Short communication

Variation in anthocyanin and total phenolics of black raspberry populations

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\section*{ABSTRACT}
Black raspberry (\textit{Rubus occidentalis}\ L.) has long been recognized as a rich source of anthocyanins. Despite renewed interest in this crop for its potential health benefits, the range of variation in anthocyanin content and other phenolic compounds has not been well examined. Here we present anthocyanin concentration and profiles, as well as total phenolics, in the fruit of 26 black raspberry seedling populations (190 samples over two growing seasons) derived from cultivated and wild parents. There was more than a twofold difference in total anthocyanin concentration between the lowest and highest pigmented populations (ranging from 244.8 to 541.3 mg 100 mL\textsuperscript{-1}). The relative amounts of the two major anthocyanins (cyanidin-3-rutinoside and cyanidin-3-xylosylrutinoside) in black raspberry fruit were significantly different. The range of total phenolics found was much lower (206.7–330.4 mg 100 mL\textsuperscript{-1}). This information will provide a valuable baseline for researchers interested in studying the health effects of these compounds, product developers in the nutraceutical market, and breeders interested in developing new cultivars with improved fruit chemistry traits.

\section*{1. Introduction}
In addition to traditional markets for fresh and processed human consumption, black raspberry has a long history of use as a natural colorant and dye because of its high anthocyanin levels (Hong & Wrolstad, 1990; Lee & Slate, 1954). Research into the pigment concentration and other chemical properties of black raspberries dates back to at least the 1950s (Lee & Slate, 1954). More recent work has shown black raspberries to be particularly high not only in anthocyanin content, but also in total phenolics (TP) (Dossett, Lee, & Finn, 2008; Moyer, Hummer, Finn, Frei, & Wrolstad, 2002; Wang & Lin, 2000). Levels of anthocyanins and total phenolics in black raspberry fruit have been found to compare favorably to a variety of other small fruits such as elderberries, huckleberries, blueberries, black currants, blackberries, etc. (Lee & Finn, 2007; Lee, Finn, & Wrolstad, 2004a,b; Moyer et al., 2002; Wu et al., 2006).

Black raspberries and other sources of dietary anthocyanins have been linked to many possible health benefits such...
as reducing eyestrain, improving night vision, helping to prevent macular degeneration, anti-inflammatory effects, protecting against DNA damage, and exhibiting anti-cancer activity (Afaq, Saleem, Krueger, Reed, & Mukhtar, 2005; Kresty et al., 2001, 2006; Lazze et al., 2003; Wang et al., 1999), and have been well reviewed by others (de Pascual-Teresa & Sanchez-Ballesta, 2008; Espin, Garcia-Conesa, & Tomas-Barberan, 2007; Rao & Snyder, 2010). Studies linking the high anthocyanin values of black raspberry with potential health benefits have led to increasing interest in black raspberry, and other anthocyanin containing berries, from various functional food and nutraceutical markets in recent years (Espin et al., 2007).

Recent studies (as described above) linking consumption of black raspberries to a number of potential health benefits have also increased awareness and interest in black raspberry anthocyanins by researchers and the public at large. Studies examining the phytochemical content and composition of black raspberry fruit have been limited to a small number of samples, from just a few closely related genotypes and nearly all have examined fruit harvested from a single location and/or in a single year (Hager, Howard, Prior, & Brownmiller, 2008; Moyer et al., 2002; Tian, Giusti, Stoner, & Schwartz, 2006; Wu et al., 2006). As a result, the range of variation in total and relative amounts of these compounds in black raspberry fruit has not been well documented. Our objective was to establish a baseline range of typical values of important phytochemicals from a large number of black raspberry genotypes (n = 190) over multiple years, which is an important step for determining the feasibility of nutraceutical usage and product development.

