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ORIGINAL PAPER

Effects of Pressurized Low Polarity Water Extraction Parameters on Antioxidant Properties and Composition of Cow Cockle Seed Extracts

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Abstract Antioxidant activity of pressurized low polarity water (PLPW) extracts of cow cockle seed and extraction residues were determined using DPPH, ABTS, and FRAP assays. The effect of extraction conditions (temperature (125, 150 and 175 °C) and time) on the antioxidant activity and the relationship amongst the antioxidant activity and extract composition (total phenolics and saponin content) were determined. The antioxidant activity of PLPW extracts increased with extraction temperature. Increasing activity with time was also observed at 175 °C. PLPW extraction residues had the highest activity suggesting antioxidant compounds were not completely extracted by PLPW. Antioxidant activity correlated well with total phenolics content of samples ($R^2 \geq 0.94$), however no correlation was observed with the saponin content. A strong correlation was observed between the antioxidant activity values obtained using different methods ($R^2 \geq 0.94$). These results point to the potential of PLPW extraction as a method to modify the activity of biological materials for the production of customized extracts.

Keywords *Vaccaria segetalis* · *Saponaria vaccaria* · Cow cockle seed · Phenolics · Saponins · Subcritical water extraction · Antioxidant activity · DPPH · ABTS · FRAP

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Introduction

Pressurized low polarity water (PLPW) extraction has emerged as an environmentally friendly technique for the processing of natural products and has been investigated for the extraction of bioactive compounds such as isoflavones [1], lignans [2], saponins [3], and anthocyanins [4]. It involves the use of high pressures which enables processing at temperatures above 100 °C, improving mass transfer rates and modifying the polarity/solvent power of water. Selective extraction of sample components with temperature, which arises from the differences in the effects of temperature on their solubility behavior, can be used to modify the composition and hence the bioactivity of PLPW extracts [5].

The exposure of biological samples to high temperatures during PLPW extraction might have important implications for bioactivity of extracts. Heat treatment may affect the interaction of bioactive components with matrix components [6] and/or result in the degradation and/or formation of bioactive compounds [7]. The increase of antioxidant activity of citrus peel extracts and the corresponding increase in the total phenolics content due to heat treatment have been attributed to the liberation of phenolic compounds from their bound states [6]. The degradation of bioactive compounds such as anthocyanins has been observed during PLPW extraction of plant materials [4]. An increase in bioactivity due to the formation of new compounds upon heat treatment has also been reported [7, 8]. While heating sugar–lysine model systems resulted in the formation of Maillard reaction products with antioxidant activity [8], the increase in radical scavenging and endothelium-dependent relaxation activities of ginseng was

attributed to the formation of new ginsenosides by steaming at high temperatures (>100 °C) [7]. The hydrolysis of saponins during hydrothermal treatment can also result in the formation of bioactive sapogenins modifying the bioactivity of extracts [9].

Cow cockle seed (*Vaccaria segetalis* Garcke, *Saponaria vaccaria* L., *Vaccaria pyramidata*) is an annual herb widespread in grain fields of the northwestern United States, the prairie provinces of Canada, Asia and Europe [10, 11]. It is known as 'Wang-Bu-Liu-Xing' in traditional Chinese medicine and is commonly used to promote diuresis and milk secretion, activate blood circulation and provide relief of carbuncle [12]. In addition to starch (55%), protein (14%) and oil (2–3%), cow cockle seeds contain bioactive compounds including saponins, alkaloids, cyclopeptides, phenolic acids, flavonoids and steroids [11, 12]. However, research on these bioactive components thus far has largely been limited to their isolation and identification [12]. Research on the processing of cow cockle seeds for the extraction/concentration of saponins and other bioactive compounds has been scarce.

