Characterization of Black Bean (*Phaseolus vulgaris* L.) Anthocyanins

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Three anthocyanins were isolated from the acidified methanol extracts of UI 911 black beans (*Phaseolus vulgaris* L.) using solid phase extraction and preparative high-performance liquid chromatography . The anthocyanins were characterized using chromatographic and spectroscopic methods as delphinidin 3-glucoside (56%), petunidin 3-glucoside (26%), and malvidin 3-glucoside (18%). The monomeric anthocyanin content was 213 ± 2 mg/100 g of black beans (moisture content was 10.04 ± 0.02%).

Keywords: *Black beans; antioxidants; pigments; anthocyanins; electrospray ionization mass spectrometry*

INTRODUCTION

Anthocyanins are the largest group of water-soluble pigments in the plant kingdom. They are responsible for most of the red, purple, and blue colors exhibited by flowers, fruits, and other plant tissues and have found application in the food industry as natural colorants (Harborne, 1967). There is current medical interest in the anthocyanins; for example, crude extracts of several types of fruits appear to have replaced rutin and its derivatives in the treatment of illnesses involving tissue inflammation or capillary fragility (Harborne and Grayer, 1988).

There is currently strong evidence indicating that free radicals and other oxidants cause oxidative damage to lipids, proteins, and nucleic acids. These oxidants may be an important factor in the development of a number of diseases including cancer and atherosclerosis (Ames et al., 1993). Antioxidants, which can neutralize free radicals, may therefore be of central importance in the prevention of these disease states (Wang et al., 1996). Recent studies on the total antioxidant capacity of fruits and vegetables at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University revealed that a large group of colorful compounds, some of which are flavonoids (including anthocyanins, flavones, isoflavones, catechin, and epicatechin), may be responsible for much of the antioxidant protection against peroxyl radicals (Cao et al., 1996; McBride, 1996). Wang et al. (1997) recently confirmed the potent antioxidant properties of anthocyanins and their aglycons against peroxyl radicals.

The common bean (*Phaseolus vulgaris* L.) is the world's second most important bean (*Encyclopedia Britannica*, 1972) after soybeans and is one of the basic foods of the indigenous population in South America. The characteristic intense red anthocyanin pigments in the skin of black beans make them an attractive potential source for natural food colors. Anthocyanin pigments may be used for coloring foodstuffs and snack food, beverages, pharmaceutical, and cosmetic products and for coloring textiles, paper, and leathers (Hoffmann, 1983). The anthocyanins malvidin 3-glucoside, petunidin 3-glucoside, delphinidin 3-glucoside, and delphinidin 3,5-diglucoside were reported in black violet colored beans (*P. vulgaris* L.) by Feenstra (1960). The pigments of black beans have been reported by several investigators (Nozzolillo, 1972, 1973; Nozzolillo and McNeill, 1985). In those studies, malvidin glycosides were the only anthocyanins found in several cultivars such as Black Wax, Black Valentine, and Royalty. Four anthocyanins, delphinidin 3-glucoside, petunidin 3-glucoside, malvidin 3-glucoside, and malvidin 3,5-diglucoside, were reported in the black bean cultivar Kurodanekinugasa by Okita et al. (1972). In another study, Stanton and Francis (1966) reported that delphinidin 3-glucoside was the major anthocyanin in the Canadian Wonder cultivar. Small amounts of cyanidin 3-glucoside, cyanidin 3,5-diglucoside, pelargonidin 3-glucoside, and pelargonidin 3,5-diglucoside were also found. However, paper chromatography was the main analytical method used in these studies. Work with complex mixtures such as plant extracts has led to the development of various preparative chromatographic techniques such as droplet countercurrent chromatography (Hostettmann and Hostettmann, 1982). The advent of high-performance liquid chromatography (HPLC) has revolutionized anthocyanin studies because of its high efficiency, sensitivity, speed, and accurate quantitation (Wilkinson et al., 1977; Wulf and Nagel, 1978; Casteele et al., 1983; Baj et al., 1983; Andersen, 1985, 1987a,b; Hong and Wrolstad, 1986, 1990a,b; Strack and Wray, 1989). In their investigation of the black bean cultivar Yamashirokurosando, Tsuda et al. (1994) used HPLC to isolate delphinidin 3-glucoside and showed that this pigment possessed strong antioxidative activity. Our group is involved in the processing of black beans by extrusion. To preserve the color of extruded black beans, the original anthocyanin content in black beans must be known. The aim of this study is to determine the anthocyanin content in black beans by using a combination of analytical methods such as preparative HPLC, HPLC coupled with photodiode array detection, thin-layer chromatography (TLC), gas chromatography (GC), ultraviolet−visible (UV−vis) spectroscopy, mass spectrometry (MS), and nuclear magnetic resonance (NMR)

