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Characterization of Polyphenolics in the Seed Coat of Black Jamapa Bean (*Phaseolus vulgaris* L.)

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The common bean contains phytochemicals, including phenolic compounds, which can provide health benefits to the consumer. Our objective was to characterize the polyphenolic compounds present in the seed coat of Black Jamapa bean and to test fractionation methods that permit the recovery of polyphenolics in their naturally occurring forms. A 100% methanol extract from seed coats was subjected to different chromatographic fractionation methods. Analysis by HPLC-MS revealed that a better separation of phytochemicals was achieved using direct silica gel fractionation, which allowed more accurate identification of compounds, especially of the flavonols. Anthocyanins, flavanol monomers, and heterogeneous flavanol oligomers up to hexamers were detected. To our knowledge, this is the first time that myricetin glycoside and proanthocyanidin oligomers containing (epi)-gallocatechin have been reported in the black bean. The fractionation methods used in this study produced large quantities of natural mixtures of flavonoids suitable for testing bioactivity and phytochemical interactions.

KEYWORDS: ***Phaseolus vulgaris*; proanthocyanidins; anthocyanins; vacuum liquid chromatography; HPLC-MS**

INTRODUCTION

The dry common bean (*Phaseolus vulgaris* L.) is widely consumed throughout the world. It is recognized for high protein content and is an important source of energy, vitamins, and minerals especially in many Latin-American and African countries (*1*). In addition to the nutritional components, the common bean is rich in a variety of phytochemicals with potential health benefits such as soluble fiber and polyphenolic compounds (*2*).

A large variability exists in common bean seeds; seed color and size are the two most important quality characteristics for consumers. The seed color of beans is determined by the presence and concentration of flavonol glycosides, anthocyanins, and condensed tannins (proanthocyanidins) (*3*). The most widely distributed group of flavonoids in beans is proanthocyanidins (*3*−*5*); the presence of anthocyanins has only been reported in black and blue-violet colored beans (*3*, *6*). Proanthocyanidins have been detected in different varieties of common bean (9.4−37.8 mg catechin equivalents per g), mainly in the seed coat (*3*−*5*, *7*, *8*); however, there is relatively little comprehensive research on the proanthocyanidin profile present in a bean. Recently, Gu et al. (*9*, *10*) identified heterogeneous B-type proanthocyanidins containing (epi)afzelechin as subunits in pinto beans, using electrospray ionization (ESI) mass spectrometry (MS) in the negative mode following separation by normal phase high-performance liquid chromatography.

Flavonoid compounds in beans are reported to have biological activity in vitro as well as in vivo. Flavonoids extracted from beans, mainly anthocyanins and proanthocyanidins, have shown antioxidant (*3*, *11*, *12*) and antimutagenic (*7*, *8*, *13*) activities. Recently, red beans were identified as having one of the highest antioxidant capacities (as measured in the ORAC assay) among over 100 common dietary fruits and vegetables examined (*14*). Epidemiological studies suggest that the consumption of flavonoid-rich foods protects against human diseases associated with oxidative stress, like coronary heart disease and cancer (*15*−*17*). In vitro, flavonoids from several plant sources have shown free-radical scavenging activity and protection against oxidative stress (*18*, *19*), protection against irradiation-induced cell damage (*20*), protection against chemical-induced cellular transformation (*21*), and selective growth inhibitory activity of cancer cells (*22*−*25*).

The biological activity of flavonoids depends on the types of phytochemical constituents and the complexity of their

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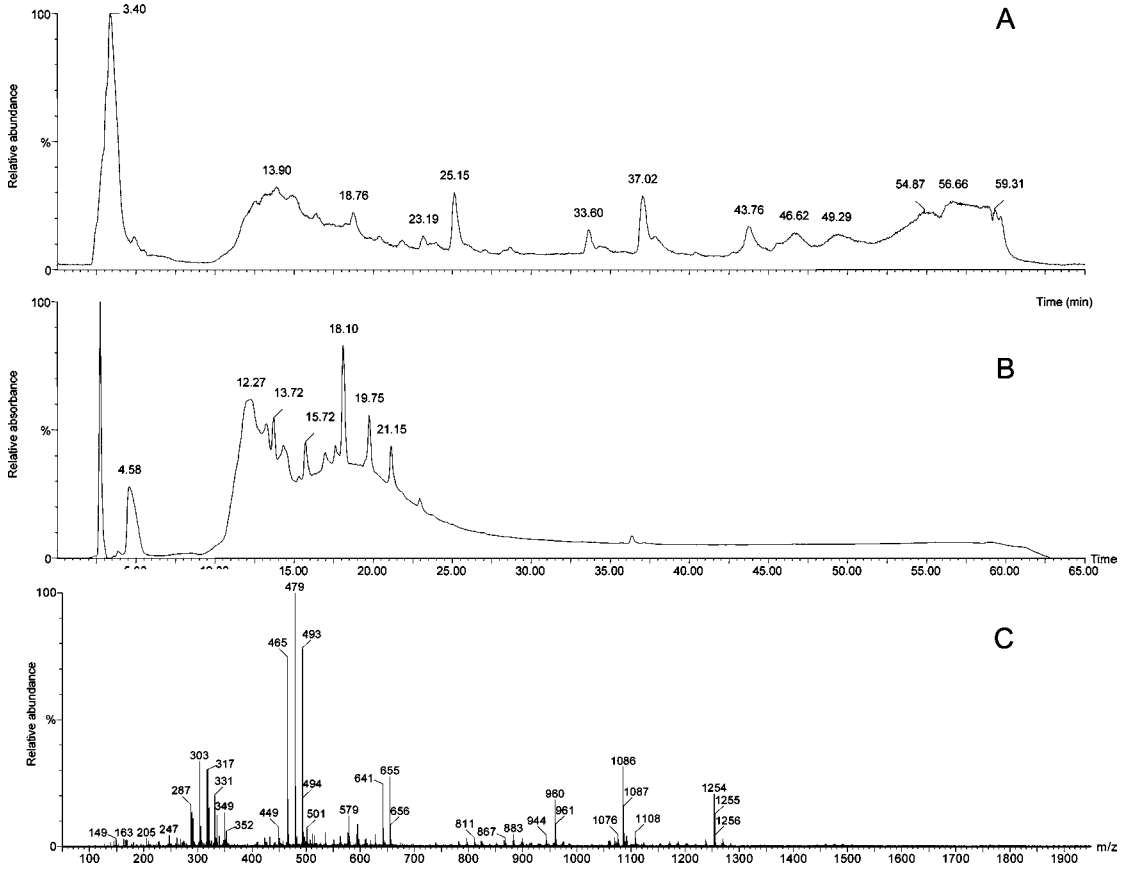

