Original article

**Effect of grape variety on the evolution of sugars, hydroxymethylfurfural, polyphenols and antioxidant activity during grape must cooking**

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**Summary**

Grape must cooking is a traditional practice used for the production of foodstuff worldwide such as traditional balsamic vinegar. The aim of this work was to reveal the effect of grape variety on the evolution of the main chemical components in grape must during cooking. To this end, two grape must varieties (red Lambrusco and white Trebbiano grapes) were cooked and analysed. The monosaccharide concentration decreased because cooking resulting in the formation of 5-hydroxymethylfurfural. At the end of cooking, the antioxidant activity and polyphenol concentration were higher and the 5-hydroxymethylfurfural was lower in Lambrusco than in Trebbiano must. Additional changes involved degradation of monomeric anthocyanins resulting in the formation of the corresponding phenolic acids. From a health point of view, the high antioxidant activity and polyphenol concentration and the low 5-hydroxymethylfurfural concentration make cooked red Lambrusco must a safer raw starting material for making traditional balsamic vinegar.

**Keywords**

5-Hydroxymethylfurfural, anthocyanins, antioxidant activity, cooking, phenolic acids, sugar degradation, traditional balsamic vinegar.

**Introduction**

Grape must cooking is a traditional practice in Mediterranean countries, and it is used in the production of different foodstuff worldwide. In Italy, the cooking of must is utilised to produce ‘saba’, which is a very concentrated grape must and also the raw starting material to make traditional balsamic vinegar (TBV) and cooked wine (‘vino cotto’). In other countries, it is used for the production of sweet wines (Spain) and pekmez (a traditional Turkish seasoning). In Italy, cooked must is produced from white or red grapes. Red grape must is used to produce ‘vino cotto delle Marche e dell’Abruzzo’. To produce TBV both white (mainly Trebbiano) and red cultivar (e.g. Lambrusco) can be utilised. Although the most used grape variety, to make TBV, is Trebbiano, the Italian and European Union regulation authorise also the employment of red grape varieties such as Lambrusco (Gazzetta Ufficiale Italiana, 2000). When Lambrusco grapes are utilised, a 24-h maceration procedure is carried out to partially extract anthocyanins and polyphenols from red skin. This is the reason why the Lambrusco must is red-purple and should contain much more polyphenols than Trebbiano must.

During cooking, grape must undergoes profound chemical and physical changes, most of them related to the heating procedure (Piva et al., 2008; Falcone et al., 2010). The most important heat-related changes are linked to the thermal degradation of sugars, resulting in the generation of 5-hydroxymethylfurfural (5-HMF) and other furanoic compounds (Falcone et al., 2010). The Maillard reaction can also occur during must cooking, resulting in the synthesis of high molecular weight melanoidins with radical scavenging activity (Di Mattia et al., 2007; Tagliazucchi et al., 2008). The above reported studies carry out analysis of the changes occurring in white grape must (Trebbiano cultivar) during cooking but, until now, only few papers (Cocchi et al., 2007, 2008) have addressed the chemical and physical modifications that take place during red grape must (Lambrusco cultivar) cooking.

The intake of HMF through food or beverage consumption is supposed to have negative effects on...
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Materials and methods

Materials

Phenolic acids standard (caffeic, caftaric, coumaric, ferulic, gallic, protoeutechic, syringic and vanillic acids), malvidin-3-O-glucoside chloride, cyanidin 3-O-glucoside chloride, 5-hydroxy methyl furfural, HPLC grade solvents, ascorbic acid (vitamin C), 4-aminoantipyrine (4-AP) and horseradish peroxidase (HRP) type II were supplied by Sigma (Milan, Italy). Delphinidin-3-O-glucoside chloride and peonidin-3-O-glucoside chloride were from Extrasynthese (Lyon, France). Petunidin-3-O-glucoside was supplied by Polyphenols Laboratories (Sandnes, Norway). 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was supplied by Calbiochem (La Jolla, CA, USA). The enzymatic Kit for D-glucose and D-fructose determination was from Roche (Darmstadt, Germany). Sephadex C-18 columns (quantity of sorbent 500 mg, volume above packing 6 mL, catalogue number 205250) were supplied by Alltech (Deerfield, IL, USA). Lambrusco grapes (Lambrusco di Sorbara cultivar) were harvested in Reggio Emilia province, and the must was immediately processed for the experiments. Trebbiano grapes (Trebbiano cultivar) were harvested in Modena province, and the must was immediately processed for the experiments.

