synthetic grape juice. The addition of selected amino acids had no considerable influence on the yeast growth and primary metabolites of the sugars. Adding BCAAs increased the production of higher alcohols, medium-chain fatty acids (MCFAs) and their corresponding ethyl esters. In comparison, adding Phe promoted the production of 2-phenylethanol, 2-phenylethyl acetate and ethyl esters of MCFAs. Nevertheless, the supplementation of BCAAs + Phe further heightened the production of MCFAs, acetate esters and ethyl esters of MCFAs compared to the single additions, but it attenuated the production of various higher alcohols (1-propanol, 2,3-butanediol and methionol) compared to the addition of BCAAs, and of 2-phenylethanol and 2-phenylethyl acetate contents compared to the Phe addition. These results suggest that adding BCAAs or Phe is an efficient way to adjust wine’s aromatic composition and complexity. Meanwhile, the combined addition of BCAAs + Phe could be a potential tool to further manipulate wine’s aromatic profile by accentuating or suppressing the formation of certain aroma compounds.

INTRODUCTION

The importance of yeast assimilable nitrogen (YAN) to yeast growth and fermentation metabolism is well known in the wine industry. The composition and amount of YAN can regulate the formation of yeast biomass and the fermentation rate (Bell & Henschke, 2005). Moreover, the nitrogen status of must can affect the production by Saccharomyces cerevisiae of many volatile compounds that contribute to wine flavour, including higher alcohols, short- to medium-chain fatty acids (MCFAs), and their ethyl ester or acetate ester derivatives (Swiegers et al., 2005). The principal sources of YAN in grape must are α-amino acids and ammonium. Insufficient nitrogen can lead to a delay in or even a cessation of fermentation. Thus, ammonium salts are usually added to grape juice to prevent these problems in wineries. However, the addition of large quantities of ammonium can result in microbiological instability and the increased production of ethyl acetate, acetic acid and carcinogenic ethyl carbamate (Vilanova et al., 2007; Garde-Cerdán & Ancín-Azpilicueta, 2008; Torrea et al., 2011).

Supplying amino acids is an alternative approach to accelerate cell growth and alter the aroma profile of the wine produced (Hernández-Orte et al., 2006; Garde-Cerdán & Ancín-Azpilicueta, 2008). Generally, the formation of higher alcohols and their corresponding acetate esters is directly proportional to the quantity of amino acids added to the juice (Trinh et al., 2010; Lee et al., 2013). As reported by Chen et al. (2014), supplementation of lychee juice with Phe resulted in significantly higher amounts of 2-phenylethyl alcohol, 2-phenylethyl acetate, 2-phenylethyl isobutyrate and 2-phenylethyl hexanoate. More 2-phenylethyl alcohol was obtained when adding Asp, Phe, Thr and Ala to Merlot juice (Hernández-Orte et al., 2006). However, inconsistent results have also been reported. Hernández-Orte et al. (2005) found that the simultaneous doubling of the level of amino acids in Airen must reduced the content of 2-phenylethanol, methionol and isoamyl alcohol in the wine and increased the contents of propanoic acid and isobutanol. These discrepancies were ascribed to the different compositions

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of the amino acids (Procopio et al., 2015). It is well known that not all nitrogen compounds that exist in grape must support yeast growth equally well. Yeast preferentially uses substrates (glutamine and ammonium) that allow the best growth, through a regulation mechanism called nitrogen catabolite repression (NCR) (Crépin et al., 2012). Thus, the variation in amino acid profiles can influence the order in which different amino acids are used by yeast, which in turn affects the ratio of secondary metabolites produced (Hernández-Orte et al., 2002).

Branched-chain amino acids (BCAAs, including L-valine, L-leucine and L-isoleucine) and L-phenylalanine are very important flavour precursors in grape must. These amino acids can be assimilated by yeast via the Ehrlich pathway to produce higher alcohols during fermentation (Hazelwood et al., 2008). These alcohols, together with acids, can form esters such as isovaleryl acetate and phenylethyl acetate, which contribute distinctly to the pleasant fruity aroma of wine. Considering the importance of BCAAs and Phe in the formation of fermented flavour compounds, this study was directed at investigating the influence of the combined addition of BCAAs and Phe on the production of yeast-derived volatiles. To facilitate this investigation, a simplified, chemically defined medium (MS300) that resembles the nutrient composition of grape juice was used.

