Nutrient Distribution and Phenolic Antioxidants in Air-Classified Fractions of Beach Pea (*Lathyrus maritimus* L.)

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Beach pea (*Lathyrus maritimus* L.) cotyledons and hulls were air-classified into different fractions. The crude protein content (%N × 6.25) of samples ranged from 32.8 to 35.3% in cotyledons and 14.7 to 16.8% in hulls. Crude fiber content was higher in hulls fraction 1 (37.13%) and fraction 2 (36.85%) than in cotyledons (2.83, 2.99, and 3.08% in fractions 1, 2, and 3, respectively). Condensed tannins of cotyledons ranged from 5.76 to 6.90% and of hulls ranged from 52.49 to 57.24%, expressed as catechin equivalents. Minerals, namely P, K, and Zn, were higher in cotyledons, but Ca and Mn were more prevalent in hulls. Nonprotein nitrogen was concentrated in hulls, whereas phytic acid was more abundant in the cotyledons. The UV absorption pattern showed that flavonoids were present in fractions (I−III) from hulls separated on Sephadex LH-20. Fraction III from hulls had the highest content of total phenolics and condensed tannins, but no condensed tannins were detected in fractions I and II from hulls. The antioxidant activity of fractions separated on Sephadex LH-20 from hulls and crude extracts in a β-carotene−linoleate model system was in the order of fraction III > crude extract > fraction II > fraction I. Spots on silica gel TLC plates, sprayed with a solution of β-carotene and linoleic acid, indicated that many of the individual compounds were antioxidative in nature. Further, separation of fraction III from hulls on a semipreparative HPLC showed the presence of (+) catechin and (−) epicatechin as the main low-molecular-weight phenolic compounds present.

Keywords: *Beach pea; Lathyrus maritimus L.; hulls; condensed tannins; antioxidant activity*

INTRODUCTION

Legumes are especially important as a complement to carbohydrate staples, such as rice, corn, and other cereals, cassava and other roots, and tuber crops. The protein contents of legumes are generally about double those of most cereals. Legumes also contain high amounts of starch and fat, and they have highly fibrous hulls (*1*). Phenolics, including condensed tannins, and chlorophyll are mostly concentrated in legume hulls. To enrich the protein content of legumes, their hulls may be removed by rapid and simple techniques. In developing countries, processing of legumes is achieved by using traditional methods such as dehulling, soaking, germination, and heat treatment (*2*). Such processing results in the improvement of nutritional value of products which are wholesome and contain a reduced level of antinutritional or toxic compounds. The degree of elimination of antinutritional or toxic constituents depends on the type of legume and the processing conditions employed.

Dehulling is a process traditionally practiced in Asia and Africa. Removal of husks is usually done in a small machine which includes both hand- and power-operated techniques under running disk shellers or blunt plate mills. The husks are removed by aspiration while dehulled grains are easily separated from split cotyledons using a sieve.

Dehulling is known to influence the nutritional and functional properties of legumes (*3*). The distribution of nutrients in different parts of whole pea seeds and beans shows that the major portion of protein, fat, phosphorus, and iron is present in cotyledons, whereas 80 to 90% of crude fiber and 32 to 50% of calcium are present in the seed coats (*4*). Hence, dehulling results in a significant decrease in crude fiber and calcium contents of food legumes. In contrast, removal of the seed coat decreases the tannin content significantly, hence improving protein digestibility of the product (*3*).

Beach pea (*Lathyrus maritimus* L.), a relatively unknown leguminous plant, grows along the sandy and gravel shorelines of Canada, Greenland, Siberia, and Japan (*5*). Unlike other legumes, beach pea is not cultivated, in part, because its nutritional value and possible presence of toxicants have not been studied previously. Recently, we have reported the compositional characteristics, nutritional value, and physicochemical properties of beach pea seeds and plant parts (*6*−*8*). Beach pea was found to serve as a good source of crude protein, starch, minerals, and vitamins. Mature beach pea seeds have fibrous hulls which may be removed, if desired, to produce specialty products for human consumption, among other items (*9*). Therefore,

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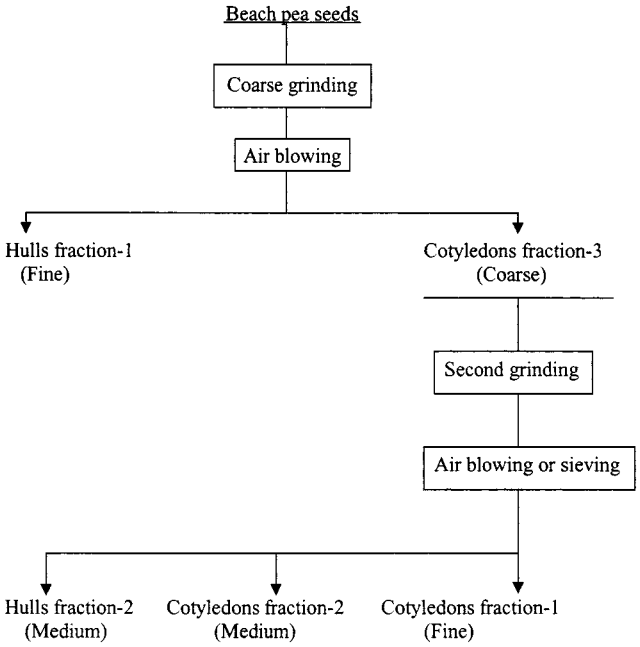

Figure 1. Flowchart for air classification of cotyledons and hulls of beach pea.

we investigated changes in chemical composition of beach pea cotyledons and hulls as a result of dehulling.

MATERIALS AND METHODS

Materials. Mature pods of beach pea were collected from Bellevue Beach in Newfoundland in September and October of 1995 and 1997. The seeds and pod shells were separated manually. The recovery of seeds and pod shells was recorded immediately after harvesting. Clean seeds were then stored in airtight glass containers for further analyses.