Must processing, cooking and dry matter determination

Lambrusco and Trebbiano grapes were washed, destemmed and immediately crushed. Trebbiano grape must was immediately cooked after skin separation, while Lambrusco grape must was cold macerated (5 °C) with skins for 24 h to extract red-coloured anthocyanins. After skin separation, the must was immediately cooked. Cooking of the musts (100 kg) was carried out in an open stainless steel vessel (diameter = 110 cm, height = 130 cm) operating at atmospheric pressure. The cooking temperature was set at 85 ± 2 °C for the whole process, and the must was cooked for 10 h until the volume was reduced by 50%. Must samples were collected at different time points during the entire process. This is the typical procedure to prepare the concentrated must representing the raw starting material to make TBV.

The amount of total dry matter (DM) was determined according to the official method recommended by the AOAC (Association of Official Analytical Chemists, 1995) and was used to calculate the must concentration factor. All the quantitative data were corrected according to must concentration factor.

Determination of D-glucose/D-fructose, 5-HMF (5-hydroxymethyl furfural) and measurements of browning

The content of D-glucose and D-fructose in the samples was assayed using a hexokinase, glucose-6-phosphate dehydrogenase and fosfo-glucos-iseromerase method. The browning index of the samples was determined by measuring the absorption at 420 nm of must. The concentration of 5-HMF was detected using the same HPLC conditions as for anthocyanins but changing the detection wavelength (270 nm instead of 520 nm). The concentration of HMF was obtained from calibrations against pure standard compound.

Determination of total phenolic compounds and radical scavenging activity

Total polyphenols were determined using an enzymatic method as previously described (Verzelloni et al., 2007), and results were expressed in mg of catechin equivalents per litre of grape must. The radical scavenging activity analysis was performed using the ABTS [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] assay (Re et al., 1999), and results were expressed in mg of vitamin C equivalent per litre of grape must.

HPLC analysis of anthocyanins and phenolic acids

Anthocyanins in Lambrusco grape must were identified and quantified using a HPLC method developed by De Villiers et al. (2004). A Jasco HPLC system equipped with a UV-Vis detector (UV-vis-2031 Plus, Jasco Corp., Tokyo, Japan), a reversed phase column Hamilton HxSil C18 (Hamilton, Reno, NV, USA; 250 mm × 4.6 mm), a volumetric injector Rheodyne (Cotati, CA, USA) and a temperature-controlled oven was utilised. Identification and quantification of monomeric anthocyanins in samples were performed comparing to chromatographic retention times and areas of external standards. Identification of acylated anthocyanins was performed on the basis of the retention...
time and order of elution as reported in De Villiers et al. (2004). The total acylated anthocyanins were quantified as malvidin-3-O-glucoside.

Phenolic acids in Lambrusco grape must were identified and quantified using a HPLC method with the same Jasco instruments as described earlier after separation using Sephadex C-18 columns. The separation method and the HPLC protocol are described in Oszmianski et al. (1988).

Identification and quantification of phenolic acids in samples were performed comparing to chromatographic retention times and areas of external standards, whereas cis- and trans-coutaric acid was identified in base to the order of elution published in literature (Oszmianski et al., 1988) and quantified as p-coumaric acid equivalents.

Statistics
All data are presented as mean ± SD for three replications for each prepared sample. The Student’s t-test was performed using GraphPad Instat (GraphPad Software, San Diego, CA, USA) when data were compared with the previous cooking time. Univariate analysis of variance (ANOVA) with Tukey’s test was applied using GraphPad Prism 5.0 (GraphPad Software) when multiple comparisons were performed. Differences of P < 0.05 were considered significant. Correlation between the total polyphenols and the radical scavenging activity was established using regression analysis (GraphPad Prism 5.0) at a 95% significance level. The correlation, expressed as Pearson r value, was considered significant when P < 0.05. Kinetic analysis of anthocyanin degradation and T1/2 calculation was performed with GraphPad Prism 5.0.

Results
Changes induced by cooking
As expected, must cooking caused a decrease in the volume due to water evaporation with a concomitant increase in the total solid content. To take into account the effect of water evaporation on solute concentration, the concentration factor was calculated on the basis of the dry matter content of must (Fig. 1) and was used to correct all the quantitative data reported in the article. The content of glucose and fructose in uncooked and cooked red and white must is shown in Table 1. Trebbiano grape must contains more reducing sugars than Lambrusco grape must. Both sugars degraded during the cooking with a different extent depending on the grape varieties. In particular, the consumption of glucose and fructose was higher during Trebbiano grape must cooking than during Lambrusco grape must cooking.

