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EXTRACTION AND CHROMATOGRAPHIC SEPARATION OF TANNIN FRACTIONS FROM TANNIN-RICH PLANT MATERIAL

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Key words: tannins, leguminous seeds, nuts, buckwheat, extraction, Sephadex LH-20, column chromatography, protein precipitating capacity

The crude phenolic extracts were obtained from red bean, adzuki bean, red lentil, green lentil, broad bean, faba bean, field bean, vetch, buckwheat, buckwheat groat, hazelnuts, walnuts, and almonds using 80% (v/v) acetone. The tannin fractions were separated from the crude extracts using Sephadex LH-20 column chromatography with ethanol and 50% (v/v) acetone. The tannin fractions were characterised by colour reaction with vanillin/HCl reagent and ability to bovine serum albumin (BSA) precipitation. The yield of extraction of phenolic compounds ranged from 3.77% (buckwheat) to 17.20% (walnuts). The content of tannin fraction in the crude extracts was differentiated and ranged from 10.5% (faba bean) to 45.5% (broad bean). The tannin fraction from buckwheat possessed the highest content of tannins determined using vanillin method (1.04 absorbance unit at 500 nm/mg) whereas the lowest content was noted for broad bean (0.066 absorbance unit at 500 nm/mg). In the model system, the highest amount of BSA was precipitated by tannin fraction of hazelnuts, whereas the lowest was noted for adzuki bean.

INTRODUCTION

According to a definition of Bate-Smith, tannins are "water-soluble phenolic compounds having molecular weight between 500 and 3 000, and besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins" [Bate-Smith & Swein, 1962]. Tannins are divided into three main classes. The condensed tannins (proanthocyanidins) are flavan-3-ol based biopolymers that at high temperature in alcohol solutions of strong mineral acid release anthocyanidins and catechins as end groups. Gallotannins and ellagitannins belong to hydrolysable tannins. Gallotaninns are comprised of galloyl esters of glucose or quinic acid whereas ellagitannins are derivatives of hexahydroxydiphenic acid [Hagerman *et al*., 2005]. Phytochemists and nutritionists are used to call tannins "a double-edged sword in biology and health" [Chung *et al.*, 1998]. Tannins act as an antinutrient compound of plant origin because they precipitate proteins, inhibit digestive enzyme, decrease the utilization of vitamins and minerals. Yet, tannins have also been considered a health-promoting component in plant-derived foods and beverages. For example, tannins have been shown to have anticarcinogenic and antimutagenic potential, and antimicrobial properties [Cos *et al*., 2004; Awika *et al.*, 2006]. Several studies have reported on antioxidant and antiradical activity of tannins [Amarowicz *et al*., 2004; Alasalvar *et al*., 2006; Amarowicz, 2007].

The aim of the study was to determine yield of tannins extraction from tannin-rich plant material (1), to compare the content of tannins fractions separated from the crude extracts using Sephadex LH-20 column chromatography (2), and to characterize tannin fractions by vanillin reaction and their protein precipitating activity (3).

MATERIALS AND METHODS

Materials. Seeds of red bean, adzuki bean, red lentil, green lentil, broad bean, faba bean, field bean, vetch, and buckwheat were obtained from the Plant Breading Station in Olsztyn. Roasted buckwheat groat, hazelnuts, walnuts, and almonds were acquired at a local market in Olsztyn.

Chemicals. Sephadex LH-20, vanillin, bovine serum albumin (BSA), sodium dodecyl sulphate (SDS), were obtained from Sigma-Aldrich Co. Ltd. (Poznań, Poland). Acetone, ethanol, and triethanolamine, all analytical grade, were purchased from P.O.Ch. Company (Gliwice, Poland).

Extraction of phenolic compounds. Ground plant material (20 g) was weighed out into sealable flasks and 160 mL of 80% acetone (v/v) was poured into [Amarowicz *et al.,* 1995]. Flasks were placed in a water bath at 70°C and shaken for 15 min. After cooling, supernatant was decanted carefully. Extraction was repeated twice more. Supernatants were combined, acetone was evaporated using rotary evaporator at 40°C, and aqueous residue was lyophilised. Hazelnuts, walnuts, and almonds were defatted using hexane before extraction.

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Isolation of tannins. The crude phenolic extract (2 g) dissolved in 20 mL of ethanol was applied on a column (5 × 40 cm) packed with Sephadex LH-20 gel. Ethanol (1L), used as first eluent, allowed removing low molecular weight phenolic compounds. Then 600 mL of 50% acetone (v/v) was used to elute tannins. Solvent from tannin fractions was removed using rotary evaporator, and water was removed during lyophilisation.