MATERIALS AND METHODS

Strain and culture medium

Fermentations were carried out with the commercial Saccharomycyes cerevisiae var. bayanus strain EC1118 (Lallemand Inc., Blagac, France). This commercial wine yeast, used worldwide for both red and white winemaking, is considered a fast and robust fermenting strain, with a neutral contribution to the wine aroma (Molina et al., 2007). The chemically defined medium MS300, similar in composition to typical grape juice (Bely et al., 1990), was used with a little modification. It contained the following ingredients: 240 g/L of glucose; 6 g/L of citric acid and 6 g/L of malic acid; mineral salts (in mg/L): KH₂PO₄ (750), K₂SO₄ (500), MgSO₄·7H₂O (250), CaCl₂·2H₂O (155), NaCl (200), MnSO₄·H₂O (4), ZnSO₄ (4), CuSO₄·5H₂O (1), KI (1), CoCl₂·6H₂O (0.4), H₃BO₃ (1), NaMoO₄·2H₂O (1); vitamins (in mg/L): myoinositol (20), nicotinic acid (2), calcium pantothenate (1.5), thiamine hydrochloride (0.25), pyridoxine hydrochloride (0.25), biotin (0.003); anaerobic growth factors: 15 mg/L ergosterol, 0.5 mL/L of Tween80; and nitrogen source (in mg/L): ammonium chloride (460), L-proline (612.6), L-glutamine (505.3), L-arginine (374.4), L-tryptophan (179.3), L-alanine (145.3), L-glutamic acid (120.4), L-serine (78.5), L-threonine (75.9), L-aspartic acid (44.5), L-leucine (48.4), L-isoleucine (32.7), L-valine (44.5), L-phenylalanine (37.9), L-histidine (32.7), L-methionine (31.4), L-tyrosine (18.3), L-glucose (18.3), L-lysine (17.0) and L-cysteine (13.1). The ammonium salt and α-amino acids (except proline) in the medium were considered yeast assimilable nitrogen. The medium pH was adjusted to 3.3. Based on MS300, BCAAs and/or Phe were added to the medium. The amino acid contents added were in the normal concentration range for these amino acids in grape must (Bell & Henschke, 2005). The nitrogen conditions of the treatments are listed in Table 1.

Fermentation conditions

The inoculum for all experiments was prepared by pre-growing the yeast in 500 mL shake flasks containing 300 mL of the YPD medium (20 g/L of glucose, 10 g/L of peptone and 5 g/L of yeast extract purchased from Oxoid) at 30°C with shaking (150 rpm) overnight. This starter culture was used to inoculate the experiment cultures with an initial viable population of 10⁶ CFU/mL. Cells were harvested by centrifuging and were washed twice with sterile water. The fermentations were carried out in 500 mL flasks filled with 350 mL of the chemically defined medium and fitted with a fermentation lock and a puncture needle for sampling. All fermentations were maintained at 25°C without shaking. All fermentations were performed in triplicate. The progress of fermentation was monitored by periodic measurement of cell density (optical density at 660 nm, OD₆₆₀) and sugar consumption. Samples were taken and centrifuged at 5 000 rpm for 10 min at 4°C, and the cell-free supernatants were stored at -20°C before analysis.

Analytical methods

In all fermentation samples, the concentrations of glucose, fructose, ethanol, glycerol, acetic acid, malic acid, citric acid, lactic acid and succinic acid were determined using the HPLC method described by Vervoort et al. (2010). The system was equipped with a HPX-87H Aminex ion-exchange column (300 mm × 7.8 mm, Bio-Rad Laboratories, Hercules, CA, USA), with 5 mM sulfuric acid as the mobile phase. Glucose, fructose, ethanol and glycerol were detected with a refractive index detector, and the column was maintained at 45°C. All of the acids were detected with a photodiode array detector at 214 nm, and the column was maintained at 60°C. Samples were diluted and filtered through 0.22 μm membrane filters (purchased from DIKMA) prior to the HPLC analysis.