2. Materials and methods

2.1. Plant materials and sample preparation

Crosses between ‘Black Hawk’, ‘Dundee’, ‘Hanover’, ‘Jewel’, ‘John Robertson’, ‘Mac Black’, ‘Munger’, and ‘New Logan’ as well as a wild selection of Rubus occidentalis (NC 84-10-3) and a bulk pollen sample from Rubus leucodermis were performed in 2002 in an incomplete partial diallel mating scheme as previously described by Dossett et al. (2008). The cultivars chosen for this study represent the best performers in trials in the Willamette Valley of Oregon (USA), the world’s largest black raspberry production region (Hall, Hummer, Jamieson, Jennings, & Weber, 2009; Strik, Oregon State University, personal communication), and include most of the black raspberry cultivars currently available to growers and breeders. Twenty-six sibling families resulting from these crosses were established in the field in a randomized complete block design with four replications each consisting of one to eight sibling seedlings, depending on the number of seedlings available.

Details regarding harvest of fruit and preparation of samples have been previously described (Dossett et al., 2008). Briefly, 25 berries from each plant (genotype) were picked and bulked by family within each block. An entire block was picked in a single day to minimize variation from the effects of differing irrigation status and weather at harvest. Fruit were packed on ice immediately after harvest and then frozen immediately (−23 °C) after arrival at the laboratory. Bulked samples were thawed at 1 °C for 24 h before being puréed in a blender for 1 min. An aliquot of the puréed sample was then centrifuged at room temperature at 3446g for 10 min to separate the juice from the pulp. The supernatant was then refrozen at −23 °C for later analysis. Over a two year period (2005, 2006) a total of 190 samples were collected and treated in this manner, for later analysis of fruit chemistry traits.

2.2. Analyses of anthocyanins and total phenolics

Total anthocyanins were determined by HPLC by adding together the total amounts of the individual anthocyanins detected. Juice samples for HPLC anthocyanin analysis were diluted with HPLC grade water, then filtered with a 0.45-µm syringe driven Millex-FH filter (Millipore, Bedford, MA, USA). Anthocyanin profiles were determined by HPLC/diode array detector/ion trap mass spectrometer (HPLC/DAD/ESI-MS/MS) on an Agilent 1100 series system (Agilent Technologies, Santa Clara, CA, USA). The analytical column, mobile phase composition, and the gradient program used for HPLC analysis were performed as described by Lee and Finn (2007). Sample injection volume was 5 µL. Anthocyanins were monitored at 520 nm and quantified with a cyanidin-3-glucoside standard (Polyphenol As, Sandnes, Norway). Ultraviolet-visible (UV-Vis) absorption spectra (190–600 nm) were collected for all peaks. ESI-MS/MS parameters were set as described in Lee and Finn (2007). Individual peak assignments were made according to UV–Vis spectra, retention times, molecular ions mass-to-charge ratio (m/z), and fragmented ions m/z. Quantification was performed with HPLC-DAD results.

Determination of TP was performed using the Folin–Ciocalteu method as described by Waterhouse (2002). Total anthocyanin (TACY) was also determined using the pH differential method described by Lee, Durst, and Wrolstad (2005). Plates (clear, flat-bottomed 96-well plate; Nalge Nunc Int., Rochester, NY, USA) containing reaction mixtures were read for absorbance at 765 (for TP), 520, and 700 nm (for TACY) on a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA). TP were expressed as gallic acid (Sigma Chemical Co., St. Louis, MO, USA) equivalents. TACY was expressed as cyanidin-3-glucoside (molar extinction coefficient = 26,900 L cm−1 mol−1, molecular weight = 449.2 g mol−1). Each sample was run in duplicate and the results averaged. The extraction procedures used were chosen because of time limitation, cost, and number of samples. As a result, TACY and TP in this study are expressed as mg 100 mL−1 of juice. For purposes of discussion, comparisons will be drawn with previous studies expressing concentrations as fresh berry weight (i.e. mg 100 g−1) with the assumption that 100 mL of juice equals 100 g fresh weight and that seed weight is negligible.