As a consequence of the sugar degradation, HMF is formed (Table 1 and Fig. 2). The HMF concentration started to increase after a lag-time period that can be ascribed to the fact that low water activity speeds HMF formation, especially during the dehydration step of the acid-catalysed sugar degradation (Falcone et al., 2010). The amount of HMF formed in Trebbiano grape must after cooking was about 30 times more than that formed at the end of the cooking of Lambrusco grape must.

Table 1 reported also data about the changes in total polyphenols and radical scavenging activity during cooking. Before cooking, the red must contains 10.7 more polyphenols than the white must, but at the end of cooking, the ratio red to white decreased to 7.5. The plot of total polyphenol concentration (corrected for the concentration factor) versus the cooking time showed that the behaviour in the two musts was different (Fig. 3). In the red must, the total polyphenol concentration decreased in the first hour of cooking remaining than constant, whereas in the white must, the total polyphenol concentration increased in the early stages of cooking and then remained constant. The radical scavenging activity in Lambrusco grape must largely followed the trend of phenol profile, and there was a clear correlation between the two values (r = 0.798, P = 0.0011). During Trebbiano grape must cooking, the radical scavenging activity showed a positive conversion trend increasing 3.6 times at the end of the cooking.

Cooking of Trebbiano must produce progressive nonenzymatic browning as demonstrated by the calculation of the Δabs420 (difference between the absorbance at 420 nm at the end and at the beginning of the cooking) that was 4.3. Visually, during Lambrusco must cooking, there was a change in the colour similar to that observed...
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Table 1 Changes in glucose, fructose, 5-hydroxymethylfurfural (5-HMF), total polyphenols and radical scavenging activity (RSA) in Lambrusco and Trebbiano must during cooking

<table>
<thead>
<tr>
<th>Lambrusco red grape must</th>
<th>Trebbiano white grape must</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration in fresh must</td>
</tr>
<tr>
<td>Glucose</td>
<td>16.9 ± 0.4⁴ *</td>
</tr>
<tr>
<td>Fructose</td>
<td>14.7 ± 0.4⁴ *</td>
</tr>
<tr>
<td>HMF</td>
<td>n.d. ¹</td>
</tr>
<tr>
<td>Polyphenols</td>
<td>749.0 ± 14.5⁶ *</td>
</tr>
<tr>
<td>RSA</td>
<td>929.9 ± 15.9⁶ *</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
</tr>
</tbody>
</table>
| Results are expressed as g/L of must for glucose and fructose, mg/L of must for HMF, mg of catechin/L of must for total polyphenols and mg of vitamin C/L of must for RSA.
| a,b,c,d,e,f,g,h,i,j,k,l,m,n,o,p,q,r,s,t,u,v,w,x,y,z | Mean ± SD, significant differences within the same row are shown by different letters (Tukey’s test, P < 0.05). |

CF, concentration factor.

Figure 2 Time of course of 5-hydroxymethylfurfural (5-HMF) formation in Lambrusco red (a) and Trebbiano white (b) grape must. The value has been corrected for the concentration factor. a Mean ± SD, significant differences are shown by different letters (Tukey’s test, P < 0.05).

Figure 3 Changes in total phenolic compounds and radical scavenging activity (RSA) as a function of the cooking time in Lambrusco red (a) and Trebbiano white (b) grape must. The value has been corrected for the concentration factor. a Mean ± SD, significant differences are shown by different letters (Tukey’s test, P < 0.05).

Anthocyanin composition of Lambrusco grape must and their degradation during must cooking

The total monomeric anthocyanins measured with the HPLC analysis in uncooked must were 986.7 ± 116.5 µmol of must and represented a subfraction of 38.2% of the total polyphenols. The HPLC analysis of monomeric nonacylated anthocyanins showed that malvidin-3-O-glucoside is the most representative anthocyanin followed by delphinidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside and cyanidin-3-O-glucoside (Table 2). The sum of the monomeric nonacylated anthocyanins measured with HPLC is 808.7 ± 93.3 µmol. Lambrusco grape must also contains acylated anthocyanins (Table 2), and the corresponding sum was 178.0 ± 23.2 µmol.