Amino acids and ammonium in the medium were analysed using the HPLC method developed previously.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Concentration of amino acids and yeast assimilable nitrogen in different treatments.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Treatments</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
</tr>
<tr>
<td>2</td>
<td>BCAAs</td>
</tr>
<tr>
<td>3</td>
<td>Phe</td>
</tr>
<tr>
<td>4</td>
<td>BCAAs + Phe</td>
</tr>
</tbody>
</table>

in our laboratory (Wang et al., 2014). Samples were pre-column derivatised with diethyl ethoxymethyleenemalonate (DEEMM). The HPLC analysis was performed using an Agilent 1200 series instrument. Chromatography separation was performed on an Agilent ZORBAX SB-C18 Rapid Resolution HT column (3.0 mm × 50 mm, 1.8 micron). For detection, a photodiode array detector monitoring at 280 nm was used.

Volatile compounds in the wines were analysed using the HS-SPME-GC-MS method developed by Zhang et al. (2011). Samples were extracted with 50/30 μm DVB/CAR/PDMS fibre (Supelco, Bellefonte, PA, USA). The GC-MS system used was an Agilent 7890A GC equipped with an Agilent 5975C mass spectrometer. The column used was a 60 m × 0.25 mm HP-INNOWAX capillary with 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA). The carrier gas was helium at a flow rate of 1.0 mL/min. The oven’s starting temperature was 50ºC, which was held for 1 min, then raised to 220ºC at a rate of 3ºC/min and held for 5 min. Electron ionisation (electron impact mode at 70 eV) mass spectrometric data from m/z 30 to 350 was collected. All determinations were performed in triplicate. The detailed identification and quantification information is listed in Supplementary Table 1.

Statistical analysis
Mean and standard deviation values were calculated on the basis of the data obtained from the triplicate fermentations performed for each treatment. Significant differences in metabolites among treatments were determined using the one-way analysis of variance (ANOVA) test, employing Duncan’s multiple range test at a significance level of p < 0.05 (SPSS 17.0 statistical software, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION
Yeast growth and fermentation profiles
As shown in Fig. 1, the supplementation of BCAAs or Phe to the medium had little effect on the fermentation kinetics, except for the combined addition of BCAAs + Phe, which slightly stimulated cell growth and the sugar consumption rate, especially for the second half of the fermentation (residual sugar less than 50%). According to previous results, once the YAN is sufficient for yeast growth (above 140 mg N/L), there is little variation in the rate of fermentation in response to nitrogen supplementation (Hernández-Orte et al., 2005; Garde-Cerdán & Aycin-Azpilicueta, 2008).

Ethanol and glycerol production were not significantly affected by amino acid addition compared to the control (Table 2). Their concentrations ranged from 11.31% to 11.85% (V/V) and 5.28 g/L to 5.48 g/L respectively. The pH value was decreased from 3.27 in the control wine to approximately 3.05 by amino acid addition. Except for acetic acid and succinic acid, the contents of malic acid, citric acid and lactic acid showed little variation with amino acid additions. The level of malic acid in the wines was nearly

![FIGURE 1](image-url)
Sugar consumption (A) and yeast growth (B) during the fermentations with the addition of BCAAs and Phe.
the same as in the initial medium, confirming that the strain EC1118 could not efficiently degrade malic acid because of the poor uptake of malic acid by simple diffusion and a very low substrate affinity of malic enzyme, which could be dysfunctional under winemaking conditions (Redzepovic et al., 2003; Chen et al., 2014; Su et al., 2014). Adding BCAAs and BCAAs + Phe led to a slight but significant decrease in the succinic acid contents compared to the control and Phe-added fermentation. This was consistent with the results of Torrea et al. (2011), who reported a decreased succinate concentration with the addition of inorganic and organic nitrogen. These results collectively suggest that adding BCAAs and/or Phe to the medium containing sufficient YAN did not have a considerable influence on the cell growth and primary metabolites of sugar.

