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Effect of various solvent systems on extraction of phenolics, tannins and sugars from beach pea (*Lathyrus maritimus* L.)

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Abstract

Phenolic compounds and sugars were extracted from beach pea (*Lathyrus maritimus* L.) seeds using methanol-water, ethanol-water and acetone-water solvent system (80:20, v/v) at 80°C. The extracted solvents were used for determining the content of phenolic compounds and sugars by colorimetrically. UV spectra were measured and TLC analysis was performed on silica gel to compare phenolic compounds extracted in particular solvent systems. UV absorbance of phenolics (280 nm) and condensed tannins were measured at 500 nm after colour development. The vanillin positive acetone extracts were pooled, concentrated and subsequently subjected to HPLC analysis. Both (+)-Catechin and (-)-Epicatechin were identified in the extraction solution. Acetone-water system extracted considerably higher amounts of phenolic compounds and condensed tannins than the ethanol-water or methanol-water systems. UV spectra of pooled extracts were similar for all solvent systems employed, but TLC plate analysis showed that the presence of higher molecular weight tannins which were not found when methanol-water or ethanol-water were used for extraction.

Introduction

Phenolic compounds classified as phenolic acids and their derivatives, are most numerous and widely distributed groups of natural products in the plant kingdom. In recent years, phenolic compounds in many edible plant products have received increasing attention as a result of their influence on nutritional and aesthetic quality of foods, their biochemical and physiological functions as well as pharmacological implications. The interest of researchers has been focused on phenolic compounds of plant origin mainly due to their biological activity (Wilska-Jeszka, 1989). This activity is demonstrated in lowering the level of histamine by inhibiting of histidine decarboxylase by procyanidines and consequently may diminish permeability of capillary blood vessels (Wilska-Jeszka, 1989), thus preventing atherosclerosis by participation in cholesterol changes and transport (Ginter, 1973), inhibiting reverse transcriptase of human immunodeficiency virus (HIV) by catechins (Nakane and Ono, 1990). Moreover, many phenolic compounds occurring in plants, such as flavonoids (Matsuzaki and Hara, 1985; Terao *et al.*, 1994), phenolic acids (Nowak *et al.*, 1992), or lignans (Amarowicz *et al.*, 1993) show antioxidative properties.

Studies on phenolic compounds in legume seeds have been concentrated mainly on tannins. Special attention has been paid to tannins capability to inhibit proteolytic enzyme activities in the digestive tract (Helsper *et al.*, 1993a, 1993b). These compounds may also affect the absorption of nutrients from the alimentary tract (Frejagel *et al.*, 1994). The type of solvent used for extraction is important for both quantification and classification of phenolic compounds occurring in plants and obtaining pure compounds for their analysis (Brun *et al.*, 1992). Various solvents generally used for extraction of different phenolic compounds include water (Matsuzaki and Hara, 1985), ethanol-water or acetone-water (Saijo, 1982; Amarowicz and Shahidi, 1995). For extraction of catechins, methanol-water or ethanol-water have been used and phenolic acids have been extracted with acetone-water, dimethylformamide-water. Meanwhile, methanol containing hydrochloric acid has been used for extraction of condensed tannins (Zadenowski, 1987; Carmona *et al.*, 1991; Nack *et*

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al., 1992; Makkow and Becker, 1994). The aim of the study was to evaluate the extraction capability of methanol-water, ethanol-water and acetone-water for phenolic compounds, condensed tannins and sugar from beach pea.

Materials and Methods

Materials

Mature pods of beach pea were collected from Bellevue Beach of Newfoundland, St. John's Canada in September-October 2010. The seeds and pod shells were separated manually. Recovery of seeds and pods shells was recorded immediately after harvesting. Fully mature seeds of beach pea were ground using a Moulinex Coffee grinder (Black and decker Canada Inc., Brockville, ON) and sieved with 60 mesh sieve and subsequently used for further analysis.

Extraction of polyphenolic compounds

Extraction of phenolic compounds along with sugars was carried out in the following manner. Twenty five grams of ground pea meal were introduced into a 100 ml dark glass bottle and suspended in 200 ml of methanol-water, ethanol-water or acetone-water (80:20, v/v). Tightly capped bottles placed in water bath at 800C. After 15 min during which the content was shaken twice, the extract was cooled and filtered under partial vacuum. The material left on the filter paper was transferred back to dark glass bottles for further extraction with 200 ml of the same extraction solution. This procedure was repeated three times over 30, 60 and 90 min of extraction, each time collecting the solution for analysis. Supernatants were combined and evaporated using rotary vacuum evaporator to remove any remaining solvent; the water was then removed by lyophilization.