During must cooking, there was a decrease in the single anthocyanin concentration (Table 2) so that the final concentration of total anthocyanins, corrected for the concentration factor, was 59.4 ± 3.4 µmol of must.

The percentage of anthocyanin degradation is more than 92% for each monomer (Table 2) and about 90% for acylated anthocyanins. The strongest decrease in anthocyanin concentration was registered in the first 30 min of heating especially for the nonacylated (Fig. 4).

The degradation of individual pigments followed 1st order reaction kinetics (Fig. 4) with correlation coefficient (R²) ranging from 0.96 to 0.99.

The half-life analysis of individual nonacylated must anthocyanins (Table 2) showed that there were some differences between anthocyanins, reflecting differences in the chemical structure.

Formation of phenolic acids during must cooking

Uncooked must contains some phenolic acids (Table 3). The most representative is trans-caftaric

during wine ageing: from red-purple to brick-red colour. The change in the colour clearly indicated that during cooking, some reactions involving anthocyanins occur. The ΔAbs 420 for red must was 0.1979 suggesting that the browning reaction was less pronounced than in the white must. The pH of uncooked must was 3.47 and 2.98 in red and white musts, respectively, remaining stable until the end of the cooking time.
Results were corrected for the concentration factor. Acylated anthocyanins were identified on the basis of the retention time and order of elution as reported in De Villiers et al. (2004) and quantified as malvidin-3-O-glucoside.

**Significantly different respect to the concentration in fresh must (t = 0 min).

**Mean ± SD, significant differences within the same column are shown by different letters (Tukey’s test, P < 0.05).

### Discussion

Grape must cooking is an ancient practice in Mediterranean countries, and in Modena and Reggio Emilia provinces, cooked must represents the raw starting material to make traditional balsamic vinegar. Only in the last years, cooked must and TBV have been considered for their content in potentially toxic molecules, for example, HMF (Theobald et al., 1998; Cocchi et al., 2007) or health-promoting components, for example, polyphenols and melanoidins (Verzelloni et al., 2007; Tagliazucchi et al., 2008; Verzelloni et al., 2010). In previous articles, Cocchi and co-workers (Cocchi et al., 2007, 2008) demonstrated that the formation of HMF is strongly dependent on the grape varieties and the cooking technology, while no data about the effect of these parameters on health-promoting compounds during must cooking are present in literature.

The first goal of our research was to study the formation of HMF as well as the behaviour of total polyphenols and radical scavenging activity as a function of grape varieties. To this purpose, two grape varieties, the red Lambrusco di Sorbara cultivar and the white Trebbiano cultivar, were cooked under the same conditions. Data showed that, during Trebbiano must cooking, there is a greater formation (about 20 times) of HMF compared to that observed during cooking of the red Lambrusco must. Furthermore, the extent of the browning reaction during cooking is much greater in Trebbiano than in Lambrusco must.

Our results confirm previously reported data (Cocchi et al., 2007, 2008), suggesting that grape variety strongly affects the HMF formation during must cooking, the red must being the one in which less HMF is formed. The differences in the HMF concentration between the two musts can be related to the different

Table 2

<table>
<thead>
<tr>
<th>$R_1$</th>
<th>Compound</th>
<th>$\mu$m t = 0 min</th>
<th>$\mu$m t = 600 min</th>
<th>Half-life (T_{1/2}) min</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.2</td>
<td>Delphinidin-3-O-glucoside</td>
<td>129.1 ± 17.0</td>
<td>4.4 ± 0.1*a</td>
<td>101.1 ± 2.4*b</td>
</tr>
<tr>
<td>24.0</td>
<td>Cyanidin-3-O-glucoside</td>
<td>64.7 ± 6.2</td>
<td>5.2 ± 0.2*a</td>
<td>141.3 ± 3.5*c</td>
</tr>
<tr>
<td>26.4</td>
<td>Petunidin-3-O-glucoside</td>
<td>100.8 ± 13.2</td>
<td>4.3 ± 0.2*a</td>
<td>106.3 ± 1.8*d</td>
</tr>
<tr>
<td>29.3</td>
<td>Peonidin-3-O-glucoside</td>
<td>84.6 ± 9.4</td>
<td>4.0 ± 0.1*a</td>
<td>110.9 ± 1.3*d</td>
</tr>
<tr>
<td>31.4</td>
<td>Malvidin-3-O-glucoside</td>
<td>429.6 ± 47.7</td>
<td>25.2 ± 0.9*a</td>
<td>117.9 ± 2.0*</td>
</tr>
<tr>
<td>/</td>
<td>Total acylated anthocyanins</td>
<td>808.7 ± 93.3</td>
<td>43.1 ± 1.5*a</td>
<td>/</td>
</tr>
<tr>
<td>/</td>
<td>Total nonacylated anthocyanins</td>
<td>178.0 ± 23.2</td>
<td>16.3 ± 1.9*a</td>
<td>123.0 ± 4.0*</td>
</tr>
<tr>
<td>57.1</td>
<td>Unidentified (tentatively pigment A)</td>
<td>n.d.</td>
<td>0.3 ± 0.1*a</td>
<td>/</td>
</tr>
<tr>
<td>Total anthocyanins</td>
<td>986.7 ± 116.5</td>
<td>59.4 ± 3.4*a</td>
<td>/</td>
<td></td>
</tr>
</tbody>
</table>
initial sugar content and to the different pH values (Muratore et al., 2006).

