Identification and Characterization of Anthocyanins in Yard-Long Beans (*Vigna unguiculata* ssp. *sesquipedalis* L.) by High-Performance Liquid Chromatography with Diode Array Detection and Electrospray Ionization/Mass Spectrometry (HPLC−DAD−ESI/MS) Analysis

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Anthocyanins play an important role in physiological functions related to human health. The objective of this study was to investigate the profiles of anthocyanins in the immature purple pods and black seeds of yard-long beans (*Vigna unguiculata* ssp. *sesquipedalis* L.) using high-performance liquid chromatography (HPLC) with diode array detection and electrospray ionization/mass spectrometry (DAD−ESI/MS) analysis. The individual anthocyanins were identified by comparing their mass spectrometric data and retention times. In the purple pods, five individual anthocyanins were identified: delphinidin-3-*O*-glucoside (**2**), cyanidin-3-*O*-sambubioside (**4**), cyanidin-3-*O*-glucoside (**5**), pelargonidin-3-*O*-glucoside (**7**), and peonidin-3-*O*-glucoside (**8**). From the black seed coat of the yard-long beans, seven anthocyanins were identified, including delphinidin-3-*O*-galactoside (**1**), cyanidin-3-*O*-galactoside (**3**), petunidin-3-*O*-glucoside (**6**), and malvidin-3-*O*-glucoside (**9**), together with compounds **2**, **5**, and **8**. In this study, we report for the first time anthocyanin profiles for the pod and seed coat of yard-long beans.

KEYWORDS: *Vigna unguiculata*; yard-long bean; anthocyanins; HPLC−ESI/MS

INTRODUCTION

Yard-long bean (*Vigna unguiculata* ssp. *sesquipedalis* L.), also known as vegetable cowpea, asparagus bean, or string bean, is an important legume species that is widely cultivated worldwide, including South Korea. The origin of the bean is possibly from the middle of west Africa, but it is primarily cultivated in southeast Asia. This plant produces long tender pods that are consumed as a vegetable before maturity in China, Japan, Korea, and southeast Asia. Subsequently, considerable variability exists in yard-long beans in terms of their pod and seed characteristics, and consumers have acquired specific preferences for various combinations of pod length, pod color, pod thickness, seed size, shape, and seed color. In particular, the pods and seeds of yard-long beans have various seed colors, such as dark green, pale green, purple, red, red and green colors creating a mosaic pattern on the pods, and black, brown, and various types of mottled colors for the seeds. Such color pigmentations are due to chlorophyll and anthocyanins, which are expected to possess various biological activities.

Anthocyanins, which belong to the flavonoid group of compounds, are natural pigments that are widely distributed in plants consumed in the human diet, including fruits and vegetables (*1*).

Every day humans ingest large amounts of anthocyanins from plants. The average intake of anthocyanins by U.S. citizens has been estimated at up to 180−215 mg/day, which is higher than that of other flavonoids, such as flavonols (*2*). In particular, these anthocyanins are associated with a wide range of biological activities, including antioxidant (*3*, *4*), anti-inflammatory (*5*, *6*), anticancer (*7*, *8*), and α-glucosidase inhibition (*9*). In addition, these pigments may reduce the risk of coronary heart disease through modulation of arterial protection (*10*), inhibition of platelet aggregation (*11*), or endothelial protection (*6*). For this reason, the food and medicinal industries have become increasingly interested in fruits and vegetables with high contents of bioactive anthocyanins for the manufacture of supplements with preventative and therapeutic uses.

To our knowledge, there are only a few reports in the literature regarding the genetic diversity (*12*) and morphological characteristics (*13*) of yard-long beans. Furthermore, the pigments of its immature purple pods and black seeds have still not been fully characterized. This prompted us to identify the anthocyanins occurring in the purple pods and black seed coats of yard-long beans by employing reversed-phase C-18 column chromatography and high-performance liquid chromatography with diode array detection and electrospray ionization/mass spectrometry (HPLC−DAD−ESI/MS) analysis.