2.3. Data analysis

Statistical analyses were performed using SAS (version 9.1; SAS Institute, Cary, NC, USA). Analyses of variance were conducted using the SAS PROC GLM procedure for analysis of family means. Mean separation was performed using Duncan’s Multiple Range Test.
Table 1 – Anthocyanin profiles and total by HPLC, TACY (by pH differential; expressed as cyanidin-3-glucoside), and TP (total phenolics; in mg of gallic acid equivalents) in 2005–2006 for the juice from 26 black raspberry populations produced from a partial diallel mating scheme and grown in Corvallis, OR, USA. All units are in mg 100 mL\(^{-1}\). Mean values followed by a standard error value in parenthesis. Proportions of the individual anthocyanin are included (in %).

<table>
<thead>
<tr>
<th>Population</th>
<th>n</th>
<th>Cyanidin-3-sambubioside</th>
<th>Cyanidin-3-xyllosylrutinoside(^a)</th>
<th>Cyanidin-3-rutinoside</th>
<th>Pelargonidin-3-rutinoside</th>
<th>Total anthocyanins by HPLC/DAD(^b)</th>
<th>TACY by pH differential</th>
<th>TP</th>
</tr>
</thead>
<tbody>
<tr>
<td>'BlackHawk' × NC 84-10-3</td>
<td>4</td>
<td>223.2 (4.6)</td>
<td>4%</td>
<td>280.8 (47.3)</td>
<td>51%</td>
<td>232.5 (36.5)</td>
<td>1%</td>
<td>541.3 (76.4) a(^c)</td>
</tr>
<tr>
<td>'MacBlack' × 'BlackHawk'</td>
<td>6</td>
<td>15.2 (2.0)</td>
<td>3%</td>
<td>186.0 (11.1)</td>
<td>40%</td>
<td>257.3 (19.7)</td>
<td>5%</td>
<td>464.9 (32.8) ab</td>
</tr>
<tr>
<td>'Dundee' × NC 84-10-3</td>
<td>8</td>
<td>23.0 (3.3)</td>
<td>6%</td>
<td>242.1 (20.7)</td>
<td>60%</td>
<td>135.1 (19.0)</td>
<td>32%</td>
<td>407.5 (41.1) bc</td>
</tr>
<tr>
<td>'MacBlack' × 'Hanover'</td>
<td>8</td>
<td>13.5 (1.9)</td>
<td>3%</td>
<td>146.1 (12.3)</td>
<td>37%</td>
<td>228.2 (16.3)</td>
<td>58%</td>
<td>393.9 (27.7) bcd</td>
</tr>
<tr>
<td>'MacBlack' × 'New Logan'</td>
<td>8</td>
<td>10.5 (0.8)</td>
<td>3%</td>
<td>140.7 (10.4)</td>
<td>36%</td>
<td>235.2 (14.4)</td>
<td>59%</td>
<td>390.2 (24.9) bcd</td>
</tr>
<tr>
<td>'BlackHawk' × 'Munger'</td>
<td>8</td>
<td>9.8 (0.7)</td>
<td>3%</td>
<td>145.4 (13.5)</td>
<td>38%</td>
<td>219.2 (12.5)</td>
<td>57%</td>
<td>382.6 (20.7) bcd</td>
</tr>
<tr>
<td>'Jewel' × NC 84-10-3</td>
<td>8</td>
<td>11.3 (1.3)</td>
<td>3%</td>
<td>204.9 (25.2)</td>
<td>54%</td>
<td>158.9 (16.2)</td>
<td>42%</td>
<td>379.5 (35.2) bcde</td>
</tr>
<tr>
<td>'Munger' × R. leucodermis</td>
<td>8</td>
<td>10.2 (1.4)</td>
<td>3%</td>
<td>127.8 (14.4)</td>
<td>35%</td>
<td>224.6 (10.4)</td>
<td>64%</td>
<td>368.5 (46.9) bcdef</td>
</tr>
<tr>
<td>'New Logan' × 'Munger'</td>
<td>8</td>
<td>10.2 (0.9)</td>
<td>3%</td>
<td>126.4 (10.1)</td>
<td>33%</td>
<td>231.2 (16.4)</td>
<td>61%</td>
<td>359.8 (22.4) cdefg</td>
</tr>
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<td>3%</td>
<td>158.1 (11.7)</td>
<td>42%</td>
<td>203.1 (9.6)</td>
<td>54%</td>
<td>376.0 (16.8) bcdef</td>
</tr>
<tr>
<td>'MacBlack' × R. leucodermis</td>
<td>8</td>
<td>13.3 (1.9)</td>
<td>4%</td>
<td>147.6 (15.1)</td>
<td>39%</td>
<td>206.4 (16.4)</td>
<td>56%</td>
<td>372.0 (21.5) bcdefg</td>
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<tr>
