Antioxidant Activity and Polyphenols of Aronia in Comparison to other Berry Species

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Introduction

Berries are delicious, low energy fruit, rich in antioxidant vitamins, fibre and various polyphenolic compounds (Beattie et al., 2005). They are studied intensively, because of possible beneficial effects on human health (Beattie et al., 2005; Kris-Etherton et al., 2002). In Croatia, berries like red raspberries, blackberries and strawberries are commonly used in diet, but aronia is almost unknown fruit. Aronia melanocarpa (black chokeberry) is small, dark violet fruit, and belongs to Rosaceae family (Benvenuti et al., 2004). Chokeberries originate from North America and are of great interest for food-coloring purposes and functional food development (Benvenuti et al., 2004; Bermúdez-Soto et al., 2004).

Polyphenolic compounds (including anthocyanins and flavonols) are important components of berries (Määttä-Riihinen et al., 2004a; Määttä-Riihinen et al., 2004b). They are potent antioxidants in vitro (Kähkönen et al., 2003; Heinonen et al., 1998; Pekkarinen et al., 1999) and may be protective against many degenerative diseases (Joshipura et al., 2001; Knekt et al., 2002; Le Marchand, et al., 2000; Garcia-Closas et al., 1999). Numerous studies have shown that anthocyanins are absorbed in their original glycosylated forms in humans (Netzel et al., 2005). They appear in urine and in human plasma after supplementation with berries or berry extracts but in very low concentrations (Netzel et al., 2005). Glucosides of quercetin are more efficiently absorbed than quercetin itself, whereas rhamnoglucosides (rutin) are less efficiently and less rapidly absorbed (Manach et al., 2005).

There is still not enough knowledge about health effects of fruits, vegetables or antioxidant concentrates. Much more research is therefore needed on the composition of antioxidants in fruits and on antioxidant effects of these products (Heinonen et al., 2002). A need for such data still exists because of increasing popularity of fruit consumption lately and because of increasing consumer awareness concerning the nutritional value of all foods (including berries).

Therefore, the objective of this work was to evaluate total polyphenols, total anthocyanins and antioxidant activity of berries which are commonly consumed in diet (red raspberry, blackberry, strawberry) and to compare these berries with aronia, berry fruit which is not so common in Croatia. Furthermore, individual flavonols (quercetin, kaempferol and myricetin) and anthocyanins of aronia were determined using HPLC method.

Materials and methods

Chemicals

For this study, anthocyanin standard (cyanidin-3-O-glucoside chloride-kuromanin chloride 0915 S) was purchased from Extrasynthese (Genay, France). Methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany) and ortho-phosphoric acid (85%, HPLC grade) was purchased from Fluka (Buchs, Switzerland). Hydrochloric acid (36.2 %), potassium chloride, sodium acetate trihydrate, Folin-Ciocalteau reagent were obtained from Kemika (Zagreb, Croatia). Myricetin (M6760), quercetin dihydrate (Q0125), kaempferol (K0133) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) (D9132) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Samples

Berries [red raspberry (Rubus idaeus), blackberry (Rubus fruticosus), strawberry (Fragaria ananassa) and chokeberry (Aronia melanocarpa)] were harvested in Slavonia region (Croatia) at the commercial maturity stage. Immediately after harvesting, fruits were frozen and stored at –20 °C until analysis.

Sample preparation

For total anthocyanin, total polyphenol, and antioxidant activity determination, three replicates of berry extracts were prepared and analyzed the day of preparation. Berry extracts for anthocyanin analysis (Kalt et al., 1999) were obtained by grinding berries (~5 g) in methanol (20 mL) acidified with HCl (0.1 %, v/v) for 2 min. The mixture was stored at room temperature in the dark for 16 h and then filtered. Berry extracts for polyphenolic analysis (Kalt et al., 1999) were obtained by grinding berries (~5 g) in hot methanol (10 mL) for 2 min. The solution was filtered. The extraction of the residue was repeated twice following above mentioned procedure. Three extracts were combined. Berry extracts for antioxidant activity determination were obtained by grinding berries (~20 g) in methanol (20mL) acidified with HCl (0.1%). After 60 min the solution was filtered. The residue was extracted again. The extracts were combined and diluted to volume of 50 mL with methanol acidified with HCl (0.1%).