Air Classification. Mature and dry beach pea seeds were ground using a Seedburo hand grinder (Seedburo Equipment Company, Chicago, IL). Ground seed fines were first separated using a mesh 30 sieve on a Seedburo portable sieve shaker; the hulls were separated from the cotyledons on a 757 South Dakota Seed Blower (Seedburo Equipment Company, Chicago, IL) equipped with a large tube set (4 in. column) (Figure 1). The separated cotyledons and hulls fractions were ground into a fine powder (60 mesh) using a coffee grinder and stored in Nasco whirl pack plastic bags (Polycello, Amherst, NS) or airtight glass containers and kept at room temperature for further chemical analyses.

Chemical Analyses. Moisture, crude protein, lipid, ash, crude fiber, and carbohydrate contents (by difference) of samples were determined by standard methods of analysis (*10*). Soluble sugars were extracted into 80% (v/v) ethanol according to the procedure of Cerning and Guilhot (*11*), and their contents, as well as those of reducing sugars, were determined using a modified version of the method of Nelson (*12*). The amount of nonreducing sugars was calculated from the difference in the content of total soluble and reducing sugars. The soluble proteins from ethanolic extract were determined according to the method of Lowry et al. (*13*). Starch from soluble sugar-free residue was obtained by extraction into 52% (v/v) perchloric acid at room temperature. Quantitative determination of starch was carried out according to the colorimetric method of McCready (*14*). The phenolics from beach pea were isolated as described by Shahidi and Naczk (*15*). One gram of sample was extracted three times with 10 mL of 70% (v/v) aqueous acetone at room temperature using a Polytron PT 3000 homogenizer (Brinkman Instruments, Rexdale, ON) at 5000 rpm for 1 min. The slurry was centrifuged at 5000*g* for 10 min; supernatants were collected, combined, and evaporated to dryness at 30 °C under vacuum. The extracted phenolics were then dissolved in 25 mL of methanol and centrifuged again, and the total content of phenolics in methanol was determined colorimetrically (*16*). To 0.5 mL of methanolic solution of phenolics, 0.5 mL of Folin-Denis reagent, 1 mL of saturated solution of sodium carbonate, and 8 mL of water were added and mixed well. Absorbance of the resultant solution was read at 725 nm after 30 min standing at room temperature, and the content of phenolics was calculated as percent trans-sinapic acid equivalents on a dry weight basis. Condensed tannins in methanolic solution were determined by the modified vanillin assay of Price et al. (*17*) and by the proanthocyanidin assay of Mole and Waterman (*18*), as described by Naczk et al. (*19*).

Determination of Mineral Constituents. Dried and ground samples (1 to 2 g) were subjected to dry ashing in well-cleaned porcelain crucibles at 550 °C in a muffle furnace (Blue M Electric Company, Blue Island, IL). The resultant ash was dissolved in 5 mL of $HNO_3/HCl/H_2O$ (1:2:3, v/v/v) and heated gently on a hot plate until brown fumes disappeared. To the remaining content in each crucible, 5 mL of deionized water was added and the mixture was heated until a colorless solution was obtained. The solution in each crucible was transferred into a 100-mL volumetric flask by filtering through a Whatman No. 42 filter paper, and the volume was made up to the mark with deionized water. This solution was used for elemental analysis by atomic absorption spectrophotometry using a Perkin-Elmer 8650 atomic absorption spectrophotometer (Perkin-Elmer Co., Montreal, PQ). The concentration of elements (Ca, Na, K, Mg, Mn, Zn, Fe, Cu, Li, Al, and Si) in each solution, prepared as described above, was then determined. Calibration curves of absorbance values versus concentration of each element at appropriate concentrations (to obey Beer's-Lambert Law) were constructed using their respective standards of 0−1000 μg/l (Fisher Scientific, Unionville, ON). A 10-cm -long cell was used, and the concentration of each element in the samples was calculated as mg/100 g of dry matter. Phosphorus content of the digest was determined colorimetrically according to the method described by Nahapetian and Bassiri (*20*). To 0.5 mL of the diluted digest, 4 mL of demineralized water, 3 mL of 0.75 M H_2SO_4, 0.4 mL of 10% (w/v) $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, and 0.4 mL of 2% (w/v) ascorbic acid were added and mixed. The solution was allowed to stand for 20 min and absorbance readings were recorded at 660 nm. The content of phosphorus in the extracts was determined using standard curves obtained for KH_2PO_4 and expressed as mg phosphorus per 100 g of sample.

Nonprotein Nitrogen. The content of nonprotein nitrogen (NPN) was determined by the method of Bhatty and Finlayson (*21*) as modified by Naczk et al. (*22*). One gram of meal was shaken with 40 mL of a 10% trichloroacetic acid (TCA) solution at 20 °C for 1 h using a wrist-action shaker (Burrel, Pittsburgh, PA). The insoluble residue was removed by centrifugation at 5000*g* for 10 min, and the residue was treated three times with 15 mL of a 10% (w/v) TCA solution. The supernatant was collected as before, its volume was made up to 100 mL with distilled water; and an aliquot of it was taken for determination of soluble nitrogen using the Kjeldahl procedure (*10*).

Phytic Acid. Phytic acid from the prepared meals was extracted according to the method of Tangkongchitr et al. (*23*) as modified by Naczk et al. (*24*). Two grams of meal were extracted with 40 mL of 1.2% HCl containing 10% Na_2SO_4 for 2 h using a wrist-action shaker. The slurry was centrifuged for 20 min at 5000*g*. Five milliliters of the supernatant was mixed with 5 mL of distilled water and 6 mL of 0.4% $FeCl_3\cdot 6H_2O$ in 0.07 M HCl solution. The mixture was heated in a boiling water bath for 45 min and then cooled to room temperature. The resulting ferric phytate precipitate was collected by centrifugation at 5000*g* for 15 min and the supernatant was discarded. The precipitate was mixed thoroughly with 5 mL of 4% Na_2SO_4 in 0.07 M HCl and the mixture was centrifuged (5000*g*) again. The recovered ferric phytate was digested using 6 mL of a 1:1 (v/v) mixture of concentrated H_2SO_4 and concentrated HNO_3. The digestion was terminated when white fumes hung over the liquid. A 10-mL portion of distilled water was added to the warm digest, and the solution was heated in a boiling water bath for 30 min to destroy

pyrophosphate; the mixture was then diluted with distilled water to 50 mL. The phytate phosphorus was determined according to the method described by Nahapetian and Bassiri (*20*). To 1 mL of diluted digest, 4 mL of distilled water, 3 mL of 0.75 M H_2SO_4, 0.4 mL 10% $(NH_4)_6$ $Mo_7O_{24}\cdot 4H_2O$, and 0.4 mL of 2% ascorbic acid were added and mixed. The solution was allowed to stand for 20 min and its absorbance was read at 660 nm. The content of phosphorus in the mixture was calculated from a standard curve using KH_2PO_4 as the standard. The phytic acid content was calculated by multiplying the phytate phosphorus content of the meal by a factor of 3.55 which is derived from the empirical formula $C_6P_6H_{18}O_{24}$.

