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DPPH Radical Scavenging Activity and Polyphenols in the Pods of 3 Common Beans

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Abstract The pods of common beans (*Phaseolus vulgaris*) are rich in various nutrients, and they could therefore be important parts of the human diet. In the present study, we have analyzed the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and the contents of polyphenols, anthocyanin, vitamin C, and carotenoid in the pods of 3 varieties of common beans. French beans had the highest DPPH radical scavenging activity and polyphenol contents among the three varieties. The anthocyanin content was 3.93 mg/g D.W. in French beans, but not detected in bush beans and Romano beans. The vitamin C and carotenoid contents ranged from 0.45-1.96 and 0.31-0.60 mg/g D.W. in the pods of these common beans, respectively. Moreover, we found French beans contained monomeric (Fra.I), oligomeric (Fra.II), and polymeric (Fra.III) polyphenols (67%, 31%, and 2%, respectively). In contrast, monomeric polyphenols are the main polyphenols (Fra.I > 96%) in bush beans and Romano beans. Oligomeric polyphenols (Fra.II) show higher DPPH radical scavenging activity than monomeric ones (Fra.I) in French beans. Oligomeric polyphenols with both B-type and A-type bonds were present in Fra.II from French beans. We identified malvidin 3,5-diglucoside as the anthocyanin in French beans by HPLC-PDA/MS. In summary, as a vegetable, French beans have the highest antioxidant activity among the three varieties of common bean pods studied here.

Keywords: *DPPH radical scavenging activity, monomeric polyphenols, oligomeric polyphenols, anthocyanin, malvidin 3, 5-diglucoside*

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1. Introduction

Antioxidants are known to have beneficial effects, such as reducing the risk of many diseases that are related to oxidative stress. Meanwhile, phenolic compounds are commonly found in plants and have been reported to have multiple biological effects, including antibacterial, anti-inflammatory, and antioxidant activities [1,2]. Therefore, fruits and vegetables containing polyphenol compounds can act as antioxidants [3].

Anthocyanins are members of the flavonoid family, which is the most important single group of phenolics. They are responsible for the bright red-orange to blue-violet colors of many fruits and vegetables [4]. The individual anthocyanins differ in the number of hydroxyl groups and the number and position of sugars attached to the molecule. Only six anthocyanidins are common in higher plants—pelargonidin (Pg), peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt), and delphinidin (Dp). The distribution of these six anthocyanidins in the edible parts of plants is: cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%) [5].

Carotenoids are the most widespread group of naturally occurring pigments, and they are also responsible for the yellow, orange, and red colors of fruits and vegetables [6]. Vitamin C, a water-soluble vitamin, is the micronutrient most readily associated with vegetables. It can increase the absorption of iron, prevent cell damage, and reduce risks for cancers and other chronic diseases [7].

As a vegetable, the pods of common beans (*Phaseolus vulgaris*) are rich in various nutrients. They can act as an anti-diabetes and weight loss food, because the bean pods are digested slowly and therefore have a stabilizing effect on blood sugar, which promotes satiety and helps to prevent food cravings. The bean pods also contain soluble fiber, which lowers cholesterol levels [8]. We are particularly interested in French beans, because its purple pods are assumed to have a higher anthocyanin content. The aim of the present work was to evaluate the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and the contents of the aforementioned bioactive compounds in the pods of 3 common beans (French, bush, and Romano beans).

2. Materials and Methods

2.1. Samples

Common beans of three varieties were grown at Obihiro University of Agriculture and Veterinary Medicine: French beans ("Murasaki saya inngenn" in Japanese), bush beans ("Turunasi inngenn" in Japanese), and Romano beans ("Romano inngenn" in Japanese). The pods were harvested after 55–70 days. The pods' length is 12-15 cm, and their width is 1–2 cm. Diaion HP-20 columns and Sephadex LH-20 columns for chromatography were supplied by the Mitsubishi Chemical Corporation (Tokyo, Japan) and GE Healthcare Bio-Sciences AB (Uppsala, Sweden), respectively. All other reagents and chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless stated otherwise.