Another evident difference between Trebbiano and Lambrusco grape must is the content in total polyphenols and radical scavenging activity, with the last one containing 10 times more polyphenols and having a radical scavenging activity 20 times more than the white one, before cooking. The behaviour of total phenolic compounds during cooking is different in the considered musts. During red must cooking, there is an initial decrease in the total polyphenol concentrations. The initial decrease in phenolic compound concentration could be related to the transformation of anthocyanins in phenolic acids or other oligomeric/polymeric compounds, which may reduce their ability to react with horseradish peroxidase/H$_2$O$_2$ under test conditions.

The increase in the polyphenol concentration during cooking of Trebbiano must have already been observed (Falcone et al., 2010) and explained as a function of a heat-induced rearrangement in their structure that enhances their reactivity in the enzymatic system. Falcone et al. (2010) also found a decrease in the polyphenol concentration after long time of cooking probably related to the polymerisation.

Figure 4 Examples of thermal degradation kinetic of acylated and nonacylated anthocyanins during must cooking. (a) Delphinidin-3-O-glucoside; (b) cyanidin-3-O-glucoside; (c) petunidin-3-O-glucoside; (d) malvidin-3-O-glucoside; (e) peonidin-3-O-glucoside; (f) total acylated anthocyanins. Results are expressed as $\mu$M of delphinidin-3-O-glucoside for (a), $\mu$M of cyanidin-3-O-glucoside for (b), $\mu$M of petunidin-3-O-glucoside for (c), $\mu$M of peonidin-3-O-glucoside for (e) and $\mu$M of malvidin-3-O-glucoside for (d and f).
Results were corrected for the concentration factor. ± represent the standard error of the mean.

**Table 3** Retention times and concentration of individual identified phenolic acids before and at the end of the cooking

<table>
<thead>
<tr>
<th>Rf</th>
<th>Compound</th>
<th>µM t = 0 min</th>
<th>µM t = 600 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.3</td>
<td>Gallic acid</td>
<td>13.0 ± 0.8</td>
<td>29.2 ± 2.1*a</td>
</tr>
<tr>
<td>11.1</td>
<td>Protocatechuic acid</td>
<td>8.1 ± 0.2</td>
<td>11.2 ± 0.9*a</td>
</tr>
<tr>
<td>13.1</td>
<td>Trans-caftaric acid</td>
<td>90.3 ± 2.2</td>
<td>53.6 ± 5.5*a</td>
</tr>
<tr>
<td>16.1</td>
<td>Cis-caftaric acid</td>
<td>8.9 ± 0.4</td>
<td>5.8 ± 0.1*a</td>
</tr>
<tr>
<td>16.7</td>
<td>Trans-coutaric acid</td>
<td>5.7 ± 0.5</td>
<td>3.3 ± 0.1*a</td>
</tr>
<tr>
<td>18.8</td>
<td>Vanillic acid</td>
<td>n.d.</td>
<td>5.9 ± 0.4*a</td>
</tr>
<tr>
<td>20.3</td>
<td>Caffeic acid</td>
<td>n.d.</td>
<td>20.6 ± 1.4*a</td>
</tr>
<tr>
<td>21.2</td>
<td>Syringic acid</td>
<td>3.9 ± 0.2</td>
<td>12.7 ± 1.3*a</td>
</tr>
<tr>
<td>28.3</td>
<td>p-coumaric acid</td>
<td>1.5 ± 0.2</td>
<td>6.1 ± 0.2*a</td>
</tr>
<tr>
<td></td>
<td>Total phenolic acids</td>
<td>131.4 ± 4.5</td>
<td>148.4 ± 12.0</td>
</tr>
</tbody>
</table>

Results were corrected for the concentration factor. *Significantly different respect to the concentration in fresh must (t = 0 min).