Protein Precipitation Assay. The protein precipitating capacity of condensed tannins of beach pea hulls was assayed as described by Hagerman and Butler (*25*) with the following modifications. To 1 mL of methanolic solution of crude tannin extract, 2 mL of a standard solution of bovine serum albumin (BSA; Sigma, fraction V, initial fractionation by cold alcohol precipitation) was added (1 mg of protein/mL in 0.2 M acetate buffer, pH 4.0 and containing 0.17 M sodium chloride) and mixed well. After the solution was left to stand at room temperaturefor 15 min, it was centrifuged at 5000*g* for 15 min. The supernatant was discarded, and the surface of the pellet and the tube walls were carefully washed with acetate buffer (pH 4.0) without disturbing the pellet. The pellet was then dissolved in 4 mL of sodium dodecyl sulfate (SDS)-triethanolamine solution [1% SDS and 5% (v/v) triethanolamine in distilled water], and 1 mL of ferric chloride reagent (0.01 M ferric chloride in 0.01 M HCl) was added to it and mixed. Fifteen minutes after the addition of ferric chloride reagent, the absorbance of the solution was read at 510 nm against a reagent blank (4 mL of SDS solution and 1 mL of ferric chloride reagent). The protein precipitating capacity of tannins was expressed as A_{510}/g hulls.

The protein precipitating activity of condensed tannins of beach pea hulls was also assayed by the dye-labeled protein assay of Asquith and Butler (*26*) as modified by Naczk et al. (*27*). One milliliter of methanolic solution of crude tannin extract was added to 4 mL of a blue BSA solution containing 2 mg of protein/mL in 0.2 M phosphate buffer, pH 3.5. The mixture was vigorously mixed at 1000 rpm for 5 min at room temperature. The protein−tannin complex was then separated by centrifugation at 5000×*g* for 20 min. The supernatant was carefully discarded, and the pellet was dissolved in 3.5 mL of a 1% (w/v) solution of SDS containing 5% (v/v) triethanolamine and 20% (v/v) 2-propanol. The absorbance was read at 590 nm against an appropriate blank. The protein precipitating capacity of tannins was expressed as milligrams of BSA precipitated per gram of hulls.

Seed Treatments. Beach pea seeds were soaked in concentrated H_2SO_4 (1:3, w/v) for 30 min, the acid was drained, and the seeds were then soaked again in distilled water (1:3, w/v) for 12 h at room temperature. Beach pea seeds were also heat processed for 30 min in boiling water (1:3, w/v). After the acid and heat processing treatments, the hulls were separated from cotyledons, freeze-dried, and then used for scanning electron microscopic studies.

Scanning Electron Microscopy (SEM). Structural morphology of beach pea hulls was studied using SEM. Hull samples were mounted on circular aluminum stubs with double-stick tape, and then coated with 20 nm of gold using Edwards S150A sputter coater, and examined and photographed using a Hitachi (S-570) scanning electron microscope (Hitachi, Ltd., Tokyo, Japan) at an accelerating potential of 20 kV.

Extraction of Phenolics and Natural Antioxidants from Beach Pea Hulls. Fine powders (60 mesh) of beach pea hulls were extracted with a 70% solution of (v/v) acetone containing 1% concentrated HCl (meal-to-solvent ratio of 1:10, w/v) at room temperature using a Polytron homogenizer (Brinkman PT 3000) for 1 min at 10 000 rpm. The slurry was centrifuged at 4000*g* for 10 min, and the supernatant was filtered through a Whatman No. 41 filter paper; the residue was extracted two more times using the same procedure. Supernatants were combined and evaporated under vacuum using a rotary evaporator to remove acetone; the water was then removed by lyophilization.

A known quantity of lyophilized sample was dissolved in absolute methanol and used for determination of total phenolic compounds and condensed tannins as described by Naczk et al. (*19*).

Fractionation of Phenolic Compounds using Column Chromatography. A 1.5 g portion of acetone extract of hulls was dissolved in 5 mL of methanol and applied to a chromatographic column (3.4 × 50 cm) packed with Sephadex LH-20 and eluted with absolute ethanol. Fractions (8 mL) were collected using a LKB Bromma 2112 Redirac fraction collector (Pharmacia, Uppsala, Sweden) and their absorbance in ethanol was read at 280 nm; the absorbance was lowest and constant in tube numbers 60−66. Then, the elution solvent was changed to acetone−water (50:50, v/v) to remove the tannin fraction, the absorbance of which was read at 280 nm. In addition, the absorbance of all fractions at 500 nm was read after color development using the modified vanillin assay (*17*). Eluates were then pooled into three major fractions on the basis of their absorbance at 280 nm and a positive test with vanillin. Pooled eluates were lyophilized and weighed. The content of total phenolic compounds and condensed tannins in each major fraction was then estimated (*19*). Standards used were transsinapic acid for phenolic acids and (+) catechin for condensed tannins.

UV Spectra. The UV spectrum of each separated fraction was recorded using a Hewlett-Packard 8452A diode array spectrophotometer (Hewlett-Packard Canada, Ltd., Montreal, PQ).

Thin-Layer Chromatography (TLC). Each separated fraction and crude extracts were examined on silica gel TLC plates (Silica gel, 60 Å mean pore diameter, 2−25 μm mean particle size, 250 μm thickness, Sigma Chemical Co., St. Louis, MO). Plates were developed in a glass chamber (22 × 22 × 10 cm; Fisher Scientific Co., Toronto, ON) using acetic acid−petroleum ether−diethyl ether (1:20:80, v/v/v) and water−acetic acid−*n*-butanol (1:1:3, v/v/v) mixtures as the developing systems (*28*). To visualize phenolic compounds, each plate was sprayed with a solution of ferric chloride (*29*). Compounds with antioxidant activity were visualized after spraying of each plate with a solution of β-carotene and linoleic acid (*30*).