We also determined the consumption profiles of amino acids in different treatments. The consumption of specific amino acids was determined by calculating the ratio of consumed amino acid content to the initial content in the medium (Supplementary Table 2). The addition of single BCAAs decreased the consumption ratios of Val, Phe, Tyr and Cys compared to the control, especially Tyr, as its consumption ratio dropped from 66.9% to 39.7%. In comparison, Phe addition only caused a decreased utilisation of Glu and Cys. The consumption of more types of amino acids was inhibited in the combined addition of BCAAs + Phe medium, including Asp, Val, Phe, Cys and Tyr. Unlike other amino acids, Ser consumption increased with the combined addition of BCAAs + Phe medium (80.2%) compared to the control (69.8%).

Volatile compounds in the wines supplemented with amino acids

After alcoholic fermentation, the concentrations of the most important aroma-active compounds were analysed (Table 3). The addition of BCAAs and/or Phe significantly affected the synthesis of wine aroma compounds. Generally, the total production of volatile aroma compounds was promoted by the addition of selected amino acids.

Higher alcohols

Higher alcohols were the main abundant group of volatiles in all wines. A positive relationship between higher alcohol production and amino acid supplementation was found. BCAA addition resulted in a 34.4% increase in 3-methyl butanol compared to the control. This was reasonable, considering the mechanism involved in the Ehrlich pathway. BCAA addition also increased the production of 2-phenylethanol, 1-propanol and 2,3-butanediol. As a result, the total production of higher alcohols was 81.6% higher than that of the control.

The highest increase in higher alcohol was observed in 2-phenylethanol with Phe-added fermentation. Its concentration increased by 157.4% and 80.6% compared to the control and the BCAA-added wines respectively. This result was consistent with the data of Chen et al. (2014), who found that supplementation of lychee juice with Phe significantly promoted the formation of 2-phenylethyl alcohol. Unlike BCAA addition, Phe addition had no obvious effect on the production of other higher alcohols.

Although they still presented a promotion effect compared to the control, most higher alcohols showed a lesser extent of promotion in wines to which BCAAs + Phe had been added compared to single additions, except for 2-methyl propanol. This suggests that the combined addition of BCAAs + Phe could weaken the promotion effect of higher alcohol production induced by the single addition of the amino acid(s). Moreira et al. (2011) also observed that branched-chain alcohols, including isobutyl alcohol and isoamyl alcohol, were decreased in wine supplemented with DAP, which could be explained by the interaction of different nitrogen sources because ammonium is preferentially taken up and consumed by yeast cells compared to BCAs (Crépin et al., 2012).

Higher alcohols could contribute a masking effect of fruity aromas in wines, and they are able to communicate alcoholic and solvent notes to the wine only in certain aromatic contexts (Camelyre et al., 2015; De-la-Fuente-Blanco et al., 2016). Compared to Phe, increasing the initial BCAA concentration in the medium could result in the higher formation of higher alcohols. As a result, controlling