Content of condensed tannins. The content of condensed tannins in such obtained materials was determined according to a modified vanillin assay [Price *et al.,* 1978]. Tannin fractions were dissolved in methanol (0.5 mg/mL). To 1 mL of prepared solution, 5 mL of vanillin/HCl reagent (0.5 g vanillin in 4% hydrochloric acid in methanol (v/v)) was added. Samples and controls (without vanillin) were allowed to stand for 20 min in darkness and then absorbance at 500 nm was read. Results were expressed as absorbance units per 1 mg of tannin fraction.

BSA precipitation method. To 1 mL of tannin solution in water (1 mg/mL), 2 mL of bovine serum albumin solution in 0.2 mol/L acetic buffer, pH 5.0 with 0.17 mol/L NaCl (1 mg/mL) was added and mixed carefully [Hagerman & Butler, 1978]. After 15 min the samples were centrifuged at 5000 g for 15 min. The supernatant was removed, and pellet was dissolved in 4 mL of aqueous solution containing 1% SDS and 4% triethanolamine. Then 1 mL of 0.01 mol/L $FeCl_3$ in 0.01 mol/L HCl was added. After 30 min the absorbance was recorded at 510 nm. Results were expressed as absorbance units per 1 mg of tannin fraction.

RESULTS AND DISCUSSION

The range of solvent used for extraction of various groups of phenolic compounds from plant material is very broad and includes water, methanol, ethanol, water-ethanol, water--methanol, acetone-water, dimethyl-formamid-water [Amarowicz *et al.,* 1995; Naczk *et al.,* 2005, 2007]. According to Amarowicz *et al.* [1995], 80% acetone (v/v) ensured the most complete extraction of phenolic compounds from lentil seeds, especially of flavonols and tannins. The high capability of acetone-water system to extract phenolic compounds and high antioxidant activity of the extracts obtained this way was confirmed by Troszyńska *et al.* [1993], Naczk *et al.* [2005], Rocha-Guzmán *et al.* [2007], and Pegg *et al*. [2007].

The yield of extraction is presented on Figure 1. The result ranged from 3.77% (buckwheat) to 17.2% (walnuts) and they were in order of walnuts > almonds ~ hazelnuts > red bean > red lentil ~ green lentil ~ vetch ~ field bean > broad bean ~ faba bean > adzuki bean > buckwheat groat ~ buckwheat. These results are in accordance with literature data. The yield of extraction from whole grains (wheat, barley, rye, oat, buckwheat) using 80% methanol (shaking at 20°C for 40 min) ranged from 3.82% (oat) to 7.44% (rye cv. *Dańkowskie Złote*) [Zieliński & Kozłowska, 2000]. The content of the 70% acetone extract in common bean cultivars reported by Rocha-Guzmán *et al.* [2007] was 6.13% – 6.83% (extraction for 24 h at room temperature). Different varieties of cow pea (*Vigna unguicula* (L.) Walp.) seeds were characterized by the content of the extracts from 4.00% to 9.73%

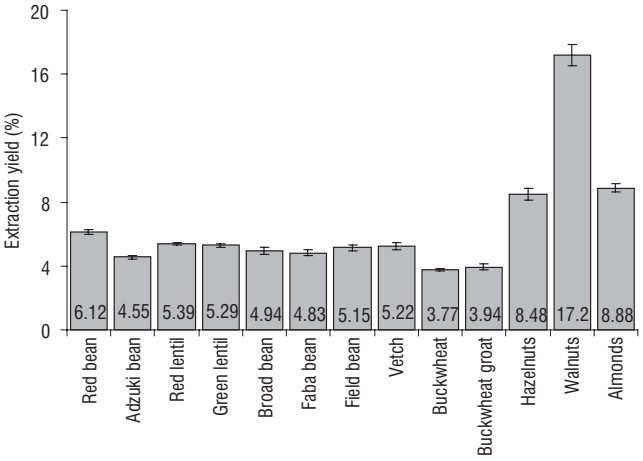

FIGURE 1. Extraction yield of crude phenolics (%) from tannin-rich plant material.

[Siddhuraju & Becker, 2007] (extraction using 70% acetone at 25°C for 24 h). A yield of extraction (10% - 15%) from sage using ethanol-water mixture was reported by Durling *et al.* [2007].