<td>'BlackHawk' × 'New Logan'</td>
<td>5</td>
<td>9.1 (2.1)</td>
<td>2%</td>
<td>112.6 (17.7)</td>
<td>30%</td>
<td>235.5 (26.3)</td>
<td>64%</td>
<td>368.5 (46.9) bcdef</td>
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<td>127.8 (16.5)</td>
<td>35%</td>
<td>213.3 (23.4)</td>
<td>59%</td>
<td>371.1 (40.6) cdefg</td>
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<tr>
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<td>8</td>
<td>9.7 (0.6)</td>
<td>3%</td>
<td>124.1 (9.3)</td>
<td>34%</td>
<td>213.0 (13.5)</td>
<td>59%</td>
<td>359.8 (22.4) cdefg</td>
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<tr>
<td>'MacBlack' × 'Munger'</td>
<td>8</td>
<td>10.1 (1.3)</td>
<td>3%</td>
<td>130.6 (12.8)</td>
<td>37%</td>
<td>198.5 (14.7)</td>
<td>58%</td>
<td>346.1 (28.6) bcdefg</td>
</tr>
<tr>
<td>'BlackHawk' × 'John Robertson'</td>
<td>8</td>
<td>9.0 (0.5)</td>
<td>3%</td>
<td>124.3 (6.2)</td>
<td>34%</td>
<td>195.5 (9.9)</td>
<td>57%</td>
<td>338.8 (12.9) bcdefg</td>
</tr>
<tr>
<td>'Hanover' × 'John Robertson'</td>
<td>8</td>
<td>8.4 (0.7)</td>
<td>3%</td>
<td>111.6 (7.4)</td>
<td>34%</td>
<td>196.4 (20.2)</td>
<td>59%</td>
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<td>'New Logan' × 'Hanover'</td>
<td>8</td>
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<td>3%</td>
<td>118.5 (20.1)</td>
<td>34%</td>
<td>193.0 (13.2)</td>
<td>57%</td>
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<tr>
<td>'John Robertson' × NC 84-10-3</td>
<td>7</td>
<td>10.8 (1.6)</td>
<td>3%</td>
<td>145.0 (24.9)</td>
<td>44%</td>
<td>159.6 (17.6)</td>
<td>51%</td>
<td>322.2 (40.8) cdefg</td>
</tr>
<tr>
<td>'Munger' × 'Dundee'</td>
<td>8</td>
<td>9.1 (0.9)</td>
<td>3%</td>
<td>107.8 (11.3)</td>
<td>35%</td>
<td>180.0 (14.0)</td>
<td>58%</td>
<td>307.5 (17.0) cdefg</td>
</tr>
<tr>
<td>'BlackHawk' × 'Hanover'</td>
<td>8</td>
<td>7.8 (0.8)</td>
<td>3%</td>
<td>107.5 (12.8)</td>
<td>35%</td>
<td>177.7 (12.9)</td>
<td>59%</td>
<td>302.8 (27.1) cdefg</td>
</tr>
<tr>
<td>'New Logan' × 'John Robertson'</td>
<td>7</td>
<td>7.7 (1.7)</td>
<td>2%</td>
<td>94.3 (17.2)</td>
<td>30%</td>
<td>186.5 (21.1)</td>
<td>62%</td>
<td>300.9 (39.6) cdefg</td>
</tr>
<tr>
<td>'MacBlack' × 'John Robertson'</td>
<td>7</td>
<td>8.0 (0.7)</td>
<td>3%</td>
<td>105.7 (8.1)</td>
<td>36%</td>
<td>170.3 (12.0)</td>
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<td>289.5 (20.7) defg</td>
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<td>'Munger' × 'John Robertson'</td>
<td>7</td>
<td>7.4 (1.6)</td>
<td>2%</td>
<td>92.9 (16.5)</td>
<td>32%</td>
<td>166.9 (17.7)</td>
<td>61%</td>
<td>276.6 (36.0) efg</td>
</tr>
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<td>'John Robertson' × R. leucodermis</td>
<td>8</td>
<td>7.5 (1.2)</td>
<td>3%</td>
<td>87.3 (11.7)</td>
<td>31%</td>
<td>168.2 (13.1)</td>
<td>62%</td>
<td>273.2 (23.5) fg</td>
</tr>
<tr>
<td>'Dundee' × R. leucodermis</td>
<td>3</td>
<td>11.4 (5.2)</td>
<td>4%</td>
<td>111.8 (21.6)</td>
<td>46%</td>
<td>113.6 (27.0)</td>
<td>46%</td>
<td>244.8 (54.5) g</td>
</tr>
</tbody>
</table>