2.2. Preparation of Polyphenol Extracts

Approximately 5 g (approximately 0.7 g of dry weight) of fresh bean pods were cut into pieces and homogenized using a Teflon homogenizer. The mixture was added with 20 mL of methanol–HCl (99:1 v/v) and treated with ultrasound for 30 min. The mixture was centrifuged at 1,006 ×g for 10 min to obtain a supernatant. The same extraction process was repeated two more times. Then, the residues were extracted three times with 80% ethanol, followed by three times with 70% acetone-water (20 mL solvent, 30 min of ultrasonication, followed by centrifugation each time). All extracts were mixed together and filtered using Advantec No. 5 filters (Tokyo, Japan). The filtrate was collected, concentrated by rotary evaporation in vacuum, and purified by chromatography through Diaion HP-20 columns. The columns were washed with distilled water and then eluted with methanol. The methanol solution was concentrated by rotary evaporation in vacuum, and dissolved in 2 mL of methanol. A part of the concentrate was dissolved in ethanol and fractionated by Sephadex LH-20 column chromatography. The column was successively eluted with ethanol, methanol, and 60% acetone to collect fraction I (Fra.I), fraction II (Fra.II), and fraction III (Fra.III), respectively.

2.3. Estimation of DPPH Radical Scavenging Activity

DPPH radical scavenging activity was evaluated by the method described by Brand-Williams et al. [9] with some modifications. A 50-µL aliquot of the sample was mixed with 100 µL of ethanol, and then the mixture was added with 150 µL of 0.5 mM DPPH in ethanol. The absorbance of the mixture was measured by a micro plate reader at 517 nm. The DPPH radical scavenging activity was expressed in µmol of trolox equivalents per gram of dry weight of bean pods (µmol/g D.W.).

2.4. Quantification of Polyphenols

The quantification of polyphenols used the method of Folin-Ciocalteu [10]. The methanol fraction (after HP-20 column) (100 µL) was treated with 300 µL of distilled water, 400 µL of Folin-Ciocalteu reagent, and 400 µL of 10% Na_2CO_3 solution. The prepared mixture in triplicate was incubated at 30 °C for 30 min, and centrifuged at 1,006 ×g for 10 min. The absorbance of the mixed supernatant was measured at 760 nm. The polyphenol content was expressed in mg of catechin equivalents per gram of dry weight of bean pods (mg/g D.W.).

2.5. Quantification of Anthocyanin

The amount of anthocyanin was estimated according to the method described by Swllappan et al. [11]. Two 0.2 mL aliquots of the methanol fraction were separately mixed with 1.8 mL of 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5). The absorbance of the mixtures was measured at 510 and 700 nm. The difference in absorbance between the two pH values and wavelengths was used to calculate the anthocyanin content as cyanidin-3-glucoside with molecular weight of 449.2 g/mol and molar absorptivity of 26,900 L/cm/mol. The anthocyanin content was expressed in mg per gram of dry weight (mg/g D.W.).

2.6. Quantification of Vitamin C

The analysis of vitamin C content used the indophenol xylene method [12]. Common bean pods (10 g) were cut into pieces and treated with 10 mL of 5% metaphosphoric acid. The mixture was grounded in a mortar, and filtered by Advantec No. 5 filter paper. The extraction process was repeated three times. The combined supernatant was diluted with 5% metaphosphoric acid up to 50 mL. 2,6-Dichlorobenzeneindophenol (DCIP) was added dropwise into 8 mL of the extract until the mixture became reddish (5 or 6 drops). Then, 4 mL of 3% thiouric acid in 5% metaphosphoric acid was added into the mixture. 200 µL of 50% ascorbic acid in 5% metaphosphoric acid was mixed with 7.8 ml of 5% metaphosphoric acid to make a standard solution (referring to "standard A, and standard B" in Section 2.6). Five or six drops of DCIP were added to this mixture until it became reddish, and then the solution of 3% thiouric acid in 5% metaphosphoric acid was added into the reference. After 10 min, the mixture was centrifuged at 1,006 ×g for 10 min. After centrifugation, 2 mL of sample A, sample B, standard A, and standard B were separately prepared. Sample A and standard A were added with 0.5 mL of 0.1 M 2,4-dinitrophenylhydrazine (DNPH), and incubated at 50 °C for 30 min. Sample B and standard B were kept at room temperature. After incubation, sample A and standard A was added with 2.5 mL of 90% H2SO4, and sample B and standard B was added with 2.5 mL of 90% H_2SO_4 and 0.5 mL of 0.1 M DNPH. This step was carried out on the ice. After keeping at room temperature for 10 min, the absorbance was analyzed at 530 nm. The vitamin C content was calculated using the following formula, and finally expressed in units of mg per gram dry weight of bean pods (mg/g D.W.).

$$X = 200(E/S) \times V/8 \times 100/W \times 1/1000 \quad (1)$$

X: vitamin C content (mg/100 g sample)
E: absorbance of sample (sample A – sample B)

S: absorbance of standard (standard A – standard B)
200: standard content (μg)
W: sample weight (g)
V/8: total constant volume of sample / volume for the experiment