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spectroscopy. The anthocyanin content in black beans and their chemical structures will be discussed. The stability of anthocyanins during extrusion will be the subject of future studies.

EXPERIMENTAL PROCEDURES

Materials. The UI 911 black beans (*P. vulgaris* L.), originally developed by the Idaho Agricultural Experiment Station at Kimberly, ID, were supplied by the Treasure Seed Co. (Homedale, ID). The moisture content of the beans was 10.04 ± 0.02% on a wet weight basis. Concord grape puree was obtained from Milne Fruit Products, Inc. (Prosser, WA). Solvents were of HPLC spectroquality grade unless otherwise stated.

Extraction Method. For quantitative studies, the black beans were ground with a Wiley mill to pass a 30−40 mesh screen. Five hundred milligrams of bean powder and 50 mL of 0.5% HCl in methanol were placed in a 125 mL Erlenmeyer flask. The flask was placed in a refrigerator at 4 °C for 24 h and was shaken occasionally. The extraction solution was filtered through Whatman No. 4 filter paper, and the residue was repeatedly reextracted with additional 50 mL aliquots of extracting solution until colorless. The combined acidified methanol extracts were evaporated to dryness and then redissolved in 10% formic acid. The sample was deposited in an Extrelut 20 cartridge (Merck, Darmstadt, Germany) and extracted with hexane and then with ethyl acetate to remove nonpolar impurities and other flavonoids (Baldi et al., 1995). The anthocyanins and hydrophilic molecules were desorbed using 0.02% HCl in methanol, and the final volume was adjusted to 100 mL before the absorbance was measured by UV−vis spectroscopy. The total anthocyanin content was determined in triplicate and expressed as percentage of bean weight. For samples to be processed by analytical HPLC and preparative HPLC, the whole beans were broken by mortar and pestle and repeatedly extracted using the above procedures. After cleanup with Extrelut 20, the final extracting solution was evaporated, redissolved in 10% formic acid, and filtered through a 0.5 μm disposable membrane filter before injection.

Ultraviolet−Vis Spectroscopy. A Hewlett-Packard (HP) UV−vis 8453 spectrophotometer controlled by an HP ChemStation (version A.02.04a) was used. The total anthocyanin content was calculated from the absorption maximum at 537 nm. Pigment content was calculated as delphinidin 3-glucoside, using a molar extinction coefficient (ϵ) of 27 300 (Andersen, 1988) and a molecular weight of 465.

Analytical HPLC. The HPLC system consisted of a HP 1050 quaternary pump, a manual injector (Model 7125, Rheodyne, Cotati, CA) equipped with a 20 μL sample loop, and an HP 1040M diode array detector. The instrument was controlled and the data were processed by an HP ChemStation (G2180AA version A.03.02). The analytical column was an ODS/B (250 × 4.6 mm i.d., 5 μm, 100 Å; Keystone Scientific Inc., Bellefonte, PA) protected by a Supelguard LC-18-DB (Supelco, Inc., Bellefonte, PA) guard column. Solvent A consisted of 10% aqueous formic acid, and solvent B was formic acid/water/methanol (10:40:50 v/v). Separations were performed with a linear gradient of 40−80% solvent B over a period of 50 min at a flow rate of 1.2 mL/min and detection at 520 nm. Sample volumes of 50 μL were used for injection.