The radical scavenging activity showed a different trend in the two tested musts. In fact, in Trebbiano must, we detected an increased radical scavenging activity that is a consequence of newly formed antioxidants coming from the Maillard reaction (Tagliazucchi et al., 2008). For example, high radical scavenging activity could be linked with the presence of high molecular weight melanoids as already proved in grape must derivatives (Tagliazucchi et al., 2008).

However, also in cooked must, the radical scavenging activity in Lambrusco was higher than that of Trebbiano. Taken together, these data show that the formation of HMF and the trend of radical scavenging activity are greatly dependent on grape variety. Red must supplies a safer raw starting material to make TBV than white must, having a lower amount of undesirable compounds and a higher concentration of health-promoting polyphenolic and antioxidant compounds.

**Anthocyanin stability during Lambrusco must cooking**

Monomeric anthocyanins are quantitatively the main representative class of phenolic compounds in Lambrusco di Sorbara grape must. Their concentration is about 1 mM but greatly decreases (>90%) during must cooking. It is known that anthocyanin stability is strongly influenced by the temperature and the duration of heating as well as by their structure (Cavalcanti et al., 2011). For example, Sadilova et al. (2006) observed that elderberry nonacylated anthocyanin half-life was about 2 h (heating at 95 °C and pH 1), while black carrot acylated anthocyanin half-life was more than 4 h in the same conditions. The authors suggested that acylation of anthocyanins prolongs their half-life. Also, the pH of the solution influenced the thermal stability of anthocyanins. The same authors (Sadilova et al., 2007) tested the stability of the same extracts at higher pH (3.5: the pH value as in the must), suggesting that high acidity improves the thermal anthocyanin stability. In another study, Yue & Xu (2008) found that the half-life of bilberry anthocyanins was about 2 h during heating at 80 °C and dropped to <30 min at 100 °C, suggesting the importance of temperature. Similarly to that reported in Woodward et al. (2009), comparing the half-life (Table 2) of cyanidin-3-O-glucoside with that of delphinidin-3-O-glucoside, it is possible to conclude that additional hydroxylation in the B-ring decreases the stability of the molecule. Methylation of a hydroxyl group in the B-ring prolongs the half-life, as can be observed by comparing delphinidin-3-O-glucoside with petunidin-3-O-glucoside, and the methylation of an additional hydroxyl group (as observed in malvidin-3-O-glucoside) further increases the stability of the molecule. Sadilova et al. (2007) reported a half-life of 109 min for cyanidin-3-O-glucoside at pH of 3.5 (same as in must) when heated at 95 °C, whereas our data showed a higher half-life at 85 °C, suggesting that this anthocyanin is less stable when heated at higher temperature. These considerations are in line with previously reported data (see Cavalcanti et al., 2011 for a recent review on the topic). Our results suggest that, during the long time cooking of the red must, anthocyanins are almost completely degraded.

Thermal degradation of nonacylated anthocyanins can result in a variety of species according to the pH of the solution and of the starting anthocyanins (Sadilova et al., 2007; Cavalcanti et al., 2011). At pH 3.5, the first step in anthocyanin degradation is the opening of the pyrylium ring (ring C) and the chalcone glycoside, followed by deglycosylation and the further instant cleavage of the B-ring, providing their respective phenolic acid (Sadilova et al., 2007). Acylated anthocyanins, instead, are cleaved into the corresponding acyl glycosides and anthocyanidin. The former is deglycosylated, generating the corresponding sugar and acid (acetic or coumaric acid), and the latter should be converted into the corresponding chalcone and finally into the respective phenolic acid. Confirming this pathway also during must cooking, we found an increase in the concentration of the parent phenolic acids.

