Note

Determination of polyphenols and antioxidant activity of *Vitis labrusca* cv. baile berries

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Received 20 March 2014; revised 21 February 2015

Grape juice and grape skin extracts are important commercial source of polyphenolic compounds which exert different functional properties such as color potential, antimicrobial, antioxidant activity, and health benefits. In this paper we describe a sensitive and specific assay for determination of bioactive polyphenolic compounds in Campbell Early (*Vitis labrusca* cv. baile). Five polyphenolic components were separated on an Agilent Zorbax Extend C\textsubscript{18} Column (250 mm × 4.6 mm × 5 μm) and detected by a diode array detector. The mobile phase was composed of (a) aqueous phosphoric acid (0.2%, v/v); and (b) acetonitrile using a gradient elution. Analytes were performed at 25°C with a flow rate of 0.8 ml/min and UV detection at 280, 360, and 520 nm. All calibration curves showed good linear regression (\(r^2 \geq 0.9999\)) within tested ranges. Overall intra- and inter-day variations were less than 1.90%, and the average recoveries were 95.5-105% for analytes. The antioxidant activity determined by DPPH radical assay, ranged from 86-105 for extracts, and 165-252 for studied standards (µM trolox/100 g dry wt.). The proposed method would be sensitive enough and reliable for quality control in functional food and modernization of Campbell Early (*Vitis labrusca* cv. baile) as potent antioxidant agent.

Keywords: Anthocyanins, Campbell Early, DPPH radical assay, Grapevine, Phenolics, Wine.

Grapes, one of the most consumed fruits, whether processed or in their natural form, stand out as a source of phenolic compounds vital for human health\(^1,2\). Although, the bioavailability and bioconversion of phenolic compounds have not yet been fully elucidated, literature suggests that these compounds, particularly polyphenolics, can act as powerful antioxidants scavenging free radicals in cells\(^3\). These polyphenolic compounds also participate in the regeneration of other antioxidants, such as vitamin E and C, which protect cellular constituents against oxidative damage. In addition, they act as chelators of metal ions capable of catalyzing lipid peroxidation, along with diverse biological activities (anti-estrogens, anti-cardiovascular diseases, anti-inflammatory, antilucer, anticancer, anti-mutagenic, and antimicrobial)\(^4,5\). Grapes contains a large amount of different phenolic compounds in skins, pulp and seeds, that are partially extracted during wine making. More than 15 phenolic compounds with antioxidant properties (flavan-3-ols, anthocyanins, cinnamic acid derivatives, flavonol derivatives and trans-resveratrol) have been shown to be separated in a single run by direct injection of red wine\(^6,7\). Anthocyanins constitute a large family of polyphenols in plants and are responsible for many of the fruit and floral colors observed in nature. These are pigments dissolved in the vacuolar sap of the epidermal tissues of flowers and fruits which impart mostly pink, red, blue, or purple colour\(^8\). Anthocyanins exhibit various health benefits, including protection from DNA cleavage, anticancer, antidiabetic, anti-inflammatory, and antioxidant activity; with prevention of cardiovascular and neurodegenerative diseases\(^9-12\).

In this study, we selected a high quality grape variety Campbell's Early (*Vitis labrusca* cv. baile). The wines are moderately vigorous and moderately susceptible to Powdery Mildew. It is important to determine the colour pigments in this variety\(^13\). Hence, we explored this grapevine variety adopting an innovative HPLC-DAD method for simultaneous determination of the anthocyanins and their antioxidant activity.

Materials and Methods

Standards and reagents—Gallic acid, malvidin, delphinidin, cyanidin, and peonidin were purchased from Sigma Chemical Co. (St. Louis, MO) and DPPH was obtained from Fluka Chemicals AG (Buchs, Switzerland). The purity of each standard compound was greater than 98% as determined by the HPLC analysis. All reagents with high grade were obtained from (Dae-Jung Chemical, Seoul, Korea). Milli-Q water (Millipore, Bedford, MA) was used throughout the study.

Plant collection and preparation—Campbell Early (*Vitis labrusca* cv. baile) was obtained from Suwon, Gyeonggi, Korea. The fruits were harvested in...
summer, stored for about 5 months at −70°C, homogenized with food processor, and lyophilized to concentrate each sample in powdered form. The powdered samples (1.0 g each) were transferred into dark brown calibrated flasks and extracted with 20 ml of 80% methanol in an ultrasonic bath for 45 min, cooled at 25 °C; and 80% of methanol was added to compensate for the lost weight. The solution was filtered through a 0.45 μm membrane filter, and 10 μl of the sample was subjected to HPLC analysis.

Preparation of standard solutions and calibration curve—Each reference standard was accurately weighed, dissolved in 80% methanol and diluted to appropriate concentration. Finally, mixed stock solution of standards (100 μg/ml, each), containing gallic acid, malvidin, delphinidin, cyanidin, and peonidin. The standard stock and working solutions were all prepared in calibrated flasks and stored at 4°C. All calibration curves were constructed from peak areas of the reference standards vs. their concentrations. The solutions were filtered through a 0.45 μm membrane prior to injection.

