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CONTENT OF PROANTHOCYANIDINS IN SELECTED PLANT EXTRACTS AS DETERMINED *VIA n*-BUTANOL/HCL HYDROLYSIS AND A COLORIMETRIC ASSAY OR BY HPLC – A SHORT REPORT

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Extracts of phenolic compounds were prepared from peas, faba beans, broad beans, red beans, adzuki beans, vetch, red lentils, green lentils, hazelnuts, walnuts and almonds using 80% (v/v) acetone. The content of proanthocyanidins was determined after *n*-butanol/HCl hydrolysis using a colorimetric assay and by an HPLC method. Extracts of red beans and adzuki beans were characterised by the highest content of proanthocyanidins. The hydrolysis of extracts of legume seeds, hazelnuts, walnuts and almonds resulted in delphinidin and cyanidin formation. A strong correlation (r=0.9877) was found between the content of proanthocyanidins in plant extracts determined using a colorimetric assay and by an HPLC method.

INTRODUCTION

Condensed tannins (proanthocyanidins) belong to a health-promoting component found in plant-derived foods and beverages. Reports in the literature have indicated that tannins demonstrate anticarcinogenic and antimutagenic potentials as well as antimicrobial properties [Chung *et al.,* 1993; Katiyar & Mukhtar, 1997; Amarowicz *et al.*, 2000b]. Several studies have also reported on the antioxidant and antiradical activity of these ubiquitous compounds [Muir, 1996; Amarowicz *et al.* 2000a; Amarowicz & Troszyńska, 2004]. Consumption of tannin-rich food and beverage, however, has a major drawback sensorially; that is, the sensation of astringency [Amarowicz *et al.,* 2005].

Analysis of condensed tannins in plant material is somewhat difficult because these phenolic compounds are characterised by different degrees of polymerisation. This, in effect, complicates the employment of HPLC methods. In practice, however, a colorimetric assay with the vanillin/HCl reagent [Price *et al.*, 1978], a bovine serum albumin (BSA) precipitation technique [Hagerman & Butler, 1978], and a colorimetric protocol based on *n*-butanol/HCl hydrolysis [Porter *et al.*, 1986] are often employed in laboratories (Figure 1). Using this last method, heat-stable anthocyanidins are liberated from proanthocyanidins and they can be determined colorimetrically at a wavelength of 550 nm. The individual anthocyanidins formed during *n*-butanol/HCl hydrolysis can be separated and quantified by reversed-phase high performance liquid chromatography (RP-HPLC) [Naczk *et al.*, 2000].

The objectives of this study were to determine the content of proanthocyanidins in acetonic extracts of selected plant materials *via n*-butanol/HCl hydrolysis and a colorimetric assay or by an HPLC method, and then to compare the results obtained from both techniques.

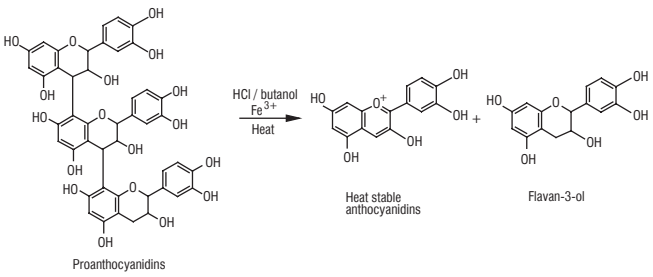

FIGURE 1. Hydrolysis of proanthocyanidins using *n*-butanol/HCl reagent.

MATERIAL AND METHODS

Chemicals. All solvents used were of analytical or HPLC grade. Acetone, hexane, *n*-butanol, and ferric ammonium sulphate were acquired from the P.O.Ch. Company (Gliwice, Poland). Cyanidin and delphinidin were obtained from Extrasynthese S.A. (Genay Cedex, France).