Previously we have demonstrated the potential of PLPW for the extraction of saponins from cow cockle seed and investigated the effects of operating parameters (extraction time, temperature, sample pre-treatment, flow rate and flow direction) on the yield, concentration and composition of bioactive compounds such as saponins and cyclopeptides [3, 13]. This study focuses on the antioxidant activity of PLPW extracts of cow cockle seeds and its correlation with the composition of extracts. Therefore the main objective of this study was to determine the effect of PLPW extraction parameters on the antioxidant activity of cow cockle seed extracts and extraction residues. The relationship amongst antioxidant activity and the total phenolics and saponin contents was further investigated.

Materials and Methods

Materials

Cow cockle seeds, grown near Saskatoon, SK, Canada, were ground through a 1 mm screen using an IKA MF 10 grinder (Rose Scientific Ltd., Edmonton, AB, Canada). Ethanol (95%) and Milli-Q grade water were used for the extractions and analyses unless specified otherwise. DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate), Trolox standard (97%), gallic acid, ABTS 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonate) (~98%), $K_2S_2O_8$ (≥99%), $FeCl_3$ (reagent grade≥98%), TPTZ (2,4,6-tris(2-pyridyl)-*s*-triazine) (≥98%), and Folin–Ciocalteu phenol reagent (2 N) were obtained from Sigma-Aldrich Canada Ltd. (ON, Canada).

Extraction Procedures

PLPW extraction of ground cow cockle seeds (2 g) was carried out at 125, 150, and 175 °C for 3 h using a water flow rate of 2 mL/min as described previously [3]. Fractions were collected every 15 min resulting in a total of 12 fractions. Fractions and residues were freeze-dried and stored at −25 °C until further analysis. The latter fractions (5–8 and 9–12 at 125 and 175 °C, and 7–8 and 9–12 at 150 °C) were combined prior to freeze-drying to ensure adequate quantities for analysis.

Ground cow cockle seeds (200 mg) were also extracted with ultrasonic solvents with varying polarities (10 mL; water, 50% ethanol, and acetonitrile) at room temperature to obtain baseline information on the antioxidant activity of cow cockle seeds. The ultrasonic extractions were carried out in triplicate for 1 h using an ultrasonic bath (Branson 3200 ultrasonic cleaner, Branson Ultrasonics Corporation, Danbury, CT). Similarly, the residues of PLPW extractions (100 mg) were extracted using 50% ethanol and acetonitrile (5 mL) for 1 h. After centrifugation (at 1,500 rpm for 20 min), the supernatant was filtered into glass tubes, dried under nitrogen, and weighed to determine the extraction yield. The extracts were then prepared for antioxidant analysis as described in the next section.

Determination of Antioxidant Activity

Sample Preparation

Trolox standard was prepared in concentrations of 0, 3.125, 6.25, 12.5, 20, 25, 40 and 50 mg/L in 50% ethanol. Freeze-dried PLPW extracts of cow cockle seed (10 mg) were dissolved in 2 mL 50% ethanol. Ultrasonic seed extracts were dissolved in 50% ethanol to make up a concentration of 5 mg/mL. Ultrasonic extracts of PLPW extraction residues (0.1–1.2 mg) were dissolved in 0.5 or 0.75 mL 50% ethanol. All samples were ultrasonicated for 30 min after addition of 50% ethanol to ensure dissolution of sample. After centrifugation, six dilutions (1/2, 1/4, 1/8, 1/16, 1/32 and 1/64) of the supernatant were prepared for each sample. A total of seven concentrations and a blank (50% ethanol) was analyzed for each sample. When the number of dilutions was limited by the amount of sample (for the analysis of residues, and one fraction at 125 and 175 °C (collected at 60 and 180 min, respectively), the samples were diluted to ensure that the absorbance values were in the range used for the Trolox standard.