2.7. Quantification of Carotenoid

The method of carotenoid quantification was described in [13]. Briefly, pods of common beans were extracted with acetone three times, each time followed by centrifugation and separation of the supernatant. The combined supernatant was adjusted with acetone to the constant volume of 100 mL. The absorbance of the extract was analyzed at 475 nm. Carotenoid was calculated using the following formula, and converted into mg per gram of dry weight of bean pods (mg/g D.W.).

$$\text{Total carotenoid } (\text{mg/L}) = 4.143 \times A_{475} - 0.561. \quad (2)$$

2.8. Thiolysis of Fra.II in French Beans for RP-HPLC Analysis

Thiolysis was conducted according to a reported method with minor modification [14]. In a small glass vial, Fra.II (250 mg/mL) was mixed with HCl (0.1% v/v in methanol) and 2-mercaptoethanol (5% v/v in methanol). The vial was sealed with an inert Teflon cap, heated at 40 °C for 60 min, and then stored at −20 °C until analysis. Reversed-phase high-performance liquid chromatography (RP-HPLC) separation was conducted on C18 columns (250 mm × 4.6 mm; Shimadzu Corporation, Tokyo, Japan) with SPD-10AD (Shimadzu Corporation) used for detection. The analysis was performed with (A) 0.1% (v/v) trifluoroacetic acid and (B) 0.1% (v/v) trifluoroacetic acid-acetonitrile, and the elution protocol was as follows: 8% B (initial), 30% B for 30 min, 30% B for 50 min, and 8% B for 60 min at a flow rate of 1 mL/min. Detection was performed at 280 nm.

2.9. Analysis of Monomeric Polyphenols for French Beans by HPLC-PDA MS

We used acid hydrolysis to characterize the anthocyanidin in French beans. Fra.I of French beans was concentrated by rotary evaporation in vacuum, and dissolved in 2 mL of distilled water. After adding 1 mL of 6 N HCl, the solution was hydrolyzed at 100 °C for 40 min. Then, 3 mL of ethyl acetate was added, and the mixture was centrifuged at 1,006 ×g for 10 min. The upper layer was separated as the acetic acid fraction, while the lower layer was added with 15 μL of isoamyl alcohol solution. Malvidin glycoside (100 μL; 0.01 mg/mL; Tokiwa Phytochemical Co., Ltd., Chiba, Japan) as a standard was also acid-hydrolyzed in the same manner. The condition of RP-HPLC was same as that for thiolysis (see Subsection 2.8, "Thiolysis of Fra.II in French Beans for RP-HPLC Analysis"), except that the detection was performed at 520 nm.

We used high-performance liquid chromatography-photo-diode array mass spectrometry (HPLC-PDA/MS) to investigate the monomeric polyphenols in Fra.I of French beans. The HPLC-PDA system was from Shimadzu Prominence. The column was Develosil C30-UG-3 (2.0 mm × 250 mm, Mfg No. 1207268), the flow rate was 0.15 mL/min, and the injection volume was 1 μL. The gradient was acetonitrile 5–95% in 90 min, 100% wash for 5 min, 5% reconditioning for 15 min. Spectral data were collected from 200 to 760 nm, and the chromatograms were obtained at 280 nm for phenolic compounds and at 520 nm for anthocyanins. The PDA data acquisition time was 0–90 min (only the range from 0 to 40 min is shown in the paper). The flow rate diverted to the mass spectrometer was 0.2 mL/min. Mass spectrometry analysis was performed under positive ion mode with tandem MS/MS (Thermo LTQ Orbitrap XL). The MS data acquisition time was 5–91 min, and the detected mass range was m/z 300–2000. Thermo Xcalibur Ver. 2.07 SP1 software was used for data analysis.

3. Statistical Data Analysis

All data were expressed as mean ± standard deviation. The data were analyzed with SAS Enterprise Guide 5.1 system using one-way ANOVA, followed by least significant difference (LSD) test at the 95% confidence level ($p < 0.05$).

4. Results and Discussion

4.1. DPPH Radical Scavenging Activity in the Pods of 3 Common Beans

Antioxidants could react with the stable free radical DPPH (deep violet color) and convert it to 1,1-diphenyl-2-picryl hydrazine, resulting in discoloration. Significant difference ($p < 0.05$) in DPPH radical scavenging activity was found among the three beans (Table 1). French beans showed the highest DPPH radical scavenging activity, followed by bush beans and Romano beans. These common beans (*Phaseolus vulgaris*) have higher DPPH radical scavenging activity than *Vicia faba*, *Cyamopsis tetragonoloba*, and *Vigna unguiculata* [15].