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Control</th>
<th>BCAAs</th>
<th>Phe</th>
<th>BCAAs + Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol (V/V, %)</td>
<td>11.85 ± 0.12a</td>
<td>11.48 ± 0.43a</td>
<td>11.31 ± 0.38a</td>
<td>11.73 ± 0.23a</td>
</tr>
<tr>
<td>Glycerol (g/L)</td>
<td>5.35 ± 0.19a</td>
<td>5.28 ± 0.19a</td>
<td>5.48 ± 0.32a</td>
<td>5.46 ± 0.12a</td>
</tr>
<tr>
<td>pH</td>
<td>3.27 ± 0.02a</td>
<td>3.07 ± 0.01b</td>
<td>3.05 ± 0.01b</td>
<td>3.06 ± 0.04b</td>
</tr>
<tr>
<td>Residual YAN (mg N/L)</td>
<td>37.17 ± 0.62c</td>
<td>39.68 ± 1.31b</td>
<td>37.18 ± 1.23c</td>
<td>43.14 ± 1.73a</td>
</tr>
<tr>
<td>Acetic acid (g/L)</td>
<td>0.77 ± 0.05b</td>
<td>0.79 ± 0.01ab</td>
<td>0.83 ± 0.02a</td>
<td>0.79 ± 0.01ab</td>
</tr>
<tr>
<td>Malic acid (g/L)</td>
<td>5.73 ± 0.40a</td>
<td>5.48 ± 0.02a</td>
<td>5.57 ± 0.21a</td>
<td>5.55 ± 0.14a</td>
</tr>
<tr>
<td>Citric acid (g/L)</td>
<td>4.44 ± 0.25a</td>
<td>4.35 ± 0.10a</td>
<td>4.41 ± 0.15a</td>
<td>4.48 ± 0.15a</td>
</tr>
<tr>
<td>Lactic acid (g/L)</td>
<td>0.23 ± 0.01a</td>
<td>0.23 ± 0.01a</td>
<td>0.26 ± 0.02a</td>
<td>0.26 ± 0.01a</td>
</tr>
<tr>
<td>Succinic acid (g/L)</td>
<td>0.36 ± 0.03a</td>
<td>0.30 ± 0.00bc</td>
<td>0.34 ± 0.02ab</td>
<td>0.29 ± 0.01c</td>
</tr>
</tbody>
</table>