Antioxidant Activity. The antioxidant activity of isolated fractions and crude extract was evaluated using a β-carotene−linoleate model system (*31*), with the following modifications. A solution of β-carotene (Sigma) was prepared by dissolving 2 mg of β-carotene in 10 mL of chloroform. One milliliter of this solution was then pipetted into a 50-mL round-bottom flask. After removing the chloroform under vacuum by using a rotary evaporator at 40 °C, 20 mg of purified linoleic acid, 200 mg of Tween 40 emulsifier (Aldrich Chem. Co., Milwaukee, WI), and 50 mL of aerated distilled water were added to the flask with vigorous manual shaking. Aliquots (5 mL) of this prepared emulsion were transferred into a series of tubes containing 2 mg of each fraction (fractions I−III), the crude extract, or 2 mg of butylated hydroxyanisole (BHA) which was used for comparative purposes. As soon as the emulsion was added to each tube, the zero time absorbance was read at 470 nm. Subsequent absorbance readings were recorded at 15 min intervals by keeping the samples in a water bath at 50 °C until the color of β-carotene in the control sample devoid of any extract or synthetic antioxidant had disappeared (approximately 120 min).

HPLC Analyses. The vanillin-positive fraction (III) was used for purity testing by HPLC using standard catechin (Sigma). (+) Catechin and (−) epicatechin from fraction number III were separated from the Sephadex-isolated fractions by semipreparative HPLC. A Shimadzu (Tokyo, Japan) chromatographic system consisting of a LC-6A pump, SPD-6AV UV−Vis spectrophotometric detector, SCL-6B system controller, CR 501 chromatopac, and a CSL-Spherisorb-ODS-2 analytical column (4.5 mm × 250 mm) (Chromatographic Specialities, Inc., Brockville, ON) was used. The mobile phase was acetic acid−methanol−dimethylformamide−water (1:3:40:157, v/v/v/v) (*32*) and the flow rate was 1.5 mL/min with

Table 1. Chemical Composition of Air-Classified Fractions of Cotyledons and Hulls of Beach Pea[a]

	cotyledons			hulls	
constituent, %	fraction 1	fraction 2	fraction 3	fraction 1	fraction 2
moisture	4.57 ± 0.08^{ab}	4.73 ± 0.13^{a}	4.64 ± 0.06^{ab}	4.22 ± 0.09^{c}	4.49 ± 0.05^{b}
ash	3.92 ± 0.05^{a}	3.77 ± 0.11^{ab}	3.53 ± 0.07^{bc}	2.99 ± 0.13^{d}	3.29 ± 0.20^{cd}
lipid	1.08 ± 0.04^{a}	0.98 ± 0.02^{b}	0.92 ± 0.01^{c}	0.45 ± 0.02^{ed}	0.48 ± 0.01^{d}
crude fiber	2.83 ± 0.05^{d}	2.99 ± 0.08^{cd}	3.08 ± 0.10^{bcd}	37.13 ± 0.60^{a}	36.85 ± 0.92^{a}
protein	35.28 ± 0.98^{a}	34.49 ± 0.53^{a}	32.82 ± 1.02^{a}	14.73 ± 0.92^{c}	16.81 ± 1.56^{bc}
soluble proteins (mg/100 g)	318.72 ± 2.92^{a}	306.13 ± 3.06^{bc}	302.37 ± 4.31^{c}	105.49 ± 0.62^{e}	134.85 ± 1.77^{d}
carbohydrates[b]	55.15 ± 1.43^{d}	56.03 ± 1.82^{cd}	58.09 ± 1.60^{bcd}	77.61 ± 1.47^{a}	74.93 ± 1.74^{a}
soluble sugars	2.97 ± 0.12^{a}	2.86 ± 0.07^{ab}	2.73 ± 0.10^{b}	0.08 ± 0.00^{d}	0.17 ± 0.01^{cd}
reducing sugars (mg/100 g)	302.13 ± 2.27^{a}	287.25 ± 4.91^{b}	267.61 ± 5.34^{c}	66.77 ± 1.74^{e}	136.88 ± 1.28^{d}
nonreducing sugars	2.67 ± 0.11^{a}	2.57 ± 0.37^{a}	2.46 ± 0.52^{a}	0.01 ± 0.00^{c}	0.03 ± 0.01^{bc}
starch	37.12 ± 1.23^{a}	34.18 ± 2.03^{a}	34.57 ± 1.43^{a}	3.56 ± 0.22^{c}	7.15 ± 0.12^{b}
total phenolics	0.93 ± 0.005^{d}	1.05 ± 0.006^{cd}	1.12 ± 0.005^{bcd}	15.80 ± 1.20^{a}	14.92 ± 1.08^{a}
condensed tannins	5.76 ± 0.11^{e}	6.46 ± 0.16^{de}	6.90 ± 0.13^{cde}	57.24 ± 1.67^{a}	52.49 ± 0.83^{b}

[a] Results are means of triplicate determinations, on a dry weight basis, ± standard deviation. Means followed by different superscripts in each row are significantly ($p < 0.05$) different from one another. [b] By difference.