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MATERIALS AND METHODS

Plant Materials. Yard-long beans (*V. unguiculata* ssp. *sesquipedalis* L.) with dark purple pods and black seed coat germplasms were collected from a field near Suncheon City, Jeollanam-do, Korea. These seeds were cultivated within an experimental field at the Yeongnam Agricultural Research Institute, NICS, RDA at Miryang, Korea, in 2007 and 2008. The fresh immature dark purple pod samples were freeze-dried and ground into powder. The powder was kept at −70 °C until analyzed. The hand-peeled seed coats of the black yard-long bean samples were also stored at −70 °C until analyzed.

Chemicals. Delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, and peonidin-3-*O*-glucoside as standards were available from our previous work (*14*). Cyanidin-3-*O*-galactoside, pelargonidin-3-*O*-glucoside, and malvidin-3-*O*-glucoside were purchased from Polyphenols Laboratories (Sandnes, Norway). Analytical-grade methanol, ethyl acetate, acetonitrile, and water were purchased from J. T. Baker (Phillipsburg, NJ). Acetic acid, formic acid, and trifluoroacetic acid (TFA) were obtained from Sigma Chemical Co. (St. Louis, MO).

Instruments. UV/vis absorption spectra were recorded on an Infinite M200 spectrophotometer (Tecan Austria GmbH, Untersbergstrasse 1A, Austria). HPLC was performed using an Agilent 1100 series (Boeblingen, Germany) quaternary pump, Agilent 1100 series DAD, and a Hypersil Gold column (150 × 4.6 mm, Thermo Scientific, Waltham, MA). ESI/MS data were obtained using an Esquire 4000 (Bruker Daltonick GmbH, Bremen, Germany).

Anthocyanin Analysis. The hand-peeled yard-long bean seed coats (0.1 g) and freeze-dried purple pods (0.5 g) were extracted with 10 mL of 40% methanol (0.1% HCl) for 2 days at 4 °C in the dark. The anthocyanin extracts were filtered through a 0.45 μm filter unit prior to HPLC analysis. The anthocyanins present in the yard-long beans were characterized by HPLC−DAD−MS analysis. A 20 μL sample of the crude acidic methanolic extract was injected onto an analytical Hypersil Gold column. The mobile phase was composed of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol. The gradient conditions were as follows: 0 min, 15% B; 30 min, 35% B; and then held for 10 min before returning to the initial conditions. The other HPLC conditions were as follows: flow rate, 0.8 mL/min; column temperature, 25 °C; detection, 530 nm; and sample size, 20 μL. The DAD spectra were measured over the wavelength range of 220−700 nm in steps of 2 nm. The mass spectrometer used was a Bruker Daltonick GmbH (Bremen, Germany) equipped with an ESI source and an ion-trap mass analyzer, which were controlled by 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; and nebulizer pressure, 60 psig. The instrument was operated in the positive-ion mode scanning from m/z 100 to 700 at a scan rate of 1.5 s/cycle. The MS revealed the positive molecular ions, and MS^2 was used to break down the most abundant species by collision-induced dissociation.

For quantification and identification purposes, seven anthocyanin standards, which included delphinidin-3-*O*-glucoside, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, and malvidin-3-*O*-glucoside stock solutions, were prepared by dissolving in 40% methanol to give a 1.0 mg/mL concentration, respectively. Calibration curves were made for each standard using seven different concentrations (5, 10, 20, 40, 60, 80, and 100 μg/mL). A high linearity ($r^2 > 0.999$) was obtained for each standard curve. The results were expressed as anthocyanidin glucoside equivalents.