Determination of phenolic compounds

A known quantity of lyophilized sample was dissolved in absolute methanol and used for the determination of total phenolics and condensed tannins as described by Naczk *et al.* (1992). The results were expressed as mg trans-sinapic acid equivalents per 100 g sample for phenolics and mg catechin equivalents per 100 g dry meal for condensed tannins. The same colorimetric methods were used to analyze the degree of extraction of phenolic compounds and sugars after extractions.

Estimation of sugars

Following evaporation of the organic solvents in a rotary vacuum evaporator at 40°C, the remaining water was removed by lyophilization followed by colorimetric determination of sugars by the method of Dubois *et al.* (1956) was carried out. A known quantity of the lyophilized extract was dissolved in a known amount of distilled water, 2 ml of the sugar solution were pipetted into a test tube and 0.05 ml of an 80% (w/v) phenol was added to the mixture. Subsequently 5 ml of concentrated sulphuric acid were added rapidly, the stream of acid being directed against the liquid surface rather than the side of the test tube in order to obtain good mixing. The tubes were allowed to stand for 10 min, then shaken and placed for 20 min at room temperature (25-30°C). The absorbance of the characteristic yellow-orange colour was read at 490 nm for hexoses. Blanks were prepared by substituting distilled water for the sugar solution. The amount of sugar present in the sample was determined by constructing a standard curve using glucose.

UV spectra

Extraction of phenolic compounds and sugars by different solvent systems was monitored by means of UV absorption at 280 nm using a diode array spectrophotometer (Hewlett Packard 8452A Diode Array Spectrophotometer, Montereal, PQ). UV spectra of the combined extracts (Extractions I-IV) in methanol were also measured.

Thin layer chromatography

The extracts were also characterized by means of thin layer chromatography on silica gel plates (Merck) using the following developing systems; A: acetic acid-water-n-butanol (10:10:30, v/v/v) (Zadernowski, 1987); B: acetic acid-petroleum ether-diethyl ether (1:20:80, v/v/v); and C: water-methanol-chloroform (10:35:65, v/v/v) (Amarowicz *et al.*, 1992). Following developing of plates, they (A and B) were sprayed with an aqueous solution of ferric chloride to visualize phenolic compounds (Barton *et al*., 1952). Sugars, glucosides and some other organic compounds were visualized on plate "c" by spraying with an aqueous solution of H2S04 (10 g/100 ml) and heating at 120°C for 10 min (Amarowicz et al., 1992).

Column chromatography

A 1.0 g portion of the acetone extract was dissolved in 5 m1 of methanol and applied to a chromatographic column (3.4 x 50 cm) packed with Sephadex LH-20 and eluted with methanol. Fractions (6 ml) were collected using a fraction collector and their absorbance in methanol was read at 280 nm.

Thin layer chromatography

The eluted fractions were characterized by means of thin layer chromatography on silica gel

plates (Merck) using water-methanol-chloroform (10:35:65, v/v/v) as the developing system followed by spraying with a 0.5% vanillin solution in methanol containing 4% (v/v) HCl. Absorbance at 500 nm was read after colour development for condensed tannins (Price *et al*., 1978). Eluates were then pooled into two major fractions based on their absorbance at 280 nm and TLC examination. The purity of each fraction was then tested using catechin as a standard (Sigma Chemical Co., St. Louis, MO).

HPLC analysis

The vanillin-positive fraction, separated on Sephadex LH-20 (tube numbers 23 to 33), was used for purity testing by semi-preparative HPLC using standard catechin (Sigma Chemicals Co., St. Louis, MO). A Shimadzu (Japan) chromatographic system was used and consisted of a LC6A 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 x 250 mm) (Chromatographic Specialties, Inc., Brockville, ON). The mobile phase was acetic acid¬ methanol-dimethylformamide-water (1:3:40:157, v/v/v/v) (Hofler and Coggon, 1976) and the flow rate was 1.5ml/min with 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 semi-preparative HPLC column under identical conditions compared to the unknowns from beach pea seed extracts.

Statistical analysis

All determinations analyses were replicated three times or more. In each case, the mean value ± standard deviation was recorded. Analysis of variance (ANOV A) was performed and differences in mean values were performed using Tukey's Studentized Test at $p < 0.05$ and employing ANOYA and Tukey's Procedures of Statistical Analytical System (SAS, 1990).