Preparative HPLC. The preparative HPLC system consisted of Gilson Model 305 and 306 pumps, a Gilson 806 manomeric module, a Gilson 811C dynamic mixer (Gilson Medical Electronics, Inc., Middleton, WI), a manual injector (Rheodyne, Model 7125) fitted with a 200 μL sample loop, and an HP 1050 variable wavelength detector equipped with a preparative flow cell. A C_{18} reversed phase Dynamax preparative column (250 mm × 21.4 mm i.d., 8 μm, 100 Å; Rainin Instrument Co., Woburn, MA) was directly coupled to a guard column (50 mm × 21.4 mm i.d.) containing the same packing material. The mobile phase was formic acid/water/methanol (10:65:25 v/v). The isocratic flow rate was 11 mL/min, and the detector was set at 520 nm. The three anthocyanin fractions of interest were isolated and their purities checked by analytical HPLC. When necessary, the compounds were rechromatographed. The fractions were evaporated to dryness, dissolved in 10% aqueous formic acid, and loaded on a C_{18} Bond Elut SPE cartridge (3 cm^3/500 mg; Varian Associates, Inc., Harbor City, CA). The cartridge was rinsed with distilled water, and the adsorbed anthocyanins were eluted with 0.1% HCl in methanol. The eluent solution was evaporated to dryness, and the resulting solid was resolubilized with suitable solvents for NMR and MS.

Identification of Anthocyanidins. Due to the limited commercial availability of anthocyanin standards, Concord grape puree was used as a source of five of the most common anthocyanidins with the exception of pelargonidin (Hong and Wrolstad, 1986). The HPLC elution order is as follows: delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin (Karppa et al., 1984; Wilkinson et al., 1977).

Acid Hydrolysis of Anthocyanins. The Concord grape puree and black bean extracts were evaporated to near dryness by rotary evaporation and then redissolved in 10% aqueous formic acid. Two milliliters of the extract was heated with 2 mL of 2 N HCl in a boiling water bath for 60 min and then cooled in an ice bath. The anthocyanidins were extracted with ethyl acetate, evaporated to dryness in a stream of nitrogen, redissolved in 10% formic acid, and finally filtered through a 0.5 μm disposable membrane filter. Since anthocyanidins are very unstable (Harborne, 1967; Iacobucci and Sweeny, 1983; Francis, 1989), HPLC analysis was performed promptly.

Identification of Carbohydrates by TLC. The anthocyanin fractions collected from preparative HPLC were subjected to acid hydrolysis and then cooled in an ice bath. The hydrolysate was adsorbed on a C_{18} Bond Elut cartridge (3 cm^3/500 mg), and the sugar fraction was eluted from the cartridge with 0.1% HCl, concentrated to dryness, and redissolved in ethanol/water (50:50 v/v). Fifteen microliters of the sugar fraction was spotted on a Whatman K5 silica gel (Alltech Associates, Deerfield, IL) TLC plate (20 cm × 20 cm, activated for 60 min at 105 °C) along with carbohydrate standards. After development with 2-propanol/acetone/0.1 M lactic acid (4:4:2 v/v), the plate was air-dried and the sugar spots were visualized by spraying with aniline/diphenylamine/acetone/80% phosphoric acid (4 mL:4 g:200 mL:30 mL) and heating for 30 min at 105 °C (Hansen, 1975).

Preparation of Methyloxime-Trimethylsilyl (MO-TMS) Derivatives of Carbohydrates for GC and GC/MS Analyses. Carbohydrate samples were isolated using C_{18} SPE cartridges using the same procedure as for analyses of sugars by TLC. A mixture of 125 μL of dry pyridine (Mallinckrodt, St. Louis, MO) and 1 mg of *O*-methylhydroxyamine−HCl (TCI America, Portland, OR) was added to 1 mg of each carbohydrate sample and standard. Carbohydrate standards were obtained from the Sigma Chemical Co. (St. Louis, MO). The solutions were mixed well, heated for 2 h at 40 °C, and then allowed to stand overnight at room temperature. Pyridine was removed in a stream of nitrogen while the sample was heated to 40 °C. When the solution approached dryness, 2 drops of benzene was added and evaporation continued to dryness to azeotropically remove the last trace of water formed in the oximation reaction. One hundred microliters of a 99:1 mixture of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) plus trimethylchlorosilane (TMCS) (Sylon BFT, Supelco, Inc., Bellefonte, PA) was added to each sample. The mixtures were shaken well, heated for 2 h at 40 °C, and allowed to stand overnight at room temperature. This procedure is a modification of the method of Laine and Sweeley (1971, 1973) for the preparation of MO-TMS derivatives of aldoses, partially methylated aldoses, deoxyaldoses, and ketoses.