RESULTS AND DISCUSSION

Anthocyanin Identification and Assignment. The HPLC chromatogram of the anthocyanin standards is represented in **Figure 1A**. The use of a Hypersil Gold column with methanol/water (0.1% formic acid) as a binary mobile gradient condition resulted in good resolution of the standards within 30 min. The anthocyanins in the purple pods and black seed coats of the domestically cultivated yard-long beans (*V. unguiculata* ssp. *sesquipedalis* L.) were analyzed by HPLC−DAD−ESI/MS. Because there are few published data available on the anthocyanin
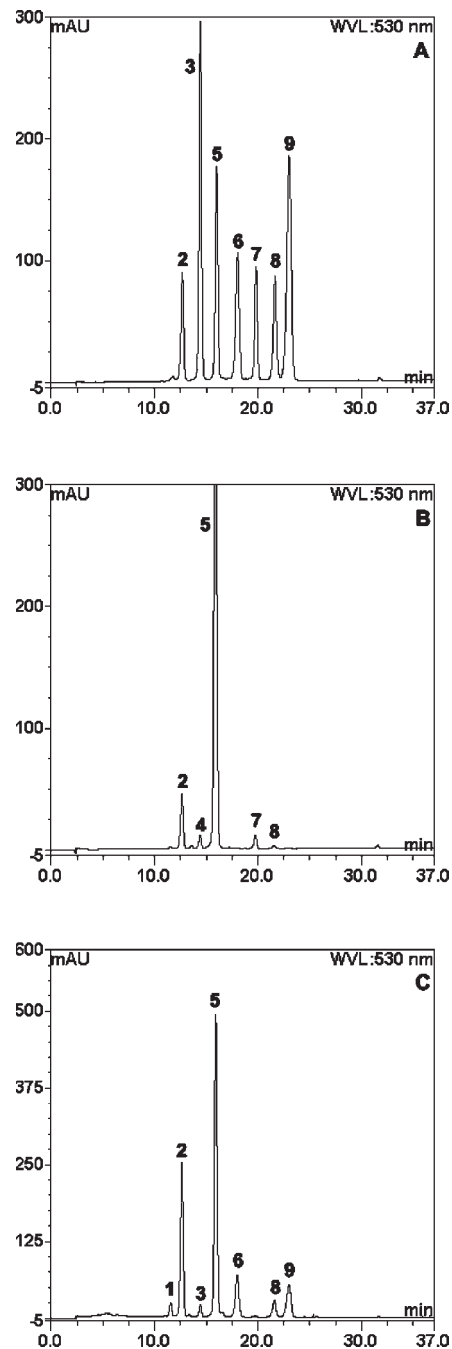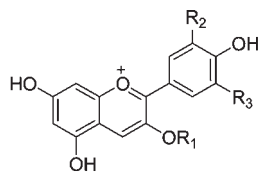

Figure 1. HPLC chromatograms of (A) anthocyanin standards mixture and the methanolic crude extract of (B) purple pods and (C) black seed coats of yard-long beans at 530 nm.

composition of yard-long beans, the peak identification and assignment were primarily based on comparisons of the retention times and mass spectrometric data to those of standards and published data.

Purple Pods. The HPLC chromatogram of the anthocyanin extract from the purple pods of the yard-long beans is shown in **Figure 1B**. The results indicated that five principal anthocyanin peaks (peaks 2, 4, 5, 7, and 8) were detected on the chromatogram by DAD at 530 nm. One major peak (5) showed a molecular ion at m/z 449, which fragmented to give ions at m/z 287 that corresponded to a cyanidin aglycone. The fragment loss [M − 162]$^+$ indicated a hexose moiety. The UV−vis spectrum (λ_{max} at 516 and 280 nm) and the retention time of peak 5 matched that of a standard (cyanidin-3-*O*-glucoside). Thus, the major peak was

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Figure 2. Chemical structures of the anthocyanins identified in the purple pods and black seed coats of yard-long bean.

tentatively identified as cyanidin-3-*O*-glucoside (**5**). The structure of this major anthocyanin, as shown in **Figure 2**, represented about 84% of the total peak area. However, four unidentified minor peaks (2, 4, 7, and 8) were also detected, which had a percentage area of less than 16%. 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 the identification and characterization of anthocyanins.

