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Analytical Methods

Characterisation of anthocyanins in the black soybean (*Glycine max* L.) by HPLC-DAD-ESI/MS analysis

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ABSTRACT

The aim of this study was to isolate and identify the anthocyanins in the black seed coated soybean (cv. Cheongja 3, *Glycine max* (L.) Merr.) using reverse phase C-18 open column chromatography and high-performance liquid chromatography (HPLC) with diode array detection and electro spray ionization/mass spectrometry (DAD-ESI/MS) analysis, respectively. Anthocyanins were extracted from the coat of black soybeans with 1% TFA in methanol, isolated by RP-C-18 column chromatography, and their structures elucidated by 1D and 2D NMR spectroscopy. The isolated anthocyanins were characterised as delphinidin-3-*O*-glucoside (**3**), cyanidin-3-*O*-glucoside (**5**), petunidin-3-*O*-glucoside (**6**), pelargonidin-3-*O*-glucoside (**7**) and cyanidin (**9**). Furthermore, four minor anthocyanins were detected and identified as catechin-cyanidin-3-*O*-glucoside (**1**), delphinidin-3-*O*-galactoside (**2**), cyanidin-3-*O*-galactoside (**4**), and peonidin-3-*O*-glucoside (**8**) based on the fragmentation patterns of HPLC-DAD-ESI/MS analysis.

1. Introduction

Anthocyanins, which belong to the flavonoid phenolic group of compounds, are natural pigments which are widely distributed in plants that are consumed in the human diet such as crops, beans, vegetables and fruits (Markakis, 1982). Every day we ingest large amounts of anthocyanins from plants. The average intake of anthocyanins by USA citizens has been estimated at up to 180–215 mg/day which is higher than that of other flavonoids such as flavonols (Clifford, 2000). In particular these anthocyanins are associated with a wide range of biological activities including antioxidant (Tsuda, Horio, & Osawa, 2003; Wang, Cao, & Prior, 1997), anti-inflammatory (Wang & Mazza, 2002; Youdim, McDonald, Kalt, & Joseph, 2002), anticancer (Bomser, Madhavi, Singletetary, & Smith, 1996; Hou, 2003) and α-glucosidase inhibition (Matsui et al., 2001). In addition, these pigments may reduce the risk of coronary heart disease through modulation of arterial protection (Colantuoni, Bertuglia, Magistetti, & Donato, 1991), inhibition of platelet aggregation (Morazzoni & Magistretti, 1990) or endothelial protection (Youdim et al., 2002). For this reason, the food and medicinal industries are increasingly interested in fruits and vegetables with a high content of bioactive anthocyanins for the manufacture of supplements with preventative and therapeutic uses.

Black soybeans (*Glycine max* (L.) Merr.) has been widely used in nutritionally rich foods and in folk medicine for hundreds of years. The black soybean has been used in traditional Chinese medicine in particular, for detoxification, and anti-inflammatory processes, and to improve the blood (Liao, Chen, & Yang, 2005). Black soybeans are found in foods such as soymilk, tofu, soy sauce, soy sprout and are cooked with rice in Korea. In addition, the sales of black soybean-based foods have grown tremendously during the past three years because of increasing consumer awareness of black soybean as a healthy food ingredient. Numerous studies have revealed that the beneficial health effects of black soybean are due to the several phytochemicals, such as isoflavones, saponin, and anthocyanins (Kim et al., 2006; Lee, Seo, Kang, Yang, & Park, 2006; Lee et al., 2006; Messina, 2000; Rao & Sung, 1995; Tham, Gardner, & Haskell, 1998). Previous studies have emphasized the role of isoflavones and the anthocyanins were generally overlooked, especially their quantification and biological activity. It should be noted, however, that the anthocyanins isolated from black seed coated soybean can be expected to possess various biological activities. Until now only the three main anthocyanins, delphinidin-3-*O*-glucoside (**3**), cyanidin-3-*O*-glucoside (**5**) and petunidin-3-*O*-glucoside (**6**) have been isolated and identified from the black soybean seed coats. The major anthocyanin in the black soybean is cyanidin-3-*O*-glucoside (**5**), as first identified by Kuroda and Wada (1933). Subsequently, delphinidin-3-*O*-glucoside (**3**) and petunidin-3-*O*-glucoside (**6**) were also identified from the black soybean variety (Choung et al., 2001; Taylor, 1976; Yoshikura & Hamaguchi, 1969). The investigation of these three major anthocyanins may not be sufficient to produce an anthocyanin profile of the black soybean from a practical point of view.