DPPH Radical Scavenging Activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was based on the method of Brand-Williams et al. [14] modified for use

with a microplate reader as described by Fukumoto and Mazza [15]. Scavenging of DPPH radicals was monitored by the decrease in absorbance at 515 nm which occurs due to reduction by the antioxidant (AH) or reaction with a radical species (R•).

$$\mathrm{DPPH}\bullet + \mathrm{AH} \rightarrow \mathrm{DPPH} - \mathrm{H} + \mathrm{A}\bullet$$
$$\mathrm{DPPH}\bullet + R\bullet \rightarrow \mathrm{DPPH} - \mathrm{R}.$$

The analysis was carried out in triplicate by adding sample or Trolox standard (22 μL) and DPPH (200 μL of 150 μM in 50% ethanol) into wells in a microplate. Absorbance of the DPPH solution at 515 nm was adjusted to read ~1.5 absorbance units. Readings were made after 30 min, 3 h and 5 h at 515 nm using a Spectramax Plus microplate reader (Molecular Devices Corporation, Sunnyvale, CA). Plates were kept covered in the dark between readings. Slopes for the samples and Trolox were calculated for the linear portion of graphs of absorbance versus concentration. Slopes at the time interval giving the highest slope value were used for calculations. DPPH radical scavenging activity was determined as Trolox equivalents (micromoles Trolox per gram dry weight) by dividing the slope of the sample by the slope of the Trolox standard.

ABTS Radical Scavenging Activity

ABTS (2,2′-azinobis 3-ethylbenzthiazoline-6-sulfonate) radical scavenging activity was determined using a modified version of the methods of Rice-Evans et al. [16] and Pellegrini et al. [17]. A concentrated solution of ABTS cation was prepared by reacting ABTS (5 mL of 7 mM solution which was made up fresh daily in deionized distilled water) and $K_2S_2O_8$ (88 μL of 140 mM, made up in deionized distilled water). The mixture was allowed to react about 16 h in the dark. The ABTS concentrate was then diluted with 80% methanol to get a 734-nm of ~1.0 absorbance units prior adding to plates. The analysis was carried out in triplicate by adding sample or Trolox standard (50 μL) and diluted ABTS concentrate (250 μL) into wells. Readings of plates were made after 5 min at 734 nm using a microplate reader. Slopes were calculated for the linear portion of graphs of absorbance versus concentration. ABTS radical scavenging activity of the samples was determined as Trolox equivalents (micromoles Trolox per gram dry weight) by dividing the slope of the sample by the slope of the Trolox standard.

Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing antioxidant power (FRAP) assay is based on the formation of a coloured ferrous–tripyridyltriazine complex with absorbance at 593 nm due to the reduction of ferric to ferrous ion at low pH. The method described by Benzie and Strain [18] was modified for use with a microplate reader. The analysis was carried out in triplicates by adding sample or Trolox standard (50 μL) and FRAP reagent (250 μL) into wells. The FRAP working reagent consisted of 25 mL of 300 mM acetate buffer at pH 3.6, 2.5 mL of 20 mM $FeCl_3$ and 2.5 mL of 10 mM 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) in 40 mM HCl. The TPTZ solution was made up fresh daily. Readings of plates were made after 5 min at 593 nm using a microplate reader. Slopes were calculated for the linear portion of graphs of absorbance versus concentration. Ferric reducing antioxidant power was determined as Trolox equivalents (micromoles Trolox per gram dry weight) by dividing the slope of the sample by that of Trolox standard.

Determination of Total Phenolics

Extracts in 50% ethanol were diluted 1/2 with 50% ethanol prior to total phenolic analysis. Total phenolics were measured by a modified version of Folin–Ciocalteu method as described by Singleton and Rossi [19] using 0–300 mg/L gallic acid as a standard. Briefly, 50 μL of sample or standard was combined with 150 μL of distilled water, 1.0 mL of 0.067 N Folin–Ciocalteu reagent and 0.8 mL of Na_2CO_3 solution (75 g/L). The mixture was allowed to sit for 60 min before reading absorbance at 765 nm. Results were expressed as gallic acid equivalents (grams gallic acid/gram dry weight).