Figure 1. HPLC-MS output of 100% methanol extract including (**A**) the total ion current, (**B**) photodiode array chromatogram, 200–600 nm, and (**C**) ESI full mass spectrum, positive mode, *m*/*z* 150–2000. Flavonoid compounds were eluted before 40 min as shown by UV maximum absorption (**B**) while nonflavonoid compounds did not have UV absorption in the 200–600 nm range.

structures (*21*, *25*) and the composition of the flavonoid mixtures, since it has been well established that complex mixtures of phytochemicals in fruits and vegetables may provide protective health benefits mainly through a combination of additive or synergistic effects (*26*, *27*). Therefore, it is important to establish fractionation methods that preserve the integrity of the structural complexes and that keep phenolic compounds in the same ratios as they are found in the original food.

Proanthocyanidin extraction from fruits has been commonly performed using 70% aqueous acetone as solvent; sometimes, a low percentage of an organic acid is added to favor the dissolution and extraction of anthocyanins as well (*9*, *10*, *28*, *29*). Cardador-Martinez et al. (*7*) reported that 100% methanol could be an ideal solvent for condensed tannin extraction in beans, as evaluated by the vanillin quantification method. To analyze the composition of crude extracts from beans, it is necessary to fractionate them into simpler mixtures. However, the process of subfractionation incurs the risk of degrading the compound's natural isomeric structures and changing the natural proportions of interacting phytochemicals. As a result, bioactivity can be attenuated in the course of chemical characterization. Proanthocyanidin and anthocyanin molecules can be sensitive to routine extraction methods and column chromatography. Commonly, HPLC is used for the separation and identification of polyphenolics. However, this involves the incorporation of acids into the extracts or mobile phase that can alter the chemical structure of polyphenolics (*7*, *9*, *10*). Also, the HPLC technique is only suitable for fractionating small quantities of plant extracts and can result in the loss of the biological activity of fractions (*30*).

Recently, various substrates have been compared for vacuum liquid chromatography of crude extracts rich in flavonoids and for obtaining larger amounts of fractions enriched in particular proanthocyanidin oligomers or mixtures of compounds with biological activity (*29*−*31*). The aim of this study was to characterize the polyphenolic compounds present in the seed coat of the Black Jamapa bean and to identify fractionation methods that permit a better characterization of proanthocyanidins and other major flavonoids in their natural state.

MATERIALS AND METHODS

Plant Material and Standards. The Black Jamapa common bean (*Phaseolus vulgaris* L. "Black Jamapa") was grown in 2003 at "El Bajío" Experimental Station of the National Research Institute for Forestry Agriculture and Livestock (INIFAP), Mexico. The mature dry seed was stored at −20 °C until separation of seed coats. The seed coats were lyophilized and stored in the dark at −20 °C until extraction and further analysis. Authentic commercial standards including anthocyanins and proanthocyanidins were used to compare retention times. The anthocyanin standards (delphinidin, petunidin, and malvidin glycosides) were obtained from Polyphenols Laboratories (Sandnes, Norway) and proanthocyanidin monomers and dimers, kaempferol, quercetin, and myricetin glycosides were obtained from Chromadex (Laguna Hills, CA).

Extraction of Polyphenolics from Jamapa Bean Seed Coats. To compare the two most commonly reported methods of proanthocyanidin extraction, samples of lyophilized seed coats from the same lot of beans were submitted, independently, to methanol (100%) and acetone extraction (70% aqueous acetone).

Methanol Extraction. The protocol for the extraction of phenolic compounds was previously described by Cardador-Martínez et al. (*7*).