Results and Discussion

Effect of different solvents on extraction capability of phenolics, tannins and sugars

The extraction capability of different solvents for phenolic compounds, tannins and sugars with time of extraction is presented in Figure 1 and Table 1. UV data after consecutive stages of extraction at 280 nm showed that acetone-water (80:20, v/v) was most effective in extracting a maximum amount of phenolic compounds and tannins from beach pea. Acetone-water mixture extracted almost 1.5 times more phenolics and condensed tannins from beach pea seeds as did methanol-water or ethanol-water

Table 1. Comparison of extraction capability of methanol, ethanol and acetone on beach pea as percentage of total amounts[1]

Extraction solvent, time (min)	Soluble sugars	Phenolic compounds[2]	Condensed tannins[3]
Methanol-Water			
15	57.65	32.00	31.98
30	23.86	26.90	27.08
60	10.54	23.32	24.43
90	7.95	17.78	16.51
Ethanol-Water			
15	42.03	32.96	30.15
30	30.63	27.35	27.85
60	19.24	22.78	23.55
90	8.10	16.91	18.45
Acetone-Water			
15	38.19	49.34	52.51
30	31.21	30.32	21.35
60	19.92	14.39	17.55
90	10.68	5.95	8.59

[1]Results are means of three determinations, [2]As sinapic acid equivalents, [3]As catechin equivalents.

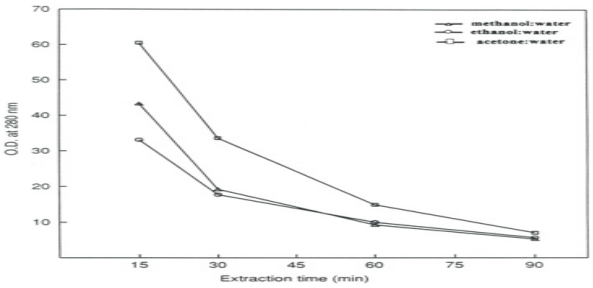

Figure 1. Comparison of extracting capability of the solutions used: extraction of compounds with UV absorbance at 280 nm; --Δ,-- methanol-water; --O-- ethanol¬-water; --□-- acetone-water extracts; all solvents at 80:20, (v/v) ratio

mixtures. Furthermore, methanol-water system extracted higher amounts of sugars than ethanol-water and acetone-water. However, methanol-water was most effective in extracting a maximum amount of sugars from beach pea seeds. This might be due to simple sugars and oligosaccharides which dissolve more easily in methanol-water than in ethanol-water or acetone-water. Price and Sipro (1985) and Price and Spitzer (1993) reported that the highest extraction of phenolic compounds from plant material with methanol-water (80:20, v/v) was achieved during the first stage of extraction. Acetone-water (80:20, v/v) was most effective in extracting the phenolic compounds from lentil seeds (Amarowicz *et al*., 1995), but this solvent system was less effective in the removal of sugars.

UV spectra of beach pea seed extracts

The beach pea seeds were extracted 4 times (I-IV) with methanol-water (A) ethanol-water (B) and acetone-water (C); their UV spectra are shown in Figure 2. Four extractions (I-IV) of each solvent combined with their UV spectra recorded between 240

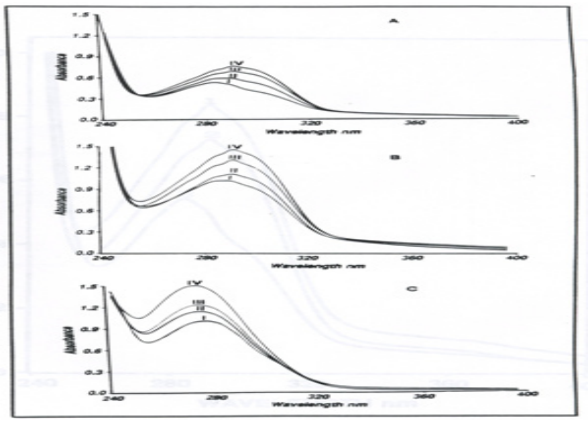

Figure 2. UV spectra of each extraction (I-IV); A, methanol-water (80:20 v/v); B, ethanol-water (80:20, v/v); C, acetone-water (80:20, v/v) from beach pea seed extracts