The MS analysis of peak 2 (t_R = 12.6 min) showed a [M]$^+$ ion at m/z 465 and a major fragmentation in MS^2 at m/z 303 (-162 amu), which would correspond to the loss of a glucose moiety. Also, the MS^2 fragmentation of the ion at m/z 303 would correspond to a delphinidin aglycone. Peak 2 had already been identified as delphinidin-3-*O*-glucoside, which is a standard compound used in our laboratory. Thus, peak 2 was tentatively identified as delphinidin-3-*O*-glucoside (**2**). Peak 4 (t_R = 14.5 min) appeared as a molecular ion [M]$^+$ at m/z 581, whose fragments gave ions at m/z 449 (-132 amu) and m/z 287 (-294 amu), which would correspond to the loss of a disaccharide constituted by hexose plus pentose. The MS^2 fragmentation pattern of the ion at m/z 517 showed only signals at m/z 287 (M $-$ 294 amu, loss of hexose plus pentose). The MS^2 fragments, which showed an ion at m/z 287, would correspond to a cyanidin aglycone moiety. Moreover, because the MS^2 spectra showed only the fragment ion of the aglycone, the sugars were linked to the same position. Because this is a minor compound, it was not possible to isolate it to perform an analysis that would allow one to confirm the identiy of the sugars that constitute it nor their exact position. Nonetheless, the data obtained from the mass spectrum and UV−vis spectrum were identical to those described by Määttä et al. (*15*) for the identification of cyanidin−hexose−pentoside (cyanidin-3-*O*-smabubioside) present in red currant. Although, until now, the presence of a sambubioside of cyanidin in beans has not been described, Wu and Prior (*16*) suggested the existence of pelargonidin-3-*O*-sambubioside in small red beans. On the basis of this evidence, peak 4 was assumed to be cyanidin-3-*O*-sambubioside (**4**) and was the only diglucoside present in the yard-long bean samples. To the best of our knowledge, this is the first time that the presence of cyanidin-3-*O*-sambubioside (**4**) in the purple pods of yard-long beans has been reported. The ESI/MS spectrum of peak 5 (t_R = 16.0 min) was characterized by an ion signal at m/z 449 with a MS^2 fragment at m/z 287 ([M $-$ 162]$^+$). Thus, peak 5 was identified as cyanidin-3-*O*-glucoside. The comparisons of the mass spectral data for the cyanidin-3-*O*-glucoside standard with the spectrum for peak 5 confirmed this identification. The ESI/MS profile of peak 7 (t_R = 19.7 min) presented a 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 a pelargonidin aglycone. On the basis of this evidence, peak 7 was assumed to be pelargonidin-3-*O*-glucoside. Peak 8 (t_R = 21.6 min) showed a molecular ion [M]$^+$ at m/z 463 with a fragmentation pattern ([M $-$ 162]$^+$ at m/z 301) that also corresponded to the loss of a hexose molecule. The MS^2 fragments, which showed an ion at m/z 301, would correspond to a peonidin aglycone moiety. Thus, peak 8 was tentatively identified as peonidin-3-*O*-glucoside. The ESI/MS spectrum and anthocyanin fragmentation patterns are illustrated in **Figure 3**. The retention times, molecular ion peaks, MS^2 fragments, and UV−vis spectral characteristics of the five identified anthocyanins are listed in **Table 1**. The total anthocyanin content of the sample was 8812.7 μg/g of purple pods, and cyanidin-3-*O*-glucoside was the most predominant anthocyanin (7377.0 μg/g), followed by delphinidin-3-*O*-glucoside (895.5 μg/g) and pelargonidin-3-*O*-glucoside (305.5 μg/g), as indicated in **Table 2**.

Black Seed Coat. The crude anthocyanin extract of the black seed coats of the yard-long beans was directly analyzed using a HPLC chromatogram, as shown in **Figure 1C**. As illustrated, seven principal anthocyanin peaks were detected in the chromatogram by DAD at 530 nm. The three major peaks identified by HPLC−MS were delphinidin-3-*O*-glucoside (**2**), [M]$^+$ = 465; cyanidin-3-*O*-glucoside (**5**), [M]$^+$ = 449; and petunidin-3-*O*-glucoside (**6**), [M]$^+$ = 479. These three major anthocyanins represented about 87% of the total peak area. However, four unidentified minor peaks (1, 3, 8, and 9) were also detected, which had a percentage area of less than 13%. 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 the identification and characterization of anthocyanins.