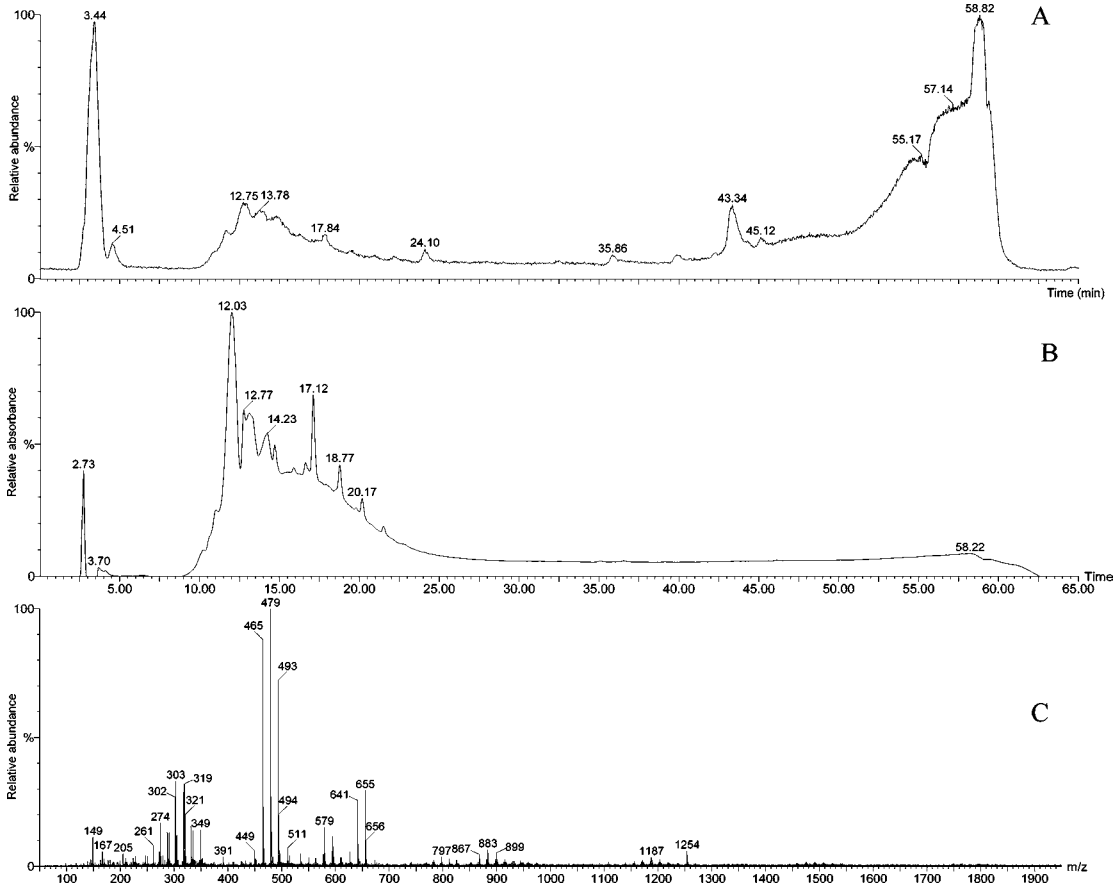

Figure 2. HPLC-MS output of 70% aqueous acetone extract including (**A**) the total ion current, (**B**) photodiode array chromatogram, 200–600 nm, and (**C**) ESI full mass spectrum, positive mode, *m*/*z* 150–2000. Flavonoid compounds were eluted before 40 min as shown by UV maximum absorption (**B**) while nonflavonoid compounds did not have UV absorption in the 200–600 nm range.

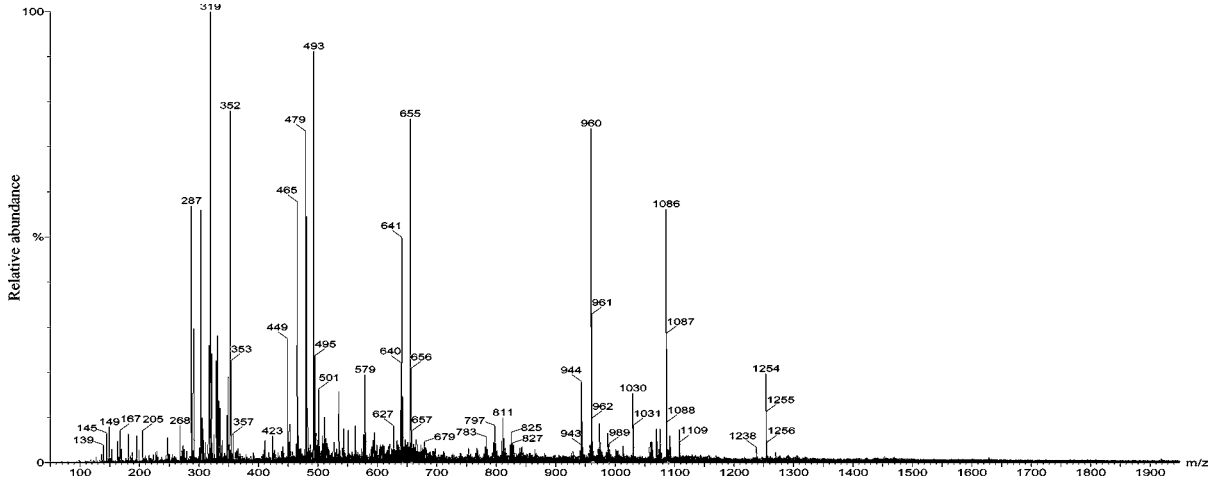

Figure 3. MS output of fraction TP3 from 100% methanol extract including the ESI full mass spectrum (*m*/*z* 150–2000), showing anthocyanins delphinidin 3-glycoside (*m*/*z* 465), petunidin 3-glycoside (*m*/*z* 479), malvidin 3-glycoside (*m*/*z* 493), and proanthocyanidin dimer *(m*/*z* 579) among other polyphenolics in this fraction.