and 400 nm as shown in Figure 3 (ethanol-water, 1; methanol-water, 2; acetone-water, 3). The UV spectra of ethanol-water extract showed a maximum at 292 nm. The first methanol-water extraction showed UV absorption maximum at 284 nm while the next three extractions and combined extracts (I-IV) exhibited a maximum at 292 nm. When acetone-water was used for the extraction, a maximum was observed at 284 nm. These UV spectral data indicate that methanol-water and ethanol-water solvent systems extract the same types of compounds, but in case of acetone-water different compounds may be extracted from the seeds. Frejnaget *et al*. (1994) reported that the absorption maxima of phenolic compounds obtained from faba bean seed coats ranged from 264 to 280 nm. Amarowicz *et al.* (1995) used similar conditions for extraction of phenolic compounds and sugars from lentil and reported that UV spectrum of the acetone extract had a maximum at 274 nm and compounds in methanol and ethanol extracts had a maximum at 272 nm. Acetone-water (8:2, v/v), used for the extraction of phenolic compounds from everlasting pea, faba bean, and broad bean exhibited an absorption maximum in the range of 260 to 282 nm (Amarowicz *et al*., 1996).

Total extracts and the content of phenolics, condensed tannins and sugars extracted by different solvent systems

The amounts of extract recovered with methanol (80:20, v/v), ethanol (80:20, v/v) and acetone (80:20, v/v) are shown in Table 2. In this table are also shown the amounts of soluble sugars, phenolics and tannins. The amount of extract recovered by employing acetone as a solvent was significantly ($p < 0.05$) higher than when methanol and ethanol solvent systems were used. Extraction of sugars was highest

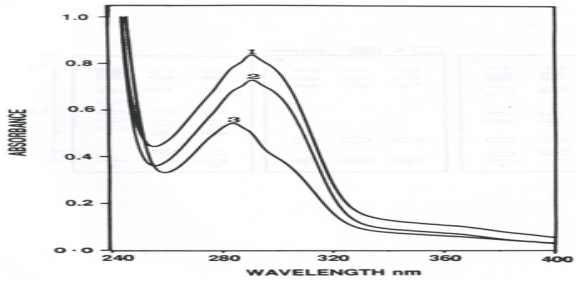

Figure 3. UV spectra of combined extracts (Extractions I-IV) of beach pea seed obtained using (1) ethanol-water (80:20, v/v); (2) methanol-water (80:20, v/v); (3) acetone-water (80:20, v/v) solvents

when methanol was employed and the content of phenolic compounds extracted from beach pea seeds by acetone were twice that recovered with methanol or ethanol. Extraction of condensed tannins with acetone-water was also ten time more effective than when methanol or ethanol was used. However, the sugar content in methanol extract was higher than that in ethanol or acetone extract. These results indicate the importance of choice of solvent in quantification of different components of the extracts.

Table 2. The content of extract (%), sugars (%), phenolic compounds (mg/100 g) and condensed tannins (mg/100 g) in beach pea seeds using different solvent systems[1]

Extraction solvent	Extract	Sugars	Phenolic compounds[2]	Condensed tannins[3]
Methanol-Water (80:20, v/v)	16.62 ± 0.60^{ab}	5.03 ± 0.35^{a}	619.64 ± 0.31^{b}	748.93 ± 2.29^{b}
Ethanol-Water (80:20, v/v)	15.01 ± 0.65^{b}	4.87 ± 0.29^{a}	421.06 ± 0.54^{c}	381.06 ± 1.70^{c}
Acetone-Water (80:20, v/v)	19.32 ± 0.90^{a}	2.95 ± 0.15^{b}	1283.87 ± 3.90^{a}	7485.74 ± 3.01^{a}
Difference significance	A=M, A>E, M=E	M=E>A	A>M>E	A>M>E

[1]Results are means of triplicate determinations, ± standard deviation, Means followed by different superscripts in each column are significantly ($p < 0.05$) different from one another, M=Methanol, E=Ethanol, A=Acetone, [2]As sinapic acid equivalents, [3]As catechin equivalents.

The content of phenolic compounds, condensed tannins and sugars in total dry extract of beach pea showed a similar trend to that present in the solvent extract (Table 3). However, these results for phenolic compounds and condensed tannins were much higher than those reported for other legumes (Salunkhe and Kadam, 1989).