The content of tannin fractions in the crude extracts was differentiated and ranged from 10.5% (faba bean) to 45.5% (broad bean) (Figure 2). The results were in order of broad bean > vetch > walnuts ~ adzuki bean ~ buckwheat > red bean ~ field bean ~ buckwheat groat > almonds ~ red lentil ~ green lentil ~ hazelnuts > faba bean. For Sephadex LH--20 column chromatography the first solvent used was ethanol. Ethanol can elute low-molecular weight phenolics together with sugars. Condensed tannins are available to be eluted with the system acetone-water (1:1; v/v). Therefore the high content of phenolic compounds in the extract can be caused also by the content of sugars in the crude extract. It seems to be interesting to compare in the future the content of sugars and tannin fractions in the crude extracts in tannin-rich plant material.

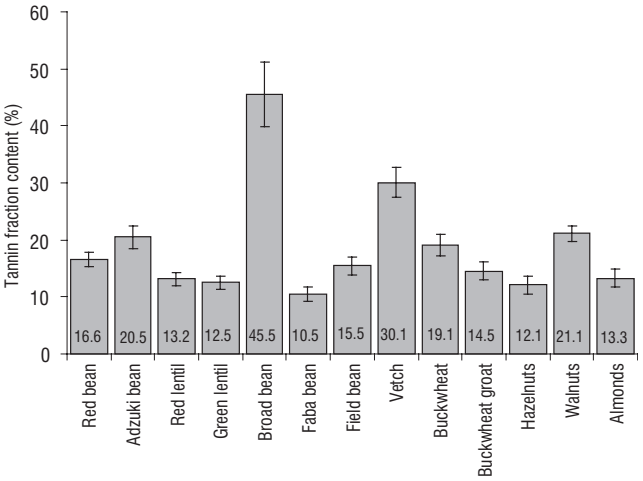

FIGURE 2. Content (%) of tannin fractions in the crude phenolic extracts.

The vanillin/HCl reaction is the principal colorimetric method used for determination condensed tannins and flavan-3-ols (catechins) in plant material. In our study only con-

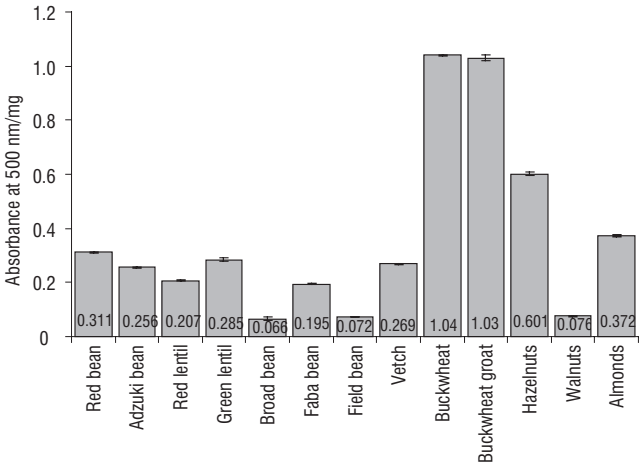

FIGURE 3. Content of tannins in tannin fractions determined using vanillin/HCl assay. The results are expressed as absorbance unit at 500 nm per mg of tannin fractions.

densed tannins gave the colour reaction with vanillin reagent because catechins were eluted with methanol from the column as the low-molecular phenolic fraction. The content of tannins in this work was expressed as absorbance units per 1 mg of tannin fraction (A_{500}/mg). The tannin fraction from buckwheat (1.04) possessed the highest content of tannins determined using vanillin method whereas the lowest content was noted for broad bean (0.066) (Figure 3). The results were in order of buckwheat ~ buckwheat groat > hazelnuts > almonds > red bean > green lentil ~ vetch ~ adzuki bean > red lentil > faba bean > walnuts ~ field pea ~ broad bean. The presence of tannins in leguminous seeds has been reported by several authors [Ariga *et al.,* 1988; Ariga & Hamano, 1990; Chavan *et al*., 1999; De Pascual *et al*., 2000; Amarowicz *et al.,* 2000; Dueñas *et al*., 2002, 2003, 2004]. The content of tannins in tannin fractions separated in this study from buckwheat, buckwheat groat, hazelnuts, almonds, and red bean was higher than that reported by Naczk *et al.* [2001] for the extracts of canola and rapeseed hulls (0.007–0.286 absorbance units at 500 nm /mg). The content of tannins in tannin fractions from beach pea and evening primrose was 0.684 and 1.328, absorbance unit at 500 nm/mg, respectively [Amarowicz *et al*., 2000].