Table 1. DPPH Radical Scavenging Activity in the Pods of 3 Common Beans

	DPPH radical scavenging activity (μmol/g D.W.)
French Bean	$40.78^{a} \pm 0.71$
Bush Bean	$19.07^{b} \pm 0.31$
Romano Bean	$14.57^{c} \pm 0.93$

D.W.: dry weight of beans. The results are means in triplicate ± standard deviation. Values followed by different letters in a column are significantly different ($p < 0.05$).

4.2. Polyphenols, Anthocyanin, Vitamin C, and Carotenoid Contents

The levels of polyphenols were found to be significantly different ($p < 0.05$) for the common beans (Table 2). French beans showed the highest polyphenol level (11.44 mg/g D.W.), followed by bush beans (4.68) and Romano beans (3.21). The anthocyanin content was 3.93 mg/g D.W. in French beans and not detected in the

other two. Vitamin C content ranged from 0.45 to 1.96 mg/g D.W. in the 3 beans, being the highest in French beans and the lowest in bush beans. Significant difference ($p < 0.05$) in carotenoid was also found among French beans (0.40 mg/g), Romano beans (0.31) and bush beans (0.60). We found a positive correlation between the contents of polyphenols and vitamin C to the DPPH radical scavenging activity; and the correlation coefficient (r^2) is 0.99 and 0.53, respectively. We did not find any correlation between carotenoid level and DPPH radical scavenging activity. Therefore, we consider polyphenols to be the main source of DPPH radical scavenging activity in these common bean pods. These results agree with those obtained for various fruits (nectarines, peaches, and plums [16]; strawberry, raspberry, and other berries [17]; and 39 edible fruits in Panama [18]) in that vitamin C is not the main antioxidant, and polyphenols are mainly responsible for the DPPH radical scavenging activity.

Table 2. Polyphenols, Anthocyanin, Vitamin C, and Carotenoid in the Pods of 3 Common Beans

	Polyphenols (mg/g D.W.)	Anthocyanin (mg/g D.W.)	Vitamin C (mg/g D.W.)	Carotenoid (mg/g D.W.)
French bean	11.44^{a}±0.33	3.93±0.25	1.96^{a}±0.17	0.40^{b}±0.02
Bush bean	4.68^{b} ± 0.05	N.D.	0.45^{c}±0.01	0.60^{a}±0.01
Romano bean	3.21^{c} ± 0.21	N.D.	1.30^{b}±0.08	0.31^{c}±0.02

D.W.: dry weight of beans. ND: not detected. Results are means in triplicate ± standard deviation. Values followed by different letters in a column are significantly different ($p < 0.05$).

4.3. Polyphenol Fractions

We performed column chromatography (Sephadex LH-20) to separate the polyphenols into three fractions: Fra.I, Fra.II, and Fra.III for each common bean. Bush beans and Romano beans had mostly Fra.I (> 96%) with small amounts of Fra.II and Fra.III (<4%). In contrast, French beans showed 67%, 31% and 2% ratios in Fra.I, Fra.II, and Fra.III, respectively (Figure 1). According to Saito et al. [19], Fra.I contains monomeric polyphenols, Fra.II contains oligomeric polyphenols, and Fra.III contains polymeric polyphenols. The DPPH radical scavenging activity of Fra.I and Fra.II (per μg polyphenols) from French beans were compared. As shown in Figure 2, Fra.II showed higher DPPH radical scavenging activity than Fra.I. Therefore, polyphenols with higher polymerization seem to possess greater antioxidant in the case of French beans. However, the activity of Fra.III was not analyzed here due to its low percentage.

4.4. Thiolysis Analysis of Fra.II in Pods of French Beans

Thiolysis is a method commonly used for the structural characterization of higher polymerization polyphenols. It supposedly only breaks the single B-type bonds without affecting the doubly linked A-type bonds, resulting in the release of the terminal units and the formation of thioether derivatives from the extension units. Proanthocyanidins belong to flavonoids, and they are oligomers or polymers of flavan-3-ols [20]. They can be classified into two subgroups, namely B-type proanthocyanidins in which monomers are linked only with B-type bonds, and A-type proanthocyanidins which have both A- and B-type bonds. To investigate the composition of Fra.II, thiolysis was carried out and the reaction products were analyzed by RP-HPLC (Figure 3). We detected (+)-catechin monomers in Fra.II, indicating that (+)-catechin constituted the terminal units of the corresponding proanthocyanidins. Moreover, since procyanidin A2 was also detected in Fra.II, we conclude that both B-type and A-type bonds were present in Fra.II of French beans. B-type bonds were previously found in peas, lentil, and faba beans [21]. We also detected other peaks in Fra.II that correspond to unknown compounds. Moreover, Fra.II contained oligomers (dimer, pentamer, hexamer) of catechin units linked through A-type bonds by MALDI-TOF/MS analysis.