**TABLE 2**

General oenological parameters of wines obtained from the fermentation of synthetic grape juice with different initial concentrations of BCAAs and Phe. Values (mean ± SD, n = 3) of the same parameter (compound) followed by the same lowercase letters indicate no significant difference (p < 0.05).
<table>
<thead>
<tr>
<th>Compound</th>
<th>Aroma descriptor</th>
<th>Control</th>
<th>BCAAS</th>
<th>Phe</th>
<th>BCAAs + Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-methyl propanol</td>
<td>Solvent, pungent †</td>
<td>87 955.5 ± 3 789.0b</td>
<td>90 719.0 ± 5 263.2b</td>
<td>90 406.2 ± 5 029.5b</td>
<td>135 438.7 ± 10 500.5a</td>
</tr>
<tr>
<td>3-methyl butanol</td>
<td>Burnt, alcohol ‡</td>
<td>63 173.5 ± 3 710.7b</td>
<td>84 916.9 ± 1 873.3a</td>
<td>67 486.6 ± 3 265.8b</td>
<td>78 031.5 ± 5 472.6a</td>
</tr>
<tr>
<td>2-phenylethanol</td>
<td>Floral, rose, honey †</td>
<td>9 227.5 ± 881.2c</td>
<td>13 149.7 ± 637.9b</td>
<td>23 748.9 ± 2 576.3a</td>
<td>15 897.3 ± 1 944.6b</td>
</tr>
<tr>
<td>1-propanol</td>
<td>Solvent, pungent †</td>
<td>36 503.4 ± 1 263.4c</td>
<td>59 827.1 ± 4 367.4a</td>
<td>35 315.2 ± 3 380.9c</td>
<td>50 055.3 ± 4 441.3b</td>
</tr>
<tr>
<td>1-butanol</td>
<td>Alcohol, solvent †</td>
<td>568.4 ± 28.9a</td>
<td>475.2 ± 8.6b</td>
<td>557.2 ± 37.5a</td>
<td>412.2 ± 21.4b</td>
</tr>
<tr>
<td>1-hexanol</td>
<td>Green, cut grass ‡</td>
<td>22.9 ± 0.2a</td>
<td>23.2 ± 1.1a</td>
<td>23.3 ± 0.4a</td>
<td>23.0 ± 0.5a</td>
</tr>
<tr>
<td>1-octanol</td>
<td>Jasmine, lemon †</td>
<td>3.8 ± 0.5c</td>
<td>4.8 ± 0.2b</td>
<td>6.1 ± 0.8a</td>
<td>5.9 ± 0.8a</td>
</tr>
<tr>
<td>1-decanol</td>
<td></td>
<td>7.9 ± 0.2b</td>
<td>8.4 ± 0.8ab</td>
<td>9.5 ± 0.5a</td>
<td>9.8 ± 1.1a</td>
</tr>
<tr>
<td>2,3-butanediol</td>
<td>Fruity †</td>
<td>118 465.7 ± 8 648.1c</td>
<td>324 731.1 ± 24 596.9a</td>
<td>113 358.9 ± 8 081.4c</td>
<td>208 773.4 ± 7 536.9b</td>
</tr>
<tr>
<td><strong>Total alcohols</strong></td>
<td></td>
<td>315 928.8 ± 14 305.7c</td>
<td>573 855.4 ± 32 588.3a</td>
<td>330 912.1 ± 15 530.5c</td>
<td>488 647.0 ± 26 148.4b</td>
</tr>
<tr>
<td>3-methyl butanoic acid</td>
<td>Sweaty, acid, rancid ‡</td>
<td>509.1 ± 20.3b</td>
<td>607.5 ± 46.0ab</td>
<td>669.3 ± 78.4a</td>
<td>644.6 ± 56.7a</td>
</tr>
<tr>
<td>butanoic acid</td>
<td>Cheese, rancid †</td>
<td>344.3 ± 26.7b</td>
<td>514.5 ± 20.8a</td>
<td>373.3 ± 39.5b</td>
<td>516.2 ± 54.0a</td>
</tr>
<tr>
<td>hexanoic acid</td>
<td>Cheese, sweaty †</td>
<td>752.0 ± 69.3c</td>
<td>985.3 ± 104.7ab</td>
<td>850.0 ± 70.4bc</td>
<td>1 033.4 ± 85.5a</td>
</tr>
<tr>
<td>octanoic acid</td>
<td>Cheese, sweaty †</td>
<td>1 428.4 ± 120.5c</td>
<td>1 894.6 ± 199.3b</td>
<td>1 641.8 ± 54.4bc</td>
<td>2 315.2 ± 207.4a</td>
</tr>
<tr>
<td>decanoic acid</td>
<td>Rancid fat †</td>
<td>672.1 ± 86.4d</td>
<td>983.3 ± 100.8b</td>
<td>832.7 ± 19.5c</td>
<td>1 054.8 ± 71.6a</td>
</tr>
<tr>
<td>dodecanoic acid</td>
<td></td>
<td>217.1 ± 4.0b</td>
<td>233.2 ± 3.2a</td>
<td>222.7 ± 5.5b</td>
<td>233.7 ± 5.1a</td>
</tr>
<tr>
<td><strong>Total acids</strong></td>
<td></td>
<td>3 923.0 ± 226.7c</td>
<td>5 218.4 ± 445.4a</td>
<td>4 589.8 ± 139.7b</td>
<td>5 797.9 ± 469.9a</td>
</tr>
<tr>
<td>ethyl acetate</td>
<td>Fruity, vinegar, solvent †</td>
<td>319 191.2 ± 15 211.5b</td>
<td>326 039.6 ± 38 914.6b</td>
<td>391 980.3 ± 21 501.1a</td>
<td>421 733.4 ± 46 923.8a</td>
</tr>
<tr>
<td>isovaleryl acetate</td>
<td>Banana †</td>
<td>925.5 ± 90.0b</td>
<td>987.5 ± 83.7b</td>
<td>1 204.4 ± 116.5a</td>
<td>1 286.4 ± 25.0a</td>
</tr>
<tr>
<td>phenethyl acetate</td>
<td>Floral, rose †</td>
<td>519.4 ± 50.2c</td>
<td>726.9 ± 60.8b</td>
<td>2 067.6 ± 189.7a</td>
<td>831.3 ± 80.2b</td>
</tr>
<tr>
<td>ethyl butanoate</td>
<td>Fruity ‡</td>
<td>65.0 ± 6.4b</td>
<td>52.2 ± 2.5c</td>
<td>80.3 ± 6.5a</td>
<td>92.5 ± 9.6a</td>
</tr>
<tr>
<td>ethyl hexanoate</td>
<td>Green apple, fruity †</td>
<td>123.1 ± 16.6c</td>
<td>92.7 ± 4.3d</td>
<td>164.8 ± 10.9b</td>
<td>189.8 ± 7.2a</td>
</tr>
<tr>
<td>ethyl octanoate</td>
<td>Fruity, fat ‡</td>
<td>221.6 ± 19.9c</td>
<td>325.2 ± 26.0b</td>
<td>397.2 ± 28.6b</td>
<td>596.3 ± 63.8a</td>
</tr>
<tr>
<td>ethyl decanoate</td>
<td>Oily, grape, floral ‡</td>
<td>739.5 ± 80.2c</td>
<td>1 217.4 ± 75.8b</td>
<td>1 348.6 ± 44.6ab</td>
<td>1 475.6 ± 106.6a</td>
</tr>
<tr>
<td>ethyl dodecanoate</td>
<td></td>
<td>440.0 ± 57.6c</td>
<td>950.4 ± 108.2b</td>
<td>807.9 ± 78.1b</td>
<td>1 494.2 ± 126.2a</td>
</tr>
<tr>
<td>ethyl lactate</td>
<td>Acid, medicine ‡</td>
<td>1 201.9 ± 64.2c</td>
<td>2 692.8 ± 273.6a</td>
<td>1 944.0 ± 172.9b</td>
<td>2 126.8 ± 189.8b</td>
</tr>
<tr>
<td><strong>Total esters</strong></td>
<td></td>
<td>322 688.4 ± 14 238.1b</td>
<td>333 084.7 ± 39 065.2b</td>
<td>399 995.1 ± 21 693.8a</td>
<td>429 826.3 ± 47 493.5a</td>
</tr>
<tr>
<td>methionol</td>
<td>Cooked vegetable ‡</td>
<td>1 879.8 ± 58.4b</td>
<td>2 587.8 ± 237.5a</td>
<td>2 336.1 ± 184.2a</td>
<td>1 541.6 ± 42.2c</td>
</tr>
</tbody>
</table>

