Evaluation of Main Anthocyanins in Skin of Forest Pear (*Pyrus pyraster*) (Sange Khouj) Fruit in Guilan Province of Iran

Ahmad Dadashpour*, Hassan MohammadAmooie, Mohammad Jouki, Naimeh Khazaei

1 Young Researchers Club, Shahr-e-Qods Branch, Islamic Azad University, Tehran, Iran.

2 Shahr-e-Qods Branch, Islamic Azad University, Tehran, Iran.

Received: 12 January 2012 Accepted: 6 June 2012

*Corresponding author’s email: dadashpour@can.ut.ac.ir

The anthocyanins of the fruit skin of Iranian forest pear (Sange Khouj) were separated, identified and quantified using high-performance liquid chromatography / diode array detection (HPLC / DAD) and HPLC / electrospray ionization / mass spectrometry (HPLC / ESI / MS). The individual anthocyanins were identified by comparing their mass spectral data and retention times with those of standards and published data. Cyanidin-3-galactoside was the major compound, taking up 93% of the total anthocyanin content. The content of Cyanidin-3-galactoside reached 23.7 ± 3.2 mg 100 g⁻¹ fresh weight, followed by the eighth peak (2.14%) and Cyanidin-3-glucoside (1.23%). Pelargonidin-3-rutinoside was identified for the first time in ordinary pear (*P. communis* L). DPPH (2, 2-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing antioxidant power) assays showed that the extract from the fruit peel of ‘Sange Khouj’ contained anthocyanins and possessed high antioxidant capacity.

**Keywords:** Anthocyanins, Antioxidant activity, ‘Sange Khouj’ pear.
INTRODUCTION

Color is an important factor determining fruit external/visual quality, which has been used successfully in the characterization of fruits (Gautier-Hion et al., 1985). This characteristic has an important effect on overall acceptability to the consumer (Gamble et al., 2006). Anthocyanins are responsible for most of the red, blue and purple colors of fruits, vegetables, flowers and other plant tissues or products (Gould et al., 2002; Manetas, 2006; Steyn et al., 2002; Stintzing and Carle, 2004). The isolation and identification of anthocyanins are difficult as a result of their ability to undergo structural transformations and complex reactions. In addition, it is difficult to measure independently the anthocyanins from other flavonoids because they have similar reactionary characteristics. Paper and thin-layer chromatography have traditionally been used for the identification of anthocyanins (Aneersen, 1985; Matysik and Benesz, 1991; Sherma, 2000; Timberlake and Bridle, 1976) but analysis and identification of anthocyanins were developed by using HPLC with mass spectrometry or with tandem mass spectrometry (Bakker et al., 1986; Hong and Wrolstad, 1990). In recent years, HPLC coupled with MS have become the standard methods for identification and separation in most laboratories (Desai et al., 2010; Huang et al., 2009). HPLC can provide a faster and improved separation of complex compounds and allows their tentative identification based on their retention characteristics. However, it is still difficult to obtain each reference compound for the same spectrum that represents the anthocyanins. Therefore, HPLC coupled with MS, especially ESI/MS are possibly the most powerful methods for identifying the structure of anthocyanins. These methods allow the sequential fragmentation of a given molecular ion and provide information on the identification of anthocyanins based on their ion fragment patterns (Wilson et al., 2005). Several studies have been reported on the structural elucidation of anthocyanin by means of HPLC-DAD-MS. Using the HPLC-DAD-ESI-MS method, Lopes-da-Silva et al (Lopes-da-Silva et al., 2002) discovered that strawberry extract contained three major anthocyanins (Pg3-gluc, Pg3-rut and Cy3-gluc) and 12 minor anthocyanins, although identity could be only assigned to five of them as Pg-3-acetylglucoside, Cy-3-rutinoside, Pg-3-malylglucoside, Pgdiglucoside, and Cy3-malonylglucosy-l-5-glucoside which the three later had been discovered in strawberry for the first time. So this technique has been shown to be appropriate for isolation and identification of complex mixtures. Many studies focused on the composition of anthocyanin in apple (DuPont et al., 2002; Shoji et al., 2006), strawberry (Aaby et al., 2007; Gil et al., 1997; Wu et al., 2006), blueberry (Gu et al., 2002; Prior et al., 2001), grape (Garca-Beneytez et al., 2003; Huang et al., 2009; Oliveira et al., 2010), and other fruits (Wu et al., 2006). However, few studies have been conducted on pear phenolic compounds, especially anthocyanins. Pear is one of the most important temperate fruits. Fruit skin color is a significant factor that affects the quality and customer acceptance.

The objective of this study was to isolate and identify the anthocyanin molecules responsible for fruit skin coloration in Iranian forest pear (Sange Khouj).