The MS analysis of peaks 1 (t_R = 11.6 min) and 2 (t_R = 12.6 min) showed the same molecular ion [M]$^+$ at m/z 465 with the same fragmentation patterns ([M $-$ 162]$^+$ at m/z 303), which corresponded to the loss of a hexose molecule. The MS^2 fragmentation of the ion at m/z 303 would correspond to a delphinidin aglycone. The MS^3 fragmentation pattern of the ion at m/z 303 showed signals at m/z 275 (M $-$ 46 amu, loss of H$_2$O and CO) together with m/z 229 (M $-$ 74 amu, partial loss of two CO and water). These two peaks (1 and 2) differed by only a single hexose moiety. Peak 2 had already been identified as delphinidin-3-*O*-glucoside, which is a standard compound used in our laboratory. Thus, peak 1 was tentatively identified as delphinidin-3-*O*-galactoside (**1**) because of its shorter retention time. These results are in agreement with other studies (*17, 18*). Peaks 3 (t_R = 14.4 min) and 5 (t_R = 16.0 min) also appeared to share the same molecular ion [M]$^+$ at m/z 449 with the same fragmentation patterns ([M $-$ 162]$^+$ at m/z 287), which also corresponded to the loss of a hexose molecule. The MS^2 fragments, which showed an ion at m/z 287, would correspond to a cyanidin aglycone moiety. The MS^3 fragmentation pattern of the ion at m/z 287 showed signals at m/z 259 (M $-$ 28 amu, loss of CO) together with m/z 213 (M $-$ 74 amu, loss of two CO and water). The nature of the hexose was determined by comparing the retention time with data reported in previous studies (*17−20*). Thus, peaks 3 and 5 were tentatively identified as cyanidin-3-*O*-galactoside and cyanidin-3-*O*-glucoside, respectively. Cyanidin-3-*O*-glucoside (peak 5) was previously identified by a comparison to our standard, and this identification can easily be confirmed by HPLC−DAD−ESI/MS data. The ESI/MS spectrum of peak 6 (t_R = 18.0 min) was characterized by an ion signal at m/z 479 with a MS^2 fragment at m/z 317 ([M $-$ 162]$^+$). Thus, peak 6 was identified as petunidin-3-*O*-glucoside. The comparisons of the mass spectral data for a petunidin-3-*O*-glucoside standard to the spectrum for peak 6
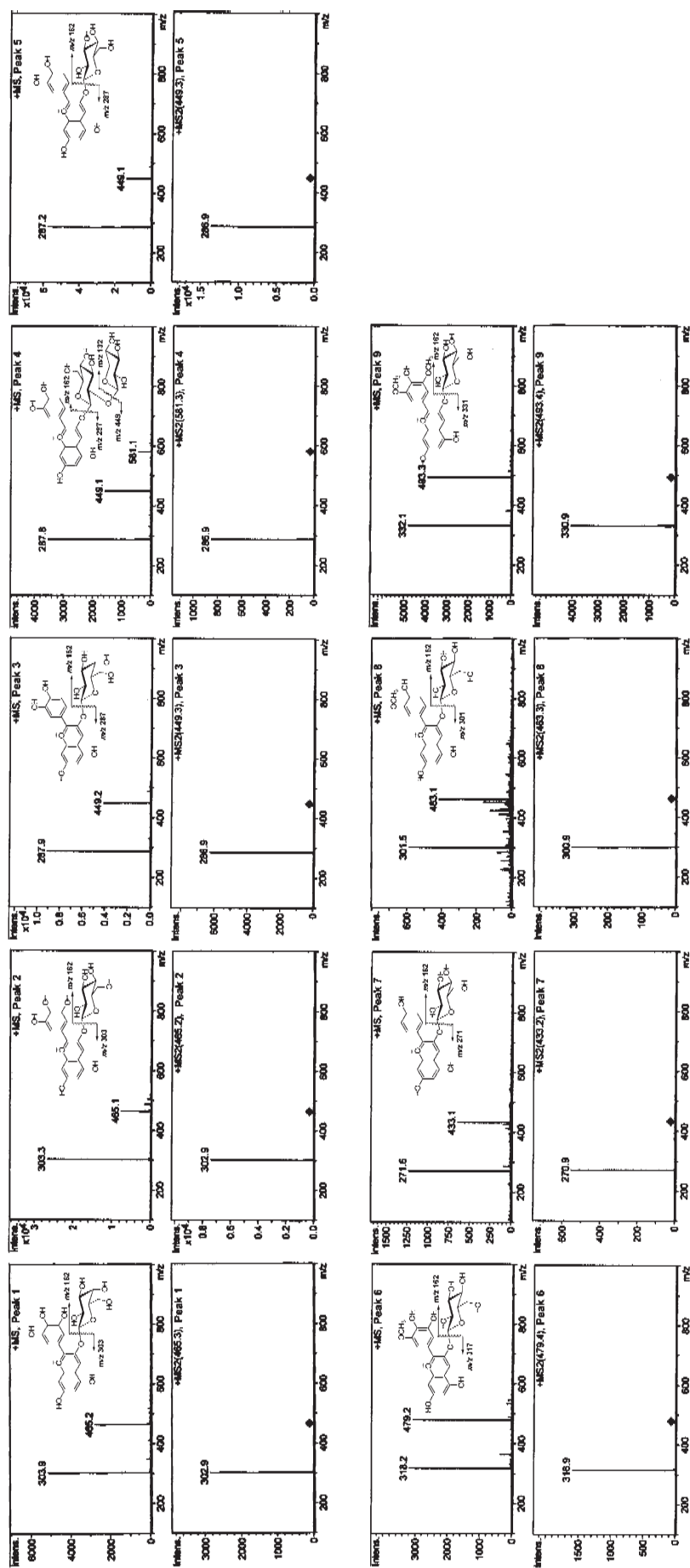
Figure 3. Mass fragmentation pattern of identified anthocyanins from yard-long beans: peak 1, delphinidin-3-*O*-glucoside; peak 2, delphinidin-3-*O*-glucoside; peak 3, cyanidin-3-*O*-galactoside; peak 4, cyanidin-3-*O*-sambubioside; peak 5, cyanidin-3-*O*-glucoside; peak 6, petunidin-3-*O*-glucoside; peak 7, pelargonidin-3-*O*-glucoside; peak 8, peonidin-3-*O*-glucoside; peak 9, malvidin-3-*O*-glucoside.