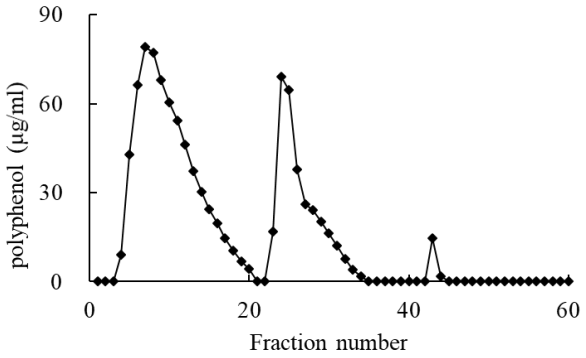

Figure 1. Polyphenols in the pods of French beans measured by an LH-20 column with successive elutions. Fra.I: ethanol fraction, 1-20, Fra.II: methanol fraction, 21–40, and Fra.III: 60% acetone fraction, 41-60

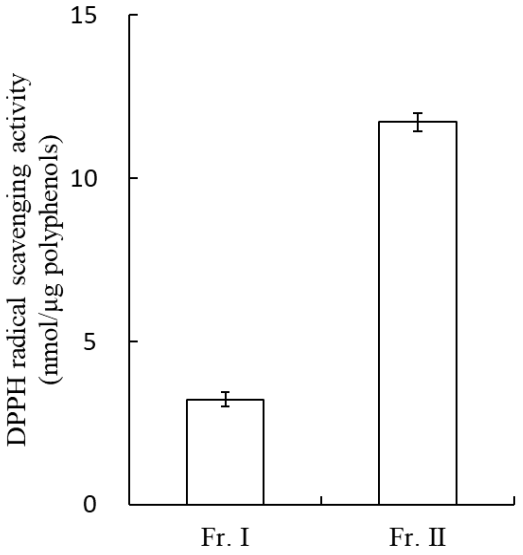

Figure 2. DPPH radical scavenging activity of monomer and oligomeric polyphenols in French beans

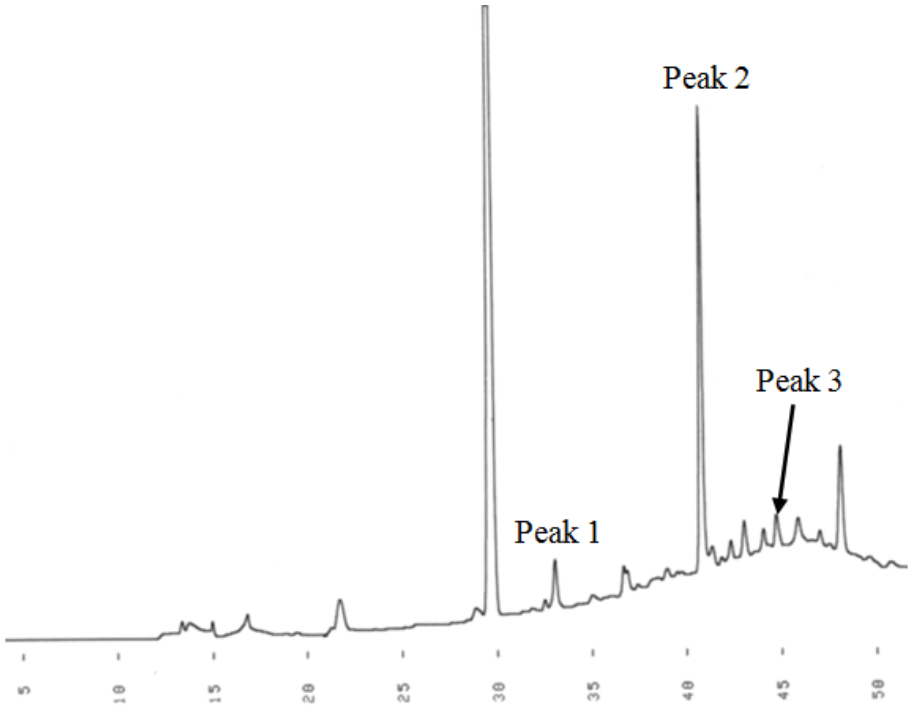

Figure 3. RP-HPLC chromatogram (280 nm) of the products of Fra.II thiolysis. Peaks: 1, (+)-catechin; 2, (+)-catechin derivative; 3, procyanidin A2