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To our knowledge there have been only a few reports in the literature regarding the constituents and compositions of the anthocyanins in black soybean however the minor pigments have still not been fully characterized. This prompted us to identify the minor anthocyanins from the black seed coated soybean. In particular, Cheongja 3 which is a variety of soybean with a black seed coat, has been used for the separation and identification of black soybean anthocyanins using reversed-phase C-18 column chromatography and high-performance liquid chromatography with diode array detection and electro spray ionization/mass (HPLC-DAD-ESI/MS) spectrometry analysis.

2. Materials and methods

2.1. Plant material

Black seed coated soybean, Cheongja 3 developed by the National Institute of Crop Science (NICS), was selected for this study. It was grown in the experimental field of the Yeongnam Agricultural Research Institute, NICS, RDA at Milyang and Muju, during 2005 and 2006.

2.2. Chemicals

Delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, and petunidin-3-*O*-glucoside used for the standards were provided by Choung et al. (2001). Analytical grade methanol, ethyl acetate, acetonitrile, and water were purchased from J.T. Baker (Phillipsburg, NJ, USA). Amberlite XAD-7, acetic acid, formic acid, and trifluoroacetic acid (TFA), CD_3OD were obtained from Sigma Chemical Co., (St. Louis, MO, USA). Silica gel 60 Rp-18 (40–63 μm) and TLC aluminium sheets RP-18 F_{254} were obtained from Merck (Darmstadt, Germany). Sep-Pak cartridges were from Waters Co., (Milford, MA, USA).

2.3. Instruments

UV–Vis absorption spectra were recorded on an Infinite M200 spectrophotometer (Tecan Austria GmbH, Untersbergstrasse 1A, Austria). ^1H and ^{13}C NMR along with 2D NMR data were obtained on a Bruker AM 500 (^1H NMR at 500 MHz, ^{13}C NMR at 125 MHz) spectrometer (Bruker, German) in CFCOOD-CD_3OD (1/19, v/v) (Sigma-Aldrich Co.,). HPLC was performed using an Agilent 1100 series (Boeblingen, Germany) quaternary pump, Agilent 1100 series diode array detector, and LichroCART 125-4 HPLC-Cartridge (Lichrophore 100 RP-18e; Merck KGaA, Darmstadt, Germany) column. ESI/MS (electro spray ionization/mass spectroscopy) data were obtained using an Esquire 4000 (Bruker Daltonick GmbH, Bremen, Germany).

2.4. Extraction and isolation of anthocyanins

Hand-peeled seed coats (200 g) from Cheongja 3, a breeding line of black soybean, were extracted twice with 1000 mL of 1% TFA (v/v) in methanol for 2 days at 4 °C, in darkness. The methanol extracts were concentrated in a rotary evaporator (35 °C) to obtain the crude extract. The concentrated crude extract was purified by partition (three times) against ethyl acetate, and further purified by Amberlite XAD-7 chromatography. The anthocyanins absorbed to the column were washed with water, and eluted from the column with methanol containing 1% TFA. The concentrated anthocyanin extract was purified by silica gel 60 Rp-18 (20 × 5 cm; 40–63 μm) column chromatography using gradient elution (MeOH/H_2O/TFA; 5:94:1 (v/v) to MeOH/H_2O/TFA; 50:49:1 (v/v)) to afford nine fractions (F1F9). The F1 (120 mg) was applied to a silica gel 60 Rp-18 (3 × 15 cm; 40–63 μm) chromatography column and eluted with MeOH/H_2O/TFA (5:94:1 → 50:49:1) and purified on a C-18 solid phase cartridge to yield delphinidin-3-*O*-glucoside (**3**) and cyanidin-3-*O*-glucoside (**5**). The F3 fraction (89 mg) was chromatographed on a silica gel Rp-18 column and eluted using a gradient of MeOH/H_2O/TFA starting with a ratio of 5:94:1 and then decreasing the polarity to a final ratio of 96:4:1 volume percentage to yield petunidin-3-*O*-glucoside (**6**). Fraction F6 (91 mg) was chromatographed on a silica gel Rp-18 column and using eluted with a gradient of MeOH/H_2O/TFA starting at 10:89:1 and then decreasing the polarity to a final ratio of 96:4:1 volume percentage to yield pelargonidin-3-*O*-glucoside (**7**). Fraction F7 (42 mg) was chromatographed using the same conditions as described for F1, F3 and F6 except that the starting ratio of MeOH/H_2 O/TFA was 15:85:1 and the polarity reduced to give a final ratio of 95:5:1 volume percentage to yield cyanidin (**9**).