Determination of Saponins

The ultrasonic and PLPW extracts were purified using SPE and analyzed for saponins using HPLC as described in Güçlü-Üstündağ et al. [3]. Spectral analysis of HPLC peaks together with information provided by LC–MS analysis of an aqueous methanol cow cockle seed extract by Balsevich et al. [20] was used for the qualitative determination of saponins in cow cockle seed extracts. Glycyrrhizic acid ammonium salt (75% purity, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) was used as an external standard for the quantitative determination of saponins.

Results and Discussion

In this study DPPH, ABTS and FRAP methods were used to assess the antioxidant activity of cow cockle seed extracts (Table 1). Ultrasonic 50% ethanol and water extracts of cow cockle seed showed antioxidant activity whereas no activity was observed for the acetonitrile extract (Table 1). The

Table 1 Antioxidant activity, saponin and total phenolics content of pressurized low polarity water (PLPW) extracts and residues of ground cow cockle seeds (2 g) at 125, 150 and 175 °C (flow rate=2 mL/min)

Extraction temp (°C) and time (min)	Antioxidant activity (μmol Trolox equivalent/g sample)			Total phenolics (g /100 g dried sample)	Total saponin conc. (g/100 g dried extract)
	DPPH	ABTS	FRAP		
Antioxidant activity and composition of PLPW fractions and residues of cow cockle seed					
125 °C					
15	27.7±2.5	29.9±0.7	20.5±1.6	0.73±0.01	4.5±1.3
30	67.4±2.5	68.1±7.5	40.7±3.1	1.19±0.17	5.4±0.2
45	88.7±5.2	83.9±24.5	54.5±8.6	1.38±0.18	5.3±1.8
60	82.7±13.9	113.9±28.5	69.7±22.6	1.66±0.40	4.9±2.2
120	71.4±13.8	65.0±3.4	44.3±5.2	1.23±0.11	6.0±1.5
180	81.4±14.3	78.2±12.4	51.9±3.6	1.46±0.08	6.7±0.8
Res_1	1733.2±251.1	1553.0±98.9		25.65±1.15	
Res_2	11.0±6.3	14.1±8.9		1.25±0.21	
150 °C					
15	58.2±5.5	60.8±1.6	36.6±1.8	1.13±0.01	5.2±0.4
30	76.6±0.1	70.9±0.9	49.3±3.3	1.37±0.05	3.6±0.0
45	75.9±15.7	67.6±10.9	48.3±9.7	1.39±0.29	5.1±1.3
60	74.4±18.0	75.0±24.3	49.0±10.5	1.45±0.24	4.6±1.0
75	72.8±8.5	67.7±7.0	44.7±5.2	1.38±0.12	
90	77.1±2.9	78.2±12.8	49.5±3.3	1.55±0.13	
120	94.4±17.0	86.1±24.1	54.9±8.1	1.72±0.31	
180	125.0±65.9	100.3±48.6	70.3±34.0	2.22±1.08	
Res_1	919.1±113.9	881.5±58.0		17.62±1.18	
Res_2	17.2±1.1	19.7±5.9		0.67±0.24	
175 °C					
15	82.6±4.8	67.1±2.0	51.0±1.2	1.46±0.02	4.9±2.6
30	125.0±7.1	99.6±5.3	72.8±6.4	2.04±0.10	5.7±2.3
45	165.2±24.0	126.9±10.8	100.2±11.2	2.66±0.29	
60	203.6±4.4	180.8±10.9	126.8±0.4	3.61±0.00	
120	344.3±56.4	260.1±7.7	165.4±7.9	4.57±0.06	
180	422.82±61.3	373.8±1.5	190.5±5.3	5.42±0.05	
Res_1	1262.6±159.1	1287.9±447.8		28.91±0.57	
Res_2	2.9±4.1	8.0±11.1		1.73±0.88	
Antioxidant activity and composition of ultrasonic extracts of cow cockle seeds[a]					
50% EtOH	44.1±0.6	59.9±5.8	38.1±0.8	1.58±0.03	35.4±3.0
Water	14.9±0.8	27.0±2.5	16.1±1.7	0.78±0.01	11.2±0.3
ACN	nd	nd	nd	0.20±0.08	