Capillary GC. An HP 5890 Series II gas chromatograph equipped with a flame ionization detector (FID) was used. A DB-1 fused silica capillary column (60 m × 0.32 mm i.d.; d_f = 0.25 μm; J&W Scientific, Folsom, CA) was employed. The linear velocity of the helium carrier gas was 32 cm/s (30 °C). Split injection was used (1:24). The oven temperature was programmed from 30 (4 min isothermal) to 110 °C at 8 °C/min and then to 200 °C at 2 °C/min (final time 35 min). The injector and detector temperatures were 190 and 300 °C,

respectively. An HP 3365 GC ChemStation (version B.02.02) was used for instrument control and data processing.

Capillary GC/MS. An HP 5890 gas chromatograph equipped with a split/splitless injector was coupled to an HP 5970B mass selective detector (capillary direct interface). The instrument was controlled and data were processed by an HP ChemStation (G1701AA version A.03.00). A 60 m × 0.25 mm i.d. ($d_f = 0.25\ \mu$m) DB-1 fused silica capillary column was used with the following temperature program: 30 (4 min isothermal) to 110 °C at 8 °C/min then to 200 °C at 2 °C/min (final time 40 min). Helium carrier gas was employed at a column headpressure of 10 psi. Splitless injection was used (purge delay time was 45 s). The injector temperature was 190 °C, and the transfer line temperature was 200 °C.

Electrospray Ionization MS. The mass spectrometer was an Extrel C-50/LQ-400 hybrid single-quadrupole (Pittsburgh, PA), fitted with an electrospray source from Analytica of Branford, Inc. (Branford, CT). Purified anthocyanins were diluted to a suitable volume in a solution of water/acetonitrile/acetic acid (40:10:50 v/v) and then introduced into the instrument at a flow rate of 1.3 μL/min via a syringe pump (Harvard Apparatus, Model 11, Southnatick, MA). Data were collected in positive ion mode.

NMR Spectroscopy. NMR spectra were taken on a Bruker ARX 400 spectrometer (400 MHz) equipped with a 5 mm ^{1}H/^{13}C dual probe.

RESULTS AND DISCUSSION

Anthocyanin Content of Black Beans. Anthocyanins were extracted from UI 911 black beans using acidified methanol (0.5% HCl in methanol). Other nonmineral acids such as citric acid were also used in combination with methanol since HCl may cause the hydrolysis of labile acyl groups (i.e. malonic acid) during the extraction procedure (Strack and Wray, 1989; Andersen and Fossen, 1995; Fossen and Andersen, 1997). However, no evidence of acylation was found using the milder extraction procedures, and HCl was used in subsequent extractions due to its higher extraction efficiency.

The total anthocyanin content of black beans (average of three determinations) was 213 ± 2 mg/100 g of beans, a level comparable to those reported in fruit such as blueberries (130 mg/100 g; Francis, 1989), Saskatoon berries (180 mg/100 g; Francis, 1989), bog whortleberries (256 mg/100 g; Andersen, 1987a), Royal Okanogan huckleberries (350 mg/100 g; Price and Wrolstad, 1995), and crowberries (300−420 mg/100 g; Karppa et al., 1984). Moisture content of the black beans was 10.04 ± 0.02%, whereas the berries were probably all >90% water. The anthocyanins in black beans appear to occur exclusively in the seed coat, which comprised 8.98 ± 0.17% of the whole bean. Thus, the anthocyanin content in the seed coat of black beans is about 2.37 g/100 g of seed coat or 2.37%.