4.5. Anthocyanin Characterization in Pods of French Beans

Acid hydrolysis is a method for anthocyanin characterization, which can convert anthocyanins to anthocyanidins. After acid hydrolyzation of French bean pods, an acetic acid fraction and an isoamyl alcohol fraction were obtained. No anthocyanidin was detected in the acetic acid fraction by RP-HPLC, while malvidin was detected in the isoamyl alcohol fraction (Figure 4 B). Before acid hydrolysis there were two peaks (Figure 4 A), the percentage of peak 1 was 85% and that of peak 2 was 15%. We considered malvidin to be the anthocyanidin, which as an aglycone connected with glycosides is responsible for the two peaks. Moreover, we used HPLC-PDA/MS to characterize the anthocyanin in the pods of French beans. The detected molecular ion at m/z 655.187 is malvidin 3,5-diglucoside (Figure 5 A). The molecular ion at m/z 655.187 had fragments at m/z 493.226 and 331.199 (Figure 5 B). The former fragment could correspond to the loss of a glucoside moiety (162u) to become malvidin 3-O-glucoside. The latter fragment (m/z 331.199) was m/z 655.187 after the loss of two glucoside moieties (324u) to become malvidin as aglycone. Anthocyanins are normally present as glycosides in plants, and anthocyanin-flavanol derivatives found in pomegranate juice are also glycosides [22]. Cyanidin-3-O-glucoside, pelargonidin-3-O-glucoside, and malvidin derivate were also detected in kidney bean seeds [23]. We could not find anthocyanin in the other two common beans by HPLC-PDA/MS.

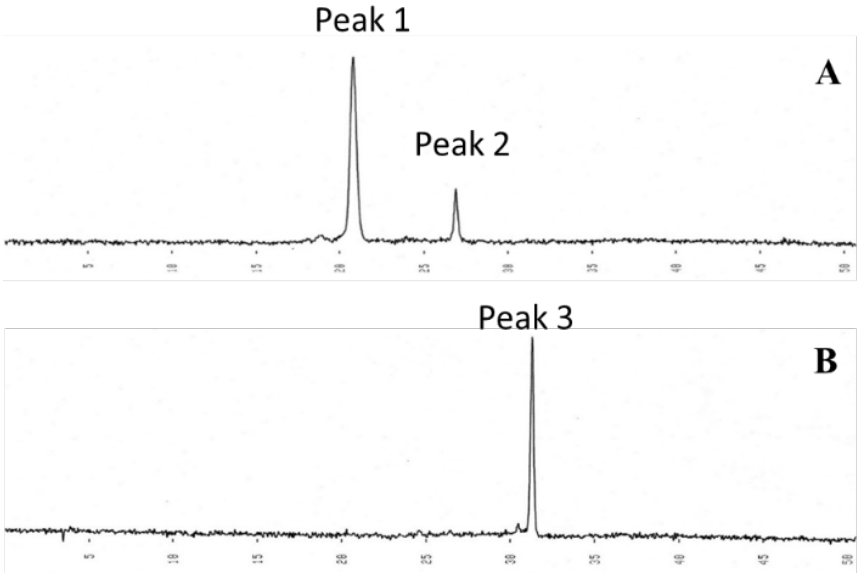

Figure 4. Acid hydrolysis of anthocyanin in pods of French beans by RP-HPLC at 520 nm. A: before acid hydrolysis; B: after acid hydrolysis. Peaks 1 and 2: glycoside of malvidin; peak 3: malvidin

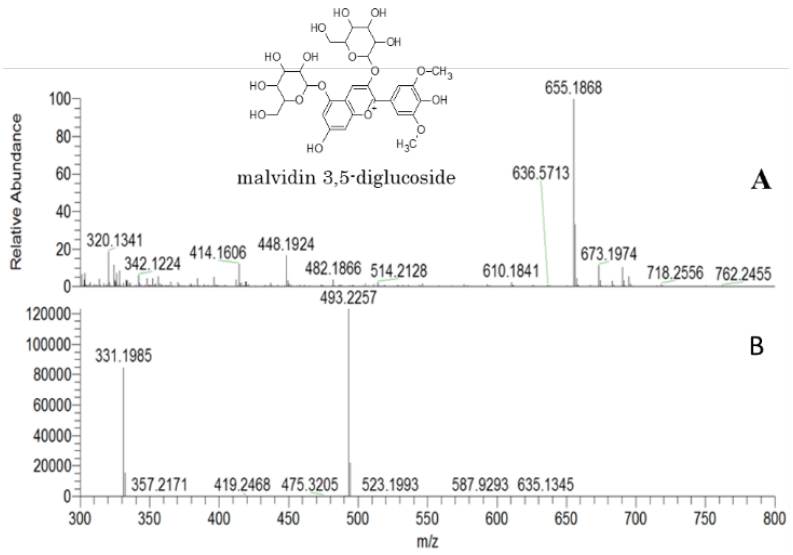

Figure 5. MS/MS fragmentation pattern of malvidin 3,5-diglucoside in pods of French beans. A: Mass spectra; B: MS/MS result at 655.187