For flavonol analysis by HPLC method, three replicates of aronia extract were prepared and analyzed the same day. Method for extraction and hydrolysis of flavonols was developed by Hertog et al., (1992), optimized by Häkkinen et al., (1998) and applied in this work with further modifications. Ascorbic acid (80 mg) was dissolved in distilled water (5 mL) and 5 g of homogenized fruit sample, 25 mL of methanol and 10 mL of 6 M HCl were added to ascorbic acid solution. Water was added to this mixture to obtain a final volume of 50 mL and final concentration of HCl 1.2 M. This solution was refluxed on water bath for 2 h at 85 °C. After cooling, extracts were filtered. A 20 mL portion of the filtrate was evaporated to dryness on a rotary evaporator using water bath and temperature of 35°C. The residue was dissolved in 2 mL of methanol and filtered.
through a 0.45 μm syringe filter (VariSep PTFE, 0.45 μm, 25 mm-Varian) prior to injection into the HPLC.

For anthocyanin analysis by HPLC method, three replicates of aronia extract were prepared and analyzed the same day. Aronia extract was obtained by grinding berries (~20 g) in methanol (20 mL) acidified with HCl (0.1%). After 60 min the solution was filtered. The residue was extracted again in the same way. The extracts were combined and diluted to volume of 50 mL with methanol acidified with HCl (0.1%). An aliquot of extract (2 mL) was filtered again through a 0.45 μm syringe filter (VariSep PTFE, 0.45 μm, 25 mm-Varian) prior to injection into the HPLC.

**Determination of total polyphenols**

Total polyphenols were determined by Folin-Ciocalteau micro method (Waterhouse, 2006). An aliquot (20 μL) of extract was mixed with 1580 μL of distilled water and 100 μL of Folin-Ciocalteau reagent. 300 μL of sodium carbonate solution (200 g L\(^{-1}\)) was added to the mixture. After incubation at 40 °C for 30 min in water bath, absorbance of the mixture was read against the prepared blank at 765 nm. Total polyphenolics were expressed as mg of gallic acid equivalents (GAE) per kg of berry. Data presented are mean ± standard deviation (SD).

**Determination of total anthocyanins**

Total anthocyanins were estimated by a pH-differential method (Giusti et al., 2001). Two dilutions of berry extracts were prepared, one with potassium chloride buffer (pH 1.0) (1.86 g KCl in 1 L of distilled water, pH value adjusted to 1.0 with concentrated HCl), and the other with sodium acetate buffer (pH 4.5) (54.43 g CH\(_3\)CO\(_2\)Na·3H\(_2\)O in 1 L of distilled water, pH value adjusted to 4.5 with concentrated HCl), diluting each by the previously determined dilution factor [strawberry and red raspberry 1:5 (v/v), blackberry 1:20 (v/v), aronia 1:100 (v/v)]. Absorbance was measured simultaneously at 510 and 700 nm after 15 min incubation at room temperature. The content of total anthocyanins was expressed in mg of cyanidin-3-glucoside equivalents (CGE) per kg of berries using a molar extinction coefficient (ε) of cyanidin-3-glucoside of 26 900 L mol\(^{-1}\) cm\(^{-1}\). Data presented are mean ± standard deviation (SD).

**Determination of antioxidant activity**

Antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. By the DPPH method (Benvenuti et al., 2004), five dilutions of each berry extract were analyzed. Reaction solution was prepared by mixing 50 μL of diluted berry extract with 300 μL of methanolic DPPH\(^{+}\) solution (1mM) and brought to 3 mL with methanol. The solution was kept in dark at room temperature for 15 minutes. The absorbance (A\(_{\text{extract}}\)) was read against the prepared blank (50 μL diluted fruit extract, 2950 μL methanol) at 517 nm. A DPPH blank solution was prepared (300 μL of 1mM DPPH solution, 2.7 mL of methanol) and measured daily. Percent inhibition of DPPH radical was calculated for each dilution of extract according to formula: % inhibition=[(A\(_{\text{DPPH}}\)-A\(_{\text{extract}}\))/(A\(_{\text{DPPH}}\))]x100, where A\(_{\text{DPPH}}\) is the absorbance value of the DPPH blank solution and A\(_{\text{extract}}\) is absorbance value of the sample solution.

**HPLC analysis of anthocyanins and flavonols**

The analytical HPLC system employed consisted of a Varian LC system (USA) equipped with a ProStar 230 solvent delivery module, ProStar 310 UV-Vis Detector, and ProStar 330 PDA Detector. Anthocyanin and flavonol separation was done in an OmniSpher C18 column (250 x 4.6 mm inner diameter, 5 μm, Varian, USA) with a ChromSep C18 guard column (1 cm x 3 mm, Varian, USA). The data were collected and analyzed on IBM computing system equipped with Star Chromatography Workstation software (version 5.52).

For anthocyanin analysis, mobile phase A was 0.5 % water solution of phosphoric acid and mobile phase B was 100 % HPLC grade methanol. The elution conditions were as follows: 3-65 % B 0-38 min; 65 % B 38-45 min; with flow rate 1 mL min\(^{-1}\). Operating conditions were: column temperature 20°C and 10 μL injection volumes of the standard and samples. A 10-minute re-equilibration period was used between individual runs. UV-Vis spectra were recorded in wavelength range from 190-600 nm (detection wavelength was 520 nm).