Table 2. Mineral Content of Air-Classified Fractions of Cotyledons and Hulls of Beach Pea (mg/100 g)[a]

	cotyledons			hulls	
mineral	fraction 1	fraction 2	fraction 3	fraction 1	fraction 2
			Macroelement		
calcium	111.34 ± 1.07^{e}	121.89 ± 1.32^{d}	124.83 ± 1.24^{cd}	270.85 ± 1.62^{a}	237.32 ± 2.06^{b}
magnesium	220.42 ± 1.11^{a}	216.42 ± 1.21^{bc}	215.98 ± 1.05^{c}	195.84 ± 1.20^{e}	202.18 ± 1.08^{d}
phosphorus	645.23 ± 3.52^{a}	590.12 ± 2.76^{b}	523.17 ± 2.31^{c}	86.95 ± 0.38^{e}	93.67 ± 0.73^{d}
potassium	1401.22 ± 8.07^{a}	1255.99 ± 9.66^{b}	1177.16 ± 9.06^{c}	1016.25 ± 10.36^{e}	1100.09 ± 10.12^{d}
sodium	178.86 ± 0.68^{e}	188.27 ± 0.29^{d}	195.40 ± 0.30^{b}	191.96 ± 0.73^{c}	203.37 ± 0.92^{a}
			Microelement		
sluminum	3.07 ± 0.28^{d}	3.29 ± 0.36^{cd}	5.47 ± 0.12^{ab}	6.41 ± 0.78^{a}	4.69 ± 0.91^{bc}
copper	1.84 ± 0.16^{ab}	1.72 ± 0.20^{b}	1.89 ± 0.10^{ab}	1.79 ± 0.21^{ab}	2.25 ± 0.18^{a}
iron	9.79 ± 0.76^{a}	10.02 ± 1.00^{a}	10.19 ± 0.93^{a}	11.23 ± 1.03^{a}	9.63 ± 0.65^{a}
lithium	1.48 ± 0.35^{a}	1.22 ± 0.30^{a}	1.22 ± 0.63^{a}	1.01 ± 0.11^{a}	1.04 ± 0.06^{a}
manganese	3.07 ± 0.24^{cd}	2.97 ± 0.06^{d}	2.97 ± 0.08^{d}	5.02 ± 0.20^{a}	4.56 ± 0.07^{b}
silicon	ND	ND	ND	ND	ND
zinc	5.26 ± 0.70^{a}	4.52 ± 0.20^{ab}	4.13 ± 0.13^{bcd}	3.42 ± 0.23^{cd}	3.41 ± 0.11^{d}

[a] Results are means of triplicate determinations, on a dry weight basis, ± standard deviation. Means followed by different superscripts in each row are significantly ($p < 0.05$) different from one another. ND, Not detected.

an injection volume of 20 μL. For preparative and analytical methods, the detector wavelength was set at 280 nm. The standard (+) catechin and (−) epicatechin were run on the same semipreparative HPLC column under the same conditions as compared to the unknowns from beach pea hulls.

Statistical Analyses. All determinations were replicated three times or more. In each case, the mean value ± standard deviation was calculated. Analysis of variance (ANOVA) was performed and significance of differences at $p < 0.05$ in mean values evaluated using Tukey's Studentized Test by employing ANOVA and Tukey's procedures of Statistical Analytical System (*33*).

RESULTS AND DISCUSSION

Nutrient Distribution in Air-Classified Fractions of Cotyledons and Hulls of Beach Pea. Chemical compositions of mechanically separated cotyledons and hulls fractions of beach pea are presented in Table 1. The crude protein content ranged from 32.82 to 35.28% in cotyledons and from 14.73 to 16.81% in hulls. Similarly, soluble proteins, soluble sugars, and starch were present in higher amounts in cotyledons. Ash content of cotyledons decreased from fraction 1 to fraction 3, but in the case of hulls it was increased, indicating a higher ash content in cotyledons compared to hulls. Similar results for ash content in cotyledons and hulls were reported for cowpea, green pea, and pigeonpea (*4*). Crude fiber content was higher in hulls fraction 1 (37.13%) and fraction 2 (36.85%) than those of cotyledons (fractions 1, 2, and 3, 2.83%, 2.99%, and 3.08%, respectively). The levels of carbohydrates, total phenolics, and condensed tannins were higher in hulls than in cotyledons. These results indicate that the latter components are mostly concentrated in the seed coats and might be easily removed by dehulling. The content of phenolics and condensed tannins in cotyledons of beach pea were somewhat higher than those reported in the literature for other seeds, perhaps due to the presence of some hulls in the cotyledons fraction. Condensed tannins in cotyledons ranged from 5.76 to 6.90% and in hulls ranged from 52.49 to 57.24%, expressed as catechin equivalents. Reddy et al. (*34*) reported that the tannin content, as catechin equivalents, in cotyledons was 28, 460−560, 22−43, and 16−38 mg/100 g of cowpea, pea, pigeonpea, and chickpea, respectively. The present results indicate that beach pea hulls contain high amounts of total phenolics and condensed tannins. This might be due to the genetic characteristics of beach pea and the fact that tannins provide a barrier for seeds against harsh and humid environmental conditions in the shorelines.

Minerals. Minerals in air-classified fractions of beach pea cotyledons and hulls are shown in Table 2. Minerals in beach pea cotyledons were dominated by potassium and phosphorus as the macroelements and iron as the microelement, whereas hulls were rich in calcium, potassium, and sodium as macroelements and again iron as the microelement. Silicon was not detected in cotyledons or in hulls of beach pea. Calcium content in hulls was lower than the reported values for peas, cowpeas, and pigeonpeas (900, 853, and 917 mg/100 g,

Table 3. Nonprotein Nitrogen (NPN) and Phytic Acid Content in Air-Classified Fractions of Cotyledons and Hulls of Beach Pea[a]

component	% NPN in total nitrogen	phytic acid (mg/100 g)
	Cotyledons	
fraction 1	17.27 ± 1.96^{c}	483.92 ± 3.76^{a}
fraction 2	18.38 ± 1.07^{bc}	478.00 ± 2.99^{a}
fraction 3	20.13 ± 0.92^{abc}	439.46 ± 2.78^{b}
	Hulls	
fraction 1	23.79 ± 1.74^{a}	68.69 ± 1.08^{c}
fraction 2	22.73 ± 1.83^{a}	67.44 ± 1.66^{cd}

[a] Results are means of four determinations, on a dry weight basis, ± standard deviation. Means followed by different superscripts in each column are significantly ($p < 0.05$) different from one another.

respectively; *4*); other elements in cotyledons and hulls were present in similar amounts.

Nonprotein Nitrogen (NPN) and Phytic Acid. The contents of NPN and phytic acid in different fractions of beach pea cotyledons and hulls are presented in Table 3. The ratio of NPN to total nitrogen was significantly higher ($p < 0.05$) in hulls (fraction 1, 23.79% and fraction 2, 22.73%) than in cotyledons (from 17.27 to 20.13%). Most of the protein nitrogen stored in cotyledons was synthesized from NPN mobilized from hulls. Ultimately the content of nonprotein nitrogen was lower in cotyledons. Singh and Jambunathan (*35*) also observed that the percentage of NPN in total nitrogen was very high in seed coats compared to that in cotyledons of chickpea (21.3 and 10.7%, respectively) and pigeonpea (27.4 and 9.5%, respectively).