2.5. HPLC DAD-ESI/MS analysis

The anthocyanins present in the seed coat of black soybean were characterised by HPLC diode array detection (DAD) mass spectrometry (MS) analysis. A sample (20 μL) of the crude acidic methanolic extract was injected onto an analytical reverse phase C-18 column (125 mm × 4 mm, LichroCART, 5 μm, Merck KGaA). The mobile phase was composed of 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B). The gradient conditions were as follows: 010 min, 15% B; 20 min, 25% B; 30 min, 40% B and then held for 10 min before returning to the initial conditions. Other HPLC conditions were as follow: a flow rate of 1 mL/min; column temperature, 25 °C; detection, 530 nm; and sample size, 20 μL. The mass spectrometer used was a Bruker Daltonick GmbH (Bremen, Germany) equipped with an electro spray ionization (ESI) source and an ion trap mass analyzer, which were controlled by the esquire 4000 control software. The mass parameters were as follows: capillary voltage, 84.9 V; fragmentation voltages, 17.4 V; drying gas temperature, 365 °C; gas flow (N_2), 9 L/min; nebulizer pressure, 60 psig. The instrument was operated in the positive ion mode scanning from m/z 100 to 1000 at a scan rate of 1.5 s/cycle. The MS revealed the positive molecular ions, and MS^2 and the subsequent MS^3 were used to break down the most abundant species by collision-induced dissociation.

3. Results and discussion

3.1. Isolated anthocyanins from black soybean

The seed coat of black soybean (cv. Cheongja 3) was extracted with acidic MeOH (1% TFA) followed by Amberlite XAD-7 column chromatography. The anthocyanins were separated by open silica gel Rp-18 column chromatography. Five anthocyanins, delphinidin-3-*O*-glucoside (**3**), cyanidin-3-*O*-glucoside (**5**), petunidin-3-*O*-glucoside (**6**), pelargonidin-3-*O*-glucoside (**7**), and cyanidin (**9**) were isolated and their chemical structures were elucidated by 1D and 2D NMR.

3.1.1. Delphinidin-3-O-glucoside (3)

Amorphous red powder; EIMS m/z 465; ^1H NMR (500 MHz, CFCOOD-CD_3OD): δ 3.52 (1H, dd, J = 9.2, 9.2 Hz, H-4 glc), 3.62 (2H, m, H-3, 5 glc), 3.75 (1H, dd, J = 8.8, 8.0 Hz, H-2 glc), 3.79 (1H, dd, J = 12.1, 5.7 Hz, H-6a glc), 3.97 (1H, dd, J = 12.1, 1.7 Hz, H-6 glc), 5.33 (1H, d, J = 7.6 Hz, H-1 glc), 6.63 (1H, s, H-6), 6.81 (1H, s, H-8), 7.69 (2H, d, J = 2.2 Hz, H-2′, 6′), 8.89 (1H, s, H-4). ^{13}C NMR (125 MHz, CFCOOD-CD_3OD): δ 163.8 (C-2), 146.1 (C-3), 136.2 (C-4), 159.6 (C-5), 103.8 (C-6), 170.7 (C-7), 95.5 (C-8), 157.8 (C-9), 113.6 (C-10), 120.3 (C-1′), 112.9 (C-2′), 147.7 (C-3′),

145.1 (C-4′), 147.8 (C-5′), 112.9 (C-6′), 103.9 (C-1 glc), 75.2 (C-2 glc), 78.5 (C-3 glc), 71.5 (C-4 glc), 79.2 (C-5 glc), 62.7 (C-6 glc).