The antioxidant analyses were carried out in triplicates and the values were reported as mean±standard deviation

Res_1 pressurized low polarity water extraction residue extracted using ultrasonic 50% ethanol, *Res_2* pressurized low polarity water extraction residue extracted using ultrasonic acetonitrile, *ACN* acetonitrile, *nd* not detected

[a] Ultrasonic extractions carried out using 200 mg ground seed and 10 mL solvent for 1 h

differences in the observed values are indicative of the varying solvent powers of these solvents for the antioxidant compounds present in the cow cockle seed. The values obtained using 50% ethanol were comparable to the activities of the fractions obtained in the early parts of the PLPW extraction (in the first 30 min) at 125 and 150 °C.

Antioxidant activities of PLPW fractions were affected by extraction time and temperature (Table 1 and Fig. 1). The highest activity was obtained at the highest temperature tested (175 °C). The activities of 125 °C fractions increased with extraction time in the first hour of extraction dropping slightly in the latter fractions. At 150 °C the activities stayed constant after an initial increase at the start of the extraction. An increasing trend of antioxidant activity with time was observed at 175 °C.

The observed differences in the antioxidant activity of the PLPW fractions can be attributed to the selective extraction of the antioxidant compounds present in cow cockle seeds and the modification of the antioxidant activity of the seeds, due to the heat treatment they were exposed to in the presence of PLPW as characterized by the temperature and time of the extraction. The liberation of

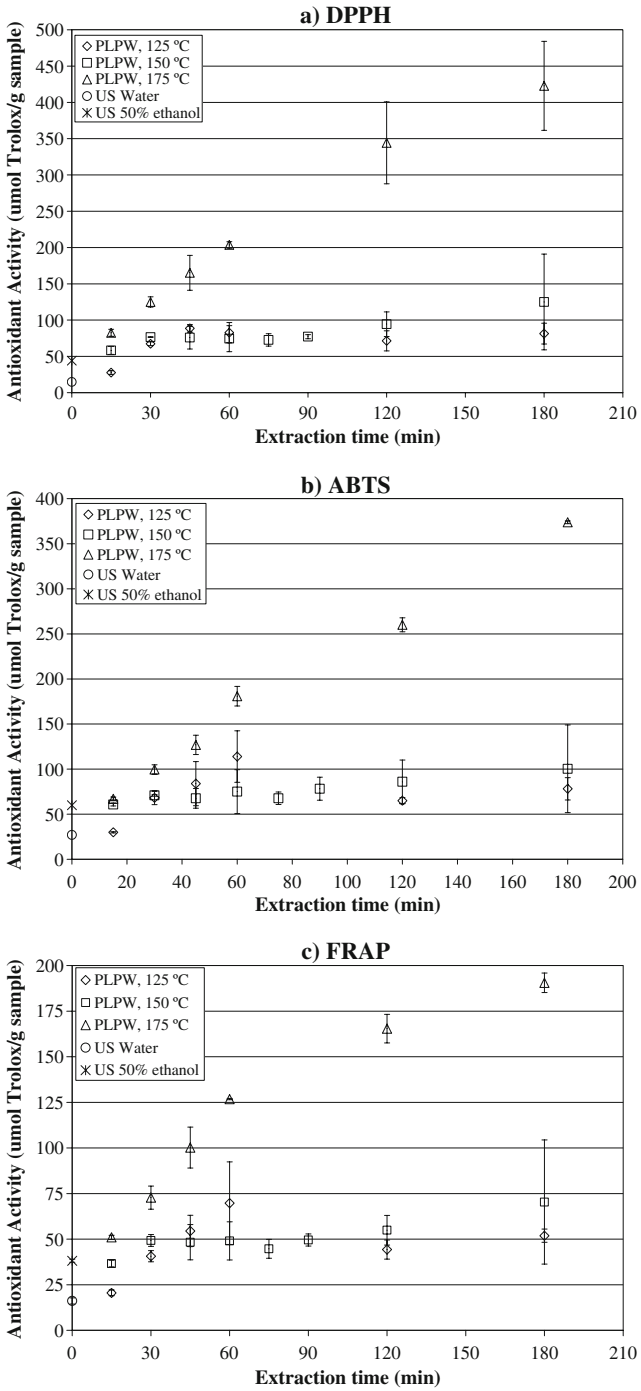

Fig. 1 Antioxidant activities of ultrasonic solvent (*US*) and pressurized low polarity water (*PLPW*) extracts of cow cockle seed determined by **a** DPPH, **b** ABTS, and **c** FRAP methods