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Table 1. Anthocyanins Identified in Purple Pod and Black Seed Coats of Yard-Long Beans Analyzed by HPLC−ESI/MS and Compared to Literature Data

yard-long bean sample	peak	t_R (min)	spectral characteristics (nm)	area (%) (530 nm)	positive ions		peak assignment	reference
					[M⁺] (*m/z*)	MS/MS (*m/z*)		
	2	12.6	278, 524	6.0	465 (303 + 162)	303	delphinidin-3-*O*-glucoside	*14* and *16−18*
	4	14.5	281, 517	5.8	581 (287 + 162+ 132)	449, 287	cyanidin-3-*O*-sambubioside	*15*
purple pod	5	16.0	280, 516	83.9	449 (287 + 162)	287	cyanidin-3-*O*-glucoside	*14* and *16−18*
	7	19.7	277, 501	58.1	433 (271 + 162)	271	pelargonidin-3-*O*-glucoside	*14* and *17*
	8	21.6	280, 517	0.9	463 (301 + 162)	301	peonidin-3-*O*-glucoside	*14* and *17*
	1	11.6	278, 524	1.3	465 (303 + 162)	303	delphinidin-3-*O*-galactoside	*14* and *16−18*
	2	12.6	278, 524	22.0	465 (303 + 162)	303	delphinidin-3-*O*-glucoside	*14* and *16−18*
	3	14.4	280, 516	1.7	449 (287 + 162)	287	cyanidin-3-*O*-galactoside	*14* and *16−18*
black seed coat	5	16.0	280, 516	58.1	449 (287 + 162)	287	cyanidin-3-*O*-glucoside	*14* and *16−18*
	6	18.0	277, 526	6.9	479 (317 + 162)	317	petunidin-3-*O*-glucoside	*14* and *16−18*
	8	21.6	280, 517	3.0	463 (301 + 162)	301	peonidin-3-*O*-glucoside	*14* and *17*
	9	23.0	277, 527	6.8	493 (331 + 162)	331	malvidin-3-*O*-glucoside	*16−18*