For flavonol analysis, mobile phase A was 0.1 % water solution of phosphoric acid and mobile phase B was 100 % HPLC grade methanol. The elution conditions were as follows: 5-80 % B 0-30 min; 80 % B 30-33 min; 80-5 % B, 33-35 min; with flow rate 0.8 mL min\(^{-1}\). Operating conditions were: column temperature 20°C and 10 μL injection volumes of the standards and samples. A 10-minute re-equilibration period was used between individual runs. UV-Vis spectra were recorded in wavelength range from 190-600 nm (detection wavelength was 360 nm).

Identification and peak assignment of anthocyanins and flavonols was based on comparison of their retention times and spectral data (190-600 nm) with those of authentic standards. Additional identification was carried out by spiking the extracts with available standards. Anthocyanin profiles of berries were compared with those found in literature (Määttä-Rihinen et al., 2004a) which gave additional information on anthocyanin identification. Calibration curves of the standards were made by diluting stock solutions in methanol to yield 1-80 mg L\(^{-1}\) (myricetin and kaempferol) and 1-250 mg L\(^{-1}\) (quercetin), or in 0.1 % methanolic HCl solution to yield 1-100 mg L\(^{-1}\) (cyanidin-3-glucoside). Identified anthocyanins
were quantified using calibration curve of cyanidin-3-
glucoside and the concentrations of anthocyanins were
expressed in mg of cyanidin-3-glucoside equivalent per
kg of berries. Flavonols were quantified using calibration
curves of quercetin, myricetin and kaempferol. Data pre-
sented are mean ± standard deviation (SD).

Results and discussion

Berry extracts were analyzed using a pH-differential
and Folin-Ciocalteau method in order to examine their
total anthocyanin (TA) and total polyphenol (TP) con-
ten. The portion of anthocyanins in total polyphenol con-
centration was evaluated by calculating TA/TP ratio. The
concentrations of total anthocyanins, total polyphenols,
and TA/TP ratios are shown in Table 1. Anthocyanins
were found in the highest concentrations in aronia (4341
mg kg⁻¹) whereas the concentrations of anthocyanins in
blackberry, red raspberry and strawberry were consider-
ably lower (1109 mg kg⁻¹, 243 mg kg⁻¹, 232 mg kg⁻¹, re-
spectively). Data presented by other authors also confi rm
that the amount of total anthocyanins were higher in aronia
than in other red fruits like blackberry, raspberry or red
currant (Benvenuti et al., 2004).

Polyphenols were found in the highest concentrations
in aronia (10637 mg kg⁻¹). High concentrations of poly-
phenols were found in blackberry as well (2485 mg kg⁻¹),
while red raspberry and strawberry had relatively lower
centrations of polyphenols (1256 mg kg⁻¹, 1005 mg kg⁻¹,
respectively). According to data presented by other authors,
aronia contained higher concentrations of polyphenols
than berries like red currant, blackberry and raspberry
(Benvenuti et al., 2004) which agree with our results.

Anthocyanins represented significant portion in total
polyphenol concentration of blackberry (45 %) or aronia (41
%). The portion of anthocyanins in strawberry (23 %) and
red raspberry (19%) was considerably lower (Table 1).

In order to evaluate antioxidant activity of chosen ber-
ries, DPPH· assay was applied and the results are presented
in Figure 1. All investigated berry extracts exhibited potent
radical scavenging activities. The strongest radical scaveng-
ing activity showed aronia, while activities of other ber-
ries were considerably lower. There are already a number
of reports on the antioxidant activity of berry extracts by
several methods such as oxygen radical absorbance capac-
ity (Wu et al., 2004) or DPPH radical scavenging capacity
(Benvenuti et al., 2004) indicating that berries like aronia,
elderberry and black currant possess strong antiradical
activities. Antioxidant activity of aronia juice concentrate
against DPPH radical was stronger than antioxidant activity
of strawberry and red raspberry concentrate (Bermúdez-
Soto et al., 2004), which agrees with our results.

In order to separate and determine individual polyphe-
nolic compounds present in aronia, HPLC method was
applied. The HPLC chromatogram of aronia recorded at
520 nm (representing anthocyanins) is shown in Figure 2.
Chromatogram recorded at 360 nm represents flavonols
and is shown in Figure 3. The concentrations and percent
distribution of individual anthocyanins and flavonols in
aronia are shown in Table 2.