*3.1.2. Cyanidin-3-O-glucoside (**5**)*

Amorphous red powder; EIMS *m*/*z* 449; ^{1}H NMR (500 MHz, CFCOOD-CD_3OD): δ 3.48 (1H, dd, *J* = 9.3, 9.3 Hz, H-4 glc), 3.59(2H, m, H-3, 5 glc), 3.70 (1H, dd, *J* = 8.7, 8.0 Hz, H-2 glc), 3.75 (1H, dd, *J* = 12.1, 5.9 Hz, H-6a glc), 3.95 (1H, dd, *J* = 12.1, 2.0 Hz, H-6b glc), 5.31 (1H, d, *J* = 7.7 Hz, H-1 glc), 6.66 (1H, s, H-6), 6.88 (1H, s, H-8), 6.99 (1H, d, *J* = 8.7 Hz, H-5′), 8.01 (1H, d, *J* = 2.1 Hz, H-2′), 8.23 (1H, dd, *J* = 8.7, 2.1 Hz, H-6′), 8.99 (1H, s, H-4). ^{13}C NMR (125 MHz, CFCOOD-CD_3OD): δ 164.5 (C-2), 146.0 (C-3), 137.2 (C-4), 159.6 (C-5), 103.8 (C-6), 170.8 (C-7), 95.5 (C-8), 158.0 (C-9), 113.7 (C-10), 121.6 (C-1′), 118.8 (C-2′), 147.8 (C-3′), 156.2 (C-4′), 117.8 (C-5′), 128.7 (C-6′), 104.2 (C-1 glc), 75.2 (C-2 glc), 78.6 (C-3 glc), 71.5 (C-4 glc), 79.2 (C-5 glc), 62.8 (C-6 glc).

*3.1.3. Petunidin-3-O-glucoside (**6**)*

Amorphous red powder; EIMS *m*/*z* 479; ^{1}H NMR (500 MHz, CFCOOD-CD_3OD): δ 3.43 (1H, dd, *J* = 9.3, 9.3 Hz, H-4 glc), 3.58(2H, m, H-3, 5 glc), 3.69 (1H, dd, *J* = 8.7, 8.0 Hz, H-2 glc), 3.75 (1H, dd, *J* = 12.1, 5.9 Hz, H-6a glc), 3.93 (1H, dd, *J* = 12.1, 2.2 Hz, H-6b glc), 4.0 (3H, s, OCH_3), 5.31 (1H, d, *J* = 7.8 Hz, H-1 glc), 6.67 (1H, s, H-6), 6.96 (1H, s, H-8), 7.71 (1H, d, *J* = 2.2 Hz, H-6′), 7.86 (1H, d, *J* = 2.2 Hz, H-2′), 8.94 (1H, s, H-4). ^{13}C NMR (125 MHz, CFCOOD-CD_3OD): δ 162.6 (C-2), 145.1 (C-3), 135.3 (C-4), 158.8 (C-5), 103.3 (C-6), 170.3 (C-7), 95.4 (C-8), 157.2 (C-9), 113.1 (C-10), 119.5 (C-1′), 109.2 (C-2′), 149.5 (C-3′), 145.6 (C-4′), 147.2 (C-5′), 113.4 (C-6′), 57.2 (OMe), 103.5 (C-1 glc), 74.8 (C-2 glc), 78.6 (C-3 glc), 71.2 (C-4 glc), 78.2 (C-5 glc), 62.5 (C-6 glc).

*3.1.4. Pelargonidin-3-O-glucoside (**7**)*

Amorphous red powder; EIMS *m*/*z* 433; ^{1}H NMR (500 MHz, CFCOOD-CD_3OD): δ 3.33 (1H, dd, *J* = 9.1, 9.5 Hz, H-4 glc), 3.45 (1H, dd, *J* = 9.1, 9.1 Hz, H-3 glc), 3.47 (1H, ddd, *J* = 2.2, 6.0, 9.5 Hz, H-5 glc), 3.56 (1H, dd, *J* = 7.8, 9.1 Hz, H-2 glc), 3.62 (1H, dd, *J* = 6.0, 12.1 Hz, H-6a glc), 3.83 (1H, dd, *J* = 2.2, 12.1 Hz, H-6b glc), 5.19 (1H, d, *J* = 7.7 Hz, H-1 glc), 6.58 (1H, d, *J* = 1.0 Hz, H-6), 6.83 (1H, d, *J* = 0.8 Hz, H-8), 6.96 (2H, d, *J* = 9.1 Hz, H-3′, 5′); 8.50 (2H, d, *J* = 9.1 Hz, H-2′, 6′); 8.98 (1H, s, H-4). ^{13}C NMR (125 MHz, CFCOOD-CD_3OD): δ 165.0 (C-2), 145.8 (C-3), 138.2 (C-4), 159.7 (C-5), 103.8 (C-6), 171.1 (C-7), 95.6 (C-8), 158.2 (C-9), 114.0 (C-10), 121.3 (C-1′), 136.1 (C-2′), 118.2 (C-3′), 166.9 (C-4′), 118.2 (C-5′), 136.1 (C-6′), 104.3 (C-1 glc), 75.2 (C-2 glc), 78.5 (C-3 glc), 71.5 (C-4 glc), 79.2 (C-5 glc), 62.8 (C-6 glc).