**Additional possible reactions involving anthocyanins and other must components**

As reported in the result section, the formation of phenolic acids may account only for a low percentage of anthocyanin disappearance during must cooking; therefore, some other reactions involving anthocyanins should occur.
Changes induced by cooking Lard reaction products during heating (Oliviero et al. used to explain the inhibitory effect of some phenolic compounds involved in Maillard reaction, we hypothesised that most of the monomeric anthocyanins may self-associate through condensation reactions generating polymeric pigments. Indeed, as happens during wine ageing, additional condensation reactions may involve anthocyanins and colourless flavanols (catechin and epicatechin) to form oligomeric coloured or colourless adducts (Cheynier, 2005). Both these condensation reactions proceed via dehydration (Salas et al., 2003), so should be favoured during must cooking. These reactions between anthocyanin monomers or between anthocyanins and flavanols may be accelerated by the presence of aldehydes such as acetaldehyde or 5-HMF. It has been reported that the presence of reducing sugars (especially fructose) enhances anthocyanin degradation during heating (Rubinskiene et al., 2005). 5-HMF was not detected at the beginning of the Lambrusco must cooking, but at the end of the process, we measured a formation of 5-HMF of 589.5 \pm 13.4 \mu M that is far from the value of lost reducing sugars (more than 20 mm). Taking into account that the stoichiometry of acid-catalysed degradation of glucose and fructose indicates that from 1 mol of sugar should be obtained 1 mol of 5-HMF, two hypotheses are possible: (i) the acid-catalysed dehydration pathway is not the predominant one or (ii) 5-HMF is continuously produced and consumed by collateral reactions such as polymerisation or reaction with anthocyanins. Considering that acid-catalysed dehydration pathway is the predominant one (Falcone et al., 2010) and that only a low browning, indicative of the Maillard reaction, was detected in our sample (\textit{A}abs\textsubscript{280} 0.1979 between the end and the beginning of the cooking), we hypothesised that most of 5-HMF reacted with anthocyanins and/or flavanols (Es-Safi et al., 2002). This hypothesis could also be used to explain the inhibitory effect of some phenolic-rich matrices on the generation of 5-HMF and Maillard reaction products during heating (Oliviero et al., 2009; Cocchi et al., 2007) and is also a possible explanation for the grape variety effect on HMF formation during must cooking.

Another interesting reaction involves anthocyanins and caffeic acid and leads to the formation of a new pigment called pinotin A (Schwarz et al., 2003). Caffeic acid is not present in fresh must (Table 3) but its concentration is 20.6 \mu M at the end of the cooking. As discussed, this increase is a consequence of the hydrolysis of the corresponding tartaric ester, caftaric acid, whose concentration decreases during cooking by 36.7 \mu M. Because the direct reaction between caftaric acid and anthocyanins has been excluded (Schwarz et al., 2003), all the lost caftaric acid should appear as caffeic acid. The missing caffeic acid could have reacted with anthocyanins leading to the formation of pinotin A. Unfortunately, under the used chromatographic conditions, pinotin A is coeluted with acylated anthocyanins and its presence could be confirmed only with further analysis.

Interestingly, we found a new peak at the end of the cooking that, on the basis of already published data (De Villiers et al., 2004), may be tentatively assigned to pigment A that is a reaction product between malvidin-3-O-glucoside and coumaric acid (Schwarz et al., 2003). On the other hand, coumaric acid concentration increased during cooking, being a hydrolysis product of caftaric acid, but its concentration is lower than expected, suggesting a possible pathway of consumption of coumaric acid as, for example, the pigment A pathway formation.

Conclusions

In conclusion, heating red grape must causes degradation of reducing sugars, that form 5-HMF, but at lower concentration than that found in cooked Trebbiano must. These low concentrations of HMF are attributable to the low sugar content and the higher pH of Lambrusco compared to Trebbiano must and also to the consumption of 5-HMF by anthocyanins. At the end of the cooking, the antioxidant activity and polyphenol concentration were higher in Lambrusco than in Trebbiano must. From a health point of view, the high antioxidant activity, the high polyphenol concentration as well as the low concentration in 5-HMF make cooked red must from Lambrusco grape a safer raw starting material for making TBV than white grape must.

Phenolic compounds are responsible for some organoleptic characteristics, particularly colour and taste properties; therefore, it is possible that the presence of a higher amount of phenolics in red must can affect the final taste of traditional balsamic vinegar. Indeed, the taste of anthocyanin reaction products remains to be investigated, especially as related to the astringency. Also, the fact that phenolic compounds, such as anthocyanins, trap furfurals, slowing the formation of advanced sugar degradation/Maillard reaction product, may affect the final taste of the product. A thorough study on the effect of grape variety on aroma compound formation and taste developing could be of great interest.

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