Table 2. Anthocyanin Contents[a] in the Purple Pods and Black Seed Coats of Yard-Long Beans

	yard-long bean	
compound (peak)	purple pod	black seed coat
delphinidin-3-*O*-galactoside (**1**)	ND	500.5
delphinidin-3-*O*-glucoside (**2**)	895.5	6399.7
cyanidin-3-*O*-galactoside (**3**)	ND	341.3
cyanidin-3-*O*-sambubioside (**4**)	159.3	ND
cyanidin-3-*O*-glucoside (**5**)	7377.0	11833.4
petunidin-3-*O*-glucoside (**6**)	ND	1944.7
pelargonidin-3-*O*-glucoside (**7**)	305.5	ND
peonidin-3-*O*-glucoside (**8**)	75.4	1254.7
malvidin-3-*O*-glucoside (**9**)	ND	2804.4
total	8812.7	25078.7

[a] Contents are expressed in micrograms per gram of dry weight.

confirmed this identification. The ESI/MS profile of peak 8 (t_R = 21.6 min) presented a molecular ion [M]⁺ at *m/z* 463, and the MS² fragment resulted from the loss of a glucose moiety (*m/z* 301) corresponding to the molecular ion of a peonidin aglycone. On the basis of this evidence, peak 8 was assumed to be peonidin-3-*O*-glucoside. Peak 9 (t_R = 23.0 min) possessed an identical molecular ion [M]⁺ at *m/z* 493. The MS² fragment resulted from the loss of a glucose moiety (*m/z* 331) corresponding to the molecular ion of a malvidin aglycone. Thus, peak 9 was tentatively identified as malvidin-3-*O*-glucoside. The ESI/MS spectrum and anthocyanin fragmentation patterns are illustrated in **Figure 3**. The retention times, molecular ion peaks, MS² fragments, and UV−vis spectral characteristics of the five identified anthocyanins are listed in **Table 1**. The total anthocyanin content of the sample was 25 mg/g of seed coat, and cyanidin-3-*O*-glucoside was the most predominant anthocyanin (11.8 mg/g), followed by delphinidin-3-*O*-glucoside (6.4 mg/g) and malvidin-3-*O*-glucoside (2.8 mg/g), as indicated in **Table 2**.

Using HPLC with ESI/MS, this study has documented and characterized for the first time the profiles of anthocyanins that can be found in the seed coats and pods of black yard-long beans. Five anthocyanins were identified in the purple pods: delphinidin-3-*O*-glucoside (**2**), cyanidin-3-*O*-sambubioside (**4**), cyanidin-3-*O*-glucoside (**5**), pelargonidin-3-*O*-glucoside (**7**), and peonidin-3-*O*-glucoside (**8**). Furthermore, nine anthocyanin derivatives, including delphinidin-3-*O*-galactoside (**1**), delphinidin-3-*O*-glucoside (**2**), cyanidin-3-*O*-galactoside (**3**), cyanidin-3-*O*-glucoside (**5**), petunidin-3-*O*-glucoside (**6**), peonidin-3-*O*-glucoside (**8**), and

malvidin-3-*O*-glucoside (**9**), were identified in the extracts of the black seed coats from the yard-long beans.

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