*3.1.5. Cyanidin (**9**)*

Amorphous red powder; EIMS *m*/*z* 287; ^{1}H NMR (500 MHz, CFCOOD-CD_3OD): δ 6.61 (1H, d, *J* = 1.5 Hz, H-6); 6.86 (1H, s, H-8); 6.99 (1H, d, *J* = 8.7 Hz, H-5′); 8.09 (1H, d, *J* = 2.3 Hz, H-2′); 8.20 (1H, dd, *J* = 8.7, 2.3 Hz, H-6′); 8.56 (1H, s, H-4). ^{13}C NMR (125 MHz, CFCOOD-CD_3OD): δ 162.5 (C-2), 146.6 (C-3), 134.2 (C-4), 158.2 (C-5), 103.2 (C-6), 169.4 (C-7), 94.9 (C-8), 157.6 (C-9), 113.7 (C-10), 122.0 (C-1′), 118.1 (C-2′), 147.5 (C-3′), 155.3 (C-4′), 117.4 (C-5′), 127.3 (C-6′).

3.2. HPLC-ESI/MS analysis

The crude anthocyanin extract was directly analysed by HPLC chromatogram as shown in Fig. 1. As can be seen, more than nine principal anthocyanin peaks were detected in the chromatogram by DAD at 530 nm. Three major peaks (**3**, **5** and **6**) have been identified as delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, and petunidin-3-*O*-glucoside, respectively, by comparison with the HPLC retention times of our standard compounds. And these three major anthocyanins, the structures of which are shown in Fig. 2,

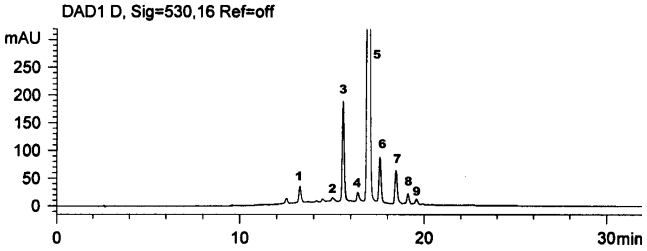

Fig. 1. HPLC chromatogram of a methanolic crude extract of black soybean (Cheongja 3: *G. max* L.) at 530 nm.

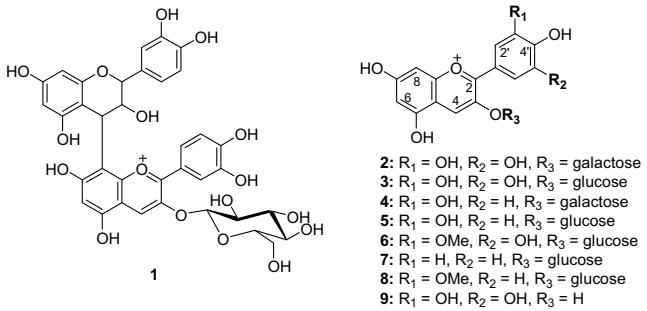

Fig. 2. Structures of the anthocyanins identified in the seed coat of black soybean (cv. Cheongja 3).

represented about 90% of the total peak area. However, six unidentified minor peaks (**1**, **2**, **4**, and **7**–**9**) were also detected which had percentage area of less then 5%. Tandem mass spectrometry (MS/MS), and in particular product–ion analysis, which acquires mass spectra from the product ions produced from the fragmentation of a selected precursor ion, have been used for identification and characterisation of anthocyanins.

The MS analysis of peak **1** (t_R = 13.2 min) showed an $[M]^+$ ion at *m*/*z* 737 and a major fragmentation in MS^2 at *m*/*z* 575 (162 amu) which would correspond to the loss of a glucose moiety. The MS^3 fragmentation pattern of the ion at *m*/*z* 575 showed signals at *m*/*z* 557 (18 amu, loss of water), 423 (151 amu, retro Diels–Alder fission of a catechin moiety), and 329 (246 amu, partial loss of a catechin unit) (Fig. 3). Fig. 4 shows the MS, MS^2, and MS^3 fragmentation scheme for peak **1**. On the basis of this evidence, peak **1** was assumed to be catechin-cyanidin-3-*O*-glucoside (**1**). The presence of catechin-cyanidin-3-*O*-glucoside was identified by comparison with the results from *Phaseolus coccineus* (González-Paramás et al., 2006; Macz-Pop, González-Paramás, Pérez-Alonso, & Rivas-Gonzalo, 2006) and *Fragaria ananassa* (Fossen, Rayyan, & Andersen, 2004), however, this is the first time its presence has been reported in black seed coated soybean (*G. max* L. cv. Cheongja 3).