Samples. Plant materials investigated were field peas, faba beans, broad beans, red beans, adzuki beans, vetch, red lentils, green lentils, hazelnuts, walnuts and almonds. Legume seeds were acquired from the Plant Breading Station in Olsztyn, whereas hazelnuts, walnuts and almonds were purchased from a local supermarket in Olsztyn (Poland).

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Extraction. Almonds, hazelnuts and walnuts were first defatted with hexane in a Soxhlet apparatus before preparation of acetonic extracts. To a 1-L dark glass bottle, 35 g of ground material (*i.e.*, prepared using a standard coffee mill) were weighed and suspended in 300 mL of 80% (v/v) acetone [Amarowicz *et al.*, 1994]. Each tightly-capped bottle was placed in an orbital-shaking water bath at 80°C. After 15 min, the extract was cooled and filtered under partial vacuum. The residue on the filter was re-extracted with 300 mL of fresh solvent. This procedure was repeated a total of three times followed by evaporation of the acetone in a rotary evaporator/water bath set at 45°C. The remaining water solutions were lyophilised.

Acid/*n*-butanol hydrolysis of extracts. Proanthocyanidins present in crude extracts were hydrolysed according to the method described by Porter *et al.* [1986]. Briefly, to a 10-mL screw cap tube, 6 mL of the *n*-butanol/HCl reagent (950 mL of *n*-butanol and 50 mL concentrated HCl, 1.0-mL aliquot of the extract, and 0.2 mL of the iron reagent {*i.e.*, 2% (w/v) ferric ammonium sulphate in 2 mol/L HCl} were added and contents vortexed. The tube was capped loosely, and placed in a boiling water bath for 50 min. Then, the tube was cooled and solution transferred to a volumetric flask and adjusted to 25 mL with the *n*-butanol/HCl reagent. The absorbance at 550 nm was recorded using a Beckman DU 7500 diode array spectrophotometer. The results were expressed as absorbance units at 550 nm per 1 mg of extract (A_{550}/mg).

HPLC analysis of anthocyanidins. After *n*-butanol/HCl hydrolysis, the liberated anthocyanidins were analysed with a Shimadzu HPLC system consisting of a LC-10AD pump, SCTL 10A system controller and SPD-M 10A photodiode array detector. Separation was achieved using a prepacked LiChrospher 100 RP-18 column (4 × 250 mm, 5 μm; Merck, Darmstad, Germany). The mobile phase comprised 4% aqueous H_3PO_4:acetonitrile (80:20; v/v) [Wang *et al.*, 1997], the flow rate was 1.0 mL/min and the detector was set at 525 nm.

Statistical analysis. Correlation analysis between the content of anthocyanidins determined according to the colorimetric assay and by the HPLC method (*i.e.* the sum of cyanidin and delphinidin) was performed using Microsoft Excel software.

RESULTS AND DISCUSSION

In this study, proanthocyanidins were present in every extract. Their content did vary markedly, however, by the colorimetric assay when results were expressed as absorbance units at 550 nm per 1 mg of extract (A_{550}/mg). The lowest result was observed for field peas (0.203) while the highest were noted for adzuki beans (1.783) and red beans (1.754).

After *n*-butanol/HCl hydrolysis, delphinidin and cyanidin were the dominant phenolics found in the extracts (Figure 2 and Table 1). The presence of delphinidin was noted for field peas, faba beans, broad beans, vetch, red and green lentils, hazelnuts and almond. The content of delphinidin ranged from 0.05 (almond) to 0.81 (red lentil). Cyanidin was absent only from the field pea extract. In all others, its content ranged from 0.38 (broad beans) to 7.53 (adzuki beans). The lowest sum of both anthocyanidins was noted in the extract of peas (0.22), whereas the highest was found in the extract of adzuki beans (7.53).