References: a. Bisson & Karpel, 2010; b. Sánchez-Palomo et al., 2010; c. Francis & Newton, 2005; d. Peinado et al., 2006; e. Sumby et al., 2010
the BCAA level in grape must is a potential way to decrease the adverse effect of higher alcohols on wine fruity aromas.

**Acids**

The formation of total volatile acids (except acetic acid) was proportional to the quantity of amino acids added to the medium. 3-Methyl butanoic acid was the only branched-chain acid quantified in this study, and it was derived from Leu metabolism. The addition of selected amino acids promoted its biosynthesis compared to the control. Medium-chain fatty acids (C6 to C12) are another significant group of volatiles that can directly contribute to the flavour of wine or serve as substrates that participate in the formation of ethyl esters. A positive relationship was observed between the production of MCFAs and the addition of amino acid(s). BCAA addition showed higher promotion of MCFA production than Phe addition did (total content 4.10 mg/L vs. 3.55 mg/L). This promotion effect became more pronounced in the mixed addition wines: 13.1%, 31.0% and 51.1% increases in the MCFA concentrations were observed in the combined additions of BCAAs + Phe compared to BCAA addition, Phe addition and the control respectively. These results suggest that BCAAs and Phe cooperatively promoted the production of MCFA. The synthesis of fatty acid could be improved by the addition of amino acids, which help yeast cells adapt their membrane fatty acid composition and resist the harsh environments during alcoholic fermentation (Torija et al., 2003). Therefore, the increased MCFAs from amino acid supplementation might be involved in the promotion of fatty acid synthesis.

Fatty acids play an important role in the complexity of wine aroma, and they could contribute a fresh flavour to wine; however, high contents can generate cheesy, sweaty or rancid odours in wine (Table 3). In the present study, the total fatty acid concentration in the wines was below the concentration that could impair wine aroma (above 20 mg/L) (Pozo-Bayón et al., 2005).