Subsequently, peaks **2** (t_R = 15.0 min) and **3** (t_R = 15.6 min) showed the same molecular ion an $[M]^+$ at *m*/*z* 465 with the same fragmentation patterns (*m*/*z* 303: $[M162]^+$) which correspond to the loss of a hexose molecule. The MS^2 fragmentation of the ion at *m*/*z* 303 would correspond to the delphinidin aglycone. These two peaks (**2**, **3**) differ by only a single hexose moiety. Peak **3** had already been identified as delphinidin-3-*O*-glucoside which is the standard compound used in our laboratory. Thus, peak **2** was tentatively identified as delphinidin-3-*O*-galactoside (**2**) because of its shorter retention time. These results are in agreement with other studies (Macz-Pop et al., 2006; Zhang, Kou, Fugal, & McLaughlin, 2004). Peaks **4** (t_R = 16.4 min) and **5** (t_R = 16.9 min) also appears to share the same molecular ion an $[M]^+$ at *m*/*z* 449 with the same fragmentation patterns (*m*/*z* 287: $[M162]^+$)

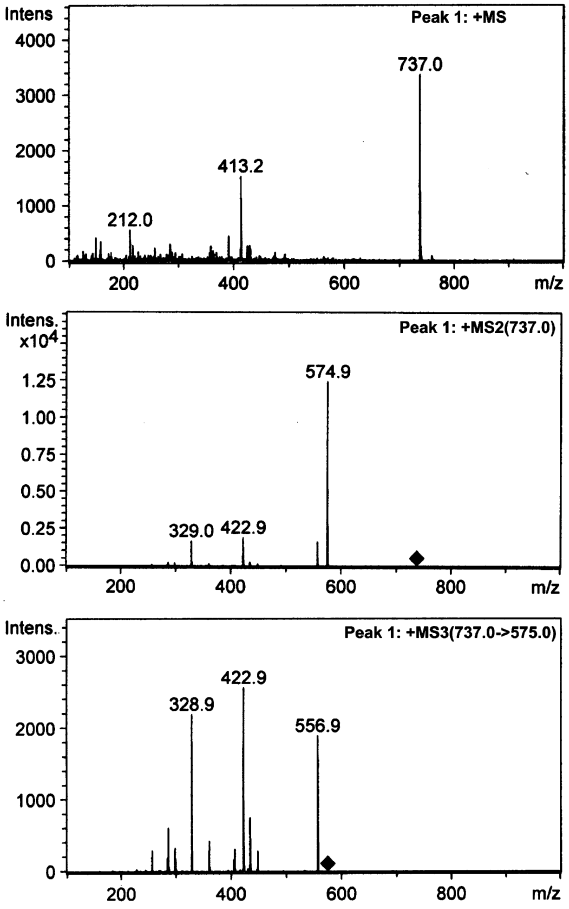

Fig. 3. MS, MS^2, and MS^3 spectra of catechin-cyanidin-3-glucoside (peak **1**).

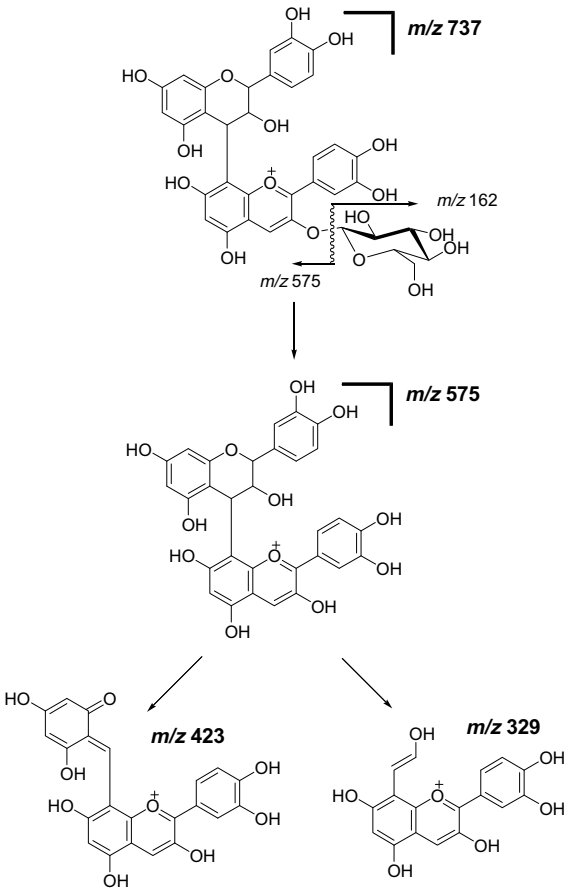

Fig. 4. Significant mass fragmentation scheme proposed for catechin-cyanidin-3-glucoside (peak **1**).