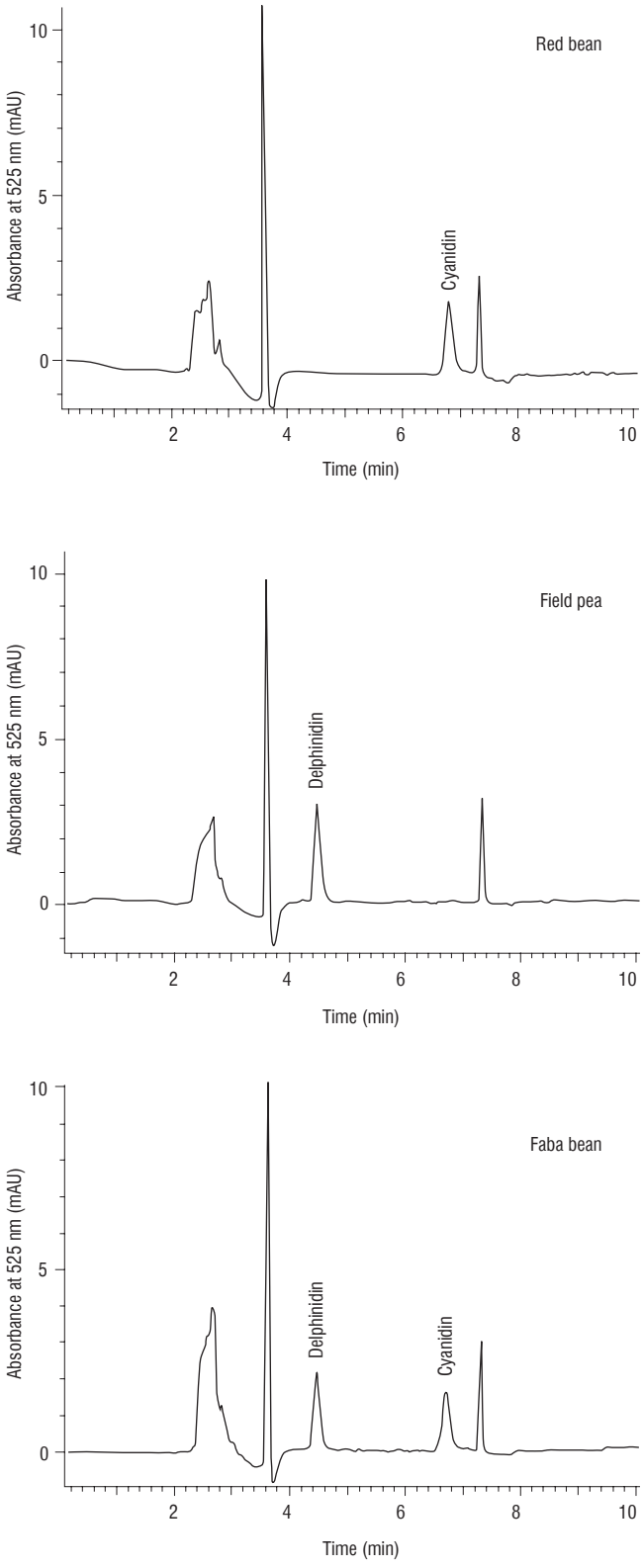

FIGURE 2. Examples of HPLC chromatograms of selected plant extracts hydrolysed using an *n*-butanol/HCl reagent.

Figure 3 depicts the strong correlation ($r=0.9877$; $p<0.01$) between the results for the colorimetric assay and by the HPLC method. The relationship can be described by the following equation:

$$y = 4.245\ x - 0.273$$

where: x – results of colorimetric method expressed as adsorbance at 550 nm/mg; y – content of the sum of cyaniding and delphinidin (mg/g).

The levels of proanthocyanidins in the selected plant materials investigated in this study are in agreement with literature data: the presence of condensed tannins in legume seeds determined using a vanillin or BSA precipitating method as reported by Wilska-Jeszka & Stasiak [1984]; the high content of tannins in faba bean as observed by Amarowicz *et al.* [2000a]; the presence of procyanidin B1 and B3 in adzuki beans as reported by Ariga *et al.* [1988] and Ariga & Hamano [1990]; the content of condensed tannins in ethanolic and acetonic extracts of hazelnuts determined using the vanillin/HCl method was 40.5 and 320 mg/g, respectively [Alasalvar *et al.*, 2006]; and the presence of condensed tannins in almonds extract as reported by Amarowicz *et al.* [2005].