**Esters**

Acetate esters are important aroma compounds and generate the desired fruity flavour in wine. They are produced by the reaction of acetyl-CoA with higher alcohols that are formed from the metabolism of amino acids or carbohydrates (Bisson & Karpel, 2010). The content of acetate esters showed an increased trend in response to amino acid(s) supplementation, but varied with the type of amino acid added. Surprisingly, BCAA supplementation slightly improved isoamyl acetate production, but significantly increased 2-phenylethyl acetate concentration (40.1% higher than the control). In comparison, Phe addition resulted in 30.2% and 298.4% increases in isoamyl acetate and 2-phenylethyl acetate compared to the control. The combined addition caused a 39.0% increase in isoamyl acetate compared to the control, but only a 60.1% increase in 2-phenylethyl acetate was obtained relative to the control, which was much lower than that of the Phe addition. 2-Phenylethyl acetate is an important volatile compound in wine and is associated with honey, fruity and floral aromas. These results suggest that the combined addition of BCAAs + Phe was unfavourable for the synthesis of 2-phenylethyl acetate compared to single Phe addition, i.e. the presence of BCAAs could attenuate the increased magnitude of 2-phenylethyl acetate caused by Phe addition. The level of 2-phenylethyl acetate was directly proportional to the quantity of Phe added to the must, and thus the increase in 2-phenylethanol accumulation (Lee et al., 2013). As a result, the reduction of 2-phenylethyl acetate in the mixed addition wines could be due to the lower level of 2-phenylethanol produced.

Fatty acid ethyl esters are important flavour-active metabolites that are responsible for the highly desired fruit aroma characters in wine. They are generated by an alcohol and acyl group derived from activated MCFAs (Bisson & Karpel, 2010). The addition of BCAAs and Phe stimulated or decreased the production of ethyl esters of MCFAs, depending on the type of amino acids added and esters. The addition of BCAAs promoted the production of ethyl octanoate, ethyl decanoate, ethyl dodecanoate and ethyl lactate, but decreased the content of ethyl butanoate and ethyl hexanoate. Phe addition enhanced all ethyl esters of MCFAs, and the total concentration was 69.9% higher than that of the control. The total content of ethyl esters was further heightened by the mixed addition of BCAAs + Phe, which obtained a 306.2% increase compared to the control, suggesting that BCAAs + Phe had a synergic promotion effect on the biosynthesis of ethyl esters of MCFAs. Sufficient MCFAs are crucial for the biosynthesis of pleasant ethyl esters (Saerens et al., 2008); therefore, the increase in ethyl esters induced by amino acid addition could be ascribed to the accumulation of the substrate MCFAs in the medium.

**S-containing volatile compound**

Methionol, the main S-containing compound, was quantified in all the wines. It contributed a cooked vegetable aroma. There was a positive relationship between its production and the single addition of BCAAs or Phe. However, the combined addition of BCAAs + Phe eliminated this effect and even decreased its concentration below the control. The combined addition of BCAAs + Phe is a potential approach to decreasing the synthesis of methionol in wine.

**CONCLUSIONS**

This study found that the mixed addition of BCAAs + Phe had a more complicated influence on the production of volatile compounds compared to the single addition of BCAAs or Phe: it further heightened the production of MCFAs, acetate esters and ethyl esters of MCFAs; lowered the production of some higher alcohols (1-propanol, 2,3-butanediol and methanol) induced by BCAA addition; and lowered 2-phenylethanol and 2-phenylethyl acetate compared to Phe addition. These results suggest that adding BCAAs or Phe was an efficient tool to manipulate the aroma sensory quality of wines. However, the combined addition of BCAAs + Phe could result in a distinct influence on the production of volatile compounds by accentuating or suppressing the formation of certain aroma compounds, which might be a potential way to produce wines with divergent aroma attributes.
Synergistic Effect of Amino Acid Addition on Wine Aroma

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