Aronia contained a mixture of four different cyanidin-
glycosides: 3-galactoside, 3-arabinoside, 3-glucoside, and
3-xyloside of cyanidin. The most abundant were cyani-
din-3-galactoside and cyanidin-3-arabinoside (2795 mg
kg⁻¹, 994 mg kg⁻¹, respectively, >93 % of total anthocyanin
content) whereas the level of cyanidin-3-xyloside (146 mg
kg⁻¹, 3.6 %) and cyanidin-3-glucoside (122 mg kg⁻¹, 3%)
were relatively low. The data presented by other authors
are showing that the main anthocyanins in aronia are cy-
anidin-3-galactoside and cyanidin-3-arabinoside; the fol-
lowing are cyanidin-3-xyloside and cyanidin-3-glucoside
(Bermúdez-Soto et al. 2004; Määttä-Riihinen et al., 2004a;
Wu et al., 2004), which is consistent with our results.

<table>
<thead>
<tr>
<th>Berries</th>
<th>Total anthocyanins (mg kg⁻¹)</th>
<th>Total polyphenols (mg GAE kg⁻¹)</th>
<th>TA/TP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red raspberry</td>
<td>242.90±3</td>
<td>1256.16±133</td>
<td>0.19</td>
</tr>
<tr>
<td>Blackberry</td>
<td>1108.87±6</td>
<td>2484.87±234</td>
<td>0.45</td>
</tr>
<tr>
<td>Strawberry</td>
<td>232.16±10</td>
<td>1005.17±56</td>
<td>0.23</td>
</tr>
<tr>
<td>Aronia</td>
<td>4341.06±22</td>
<td>10637.20±571</td>
<td>0.41</td>
</tr>
</tbody>
</table>

* values are means ± SD (n=3)

Figure 1. Radical scavenging activities of berry extracts.
The berry extracts were incubated with DPPH for 15 minutes

Figure 2. HPLC Chromatogram of aronia recorded at 520 nm (represen-
ting anthocyanins). The HPLC chromatogram of aronia recorded at 520
nm (representing anthocyanins) is shown in Figure 2.

Figure 3. HPLC Chromatogram of aronia recorded at 360 nm (represen-
ting flavonols). The concentrations and percent distribution of indi-
vidual anthocyanins and flavonols in aronia are shown in Table 2.
Two flavonols were identified in aronia: quercetin and kaempferol. The main flavonol was quercetin (71.13 mg kg⁻¹; 93.07 %) while the concentration of kaempferol was low (5.3 mg kg⁻¹; 6.9 %) (Table 2). These results are in accordance with those reported by Määttä-Riihinen et al. (2004a). Häkkinen et al. (1999b) found only quercetin while kaempferol was not identified. According to Häkkinen et al., (1999a) aronia contains quercetin as the main flavonol and the relative content of myricetin and kaempferol was low.

Conclusion

Blackberry, red raspberry and strawberry have considerably lower amount of polyphenols and anthocyanins in comparison to aronia which is very rich in these phytochemicals. Aronia possesses the highest antioxidant activity against DPPH radical, as well. The main anthocyanins of aronia are derivatives of cyanidin among which cyanidin-3-galactoside and cyanidin-3-arabinoside were the most abundant. The main flavonol in aronia is quercetin. Although all berries studied can serve as a good source of bioactive phytochemicals in the human diet, aronia stands out in high polyphenol and anthocyanin content and high antioxidant activity. From the view of the phenolic content and antioxidant activity aronia can be regarded as good candidate for raw materials in production of health beneficial functional foods.

References


Table 2. Concentrations of anthocyanins and flavonols in aronia (mg kg⁻¹) determined by HPLC method and percentage distribution of anthocyanins and flavonols.

<table>
<thead>
<tr>
<th>Berry</th>
<th>Anthocyanins (mg kg⁻¹)</th>
<th>Percentage of total anthocyanins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aronia</td>
<td>cy-3-gal 2794.74±4.0</td>
<td>68.9</td>
</tr>
<tr>
<td></td>
<td>cy-3-glu 121.69±0.1</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>cy-3-ara 993.77±1.1</td>
<td>24.5</td>
</tr>
<tr>
<td></td>
<td>cy-3-xyl 146.02±0.3</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>Total 4056.22±5.5</td>
<td>100.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Berry</th>
<th>Flavonols (mg kg⁻¹)</th>
<th>Percentage of total flavonols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>71.13±1.5</td>
<td>93.07</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>5.30±0.5</td>
<td>6.93</td>
</tr>
<tr>
<td>Total</td>
<td>76.43±2.0</td>
<td>100.00</td>
</tr>
</tbody>
</table>

* values are means ± SD (n=3); cy, cyanidin; glu, glucoside; gal, galactoside; ara, arabinoside; xyl, xyloside.


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