Lyophilized ground seed coats (70 g) were placed in a flask and mixed with 100% methanol (1:50, w/v ratio). The flask was shaken for 24 h at 20 °C while wrapped in aluminum foil to protect extract from light. The sample was then filtered using Whatman #4 paper and methanol was removed under reduced pressure. The methanol extract was lyophilized and stored in the dark at −20 °C until HPLC-MS analysis.

Acetone Extraction. One gram of lyophilized ground seed coat was sonicated five times with 70% aqueous acetone (15 mL, 4 min each, followed by filtration on Whatman #4). By the fifth extraction, there were no more colored substances in the solvent. The extracts were pooled, the solvent was evaporated under reduced pressure, and the sample was lyophilized and stored in the dark at −20 °C until HPLC-MS analysis.

Isolation of Polyphenolics for Identification. Three fractionation methods were independently applied to separate samples of the 100% methanol extract, using vacuum liquid chromatography (VLC) with different column supports including fractionation on Toyopearl (TP), fractionation on Toyopearl followed by silica gel chromatography (TPSG), and direct fractionation on silica gel (SG).

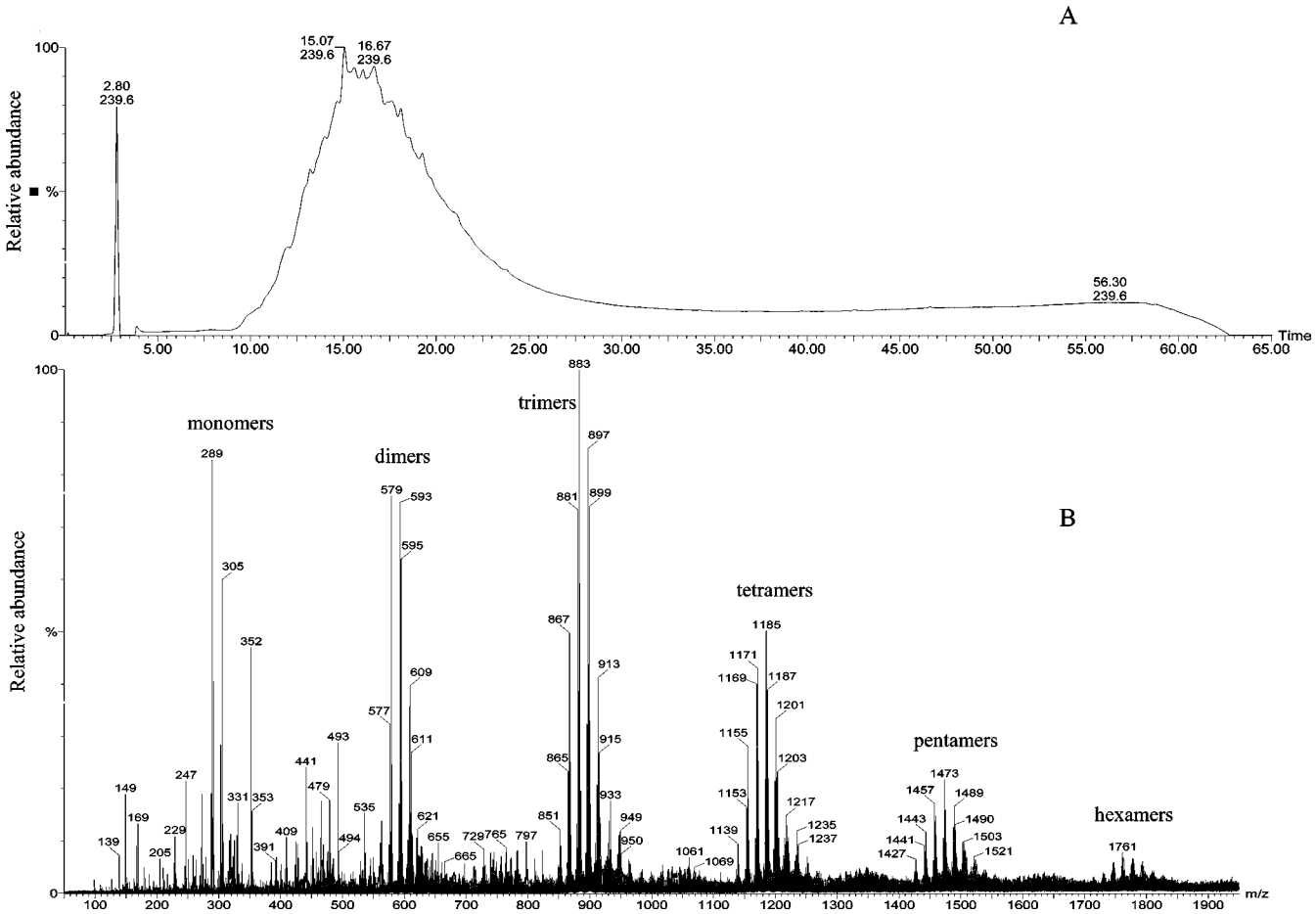

Figure 4. HPLC-MS output of fraction TP5 from 100% methanol extract including (**A**) the photodiode array chromatogram, 200–600 nm, and (**B**) ESI full mass spectrum, *m*/*z* 150–2000.