MATERIALS AND METHODS

Plant Samples, Chemicals, Extraction of Anthocyanins, HPLC-MS Analysis

The sample fruits were picked at commercial maturity from forests of Guilan province by local named “Sange Khouj” in Roudbaneh region, Lahijan, Guilan, in the north of Iran during 2009-2010 and transported to laboratory of postharvest technology of University of Tehran immediately. Peel external color of fruits was red. Fruits free of defects and without evidence of mechanical damage were selected. Fruits were peeled and frozen in liquid nitrogen and kept at -70°C until analysis. HPLC-grade methanol and acetonitrile were obtained from Merck KGaA, Darmany, Germany. Formic acid, cyanidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-arabinoside, peonidin-3-glucoside, peonidin-3-galactoside, DPPH, and 2,4,6-tripyridyl-s-triazine (TPTZ) were obtained from Sigma Chemical co. All other chemicals used in this study were analytical grade.
The separation of anthocyanins was performed according to Wu (Wu et al., 2006), with some modification. 20 mL methanol with 1% (v/v) formic acid was added into 100 mL Erlenmeyer flask containing 1g peel powder. Anthocyanins were extracted at 4°C for 60 min in dark chamber; this procedure was repeated three times. The extract was concentrated under vacuum at 30°C using a rotary evaporator until dryness. The dried extract was resolved in 5 mL solvent of 1% formic acid in distilled water. About 1 mL of extracted solution was strained through a 0.45 μm millipore filter. The solvents for chromatography were (A) aqueous 2% formic acid, and (B) acetonitrile: water (1:1 v/v) containing 2% formic acid. The gradient was from 6% to 10% B for 4 min, from 10% to 25% B for 8 min. isocratic 25% B for 1 min. from 25% to 40% for 7 min. from 40% to 60% for 15 min. from 60% to 100% for 5 min. from 100% to 6% for 5 min, at a flow rate of 1.0 mL/min. Injection volume was 15 μL, and the detection wavelength was 516 nm. MS conditions were as follows: ESI interface, positive ion model, 35 psi nebulizer pressure, 10 L/min. dry gas flow rate, 350 °C dry gas temperature, and scans at m/z 150-1000. All analyses were replicated twice.

Antioxidant Capacity Determined by FRAP

This procedure involved the reduction of ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}) to a blue colored complex Fe^{2+}/TPTZ in the presence of bioactive compounds (antioxidants), which was revealed as the increase of absorption at 593nm (Benzie and Strain, 1999). Extracts were added to the FRAP reagent. The FRAP reagent contained 10 mM TPTZ in 40 mM HCl, 20 mM FeCl$_3$ and 300 mM acetate buffer (pH=3.6), that was freshly prepared in the ratio of 1:1:10 and warmed 30 min at 37.8°C before and after adding of sample extract. The FeSO$_4$•7H$_2$O compound was used to prepare the increasing concentrations of standard solutions. Results were expressed as ascorbic acid equivalent antioxidant capacity (mg ascorbic acid /100g fresh weight).

Antioxidant Capacity Determined by DPPH-radical Scavenging Activity (DPPH assay)

The ability of DPPH free radical scavenging was determined based on the method of Brand Williams (Brand-Williams et al., 1995), with minor modification. Briefly, reaction mixtures containing 25μL extracts and 2 ml 6.25×10^{-5}M DPPH solution, mixed, and then reacted in the dark for 30 min. A control sample containing the same volume of solvent in place of extract was used to measure the maximum DPPH absorbance. The absorbance at 517 nm was recorded to determine the concentration of remaining DPPH. Results were expressed as ascorbic acid equivalent antioxidant capacity (mg ascorbic acid /100g fresh weight).

RESULTS AND DISCUSSIONS

Anthocyanins Measurement

The red, purple, and blue colors found in many plants are due to two classes of water soluble pigments: anthocyanins and betacyanins. The anthocyanins are flavonoids, a class of phenolic molecules that are synthesized via the shikimic acid pathway which are widespread in the plant kingdom. Betalains, a group of pigments that includes the betacyanins are indole-derived alkaloids and contain nitrogen. Pear peel extracts (99 % methanol with 1 % HCl) were tested for the presence of anthocyanins by observing pigment color under acidic or alkaline conditions by adding HCl or NaOHsodium hydroxide. 3 ml of extract and 3 ml HCl were mixed in a test-tube and then placed in boiling water bath for 5 min. The mixture was stable and did not lose color when boiled. A blue-green color was observed when adding sodium hydroxide to the extract, which indicated the presence of anthocyanins in the extracts.