TABLE 1. Content of anthocyanidins in acetonic extracts of selected plants formed during *n*-butanol/HCl hydrolysis and determined by a colorimetric assay (A_{550}/mg) as well as by an HPLC method.

Plant	A_{550} / mg	Delphinidin (mg/g)	Cyanidin (mg/g)	Delphinidin + Cyanidin (mg/g)
Field peas	0.203	0.22	–	0.22
Faba beans	0.545	0.56	1.37	1.93
Broad beans	0.156	0.05	0.38	0.43
Red beans	1.754	–	6.85	6.85
Adzuki beans	1.783	–	7.53	7.53
Vetch	1.347	0.80	5.22	6.02
Red lentils	0.934	0.81	2.98	3.79
Green lentils	0.829	0.66	2.85	3.57
Hazelnuts	1.240	0.14	4.08	4.22
Walnuts	0.654	–	2.44	2.44
Almonds	0.403	0.05	1.76	1.81

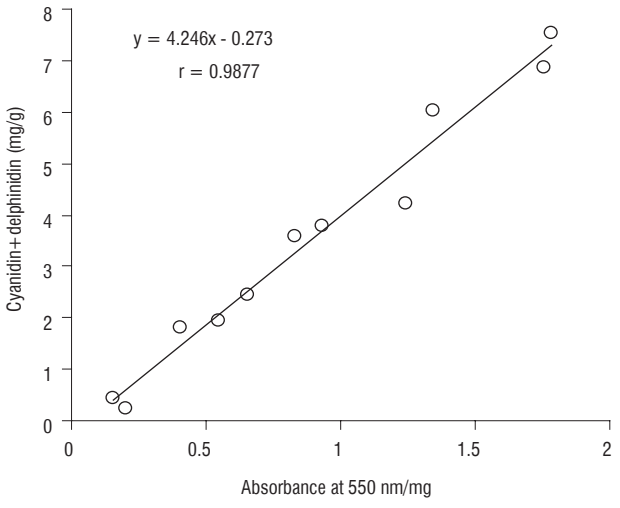

FIGURE 3. Correlation between results of the colorimetric assay and by the HPLC method.

CONCLUSIONS

1. Extracts of legume seeds, nuts and almonds were characterised by different contents of anthocyanidins, formed after *n*-butanol/HCl hydrolysis.

2. The hydrolysis of extracts of legume seeds, nuts and almonds resulted in the liberation of delphinidin and cyanidin.

3. A strong correlation was found among the content of proanthocyanidins in plant extracts determined using a colorimetric assay and by an HPLC method.

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ZAWARTOŚĆ PROANTOCYJANIDYN W WYBRANYCH EKSTRAKTACH ROŚLINNYCH OZNACZONA POPRZEZ HYDROLIZĘ *n*-BUTANOL/HCL I METODĘ KOLORYMETRYCZNĄ ORAZ HPLC – KRÓTKI KOMUNIKAT

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Ekstrakty związków fenolowych uzyskano z peluszki, bobiku, bobu, czerwonej fasoli, fasoli adzuki, wyki, czerwonej soczewicy, zielonej soczewicy, orzechów włoskich, orzechów laskowych i migdałów stosując 80% (v/v) aceton. Zawartość proantocyjanidyn oznaczono po hydrolizie *n*-butanol/HCl stosując metodę kolorymetryczną i HPLC. Ekstrakty z czerwonej fasoli i fasoli adzuki charakteryzowały się najwyższą zawartością proantocyjanidyn. W wyniku hydrolizy ekstraktów nasion roślin strączkowych, orzechów włoskich, orzechów laskowych i migdałów tworzyły się delfinidyna i cyjanidyna. Zanotowano silną korelację (r=0,9877) pomiędzy wynikami uzyskanymi metodą kolorymetryczną i HPLC.