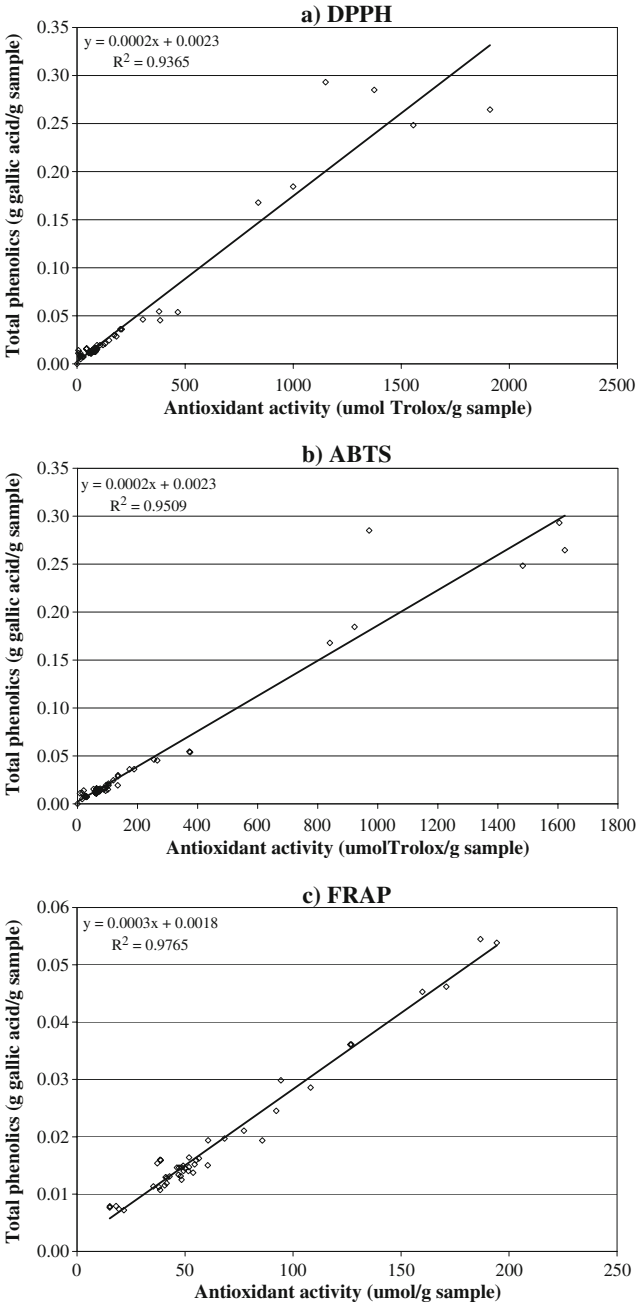

Fig. 2 Correlation between antioxidant activity (as determined by **a** DPPH, **b** ABTS, and **c** FRAP methods) and total phenolics content

bioactive compounds (such as bound phenolic compounds) with heat treatment, for example, has been shown to contribute to the antioxidant activity of heat treated sweet corn [21] and citrus peels [6]. Heating may also lead to degradation of phenolic compounds [22] modifying their bioactivity.