Identification of Pear Anthocyanins

Table 1 shows the anthocyanin profile of the extract from fruit skin of ‘Sange Khouj’ using the HPLC-DAD chromatograms at 516 nm. A total of eight anthocyanin compounds were identified...
by their elution order, UV / vis and by comparing the m / z of each anthocyanin molecule and its fragmentation to prepared standards and previous reports (Table 1). Five peaks (peak 1, 2, 3, 4 and 8) showed fragment ions at m / z 287 in MS2 analyses that could be tentatively identified as cyanidin derivatives. Peak 1 and 2 showed identical molecular ions at m / z 499 and fragmentation patterns, but their retention times in the HPLC system were 28 and 32 min. for peak 1 and 2, respectively. Their elution order and mss characteristics suggest that they should be cyaniding-3-galactoside and cyanidin-3-glucoside, respectively, which concurs with findings in previous study (Cerezo et al., 2010), and also coincides with the available standard used for confirmation purposes (Table 2). Peaks 3 and 8 showed the same major cyanidin fragment ions at m / z 287 but different molecular ions at m / z 416 and 549, respectively. Peak 3 produced a MS pattern corresponding to cyanidin-3-arabinoside, which coincides with the confirmation marker. Peak 8 might correspond to the methyl derivative of cyanidin-3-malonylglucoside. However, other structures that also match the molecular ion of peak 8 are cyanidin-malylrhamnoside and cyanidin-3-succinylglucoside (Da Silva et al., 2007). Unfortunately, no information could be obtained to confirm suggestions about its structure. Peak 4 produced a fragment pattern matching cyanidin-3-rutinoside. Peak 6 and peak 7 were assigned as peonidin-3-galactoside and peonidin-3-glucoside, both with the mass spectrometric characteristics and elution orders matching the reference compounds and those that had previously been reported in grape (Huang et al., 2009). Peak 5 showed an [M+H]+ at 579, which gave MS2 fragments that were at m / z 271 and 433 and also was identified as pelargonidin-3-rutinoside. Due to the lack of correspondent reference compounds for cyanidin-3-rutinoside, pelargonidin-3-rutinoside and the compound representing peak 8, the anthocyanins identified were quantitated via a calibration curve determined using cyanidin-3-glucoside. Other identified anthocyanins were quantitated via the calibration curves of corresponding reference compounds. Table 2 shows the individual concentration of the anthocyanins in the extracts of ‘Sange Khouj’ peel. cyanidin-3-galactoside, the major compound, accounts for 93.7% of total anthocyanin, which confirms what was found in previous study (Sanchez et al., 2003). It is followed by the compounds representing peak 8 (2.14%), cyanidin-3-glucoside(1.23%), pelargonidin-3-rutinoside (1.11%), peonidin-3-glucoside (0.87%) and peonidin-3-galactoside (0.75%). Percentages of 94.2% for cyanidin-3-galactoside and 4.5% for cyanidin-3-rutinoside, were found by Andrea et al. (2003) in Red D’Anjou, pelargonidin-3-rutinoside has also been previously reported in Concord grape (Baublis et al., 1994), cranberry (Heinonen, 2007), strawberry (da Silva et al., 2007), blackberry (Wrolstad, 1993), sweet cherry (Gao & Mazza, 1995) and black plum (Wu et al., 2006) but not in pear (Lin and Harnly, 2008; Sanchez et al., 2003). This study is tentatively proposing, for the first time, that pelargonidin-3-rutinoside is present in pear.

Antioxidant Capacity

The antioxidant capacity of the total anthocyanins obtained from fruit peel of ‘Khouj’ was evaluated with the DPPH and FRAP test. The free radical scavenging activity determined by DPPH was 165.23 ± 11.6 mg / 100 fresh weight and the value of total antioxidant capacity determined by FRAP was 87.88 ± 9.8 mg / 100 fresh weight (ascorbic acid equivalent antioxidant capacity). The extract possessed higher antioxidant capacity than that founds in previous studies. This difference might be due to the differing maturation rate of individual fruit and different methods of extraction and analysis. HPLC coupled with mass spectrometry has been become the standard and most powerful methods used for anthocyanins analysis. However, mass spectra alone are not 100% effective because MS cannot provide complete structural information for different anthocyanins with the same mass spectra. Therefore, it is necessary to combine it with sources of other useful information that can be obtained in order to identify peaks. Retention time is very important for the determination of anthocyanins even with MS data. A simple elution order for some common anthocyanidin glycosides using reverse-phase HPLC that seems to fit most experimental conditions are delphini-
din, cyanidin, petunidin, pelargonidin, peonidin, and malvidin. For different glycoside and/or acylated groups with the same anthocyanidin (cyanidin), it is cyanidin 3,5-diglucoside, cyanidin 3-diglucose, cyanidin 3-galactosid, cyanidin 3-sambubioside, cyanidin 3-glucoside, cyanidin 3-arabinoside, cyanidin 3-rutinsode and cyanidin 3-(maloyl) glucoside (Wu and Ronald, 2005).