HPLC Separation of Black Bean Anthocyanins and Anthocyanidins. The HPLC chromatogram of black bean extract detected in the visible spectral region (520 nm) revealed three major anthocyanins with retention times of 6.5, 9.7, and 13.5 min (Figure 1). A trace amount of unknown anthocyanin appeared as a small peak eluting before peak 1. The UV−vis on-line spectra using a diode array HPLC detector showed that all three anthocyanins had their λ_{max} in the 525 nm region. None of the pigments were acylated with aromatic acids as evidenced by the absence of peaks in the 300−350 nm region (Andersen, 1985). Acid hydrolysis of the acidified methanol extract of black beans showed three anthocyanidins with retention times of 13.5, 21.3, and 29.6 min (Figure 2). Tentative identification of the anthocyanidins was achieved by analyzing anthocyanidins obtained from natural sources. Concord grapes contain five of the six common anthocyanidins. Analysis of Concord grape puree anthocyanidins gave five well-separated peaks with retention times of 13.5, 18.8, 21.3, 27.6, and 29.6 min. Previous studies indicated that the elution order of these five aglycons was as follows: delphinidin, cyanidin, petunidin, peonidin, and malvidin (Wulf and Nagel, 1978; Wilkinson et al., 1977; Casteele et al., 1983; Hong and Wrolstad, 1986). Cochromatography of the anthocyanidins of black beans and Concord grape puree showed that the three aglycons of black beans had identical retention times with three from acid-hydrolyzed Concord grape puree. It was concluded that the acidified methanol extract of black beans contained three anthocyanins with three different anthocyanidins, tentatively identified as the glycosides of delphinidin, petunidin, and malvidin.

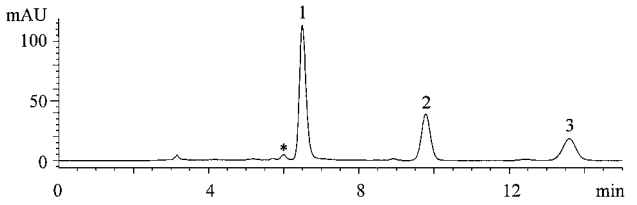


Figure 1. HPLC chromatograms (detection at 520 nm) of black bean anthocyanins. Conditions: column, Keystone ODS/B 25 × 4.6 mm i.d.; solvent A, 10% formic acid; solvent B, formic acid/methanol/water (10:40:50 v/v); linear gradient from 40% to 80% solvent B in 50 min. Peaks: 1, delphinidin 3-glucoside (56%); 2, petunidin 3-glucoside (26%); 3, malvidin 3-glucoside (18%); *, unknown anthocyanin.

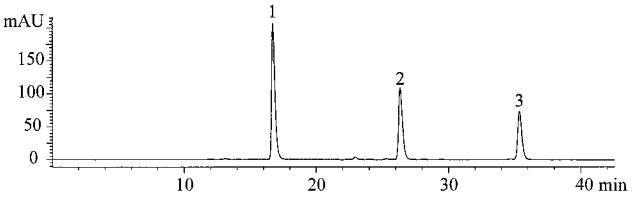


Figure 2. HPLC chromatogram (detection at 520 nm) of black bean anthocyanidins (aglycons). Conditions were as in Figure 1. Peaks: 1, delphinidin; 2, petunidin; 3, malvidin.

The on-line UV−vis spectrum of the three anthocyanins from analytical HPLC showed a visible maximum at the 525 nm region. The ratios of A_{440}/A_{max} of peak 1 = 29%, peak 2 = 29.4%, and peak 3 = 29.2% indicated the presence of 3-glycosides of delphinidin, petunidin, and malvidin, respectively (Harborne, 1967).