(a) Fractionation on Toyopearl (TP). Lyophilized methanol extract (0.5 g) dissolved in about 15 mL water was fractionated by VLC on HW-F40 Toyopearl resin polymer (150 g) (TOSOH Bioseparation Specialists, Montgomeryville, PA) using a sequential series of solvents as follows: (1) water, (2) 50% aqueous methanol, (3) 100% methanol, (4) methanol:acetone (1:1; v/v), (5) 100% acetone, and (6) 50% aqueous acetone (*29*−*30*). The first fraction volume was 250 mL, while the rest of the fractions were 500 mL. By the end of fractionation, all colored substances had been removed from the column. The six fractions obtained (coded TP1, TP2, TP3, TP4, TP5, and TP6) were evaporated under reduced pressure, lyophilized, and stored at −20 °C until HPLC-MS analysis.

(b) Fractionation on Toyopearl Followed by Silica Gel (TPSG). The lyophilized methanol extract (0.5 g) was subjected to Toyopearl VLC fractionation (150 g) as previously indicated, and then fractions TP2, TP3, TP4, TP5, and TP6 were remixed, the solvent was removed under reduced pressure, and the sample was lyophilized. The lyophilized sample was loaded onto a silica gel column (100 g; silica gel type 60, 10−40 μm with $CaSO_4$ binder) (Sigma). From the VLC on silica gel, 22 fractions were obtained as follows: (1) 100% petroleum ether, (2) petroleum ether:ethyl acetate (1:1, v/v), (3) 100% ethyl acetate, solvents used for fractions 4−19 were ethyl acetate with an increasing gradient of methanol and water (1:1, v/v) (2%, 5%, 8%, 12%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90% of methanol:water (1:1, v/v), respectively), (20) methanol:water (1:1, v/v), (21) 100% MeOH, (22) 100% water (*29*). From the series of 22 subfractions (100 mL each) collected, 7 major fractions were created by recombination of adjacent subfractions with similar composition on the basis of TLC (thin-layer chromatography) profiling and R_f values of the spots observed after vanillin-HCl reaction as described below (fractions TPSG 1−5, TPSG 6−8, TPSG 9−11, TPSG 12−14, TPSG 15−16, TPSG 17−19, and TPSG 20−22).

(c) Direct Silica Gel Fractionation (SG). The 100% methanol extract (1 g) was directly subjected to silica gel VLC; from the series of subfractions created, six major fractions were obtained by recombining adjacent subfractions with similar composition (fractions SG 1−3, SG 4−8, SG 9−12, SG 13−17, SG 18−20, and SG 20−22) as described above. The major recombined fractions were evaporated under reduced pressure, lyophilized, and stored at −20 °C until HPLC-MS analysis.

Thin-Layer Chromatography of Extracts and Fractions. Thin-layer chromatography (TLC) was used routinely during fractionation to monitor the chromatographic separation of phenolic compounds. All extracts and fractions were tested by TLC using 200-μm thick, 2−25 μm particle size, 60 Å silica gel plates on aluminum (Sigma-Aldrich, Germany) run with a solvent ratio of ethyl acetate/methanol/water (79:11:10) as a qualitative test to detect the presence of anthocyanins and proanthocyanidins (*29*). TLC plates were visualized separately with the spray reagents dichromate solution and vanillin-HCl and were followed by heating at 100 °C for 10 min. The first reagent is used as a qualitative measure of organic material in samples, while the second one reacts with flavan 3-ols producing a red coloration indicating the presence of proanthocyanidins (*32*).

HPLC-MS Analysis of Composition of Flavonoid-Rich Fractions. Five mg of each lyophilized sample (crude 100% methanol and 70% aqueous acetone extracts, TP, TPSG, or SG recombined fractions) was dissolved in 1 mL aqueous methanol (1:1; v/v) and used for HPLC-MS analysis. Commercial standards were prepared at a concentration of 0.5 mg/mL in 50% methanol. The HPLC-MS analyses were made with an LCQ Deca XP mass spectrometer (Thermo Finnigan Corp., San Jose, CA), MS version 1.3 SRI, electrospray ionization (ESI) in the positive ion mode (*m*/*z* 150−2000), with a photodiode array (PDA) detector (200−600 nm), version 1.2, autosampler version 1.2, and Xcalibur software for data processing. The HPLC separations were

Table 1. Approximate Ratio of Main Flavonoids Detected by HPLC-MS in Bean 100% Methanol Extract Obtained Using Toyopearl Followed by Fractionation on Silica Gel (TPSG)

fraction	1–5	6–8	9–11	12–14	15–16	17–19	20–22
	%[a]						
	Proanthocyanidins[c]						
monomers (C, GC)			6.7	2.5			
dimers (A, C, GC)			35.6	9		0.6	
trimers (A, C, GC)			25.2	22.1	1.3		
tetramers (A, C, GC)			6.3	18.6	3.1	10.5	5.6
pentamers (A, C, GC)				8.6	5.9		
hexamers (A, C, GC)				4.5	4.1		
total			73.8	65.3	14.4	11.1	5.6
	Anthocyanins						
delphinidin 3-glycoside				9.3	10.5	8.8	14.5
petunidin 3-glycoside				7.6	7.8	5.2	7.6
malvidin 3-glycoside				9.3	5.7	3.1	4
petunidin diglycoside					4	7.1	6.9
malvidin diglycoside			3.2		6	7.3	5.8
total			3.2	26.2	30.9	31.5	38.8
	Flavonols						
kaempferol glycoside			8.5	3.5			
total			8.5	3.5			
not identified[b]					41.5	30.8	29.5

[a] Values presented to illustrate the relative abundance of compounds, estimated on the basis of corresponding MS peak heights as explained in text. [b] Unidentified compounds of *m*/*z* 1069.4, 1085.4, 1253.3, and 1269.3. [c] C = catechin/epicatechin; GC = gallocatechin/epigallocatechin; A = afzelechin/epiafzelechin.