HPLC analysis—The HPLC/DAD analysis were performed on a Shimadzu HPLC equipped with a diode array detector (SPD-M10A, Shimadzu, Japan) at 520 nm for quantification. The column used was a C18 HPLC column (Zorbax 300SB-C18, Agilent Technologies, Rising Sun, Md., USA). A temperature programmable column oven (Younglin Instrument, Seoul, Korea) was used to maintain the column temperature at 40°C during the HPLC analysis. Detection wavelengths were set at 280, 360, and 520 nm. An Agilent Zorbax Extend C18 column (250×4.6×5 μm) was used with a flow rate of 0.8 ml/min. The injection volume was 10 μL, and the column temperature was maintained at 25°C. The mobile phase was composed of (a) aqueous phosphoric acid (0.2%, v/v); and (b) acetonitrile using a gradient elution of 0-50 min (5-20%).

Determination of total anthocyanins—Total anthocyanin contents of samples were determined using previously described method by Rigo et al. 2000. Briefly, Campbell Early extracts were appropriately diluted with ethanol: water: hydrochloric acid 0.12 M (70:29:1 v/v/v) and the absorbance was measured by using a UV–vis spectrophotometer (Lambda 25, PerkinElmer, Korea) at the wavelength of 540 nm. Malvidin was employed as a calibration standard and results were expressed as malvidin equivalents (ME) (mg ME/100 g of fruit DM).

DPPH free radical scavenging activity—This activity was measured using Campbell Early (Vitis labrusca cv. baile) skin and pulp extracts. This method was based on the mechanism in which the odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color, upon reduction by a chemical antioxidant. Briefly, 100 μM solution of DPPH was prepared in methanol and 2.7 mL of this solution was added to 0.3 mL of Campbell Early extract and gallic acid, malvidin, delphinidin, cyanidin, and peonidin solution in methanol at the same concentration (0.2 mg/mL). After 10 min, the absorbance was measured at 517 nm. The percentage of remaining DPPH was calculated as follows:

DPPH scavenging effect (%) =

\[
\frac{[A_{\text{Control}} - A_{\text{Sample}}]}{A_{\text{Control}}} \times 100
\]

where \( A_{\text{Control}} \) is the absorbance of the DPPH reaction and \( A_{\text{Sample}} \) is the absorbance in the presence of grape extracts.

Results and Discussion

Calibration curves—The linearity calibration curves were constructed by five concentration assays of each reference compound in triplicate. An aliquot (10 μl) of each standard solution was subjected to HPLC analysis. The regression equations were calculated in the form of \( Y = ax + b \), where \( Y \) and \( X \) were the concentration of each reference compound and values of the peak area, respectively. The regression equations (linear ranges) were \( y = 0.1845x + 1.4252 \) (6.52-85.40 μg/mL, gallic acid), \( y = 0.1.264x + 0.9155 \) (5.26-65.36 μg/mL, malvidin), \( y = 0.864x + 1.2.35 \) (4.5-42.31 μg/mL, delphinidin), \( y = 1.2524x + 1.8542 \) (5.8-76.24 μg/mL, cyanidin), and \( y = 0.0856x + 1.3210 \) (4.1-38.6 μg/mL, peonidin). All the marker substances showed good linearity (\( r^2 \geq 0.987 \)). The limit of detection (LOD) and the limit of quantification (LOQ) of the five analytes were 0.035-0.085 and 0.092-0.325 μg/mL, respectively.

Precision, repeatability, stability and accuracy—The intra and inter-day precision were determined by analyzing calibration samples during a single day and on three different days, respectively. The intra-day variation was determined by analyzing the five replicates on the same day, and the inter-day variation was determined on three consecutive days. The
relative standard deviation (RSD) was taken as a measure of precision, and the overall intra- and inter-day variations were less than 1.90%. To further evaluate the repeatability of the developed assay, Campbell Early was analyzed in five replicates as described above. The contents of five compounds in Campbell Early extracts were calculated from the corresponding calibration curves. The RSDs were taken as measurements of repeatability. The stability was tested with Campbell Early extracts at 25 °C and analyzed every 0, 2, 4, 8, 12, 24, and 48 h. The RSDs of the repeatability test and stability were not more than 2.50% for all analytes. Accuracy was determined by the recovery test. An appropriate amount of Campbell Early powder was weighed and spiked with the known amount of each standard compound. They were then treated and analyzed as described above. The total amount of each analyte was calculated from the corresponding calibration curve.

\[
\text{Recovery (\%)} = \frac{\text{Amount \ determined} - \text{Amount \ original}}{\text{Amount \ spiked}} \times 100
\]

where, amount determined was the determined total of each analyte, amount original was the original amount of each analyte in Campbell Early samples measured, and amount spiked was the spiked amount of each analyte. For comparison, an unspiked sample was prepared and analyzed simultaneously. Mean recoveries of the compounds were 95.5-105% for analytes %, with RSD values ranging from 3.25 to 3.75% (n = 5).