\(^a\) Coelution with a minor amount of cyanidin-3-glucoside.
\(^b\) Peonidin-3-rutinoside was found at trace levels and was not included in the quantification.
\(^c\) Mean separation by Duncan’s Multiple Range Test \(p \leq 0.05\).
Results for total anthocyanins are summarized in Table 1. The 'Dundee' NC 84-10-3 as measured by pH differential and 244.8 vs 62.1 significantly correlated (Spearman rank order correlation measured by the pH differential method were lower than total Hawk' noside) compared to the other anthocyanins present in black antioxidants (cyanidin-3-xylosylrutinoside > cyanidin-3-rutinoside). The two main anthocyanins were also more potent phenolic compounds under these sample preparation and HPLC conditions. Cyanidin-3-xylosylrutinoside and cyanidin-3-rutinoside were not quantified separately because it co-eluted with the anthocyanin profile of most parents and progenies were generally quite similar to each other, with cyanidin-3-rutinoside as the dominant pigment. This result has also been noted by every study using HPLC to quantify individual anthocyanins in black raspberry fruit and has led, in part, to a focus on the importance and effects of this particular anthocyanin in black raspberry (Ozgen et al., 2008; Tulio et al., 2008; Wyzgoski et al., 2010). In contrast, in NC 84-10-3 is a wild black raspberry selection with higher total anthocyanins in its progeny may be an indication that wild black raspberry germplasm should be surveyed for improvement in total anthocyanins and other phytochemicals of interest.

The anthocyanin profile of most parents and progenies of 'MacBlack' are unknown, however it is quite different phenotypically from other black raspberry cultivars, being much later, somewhat more erect growing, and having flatter cap-shaped fruits than most other cultivars (Finn and Dossett, personal observation). Similarly, NC 84-10-3 is a wild black raspberry selection and higher total anthocyanins in its progeny may be an indication that wild black raspberry germplasm should be surveyed for improvement in total anthocyanins and other phytochemicals of interest.