Table 2. Approximate Ratio of Main Flavonoids Detected by HPLC-MS in Bean 100% Methanol Extract Obtained Using Direct Silica Gel Fractionation (SG)

fraction	1–3	4–8	9–12	13–17	18–20	21–22
	%[a]					
	Proanthocyanidins[c]					
monomers (C, GC)		13.5				
dimers (A, C, GC)		44.7	15.6			
trimers (A, C, GC)		8.3	24.7			
tetramers (A, C, GC)		12	12.1	6.4	7.3	8.2
pentamers (A, C, GC)		0.8	4.9	6.0		
hexamers (A, C, GC)		5.7	4.6	5.1		
total		85	61.9	17.5	7.3	8.2
	Anthocyanins					
delphinidin 3-glycoside			4.1	24.3	6.9	6.1
petunidin 3-glycoside			3.9	16.9	3.1	2.6
malvidin 3-glycoside			4.6	11.8	1.5	1.5
petunidin diglycoside				12	3.1	3.3
malvidin diglycoside				10	3.1	3.1
total			12.6	56.2	17.7	16.5
	Flavonols					
kaempferol glycoside			5.8			
quercetin 3-glycoside			4.1			
myricetin glycoside			6.7			
total			16.6			
not identified[b]				23.1	59.9	58.4

[a] Values presented to illustrate the relative abundance of compounds, estimated on the basis of corresponding MS peak heights as explained in text. [b] Unidentified compounds of *m*/*z* 1069.4, 1085.4, 1253.3, and 1269.3. [c] C = catechin/epicatechin; GC = gallocatechin/epigallocatechin; A = afzelechin/epiafzelechin.

carried out on a 150 × 2.1 mm i.d., 5-μm C_{18} reversed-phase column Vydac, Western Analytical, Murrieta, CA. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). A step gradient of 0%, 5%, 50%, 80%, 100%, and 0% of solvent B at 3, 4, 30, 42, 50, and 58 min, respectively, a flow rate of 200 μL/min, and an injection volume of 10 μL were employed (*29*). The injected volume contained approximately 50 μg of each sample or 5 μg of each standard.

RESULTS AND DISCUSSION

Methanol versus Aqueous Acetone Extraction. Extracting bean seed coats using 100% methanol or 70% aqueous acetone resulted in about 5.7% and 16.6% of dry crude extract (extract g/seed coats g × 100), respectively. Although aqueous acetone extraction resulted in a higher percentage of crude extract, it appeared from HPLC-MS spectra that more nonflavonoid compounds were present, which may result in interference with bioactive phenolics in certain bioactivity assays. Initial testing using thin-layer chromatography (TLC) showed a higher color intensity and fluorescence of the spots in 100% methanol, and darker spots after treatment of TLC with dichromate reagent, from oxidation of the organic material in the extracts. The number and location of spots on TLC plates were similar between the two extraction methods. While 70% aqueous acetone is a widely reported solvent for proanthocyanidin extraction in many other foods (*9*, *10*, *29*), the 100% methanol extraction method was previously reported for flavonoid extraction in common bean seed coats and other legumes (*7*). We concluded that methanol extraction resulted in a cleaner crude extract, allowing easier identification and purification of polyphenolics from bean seed coats. In this study, subsequent fractionation and identification of polyphenolics in bean seed coats were performed using the lyophilized 100% methanol extract.

HPLC-MS spectra showed that flavonoid compounds were eluted before 40 min while nonflavonoid compounds eluted later (**Figures 1** and **2**). The figures show total ion current (TIC) which represents the relative abundance of all compounds in the extract, the maximum wavelength absorption at a range from 200 to 600 nm, and electrospray ionization (ESI) spectra plotted using a time range from 3 to 40 min. The phenolic compounds with 200−600 nm absorption were eluted from the HPLC column within this time range. HPLC-MS spectra indicated that compounds with the same elution characteristics and molecular weight were present in both methanol and acetone extracts, suggesting they were similar in composition.

Composition of Methanol Extracts. *(a) Toyopearl Fractionation (TP).* After fractionation of the 100% methanol extract of seed coats by vacuum liquid chromatography (VLC) on Toyopearl, the highest quantity of mass was found in fractions TP1 and TP2 (33% and 28% of total extract, respectively). Under UV light, TLC plates showed fluorescent spots on the six fractions indicating the presence of flavonoids. Anthocyanins in the fractions were examined under visible light where blue-purple spots on TP2, TP3, and TP4 were observed. Upon reaction with vanillin-HCl reagent (*31*), formation of red spots on TP3, TP4, TP5, and TP6 indicated the presence of proanthocyanidins.