Phytic acid was present in significantly ($p < 0.05$) higher amounts in cotyledons than in hulls, ranging from 439.46 (fraction 3) to 483.92 mg/100 g (fraction 1) in cotyledons, and being 68.69 and 67.44 mg/100 g in fractions 1 and 2 of hulls, respectively (Table 3). These results are in agreement with literature values for black gram (*36*) and cowpea (*37*).

Tannins and their Properties. The contents and properties of beach pea hulls tannins from air-classified fractions 1 and 2 and manually separated hulls are presented in Table 4. Condensed tannins, expressed as catechin equivalents, were present at very high levels in fraction 1 (572.4 mg/g) as compared to those in fraction 2 (524.9 mg/g) and 12-h-soaked seed hulls (396.8 mg/g). The contents of condensed tannins, as determined by the proanthocyanidin assay, and phenolics precipitated by bovine serum albumin (BSA), as determined by the protein precipitation assay, were also very high for fraction 1 and fraction 2 of mechanically separated hulls as compared to soaked and manually separated hulls from beach pea. The lower values for the content of tannins of soaked hulls might be due to their leaching out during soaking. Beach pea hulls were found to contain very high amounts of condensed tannins compared to those reported for other legumes such as pigeonpea (11.41 mg/g), chickpea (1.65 mg/g) (38), cowpea (2.62 mg/g) (*39*), and green pea (5−10 mg/g) (*34*). The biological and ecological roles of tannins are attributed to their ability to bind or precipitate proteins (*25*, *40*, and *41*). Dye-labeled BSA assay (*26*) allows for direct measurement of protein precipitation by tannins and was used in the present study. Condensed tannins extracted from beach pea hulls fractions precipitated 793 to 1131 mg of BSA/g hulls. Fraction 2 gave lower values for all assays than those for fraction 1, possibly due to the presence of some cotyledons in the hulls fractions.

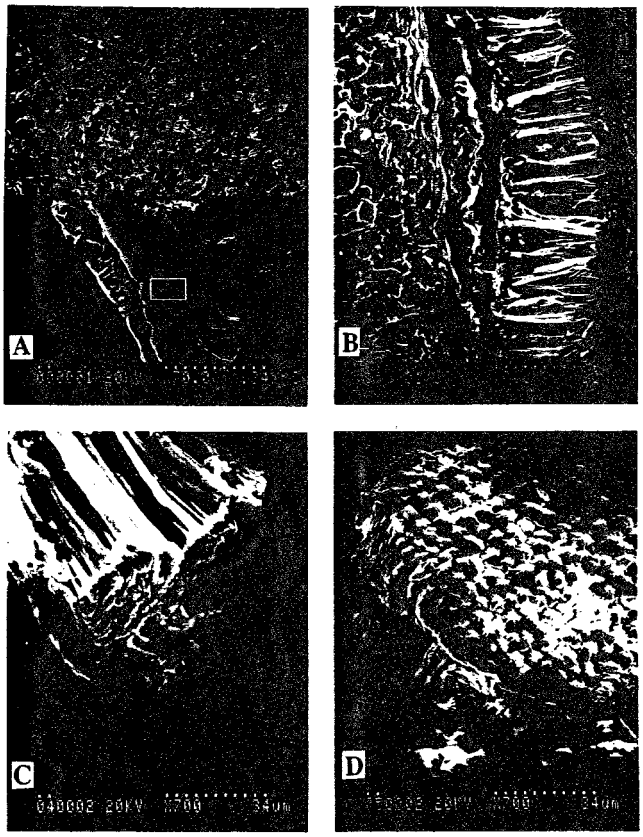

Figure 2. Scanning electron micrograph of beach pea seed coat structure (A, seed coat structure; B, cross section of seed coat; C, after soaking 30 min in H_2SO_4 and then 12 h in distilled water; and D, after heat processing for 30 min).

Seed Coat Structure of Beach Pea. Beach pea seed coat structure, studied using scanning electron microscopy (SEM), is shown in Figure 2A. The cross section of the seed coats, as shown in Figure 2B, indicates a highly compact and a very hard structure. Preliminary studies showed that beach pea seeds are resistant to water imbibition and germination. Soaking seeds in concentrated H_2SO_4 for 30 min and then in distilled water for 12 h at room temperature showed complete destruction of the compact structure of beach pea seed coat (Figure 2C). Beach pea seeds that were heat-processed for 30

Table 4. Tannin Content of Mechanically (Fractions 1 and 2) and Manually Separated Beach Pea Hulls[a]

	fraction 1		fraction 2		manually separated hulls	
assay	absorbance/g	content, mg/g	absorbance/g	content, mg/g	absorbance/g	content mg/g
vanillin	340 ± 9.0	572.4	311 ± 5.0	524.9	500 ± 3.0	396.8
proanthocyanidin	822 ± 17.0	(catechin equiv.)	614 ± 23.0	(catechin equiv.)	212 ± 15.5	(catechin equiv.)
protein precipitation	91.3 ± 7.5		57.8 ± 1.5		64.3 ± 1.3	
dye-labled BSA[b]	232 ± 20.0	1131 mg BSA/g	175 ± 26.0	851.8 mg BSA/g	163 ± 6.3	793.4 mg BSA/g
total phenol (Folin-Denis)	444 ± 15.0		284 ± 5.0		28.5 ± 2.0	

[a] Results are means of three determinations, on a dry weight basis, ± standard deviation. [b] Bovine serum albumin.