which also corresponds to the loss of a hexose molecule. The MS^2 fragments which show an ion at *m*/*z* 287 would correspond to the cyanidin aglycone moiety. The nature of the hexose was determined by comparing the retention time with data reported in previous literature (Macz-Pop et al., 2006; Slimestad & Solhem, 2002; Talavéra et al., 2004; Zhang et al., 2004). Thus, peaks **4** and **5** were tentatively identified as cyanidin-3-*O*-galactoside and cyanidin-3-*O*-glucoside, respectively. Cyanidinn-3-*O*-glucoside (peak **5**) was previously identified by comparison with our standard, and this identification can be easily confirmed by HPLC-DAD-ESI/MS data. The ESI/MS spectrum of peak **6** (t_R = 17.6 min) was characterised by an ion signal at *m*/*z* 479 with an MS^2 fragment at *m*/*z* 317 ($[M162]^+$). Thus, peak **6** was identified as petunidin-3-*O*-glucoside. The comparisons of the mass spectral data for a petunidin-3-*O*-glucoside standard with the spectrum for peak **6** confirmed this identification. The three major anthocyanins in black soybean seed coats, delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside and petunidin-3-*O*-glucoside, were previously identified by Kuroda and Wada (1933); Yoshikura and Hamaguchi (1969) and Taylor (1976). Among them cyanidin-3-*O*-glucoside has been identified as the principle anthocyanin in the black soybean seed coats comprising ca. 75% of the total anthocyanins. However, the minor anthocyanins, delphinidin-3-*O*-galactoside (**2**) and cyanidin-3-*O*-galactoside (**4**) were the first to be identified from black seed coated soybean (*G. max* L. cv. Cheongja 3). The ESI/MS spectrum and anthocyanin fragmentation patterns are illustrated in Fig. 5.

The ESI/MS profile of peak **7** (t_R = 18.5 min) presented the molecular ion $[M]^+$ at *m*/*z* 433 and the MS^2 fragment resulted from the loss of a glucose moiety (*m*/*z* 271) corresponding to the molecular ion of pelargonidin aglycone. On the basis of this evidence, peak **7** was assumed to be pelargonidin-3-*O*-glucoside. This anthocyanin has been identified from the reddish-buff seed coats of the T236 soybean line (Taylor, 1976), however, this is the first time its presence has been reported in the black seed coated soybean. Peak **8** (t_R = 19.1 min) possessed an identical molecular ion $[M]^+$ at *m*/*z* 463. And the MS^2 fragment resulted from the loss of a glucose moiety (*m*/*z* 301) corresponding to the molecular ion of peonidin aglycone. Thus, peak **8** was tentatively identified as peonidin-3-*O*-glucoside. This anthocyanin has been identified in *Ribes nigrum* (Slimestad & Solhem, 2002), *Zea mays* (Fossen, Slimestad, & Andersen, 2001), and *Phaseolus vulgaris* (Macz-Pop et al., 2006), however, this is also the first time its presence has been reported in black seed coated soybean (*G. max* L. cv. Cheongja 3). Peak **9** (t_R = 19.6 min) showed a molecular ion $[M]^+$ at *m*/*z* 287. Thus, peak **9** was tentatively identified as cyanidin. This is also the first time that this anthocyanin has been reported in black seed coated soybean (*G. max* L. cv. Cheongja 3). The retention times, molecular ion peaks, MS^2 and MS^3 fragments, and per cent area at 530 nm of the nine identified anthocyanins are listed in Table 1.

In conclusion, we characterised the anthocyanins profile of the pigmented black soybean (cv. Cheongja 3) using HPLC and ESI/MS. As a result, this study has documented for the first time the presence of nine anthocyanin derivatives, including catechin-cyanidin-3-*O*-glucoside (**1**), delphinidin-3-*O*-galactoside (**2**), delphinidin-3-*O*-glucoside (**3**), cyanidin-3-*O*-galactoside (**4**), cyanidin-3-*O*-glucoside (**5**), petunidin-3-*O*-glucoside (**6**), pelargonidin-3-*O*-glucoside (**7**), peonidin-3-*O*-glucoside (**8**), and cyanidin (**9**) in the extract of black soybean seed coats (cv. Cheongja 3).