HPLC-MS analysis revealed that Toyopearl fractions were very complex mixtures of flavonoid and nonflavonoid compounds. Some of the major compounds were characterized according to their *m*/*z* value, UV spectrum absorbance characteristics, retention time as compared to commercial standards, and comparison to previous reports on other food sources of polyphenols (*6*, *9*, *10*, *29*, *33*−*35*). Standard retention times were delphinidin glycoside (11 min), petunidin glycoside (13 min), malvidin glycoside (14 min), petunidin diglycoside (11 min), malvidin diglycoside (12 min), kaempferol glycoside (19 min), quercetin glycoside (12 min), myricetin glycoside (16 min), proanthocyanidin monomers (20 min), and proanthocyanidin dimers (11 min). The maximum UV absorption for anthocyanins

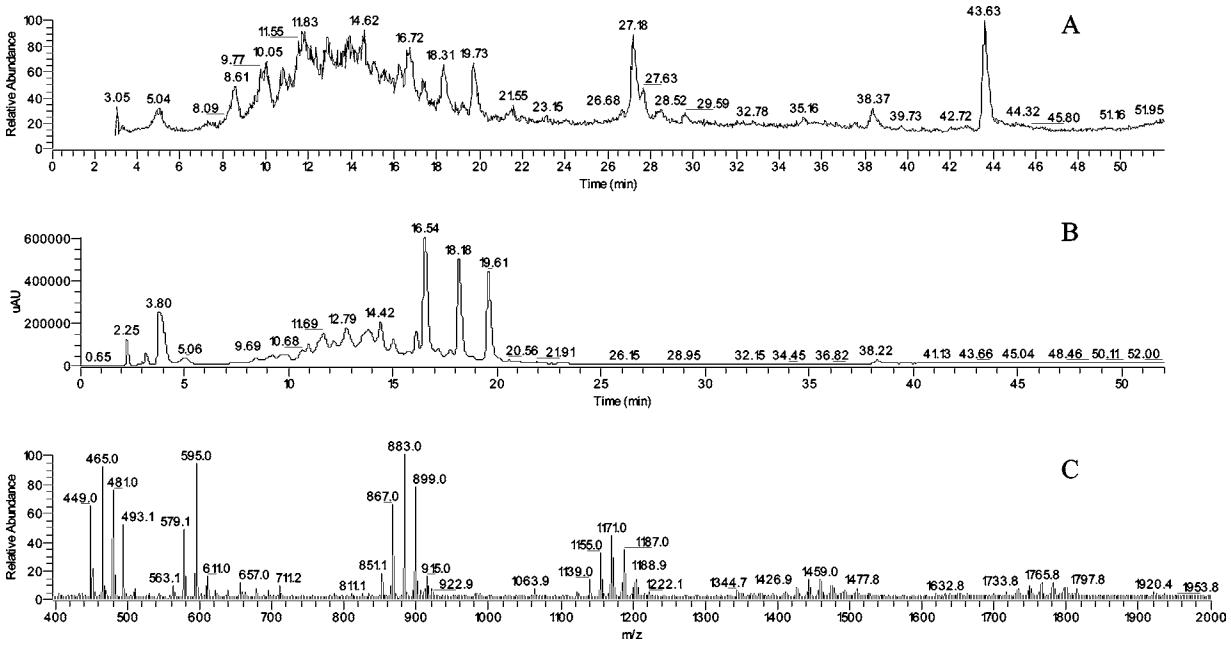

Figure 5. Representative HPLC-MS output of SG 9–12 fraction including (**A**) the total ion current, (**B**) photodiode array chromatogram, 200–600 nm, and (**C**) ESI full mass spectrum, positive mode, *m*/*z* 400–2000. Proanthocyanidins: dimers (*m*/*z* 563–611), trimers (*m*/*z* 851–899), tetramers (*m*/*z* 1139–1187). Anthocyanins: delphinidin 3-glycoside (*m*/*z* 465), petunidin 3-glycoside (*m*/*z* 479), and malvidin 3-glycoside (*m*/*z* 493). Kaempferol, quercetin, and myricetin glycosides, *m*/*z*: 449, 465, and 481, respectively.

was 235 and 525 nm; for proanthocyanidins, 280 nm; for kaempferol, quercetin, and myricetin glycosides, 360 nm.

Proanthocyanidin dimers in the form of catechin/epicatechin (*m*/*z* 579) and anthocyanins in the form of delphinidin 3-glycoside (*m*/*z* 465), petunidin 3-glycoside (*m*/*z* 479), and malvidin 3-glycoside (*m*/*z* 493) were identified in fraction TP3 (**Figure 3**). These results agree with previous reports on the presence of anthocyanins in black and blue-violet beans (*3*, *6*, *33*). Romani et al. (*6*) reported traces of diglycosylated anthocyanins in black Zolfino landraces (*P. vulgaris* L.). Other phenolic compounds with *m*/*z* values of 1069.4, 1085.4, 1253.3, and 1269.3 were detected in fraction TP3, with UV maximum absorption of 280 nm, which suggested they could be proanthocyanidin oligomers. The difference of 16 mass units between these compounds suggests that these compounds could be a series of structurally related compounds with the addition of an oxygen atom. Further research is needed to completely identify these compounds.

TP4−TP6 fractions contained higher concentrations of flavan-3-ol oligomers. TP4 appeared rich in heterogeneous dimers mainly composed of (epi)afzelechin, (epi)catechin, and (epi)-gallocatechin in addition to some anthocyanins. TP5 contained a mixture of proanthocyanidin oligomers up to trimers and smaller quantities of tetramers, pentamers, and hexamers. TP6 contained higher concentrations of tetramers, pentamers, and hexamers as well as (epi)gallocatechin monomers, dimers, and trimers compared to TP4 and TP5. Although HPLC reversed-phase C_{18} columns have the ability to separate oligomers of equivalent molecular weight into their isomers, isolation of higher oligomeric proanthocyanidins (≥ tetramers) was not feasible. These isomers coeluted in a large unresolved peak (**Figure 4**) because of overlapping of the isomers with different degrees of polymerization or steriochemistry (*29*, *36*).