4.6. Polyphenols Characterization of 3 Common Beans by HPLC-PDA/MS

We used HPLC-PDA/MS to characterize the polyphenols. Each common bean showed three peaks in the absorption at 280 nm (Figure 6). The masses of molecular ions are similar for the three beans: 342 and 370 at 7 min, 414 at 15 min, and 324 at 21 min (Supporting Information, Table 1). Therefore, we assumed the compositions of their polyphenol compounds are similar. The three peaks affect the DPPH radical scavenging activity in the cases of bush beans and Romano beans. In French beans, all the peaks showed a characteristic UV spectrum with maxima at 229 and 280 nm, the similarity of their UV spectra is shown in Supporting Information Figure 1. Flavonoids are widely distributed phenolic compounds in the plant kingdom. However, it is rather difficult to quantify every flavonoid due to the enormous diversity of plant species [24]. Their structures have a pyran ring (c ring) containing oxygen in the center, which is fused with the aromatic ring (A ring), connected to the other aromatic ring (B ring) by a single bond, and linked with hydrogen and electron donors in A ring and B ring. Thus, flavonoids are effective scavengers of free radicals. As a kind of flavonoids, catechin has two maximum absorptions in the UV at 225 and 278 nm [25]. We hypothesize that the polyphenol compounds in French beans were flavonoids.

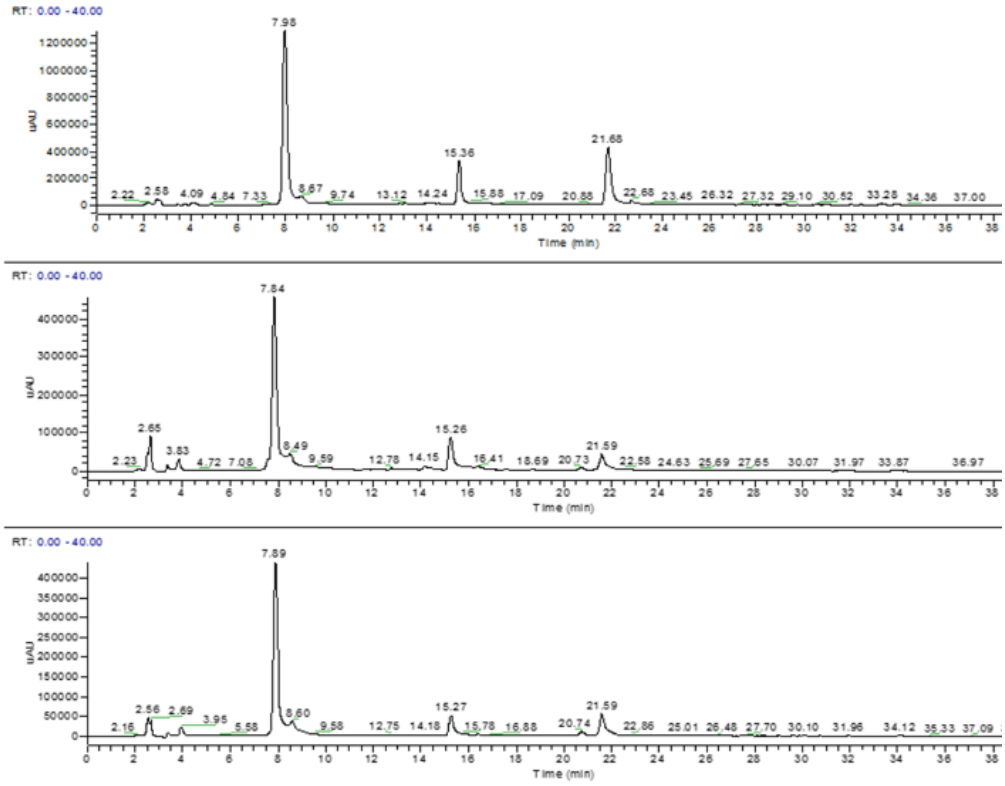

Figure 6. Chromatograms of pods of common beans by monitoring the absorbance at 280 nm by LC-ESI/MS. A: French beans; B: Romano beans; C: bush beans

5. Conclusion

Among the pods of 3 common beans, French beans showed the highest DPPH radical scavenging activity, which is mainly relevant to the higher polymerization polyphenols. Both B-type and A-type bonds were found in the polyphenols from French beans. We also identified malvidin 3,5-diglucoside as the anthocyanin in French beans. Therefore, as a part of human diet, French beans have the highest antioxidant activity among the pods of common beans studied here.