Ozgen et al. (2008) found only minor differences in anthocyanin content (measured by pH differential) between the cultivars 'Bristol', 'Jewel', 'MacBlack', and 'Haut', particularly in comparison with differences found among eight different production sites in Ohio (USA). Our data show somewhat larger differences between samples at a single location, however this should be expected with the greater diversity in samples. Ozgen et al. (2008) attributed the similarity of their samples to a lack of genetic diversity between cultivars, noting that two of the cultivars they tested ('Bristol' and 'Jewel') are closely related, and noting molecular data (Weber, 2003) that indicate a general lack of genetic diversity among black raspberry cultivars. Our results indicate that similar values for total anthocyanins may be expected from a range of cultivars and that breeding for higher anthocyanin levels in black raspberries using the currently available germplasm is unlikely to be successful. However, there are some trends in our data that indicate the possibility for future improvement of this trait. Eight of the 11 families with the highest total anthocyanins as determined by HPLC had either 'MacBlack' or the wild selection NC 84-10-3 as one or both of their parents. While environmental differences overwhelmingly accounted for most of the variation found by Ozgen et al. (2008), 'MacBlack' had the highest TACY of the cultivars they examined. The origins of 'MacBlack' are unknown, however it is quite different phenotypically from other black raspberry cultivars, being much later, somewhat more erect growing, and having flatter cap-shaped fruits than most other cultivars (Finn and Dossett, personal observation). Similarly, NC 84-10-3 is a wild black raspberry selection and higher total anthocyanins in its progeny may be an indication that wild black raspberry germplasm should be surveyed for improvement in total anthocyanins and other phytochemicals of interest.

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compounds and their related effects should be examined in the future.

TP in black raspberry juice from these populations (207–330 mg 100 mL⁻¹) varied less than TACY, with the highest means being only about 60% more than the lowest (Table 1). TP was slightly lower in 2005, than in 2006 (259 vs. 275 mg 100 mL⁻¹, p = 0.03). Values for TP were similar to some reports (267 mg 100 g⁻¹; Wang & Lin, 2000) but somewhat lower than others (376–1244 mg 100 g⁻¹, n = 29; Moyer et al., 2002; Ozgen et al., 2008; Wang & Zheng, 2005), and may be due in part to the lack of an acidified chemical extraction procedure and the extraction excluding seeds while also leaving them largely intact. Also, variations in the TP method used by the researchers could add to these differences. Nevertheless, the results in comparison with levels of anthocyanins suggest that the bioactivity present in the juice of black raspberry could be due to high levels of anthocyanins (Tulio et al., 2008). Ellagic acid derivatives (normally present at higher levels than anthocyanins in Rubus fruit; Rao & Snyder, 2010), probably contribute to the additional bioactivity of black raspberry phenolics (Rao & Snyder, 2010; Stoner et al., 2005), and should be further investigated. While the observed differences between families in TP were relatively small it is worth noting that six of the top eight values had either NC 84-10-3 or, the industry standard, ‘Munger’ as parents.

This is the first study to characterize anthocyanin and TP content of black raspberry fruit from a large number of samples over multiple years. The only previous study that attempted to do so used black raspberry germplasm that had been crossed with red raspberry (genotypes n = 124 evaluated) to introduce thornlessness (Scalzo, Currie, Stephens, McGhie, & Alspach, 2008), although, they refer to these crosses as black raspberry. In doing so, cyanidin-3-sophoroside present to introduce thornlessness (Scalzo, Currie, Stephens, McGhie, & Alspach, 2008) and the concentration of anthocyanins in the fruit may have been similarly affected.

4. Conclusions

As mentioned, this is the first study to characterize anthocyanins from a large number of samples of pure black raspberry fruit over multiple years. In addition, the large number of unique black raspberry genotypes sampled help to give a realistic picture of what values might be expected from growing available black raspberry cultivars as well as from new ones developed through breeding in the near future. These data will help provide a baseline for researchers studying the health effects of phytochemicals in black raspberry fruit as well as product developers for nutraceutical, natural colorant, and other industries interested in these properties.

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REFERENCES