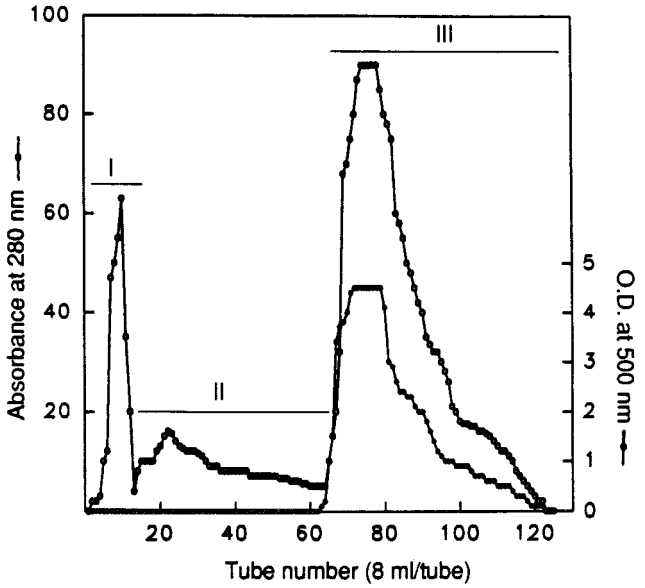

Figure 3. Separation of phenolic fractions of beach pea hulls extracts by Sephadex LH-20 column chromatography: UV absorbance of phenolics (280 nm) and condensed tannins (500 nm) following color development [Fractions I and II ethanol mobile phase, and fraction III with acetone−water, 50:50 (v/v) mobile phase].

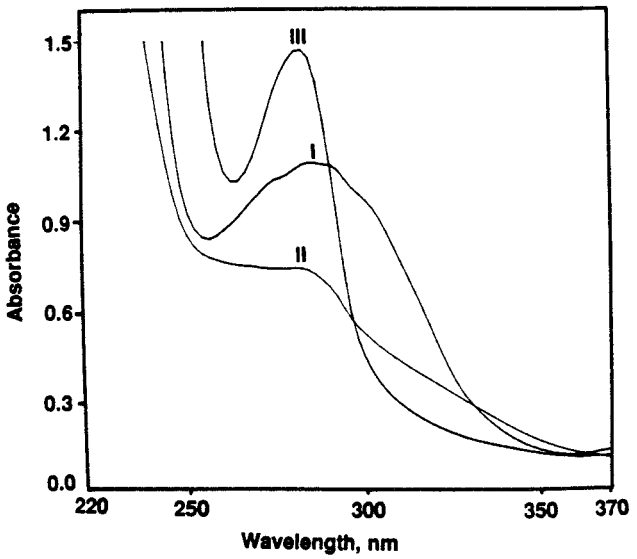

Figure 4. UV spectra of individual fractions of beach pea hulls extracts separated on a Sephadex LH-20 column [fractions I and II from ethanol as mobile phase and fraction III from acetone−water, 50:50, (v/v) as mobile phase].

Table 5. Percentage Recovery of Beach Pea Hulls Extract and Their Total Phenolic and Condensed Tannins[a]

fraction	relative content (% of total)	total phenolic (% of extract)[b]	condensed tannins (% of extract)[c]
crude extract	21.53 ± 1.23[c]	54.15 ± 1.26[b]	156.73 ± 1.53[b]
fraction I	23.87 ± 0.87[b]	3.11 ± 0.41[d]	ND
fraction II	19.22 ± 0.42[d]	19.21 ± 0.23[c]	ND
fraction III	56.89 ± 1.03[a]	79.06 ± 1.06[a]	220.19 ± 3.83[a]

[a] All fractions separated on Sephadex LH-20 column. Results are means of three determinations, ± standard deviation. Means followed by different superscripts in each column are significantly ($p < 0.05$) different from one another. ND, Not detected. [b] As sinapic acid equivalents. [c] As catechin equivalents.

min in boiling water also were swollen and showed loosened seed coat structure (Figure 2D). These two treatments make beach pea seed coat permeable to water and help imbibition as well as leaching out, and thus lowering of the antinutritional factors by diffusion. These two treatments (soaking in H_2SO_4 and heat processing) were found to be useful for separation of hulls from cotyledons.

Separation of Phenolic Fractions from Beach Pea Hull Extract. Three phenolic fractions (fractions I−III) were separated from the crude acetone extract (70%, v/v, containing 1% concentrated HCl) of beach pea hulls via Sephadex LH-20 column chromatography, and each fraction was tested for the presence of condensed tannins (Figure 3). One main peak (III) and two minor peaks (I and II) were characterized when measuring absorbances at 280 nm (Figure 4). The relative content of fraction III in the total extract was highest. For beach pea hulls, the highest content of phenolic compounds and condensed tannins was observed in fraction III. Furthermore, the crude extract possessed considerably more phenolic compounds than fractions I and II, whereas condensed tannins were not detected in fractions I and II (Table 5). Phenolic compounds from Polish white bean, green pea, everlasting pea, lentil, broad bean, and faba bean seed hulls showed higher amounts of crude extract compared with that of the whole seeds, but less than beach pea hulls. Hull extracts also had high antioxidative properties, compared with the antioxidative activity of seeds of mustard, canola, rape, and flax. The extract obtained from seed coats contained 2.5−13 times higher amounts of total phenolics than the extracts from whole seeds of Polish white bean, green pea, everlasting pea, lentil, broad bean, and faba bean (*42*).

The absorption maxima of separated fractions (Figure 4) occurred mainly in the range of 280 to 290 nm. Fractions I, II, and III had only one maximum at 284, 284, and 282 nm, respectively. This suggests that flavonoids are potentially the main phenolics present in beach pea hulls extracts. Mabry et al. (*43*) reported that flavones and flavonols produced two major absorption peaks in the 240 to 400 nm range in methanol. Amarowicz and Raab (*44*) separated five phenolic fractions from the acetone extract of everlasting pea, faba bean, and broad bean using Sephadex LH-20 column chromatography. They also reported that UV spectra of separated fractions from these legumes had most of their absorption bands in the range of 270 to 280 nm.

TLC Separation of Phenolic Fractions of Beach Pea Hulls Extract. Thin-layer chromatography of the three isolated fractions and crude extract from beach pea hulls indicated that the separated fractions contained several phenolic compounds (Figure 5). Compounds from fraction III of beach pea hulls extract, close to the solvent front in both developing systems, exhibited the highest antioxidant activity. Several phenolic compounds were visualized on silica gel TLC plates. Therefore, fraction III with the highest antioxidant activity may contain several kinds of antioxidative phenolics. The total number of hydroxyl groups present in compounds of fraction III may be higher than those in compounds of other fractions or different compound(s) may be present; this could be, in part, responsible for better antioxidative properties of this fraction as shown in the inhibition of the bleaching of β-carotene.