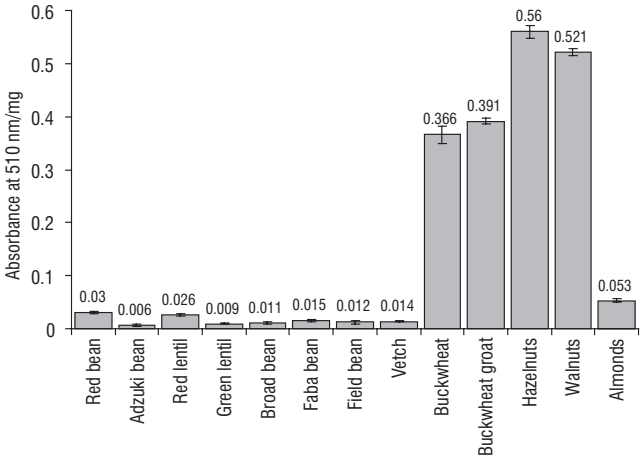

FIGURE 4. BSA precipitation capacity of tannin fractions. The results are expressed as absorbance unit at 510 nm per mg of tannin fractions.

Figure 4 characterises the ability of tannin fraction to precipitate bovine serum albumin (BSA), results are reported as absorbance units at 510 nm/mg. In the model system the highest amount of BSA was precipitated by tannin fraction of hazelnuts (0.560), the lowest result was noted for adzuki bean (0.006). The results were in order of hazelnuts > walnuts > buckwheat groat > buckwheat > almonds > red bean ~ red lentil > faba bean ~ vetch ~ field bean ~ broad bean > green lentil > adzuki bean. Naczk *et al.* [1996] using the same method reported the protein precipitating capacity of crude canola tannins app. 0.03 absorbance units at 510 nm/mg. Tannin fractions separated from wild blueberry leaves and fruits exhibited BSA precipitating activity of 0.651 and 0.742 absorbance units at 510 nm/mg [Naczk *et al.*, 2006].

In this study the high content of condensed tannins determined using vanillin method in the case of buckwheat, buckwheat groats, and hazel-nuts was correlated well with the protein precipitating capacity (Figures 3 and 4). Very week BSA precipitating activity of the tannin fractions separated from the almonds and leguminous seeds was caused by the low degree of polymerisation of tannins. Walnuts tannins showed a week colour reaction with vanillin reagent but strong BSA precipitating activity (Figures 3 and 4). This phenomenon can be explained by the presence of hydrolysable tannins in walnuts. This class of tannins does not react with vanillin but is able to make complexes with BSA. This explanation is in accordance with results of Amarowicz *et al.* [2007] who found in walnuts the presence of gallotannins and ellagotannins.

CONCLUSIONS

The extraction with 80% acetone (v/v) together with a Sephadex LH-20 column chromatography with ethanol and 50% acetone as mobile phases are a useful method for tannins separation from tannin-rich plant material. The method is characterized by a high repeatability. Among investigated plants the richest source of tannins were buckwheat and nuts. Most probably hydrolysable tannins are responsible for protein precipitating activity of the walnut extract.

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EKSTRAKCJA I CHROMATOGRAFICZNE WYODRĘBNIANIE FRAKCJI TANINOWYCH Z BOGATEGO W TANINY MATERIAŁU ROŚLINNEGO

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Surowe ekstrakty związków fenolowych uzyskano z czerwonej fasoli, fasoli adzuki, zielonej i czerwonej soczewicy, bobu, bobiku, peluszki, wyki, gryki, kaszy gryczanej, orzechów laskowych, orzechów włoskich i migdałów stosując 80% (v/v) aceton. Frakcje taninowe wyodrębnione z ekstraktów na drodze chromatografii kolumnowej na żelu Sephadex LH-20 z etanolem i 50% (v/v) acetonem scharakteryzowano reakcją barwną z odczynnikiem wanilinowym oraz poprzez zdolność do precypitacji albuminy surowicy bydlęcej (BSA). Ekstrakcyjność wahała się od 3,77% (gryka) do 17,20% (orzech włoski). Zawartość frakcji taninowej w surowym ekstrakcie wahała się od 10,50% (bobik) do 45,50% (bób). Posługując się metodą wanilinową największą zawartość tanin stwierdzono we frakcji acetonowej z gryki (1.04 jednostki absorbancji przy 500 nm/mg), najniższą w przypadku bobu (0.066 jednostki absorbancji przy 500 nm/mg). W badaniach modelowych najwyższą zdolnością do precypitacji BSA charakteryzowała się frakcja taninowa z orzechów laskowych, najniższą zdolność zanotowano w przypadku frakcji taninowej z fasoli adzuki.