Identification of Glucose from the Acid Hydrolysis of Black Bean Anthocyanins. Individual anthocyanins were isolated by preparative HPLC and then subjected to acid hydrolysis to liberate anthocyanidins and carbohydrates. The carbohydrates were isolated from the other hydrolysis products using SPE. TLC of carbohydrate samples and standards showed that all three isolates showed one blue spot with an R_f value of 0.45, identical to that of the D-glucose standard. The carbohydrates in the three hydrolysates were converted to MO-TMS derivatives and analyzed by capillary GC. The GC chromatograms of all three fractions showed two peaks (one major and one minor; peak area ratio was about 5.8:1) with Kovats indices of 1949 (major peak) and 1967 (minor peak), which had the identical retention indices and peak area ratios as the derivatized D-glucose standard. The two peaks had nearly identical mass spectra and were believed to be the *syn* and *anti* isomers of the *O*-methyloxime (Laine and Sweeley, 1971, 1973). The mass spectra of the MO-

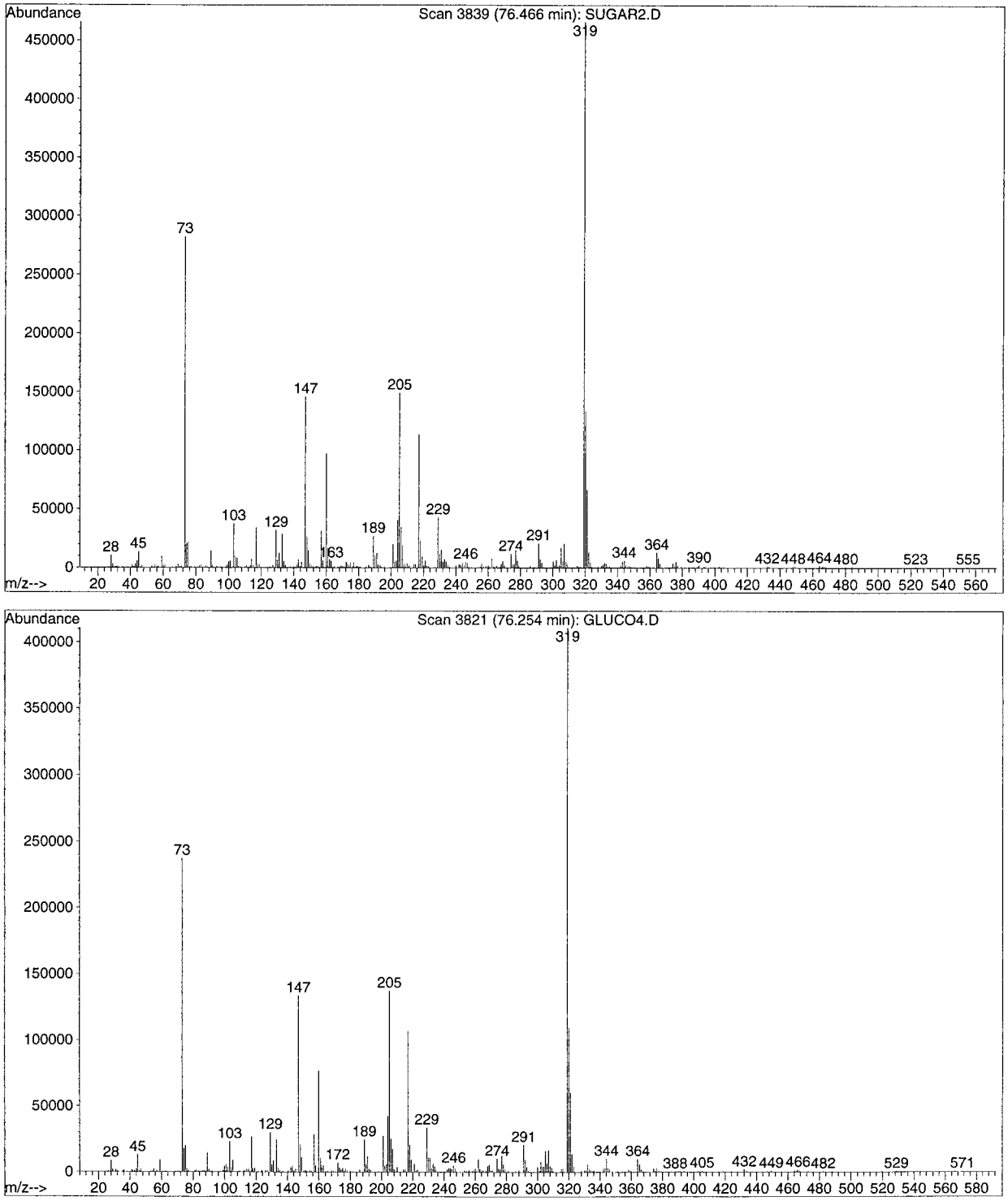


Figure 3. Mass spectra of the MO-TMS derivatives of the sugar released by the hydrolysis of **1** (top) and the D-glucose standard (bottom). The conditions are described under Experimental Procedures.

TMS derivatives of the carbohydrates liberated from the isolated anthocyanins closely matched those of the D-glucose standard (Figure 3). These data indicated that only D-glucose was present in all three hydrolysis fractions. Therefore, the major anthocyanins in black beans are delphinidin 3-glucoside (**1**, 56%), petunidin 3-glucoside (**2**, 26%), and malvidin 3-glucoside (**3**, 18%) (Figure 4).

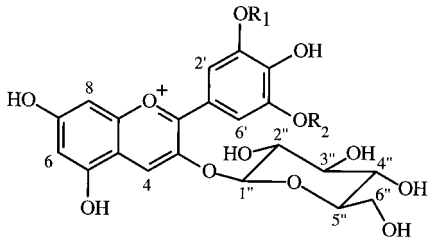