**Sample analysis**—Figure 1 shows the typical separation of a standard mixture and Campbell Early extracts obtained under the above-mentioned HPLC conditions. The contents were calculated and summarized in Table 1.

### Sample analysis

**HPLC Separation**—HPLC separation of known anthocyanins including gallic acid, malvidin, delphinidin, cyanidin, and peonidin was achieved within 30.5 min, on the basis of their HPLC retention times (peak 1-5). The contents of individual anthocyanidins in Campbell Early grape samples are presented in (Fig. 1).

**Antioxidant activity**—The DPPH radical scavenging assay demonstrated the effective antioxidant activity of the skin and pulp extract compared to the marker compounds, in alignment with our previous studies. The isolated phenolic acid from grapes i.e., gallic acid and cyanidin, showed high antioxidant activity compared to other anthocyanins and grape skin and pulp extract (Table 2). The results have given the anthocyanin composition of the technologically important wine grape variety, Campbell Early. The vinification of grapes with high anthocyanin potential has been regarded as a principal criterion for high quality grape identification which was enriched with biologically active phenolic compounds. Antioxidant activity of fruits can be traced to the polyphenolics present in plant extracts. Researchers have already established the correlation among antioxidant capacity, total phenols and anthocyanins; the latter with less significance. The present study, with results similar to the earlier studies, apart from providing a valuable data of total anthocyanin content and antioxidant activity of Campbell Early further,

### Table 1—Contents of five active compounds in Campbell Early extract (mg/g)

<table>
<thead>
<tr>
<th>Components</th>
<th>Conc. (mg/g)</th>
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<tbody>
<tr>
<td>Gallic acid</td>
<td>0.20</td>
</tr>
<tr>
<td>Malvidin</td>
<td>0.16</td>
</tr>
<tr>
<td>Delphinidin</td>
<td>0.09</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>0.12</td>
</tr>
<tr>
<td>Peonidin</td>
<td>0.08</td>
</tr>
</tbody>
</table>

### Table 2—Antioxidant activity of Campbell Early extract and marker compounds expressed as TEAC

<table>
<thead>
<tr>
<th>Samples</th>
<th>TEAC (µM trolox/100 g dry wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>105.0 ± 1.4</td>
</tr>
<tr>
<td>Pulp</td>
<td>86.5 ± 2.0</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>252.0 ± 1.9</td>
</tr>
<tr>
<td>Malvidin</td>
<td>185.5± 2.2</td>
</tr>
<tr>
<td>Delphinidin</td>
<td>172.4 ± 2.3</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>194.5 ± 1.8</td>
</tr>
<tr>
<td>Peonidin</td>
<td>165.0 ± 2.6</td>
</tr>
</tbody>
</table>

*Values are expressed as means of three determinations ±standard deviation
Correlation of total anthocyanin content and antioxidant activity (%) values demonstrated the significant correlation between the antioxidant activity and total anthocyanins (Fig. 2).

As the grapevines are moderately vigorous and moderately susceptible to Powdery Mildew, determination of the color pigments in their varieties is important. The HPLC-DAD method, suggested here, is simple and accurate. It excels in simultaneous determination of the five anthocyanins components (gallic acid, malvidin, delphinidin, cyanidin, and peonidin), with their antioxidant activity. Further, the characterization and quantification of anthocyanins in grape cultivars viz., Oll-Meou (Vitis coignetiae × Vitis labrusca), Neut-Meou (Vitis coignetiae × Vitis labrusca), Muscal Bailey A. (Vitis labrusca), and Campbell Early (Vitis labrusca × V. vinifera) cultivated in Korea were carried out by partial purification through XAD-7 column chromatography followed by C-18 HPLC/diode array detector (DAD), HPLC/MS, and HPLC/MS/MS analyses. Thus, the many researchers tried to develop a simple method for the determination of different phenolic compounds in grapes and wines, using a binary gradient and photodiode array detection but the results obtained with chromatographic procedure should not be a real picture of the actual state of anthocyanins in grapes and wines, So there is need to be complemented with different advanced analysis techniques like fluorimetric, colorimetric or mass spectra to give an accurate description of the different types of anthocyanins present in grapes and wines.

Conclusion
An innovative simple and accurate HPLC-DAD method has been developed for simultaneous determination of five phenolic components in Campbell Early, a high quality grape variety, widely cultivated in many countries. It can be used as a valid analytical method for the intrinsic quality control of Campbell Early. The results have demonstrated higher total polyphenolic content and antioxidant capacity of Campbell Early. The anthocyanins and other bioactive compounds shown to be present in the skin and pulp may serve as a new potential source nutraceuticals and functional foods.

Acknowledgment
This research work was supported by Konkuk University (KU) Research Professor Program-2015, Konkuk University, Seoul, South Korea.

References
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