By comparing their elution orders and mass spectrometric characteristics with reported data in the literature and the values of a standard substance, seven anthocyanidins were tentatively identified in the ‘Khouj’ pear samples. Most anthocyanins showed cyanidin aglycon, although peonidin and pelargonidin derivatives were also present. Cyanidin-3-galactoside was the major anthocyanin pigment in red skin of pear fruit, accounting for 93.7% of total anthocyanin. This agreed with the results of Muchuweti et al., (Muchuweti and Chikwambi, 2008). Anthocyanin compositions vary greatly with genotype. For example, pelargonidin 3-glucoside was the major anthocyanin in strawberry (Fragaria x ananassa) (Cerezo et al., 2010). Delphinidin, petunidin and malvidin were the major contributors to total anthocyanin content but the proportions of each compound were cultivar-dependent. Highbush has more polar anthocyanins than rabbiteye cultivars (Lohachoompol et al., 2008). The anthocyanin differences in fruit peels constitute a biochemical marker, so the gathered information in this study helps towards characterizing pear cultivars and their mutants. This will assist in breeding good sensory quality attributes and defense against ultraviolet light or aggression by pathogens (Hamauzu et al., 2005).

ACKNOWLEDGMENT

We would like to gratefully thank the members of section of postharvest technology of University of Tehran for their facilities and suggestions.

Literature Cited


Wrolstad, R. E. 1993. Color and pigment analyses in fruit products. Agricultural Experiment Station Oregon State University.


### Tables

**Table 1.** HPLC/DAD and HPLC/ESI-MS of anthocyanins in fruit skin of ‘Sange Khouj’

<table>
<thead>
<tr>
<th>Peak number</th>
<th>tR(min)</th>
<th>[M+] m/z</th>
<th>MS2 m/z</th>
<th>compounds</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>28.82</td>
<td>449</td>
<td>287</td>
<td>Cyanidin-3-galactoside</td>
</tr>
<tr>
<td>2</td>
<td>32.42</td>
<td>449</td>
<td>287</td>
<td>Cyanidin-3-glucoside</td>
</tr>
<tr>
<td>3</td>
<td>34.50</td>
<td>419</td>
<td>287</td>
<td>Cyanidin-3-arabinoside</td>
</tr>
<tr>
<td>4</td>
<td>35.23</td>
<td>595</td>
<td>449/287</td>
<td>Cyanidin-3-rutinoside</td>
</tr>
<tr>
<td>5</td>
<td>37.64</td>
<td>579</td>
<td>433/271</td>
<td>Pelargonidin-3-rutinoside</td>
</tr>
<tr>
<td>6</td>
<td>38.51</td>
<td>463</td>
<td>301</td>
<td>Peonidin-3-galactoside</td>
</tr>
<tr>
<td>7</td>
<td>40.17</td>
<td>463</td>
<td>301</td>
<td>Peonidin-3-glucoside</td>
</tr>
<tr>
<td>8</td>
<td>44.52</td>
<td>549</td>
<td>287</td>
<td>Cyanidin-3-methylmalonylglucoside?</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Compounds</th>
<th>content*(mg/100g fresh weight)</th>
<th>%b</th>
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<tbody>
<tr>
<td>Cyanidin-3-galactoside</td>
<td>23.7±3.2</td>
<td>93.71</td>
</tr>
<tr>
<td>Cyanidin-3-glucoside</td>
<td>0.31±0.05</td>
<td>1.23</td>
</tr>
<tr>
<td>Cyanidin-3-arabinoside</td>
<td>0.03±0.00</td>
<td>0.12</td>
</tr>
<tr>
<td>Cyanidin-3-rutinoside</td>
<td>0.02±0.00</td>
<td>0.08</td>
</tr>
<tr>
<td>Pelargonidin-3-rutinoside</td>
<td>0.28±0.02</td>
<td>1.11</td>
</tr>
<tr>
<td>Peonidin-3-galactoside</td>
<td>0.19±0.03</td>
<td>0.75</td>
</tr>
<tr>
<td>Peonidin-3-glucoside</td>
<td>0.22±0.02</td>
<td>0.87</td>
</tr>
<tr>
<td>Peak 8</td>
<td>0.54±0.03</td>
<td>2.14</td>
</tr>
</tbody>
</table>

*a* individual anthocyanins were analyzed using HPLC-ESI-MS. Each anthocyanin was quantified in cyanidin equivalents (mg/100g fresh weight).

*b* Percentage of the total content.