To confirm the identities of these aglycons and the sugar, MS and NMR spectroscopy were applied. The three anthocyanins isolated using preparative HPLC were rechromatographed to improve their purity. Anthocyanins were checked by analytical HPLC before they were subjected to MS and NMR studies.

MS. Individual anthocyanins were diluted to a suitable volume in a solution of water/acetonitrile/acetic acid (40:10:50 v/v). The electrospray (ES) mass spectra of the purified black bean anthocyanins exhibited the molecular peaks [M^+] as the flavylium cations of the intact constituents and the aglycon fragments, resulting from the loss of glucose. The ES mass spectrum of the major anthocyanin **1** (delphinidin 3-glucoside) showed the molecular cation M^+ at *m/z* 465 [in good agreement with the mass calculated for $C_{21}H_{21}O_{12}$ (*m/z* 465.394

Table 1. ^{1}H NMR Spectral Data for Black Bean Anthocyanins (in CD_3OD/CF_3CO_2D, 95:5, at 25 °C)

	1, δ_H (*J*, Hz)	ref[a]	**2**, δ_H (*J*, Hz)	**3**, δ_H (*J*, Hz)	ref[b]
anthocyanidin					
4	8.96, 1H, s	8.76	9.00, 1H, s	9.05, 1H, s	9.04
6	6.65, 1H, d (2.0)	6.67	6.67 1H, d (2.0)	6.69, 1H, d (2.0)	6.67
8	6.87, 1H, dd (2.0, 0.8)	6.73	6.91, 1H, dd (2.0, 0.8)	6.97, 1H, dd (2.0, 0.8)	6.99
2′	7.76, 1H, s	7.74	7.96, 1H, d (2.0)	8.00, 1H, s	8.00
6′	7.76, 1H, s	7.74	7.78, 1H, d (2.0)	8.00, 1H, s	8.00
3′,5′-OCH_3			4.00, 3H, s	4.02, 6H, s	4.01
glucose					
1	5.32, 1H, d (7.6)	5.25	5.35, 1H, d (7.6)	5.36, 1H, d (7.6)	5.36

[a] Delphindin 3-glucoside run in 35% CF_3CO_2D/65% DMSO-d_6 (Anderson, 1988). [b] Malvidin 3-glucoside run in CD_3OD containing 0.5% v/v 20% DCl in D_2O (Bakker and Timberlake, 1985).



1 $R_1 = R_2 = H$

2 $R_1 = CH_3$; $R_2 = H$

3 $R_1 = R_2 = CH_3$

Figure 4. Structures of delphinidin 3-glucoside (**1**), pentunidin 3-glucoside (**2**), and malvidin 3-glucoside (**3**).