Acknowledgements

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Statement of Competing Interests

We declare that we do not have any conflict of interest.

List of Abbreviations

DCIP: 2,6-dichlorobenzeneindophenol
DNPH: 2,4-Dinitrophenylhydrazine
DPPH: 2,2-diphenyl-1-picrylhydrazyl
HPLC: high-performance liquid chromatography
LSD: least significant difference
MS: mass spectrometry
PDA: photo-diode array
RP: reversed-phase

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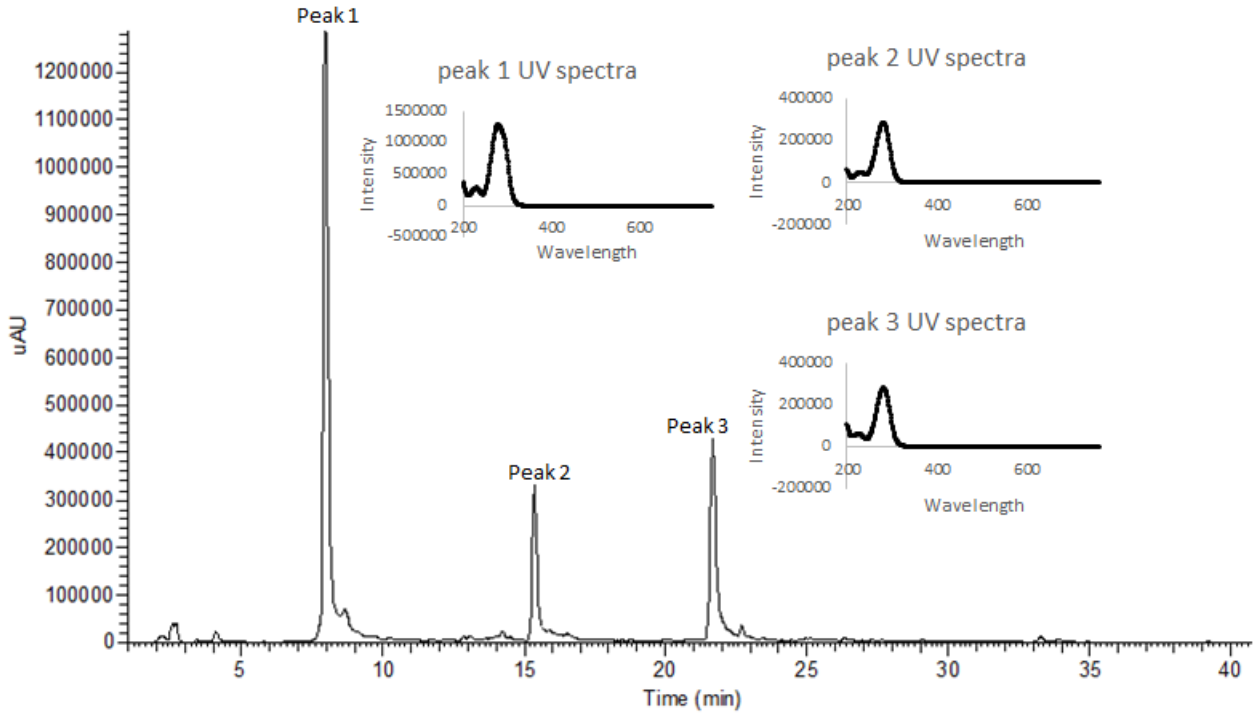
Supporting Information

S Table 1. Mass spectra for pods of 3 common beans by LC-ESI/MS at 280 nm

Sample	$R_{(t)}$min	m/z $[M-H]^+$	MS^2(m/z)
French bean	7.97	342.14	239.13
		370.17	293.08, 334.89, 279.16
	15.38	720.23	508.97, 347.07
		414.16	379.15, 365.04
	21.68	727.21	287.07, 449.08, 594.97
		324.22	279.14
Bush bean	7.84	342.14	293.09
		370.17	293.07
	15.26	414.16	379.08, 365.01, 396.86
		379.12	361.17, 221.00, 127.02, 175.09
	21.59	324.22	279.08
Roman bean	7.89	342.14	293.09
		370.17	293.10, 225.16
	15.27	374.23	176.07
		414.16	379.06, 365.10, 396.80
	21.59	324.22	279.10
		623.14	373.15, 433.04



S Figure 1. UV spectral patterns for peaks 1, 2, and 3 of French bean at 280 nm