Table 2 Correlation between antioxidant activity values determined using different assays

Assay	Correlation between different antioxidant assay results	R^2
DPPH–ABTS	ABTS=0.7877 (DPPH)+12.484	0.95
ABTS–FRAP	FRAP=0.5348 (ABTS)+11.944	0.95
FRAP–DPPH	FRAP=0.4308 (DPPH)+17.473	0.94

The production of new compounds with antioxidant activity due to heat treatment has also been demonstrated. Steaming of ginseng (at $T \geq 100$ °C for 2 h) increased its antioxidant activity due to the formation of new ginsenosides [7]. At high temperatures reducing sugars reacted with proteins, amino acids/amino groups forming Maillard reaction products with antioxidant activity [8]. Cow cockle seeds contain large amounts of proteins and starch (55% and 14%, respectively) [11], which could possibly form Maillard reaction products that could contribute to activity.

Phenolic compounds may react with Maillard reaction products further modifying antioxidant activity [23]. The investigation of the effect of heating temperature and time on the antioxidant activity of a ferulic acid–glucose–proline model system at 60–220 °C revealed varying contributions of ferulic acid and reaction products to antioxidant activity [23]. At 60 °C the significant increase in activity was attributed to the reaction of ferulic acid with Maillard reaction products, whereas at 90 °C both ferulic acid and Maillard reaction products contributed to activity. The Maillard reaction products were determined to be the main contributors to antioxidant activity at 220 °C.

The antioxidant activities of PLPW extraction residues obtained at 125, 150 and 175 °C after 3 h of PLPW extraction were determined after ultrasonic extraction (1 h) with 50% ethanol and acetonitrile (Table 1). The highest antioxidant values in this study was observed for the residues which were extracted with 50% ethanol. The activities of the residues (as determined by DPPH) were higher than those of the final fraction collected (#9–12) by a factor of 21, 7, and 3 at 125, 150 and 175 °C, respectively. These differences can in part be attributed to the long exposure of the residues to the extraction temperatures (≥1 h) and to the solubility behavior of antioxidant compounds. While some of the antioxidant compounds have been liberated and/or generated throughout the process and extracted by PLPW, some compounds were probably concentrated in the residue because they were not solubilized by PLPW. These compounds were recovered by ultrasonic 50% ethanol extraction but not by acetonitrile extraction as evidenced by the very low activities of acetonitrile extracts (Table 1). FRAP analysis results for residues were not included in the analysis as the samples showed high levels of turbidity.

The results correlate well with the phenolics content of these samples (Table 1), such samples with similar antioxidant activities also had similar phenolics content. The correlation between the total phenolics content and antioxidant activity of ultrasonic and PLPW extracts and residues as determined by the three methods is given in Fig. 2. The antioxidant activity correlated well with the total phenolics content resulting in correlation coefficients≥ 0.94 (Fig. 2). A significant correlation between antioxidant activity and total phenolic content has been reported for various fruits, medicinal plants and cereals [24].

However, no correlation was observed between the saponin content and antioxidant activity of the samples. While purified saponins from natural sources (such as soyasaponin β g [25]) have been shown to have antioxidant activity, the relationship between the saponin content of plant extracts and their antioxidant activity has not been well established. The differences between the antioxidant activities of Asian and North American ginseng extracts were attributed to the differences in the ginsenoside compositions of these extracts [26]. The antioxidant activity of the extracts (as determined by the peroxyl radical induced bilayer lamella suspension peroxidation model) was further related to both total phenolics and total ginsenoside content of the extracts. The low correlation between the phenolics content and primary oxidation inhibition capacity of asparagus extracts was explained by the contribution of other compounds such as saponins to antioxidant activity, however, this relationship was not explored further [27].

The correlation between the results of three antioxidant assays was also examined (Table 2). A strong correlation was observed between the results of DPPH–ABTS, FRAP–ABTS and DPPH–FRAP assays ($R^2 \geq 0.94$).

The findings of this study show that the antioxidant capacity of PLPW extracts of cow cockle seed was affected by the extraction time and temperature. Higher extraction temperatures which were favored from an efficiency stand point also resulted in high antioxidant activities. These results demonstrate the potential of PLPW extraction as a method to modify the activity of biological materials for the production of extracts customized for specific applications.

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