TLC separation of beach pea seed extracts

TLC plates, to which beach pea seed extracts were applied, were developed using different solvent systems and subsequently sprayed with different reagents. Chromatograms of extracts developed

Table 3. The content sugars, phenolic compounds and condensed tannins in total dry extract of beach pea (g/100 g) determined with various solvent systems[1]

Solvent system	Soluble sugars	Phenolic compounds[2]	Condensed tannins[3]
Methanol-Water (80:20, v/v)	31.42 ± 1.92[a]	6.24 ± 0.14[b]	12.22 ± 0.44[b]
Ethanol-Water (80:20, v/v)	29.49 ± 2.17[a]	5.94 ± 0.21[b]	9.28 ± 0.64[c]
Acetone-Water (80:20, v/v)	18.26 ± 0.65[b]	11.64 ± 0.62[a]	59.99 ± 1.05[a]
Difference significance	M=E>A	A>M=E	A>M>E

[1]Results are means of triplicate determinations, ± standard deviation, Means followed by different superscripts in each column are significantly ($p < 0.05$) different from one another, M=Methanol, E=Ethanol, A=Acetone, [2]As sinapic acid equivalents, [3]As catechin equivalents.

with acetic acid-water-n-butanol (10:10:30, v/v/v), a high polarity solvent system, showed three intense spots from ethanol extract and these were close to the solvent front. Methanol extract also showed three intense spots, one very close to the origin and the other two at R_f values of 0.3 and 0.9. Acetone extract also showed two intense spots at R_f values of 0.3 and 0.9. Chromatograms developed with acetic acid-petroleum ether-diethyl ether (1:20:80, v/v/v), a non-polar solvent, and sprayed with a ferric chloride solution showed only two intense spots for ethanol at R_f values of 0.8 and 0.9. Same extracts used for development of TLC plates in water-methanol-chloroform (10:35:65, v/v/v) a polar solvent and sprayed with 10% sulphuric acid and heated at 120°C showed four very intense spots close to the solvent front in ethanol extract, two spots in methanol at Rf values of 0.1 and 0.8, respectively, for the presence of sugars and no intense spots were observed for acetone extract. Ferric chloride reacts with phenolic compounds and gives blue or greenish coloured spots while sulphuric acid char the sugars at higher temperatures to yield black spots.

Separation of phenolic/tannin fraction of beach pea extract

The phenolics of beach pea extracted by acetone-water were further separated using Sephadex LH-20 column chromatography. UV absorbances of extracted phenolics at 280 nm and condensed tannins at 500 nm, following colour development, are presented in Figure 4. Major phenolics were found in tube numbers 10-23 and condensed tannins in tube numbers 23-30. The condensed tannins fraction, separated on Sephadex LH-20, was further separated by semipreparative HPLC. The major fraction (tube numbers 23-30) of condensed tannins of beach pea seed extract contained (+) catechin and (-) epicatechin as its major compounds (Figure 5). Similar type of separation of (+) catechin and (-) epicatechin were reported for pea bean (*Phaseolus vulgaris* L.) extract (Tsuda *et al*., 1993).

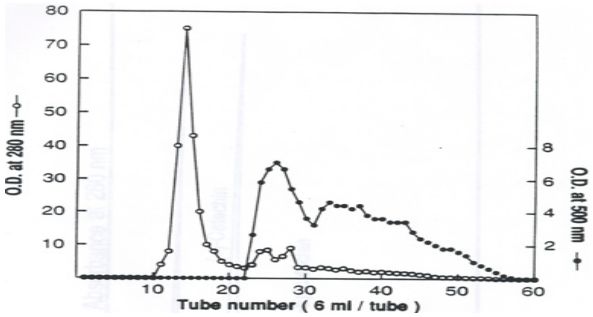

Figure 4. Separation of phenolic fractions of beach pea seed extracts by Sephadex LH-20 column chromatography: UV absorbance of phenolics (280 nm) and condensed tannins (500 nm) following colour development

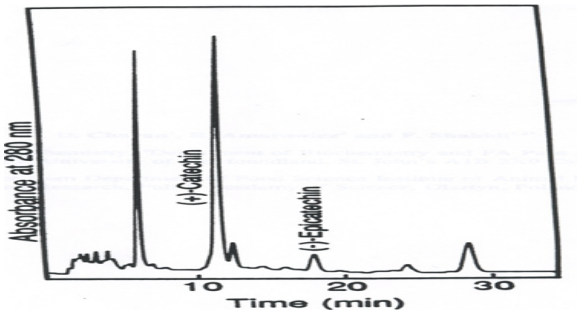

Figure 5. Chromatogram of the analytically separated pure (+) catechin and (-) epicatechin obtained from Sephadex LH-20 fractions (tube numbers 23-30, Figure 5) followed by semi-preparative HPLC separation

Conclusion

For the extraction of phenolics and tannins with acetone-water (80:20, v/v) was found more effective than other solvents. UV spectra and TLC analysis are very good indicators for identifying phenolics and tannins present in the pea extracts. HPLC analysis gives the presence of (+)-Catechin and (-)-Epicatechin in the extraction solution. These compounds can be isolated from the different food sources for utilization in other various food products as a neutraceutical or antioxidants.

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