Antioxidant Activity of Phenolic Fractions of Beach Pea Hulls Extract. The antioxidant activity of each fraction and crude extract, as compared with that

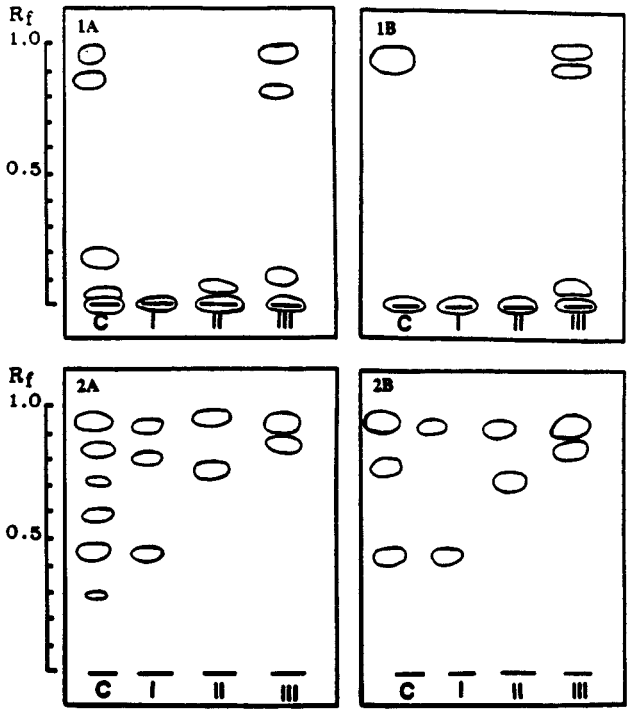

Figure 5. TLC of phenolic fractions separated from beach pea hulls extracts; chromatograms were developed using (1) acetic acid−petroleum ether−diethyl ether (1:20:80, v/v/v) and (2) acetic acid−water−*n*-butanol (10:10:30, v/v/v). Plates were sprayed with a solution of ferric chloride (A) to give spots of phenolic compounds; and with (B) β-carotene−linoleate in order to evaluate antioxidant activity of crude extract (C) and fractions (I−III).

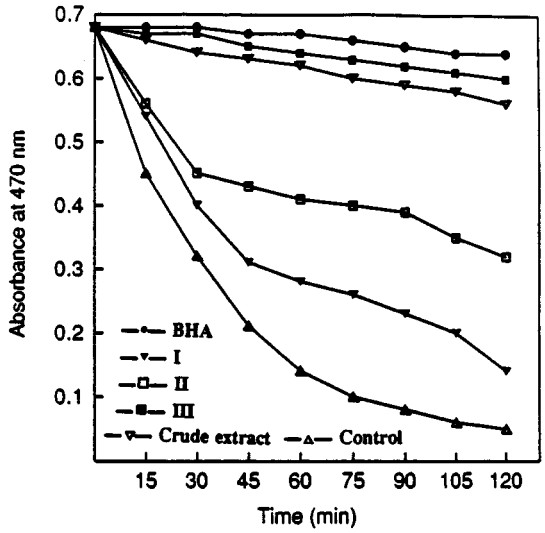

Figure 6. Inhibition of bleaching of β-carotene in a model β-carotene−linoleate system containing the crude extract or individual fractions of beach pea hulls extracts separated on a Sephadex LH-20 column.

of BHA, is presented in Figure 6. Fraction III exhibited the highest antioxidative activity against the bleaching of β-carotene. The crude extract had a better antioxidative effect than fractions I and II. The activity of fraction III was higher than that of fractions I and II as well as that of the crude extract. Fraction III, which exhibited the best antioxidative activity, contained 79.1% phenolics, as sinapic acid equivalents, and 220% condensed tannins, as catechin equivalents (Table 5). The very high values of condensed tannins obtained

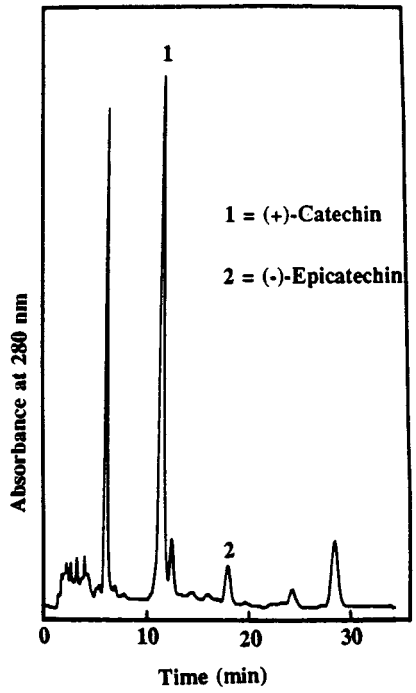

Figure 7. HPLC chromatogram of separated (+) catechin and (−) epicatechin from Sephadex LH-20 fractions (tube numbers 66−120, Figure 3).

(220%) might be due to extensive condensation of the tannins involved, thus, use of catechin as a standard would lead to overestimation of condensed tannins involved. This indicates that the amount of phenolic compounds and their molecular structures play an important role in their antioxidative activity (*45*).

Separation of Phenolic/Tannin Fraction. Fraction III of the beach pea hulls extract, which contained the highest amount of phenolics and condensed tannins, and possessed strong antioxidant activity, was further separated on a semipreparative HPLC. Presence of (+) catechin and (−) epicatechin as main phenolic compounds in this fraction was confirmed (Figure 7).

CONCLUSIONS

For beach peas, the protein content was enriched and the content of phenolics and condensed tannins (which are mostly concentrated in the seed coats) was reduced in the air-classified cotyledons. The extract of beach pea hulls exhibited a strong antioxidant activity in a β-carotene−linoleate model system. The extract contained different classes of phenolic compounds with varying antioxidative strengths. Fraction III, separated on a Sephadex LH-20 column, exhibited the highest antioxidative activity and contained several phenolic compounds. Thus, beach peas may present an alternate source of legumes for food use, while the pea hulls and their extracts could potentially be employed as nutraceutical ingredients.

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