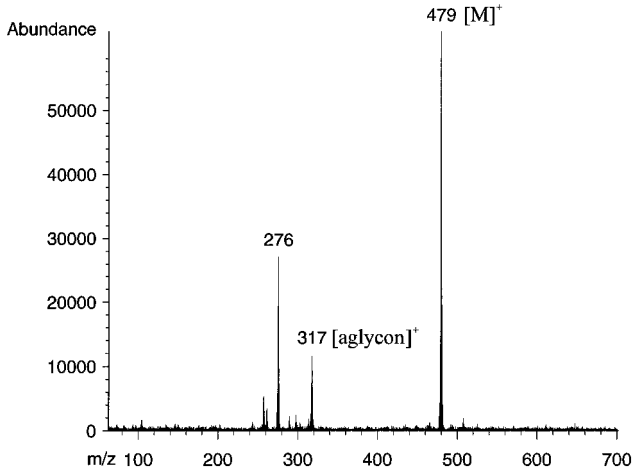


Figure 5. Electrospray mass spectrum of petunidin 3-glucoside (**3**) isolated from black beans by SPE and preparative HPLC.

m/z)] and an ion at *m/z* 303 corresponding to the aglycon delphinidin, which was formed by loss of glucose. The ion at *m/z* 479 (the mass calculated for $C_{22}H_{23}O_{12}$ was *m/z* 479.421) for **2** (petunidin 3-glucoside) represented the molecular cation M^+, while the ion at *m/z* 317 was the petunidin cation (Figure 5). The third anthocyanin **3** (malvidin 3-glucoside) displayed ions at *m/z* 493 [corresponding to the mass calculated for $C_{23}H_{25}O_{12}$ (*m/z* 493.448)] and 331 representing the molecular cation M^+ and the malvidin cation, respectively.

NMR Spectroscopy. Further, ^{1}H NMR measurements confirmed the identities and structures of delphinidin 3-glucoside, petunidin 3-glucoside, and malvidin 3-glucoside (Table 1). The ^{1}H NMR of **1** showed the presence of delphinidin. Analysis of the ^{1}H−^{1}H COSY spectrum revealed that H-4 (δ 8.96) of the flavylium nucleus is spin-coupled with H-8 (δ 6.87). The small coupling (0.8 Hz) was consistent with long-range coupling (via a zigzag coupling path) which has also been observed between the H-4 and H-8 protons in cyanidin 3-sambubioside (Andersen et al., 1991). The large coupling (7.6 Hz) of the anomeric proton (δ 5.32) indicates a β configuration of the sugar. The presence of glucose in **1** was confirmed by ^{13}C NMR observing the following chemical shifts (δc in ppm; spectrum was determined in CD_3OD/CF_3CO_2D, 95:5 at 100 MHz) for the sugar moiety: C-1″ (103.8), C-2″ (74.8), C-3″ (78.1), C-4″ (71.1), C-5″ (78.9), C-6″ (62.4). The ^{1}H NMR spectrum of **2** showed clear evidence of petunidin. In contrast to **1** and **3** the H-2′ (δ 7.96) and H-6′ (δ 7.78) proton signals of **2** are separated, reflecting their lack of symmetry of the B-ring about the C-1′—C-4′ axis. The β configuration of the sugar was evidenced by the large coupling (7.6 Hz) of the anomeric proton (δ 5.35). To our knowledge, these are the first reported ^{1}H NMR data on petunidin 3-glucoside. The ^{1}H NMR spectrum of **3** closely matched that previously reported by Bakker and Timberlake (1985) for malvidin 3-glucoside. The presence of glucose in **3** was confirmed by ^{13}C NMR observing the following chemical shifts (δ in ppm; spectrum was determined in CD_3OD/CF_3CO_2D, 95:5, at 100 MHz) for the sugar moiety: C-1″ (104.1), C-2″ (75.2), C-3″ (78.4), C-4″ (71.4), C-5″ (79.1), C-6″ (62.6).

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