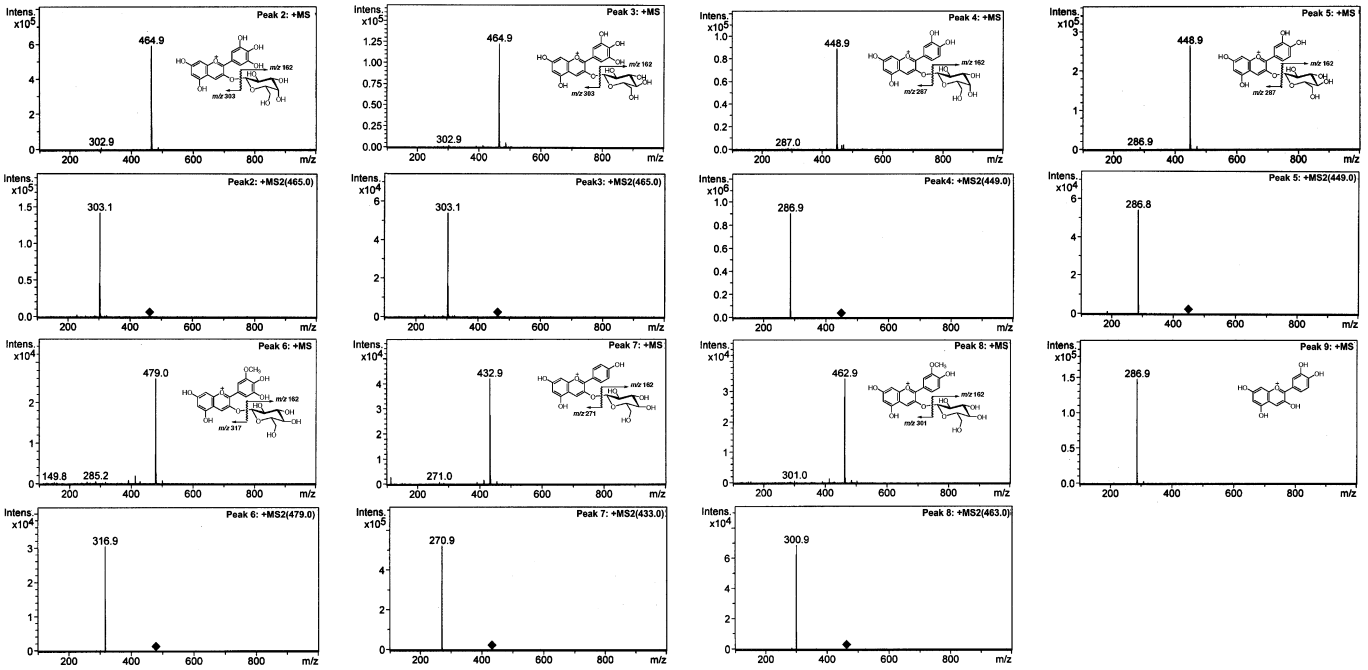

Fig. 5. Mass fragmentation pattern of identified anthocyanins. Peak **2**: delphinidin-3-*O*-galactoside; Peak **3**: delphinidin-3-*O*-glucoside; Peak **4**: cyanidin-3-*O*-galactoside; Peak **5**: cyanidin-3-*O*-glucoside; Peak **6**: petunidin-3-*O*-glucoside; Peak **7**: pelargonidin-3-*O*-glucoside; Peak **8**: peonidin-3-*O*-glucoside; Peak **9**: cyanidin.

Table 1
Chromatographic and spectroscopic characteristics of anthocyanins from black soybean seed coat

Peak	t_R (min)	Area (%) (530 nm)	$[M^+]$ (m/z)	MS^2 (m/z)	MS^3 (m/z)	Identity
1	13.2	2.0	737	575, 423, 329	557, 423, 329, 287	catechin-cyanidin-3-*O*-glucoside
2	15.0	0.8	465	303	–	delphinidin-3-*O*-galactoside
3	15.6	8.8	465	303	–	delphinidin-3-*O*-glucoside
4	16.4	1.0	449	287	–	cyanidin-3-*O*-galactoside
5	16.9	75.8	449	287	–	cyanidin-3-*O*-glucoside
6	17.6	4.4	479	317	–	petunidin-3-*O*-glucoside
7	18.5	3.4	433	271	–	pelargonidin-3-*O*-glucoside
8	19.1	1.1	463	301	–	peonidin-3-*O*-glucoside
9	19.6	0.7	287	–	–	cyanidin

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