(b) Toyopearl Fractionation Followed by Silica Gel Fractionation (TPSG). The percentages of dry weights of the major fractions TPSG 1−5, 6−8, 9−11, 12−14, 15−16, 17−19, and 20−22 were 4.4%, 0.4%, 2.2%, 4.7%, 25.7%, 35.2%, and 27.4%, respectively, recovered from 0.5 g of dry crude extract. The main flavonoids detected by mass spectrometry in TPSG fractions from bean methanol extract are shown in **Table 1**. The major fraction TPSG 1−5 contained oily material of molecular weight and UV absorption not characteristic of flavonoids, while TPSG 6−8 contained trace amounts of proanthocyanidin monomers. In TPSG 9−11 fraction, the most abundant oligomers were those based on (epi)catechin and small amounts of heterogeneous oligomers, including (epi)-afzelechin and (epi)gallocatechin, and also proanthocyanidin dimers and trimers, malvidin diglycoside, and kaempferol glycoside **(Table 1)**.

TPSG 12−14 contained a large number of proanthocyanidins up to hexamers and anthocyanins. Subsequent TPSG fractions contained fewer proanthocyanidins but more anthocyanins with increasing amounts of nonidentified compounds. To estimate the relative ratio of the proanthocyanidins and anthocyanins in the TPSG fractions, the HPLC-MS peaks plotted were measured in millimeters and summed, and each peak was expressed as a percentage of the total sum (*29*). These approximate ratios are presented to show the relative abundance of the detected polyphenolics in the fractions.

(c) Direct Silica Gel Fractionation (SG). The main flavonoids detected with this fractionation method are presented in **Table 2**. The percentages of dry weights of the major fractions SG 1−3, 4−8, 9−12, 13−17, 18−20, and 20−22 were 3.9%, 0.8%, 4.7%, 11.5%, 24.9%, and 54.2%, respectively, recovered when 4 g of dry crude extract was fractionated. The major fraction SG 4−8 comprised a large number of proanthocyanidins ranging from monomers to hexamers. Dimers of proanthocyanidin were the most abundant form in this fraction. SG 9−12 contained a larger number of higher molecular weight proanthocyanidins and anthocyanins including delphinidin, petunidin, and malvidin glycosides. Three flavonols were detected in this major fraction including kaempferol, quercetin, and myricetin glycosides. Subsequent SG fractions contained less proanthocyanidins but more anthocyanins with increasing amounts of nonidentified

compounds as was observed above with TPSG fractions. An HPLC-MS output showing the total ion current chromatogram for the major SG 9−12 fraction and the corresponding photodiode array and ESI mass spectrum is shown in **Figure 5**.

Gu et al. (*9*−*10*) reported the presence of proanthocyanidins made up of heterogeneous subunits in three varieties of *P. vulgaris*: pinto bean, small red bean, and red kidney bean. However, they found (epi)catechin as terminal units and (epi)-afzelechin (propelargonidins) and (epi)catechin (procyanidins) as extension units but did not detect (epi)gallocatechin (prodelphinidins) as we did in this report. The presence of prodelphinidins has been reported in lentils (*34*). Kaempferol glycoside, myricetin, and quercetin glycosides were present in SG 9−12 fractions (**Table 2**). The presence of kaempferol and quercetin glycosides has been detected previously in different varieties of common bean (*3*, *6*), but to our knowledge, this is the first time that myricetin glycoside has been detected in bean.

Toyopearl resin polymer is typically used to remove sugars and pectins from phenolic compounds in plant natural product extracts (*29*). However, no significant amount of sugar was detected in these bean seed coats. For this reason, and on the basis of our results from this comparative study, we suggest that direct fractionation using silica gel results in faster and more efficient fractionation of bean flavonoids. The inclusion of the initial Toyopearl fractionation step increases the time and number of steps required to accomplish separation, which could diminish the bioactive potential of some extracts. While a Toyopearl initial fractionation has been extremely useful for separation of pectin-rich berry fruit extracts, it was not essential for bean seed coat extracts.

Although HPLC separation of compounds from direct silica gel fractionation (SG) was better than those from TP and TPSG, SG fractions were still a complex mixture of flavonoid and nonflavonoid compounds. To completely characterize the 100% methanol extracts from beans, it will be necessary to further fractionate silica gel fractions, possibly using repeated fractionation steps on Sephadex LH20. We have repeatedly used silica gel for successful subfractionation of flavonoid-rich fractions in our laboratory (*29*−*31*). Similarities within the proanthocyanidin profiles obtained here and reported previously suggest the fractionation method is reproducible and reliable for obtaining similar fractions each time.

The direct silica gel fractionation (SG) yielded a more streamlined and faster separation of phenolic compounds in natural mixtures that can be suitable for testing in bioactivity assays, especially those which require large quantities for valid activity determinations. Because potentiating interactions (synergies, additive effects) may well play a role in the bioactivity of fruit and vegetable natural extracts (*26*−*27*), it is particularly important to maintain natural mixtures at least during early stages of the fractionation and purification process.

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