Antioxidant and Radical-Scavenging Activities of Buckwheat Seed Components

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ABSTRACT: The search for endogenous components in food ingredients exhibiting antioxidant activity has been intensified in order to eliminate synthetic antioxidants. Tocopherols are widely used as natural antioxidants, although their protective ability is not always sufficient. Buckwheat seed components were evaluated for antioxidant and free radical-scavenging activities using solvents of different polarities to isolate components from hulls and groats. Components extracted from buckwheat hulls were pro-oxidant in canola oil. Antioxidant activity of extracts from buckwheat groats increased when more polar solvents were used for extraction. The highest activity was observed for the methanolic extract. Radical-scavenging activity of buckwheat extracts was analyzed with DPPH (2,2-diphenyl-2-picryl-hydrazyl). This activity increased when the more polar solvents were used for extraction, with the highest activity observed for the methanolic extract. It was also observed that the radical scavenging effectiveness of extracts was concentration dependent. Analysis revealed the presence of tocopherols in the hexane extract, while methanolic extracts were rich in phenolic acids and flavonoids.

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KEY WORDS: Antioxidants, buckwheat, DPPH, phenolics, radicals, scavengers, solvents, stability, tocopherols.

Buckwheat (*Fagopyrum esculentum* Möench L.), a crop adapted to cool, moist climates and short growing seasons, is used extensively in Asia for the manufacture of noodles (1). The buckwheat seed is actually a fruit, an achene which, when dehulled (pericarp removed), is referred to as groat. Buckwheat hulls are used as pillow filling, traditionally in Japan but recently also in North America.

Antioxidants play an important role in preventing undesirable changes in flavor and nutritional quality of foods. Antioxidants protect against tissue damage associated with various human diseases (2,3). Synthetic antioxidants are widely used as food additives, but their application has been reassessed because of possible toxic or carcinogenic components formed during their degradation (2,3). Mixtures of natural tocopherols, as found in different vegetable oils, are widely used as safe antioxidants, but they are not always as effective as synthetic antioxidants (3,4). Consequently, the search for endogenous protective ingredients in accepted foods has been intensified, as their utilization will require only manipulation of food formulations. Recent reports have described antioxidants and compounds with radical-scavenging activity (RSA) present in peabean (5), peanut (6), ajowan (7), rice (8), and tamarind hulls (9). Oomah and Mazza (10) characterized antioxidant activity in methanolic extract prepared from whole buckwheat seed using an emulsion of linolenic acid and carotene. Watanabe *et al.* (11) isolated flavonoids from buckwheat hulls and observed that some of these compounds had RSA when analyzed in purified form. Polyphenols, including *p*-hydroxybenzoic, syringic, vanillic, and *p*-coumaric acids, are present in the bran–aleurone layer of buckwheat seed. Polyphenols are present in free and bound forms and the latter can be liberated by either alkaline or acid hydrolysis (12).

The objectives of this study were to isolate and characterize antioxidant in buckwheat seed and to compare the antioxidant and free RSA of buckwheat extracts to commercial antioxidants.

MATERIALS AND METHODS

Materials. The buckwheat seed, Mancan variety, was provided by Agriculture Canada, Research Station Morden, Canada. Hulls and groats were separated manually by sieving through a 20-mesh sieve after grinding with a mortar and pestle. The separated parts were stored at −30°C until extracted. All chemicals used were of analytical purity and purchased from Fisher Scientific (Edmonton, Canada).

Extraction. Buckwheat groats (BWG) and hulls (BWH) were extracted sequentially with different polarity solvents (Fig. 1). Extraction was done in a Soxhlet extractor using 20 g of seed and 200 mL of solvent. Extraction was performed using the following sequence of solvents with increasing polarity: hexane (HX), diethyl ether (DE), ethyl acetate (EA), acetone (AT), and methanol (ME). After each solvent extraction, which lasted for 15 h, the seed residue was dried under a stream of nitrogen at room temperature to remove the majority of residual solvent before extraction with the next solvent.

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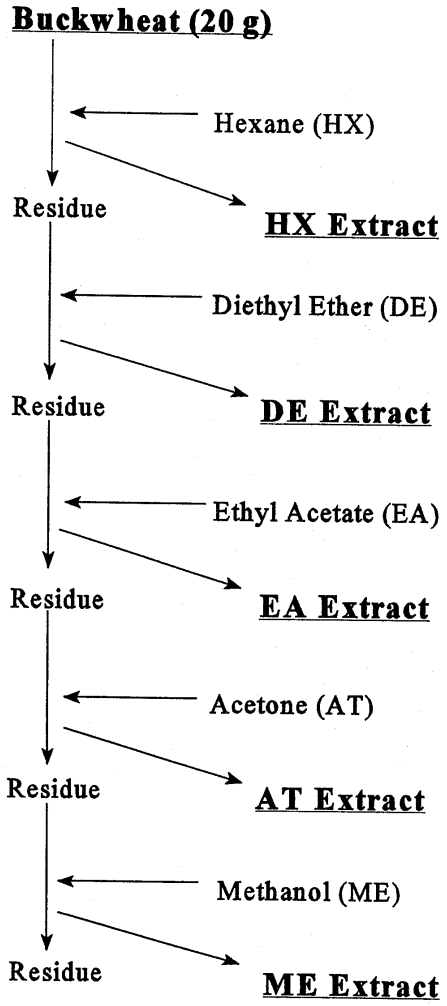

FIG. 1. Sequential extraction of buckwheat groats and hulls.

All extracts were concentrated under vacuum in a rotary evaporator at 45°C under nitrogen, weighed, and the yields calculated. Each extraction was duplicated.

Storage test. All storage tests were conducted using refined, bleached, and deodorized canola oil. The concentrated extracts were added to canola oil at a level of 200 ppm and their antioxidant activity examined. For comparison, Pristene 180 containing a mixture of natural tocopherols (70% in oil) and Sustane 20A containing TBHQ (tertiary butylhydroquinone; 20% TBHQ with 3% citric acid in a mixture of oil and polyethylene glycol) as a synthetic antioxidant, both obtained from UOP (Des Plaines, IL) were used, with the active component applied at 200 ppm. Canola oils with an equivalent volume of concentrated solvents, as per extracts, were used as controls. Each oil sample (5 g) was placed into a vial and stored in a forced air oven at 65°C for 8 d. Samples were removed every second day of storage and peroxide values determined in duplicate using the AOCS Method Cd 8-53 (13). All storage experiments were run in duplication.

RSA of extracts. RSA and presence of hydrogen donors in extracts were examined by reduction of radicals formed by ionization of 2,2-diphenyl-2-picrylhydrazyl (DPPH) when dissolved in solvent (14,15). Solutions of DPPH were prepared in methanol at a concentration of 7.5765×10^{-5} mol/L. Tocopherols and TBHQ were dissolved in ethyl acetate at a similar concentration to that of buckwheat extracts. For evaluation, 1 mL of extract or antioxidant solution was mixed with 3 mL of DPPH solution. The decrease in absorption at 515 nm was measured every 5 min, using Milton Roy spectrophotometer model Spectronic 3000 Array (Columbus, OH) until a plateau (steady state) was reached. The steady state was defined as the time when absorption readings remained the same for 30 min. The exact initial concentration of DPPH was calculated from a calibration curve prepared for concentrations ranging from 0.5051 to 7.5765×10^{-5} mol/L. The color of the extract was compensated by running a blank sample consisting of solvents and extracts without DPPH. The concentration effect on RSA was assessed by addition of 25 to 700 ppm of buckwheat extracts to DPPH solution and the absorption measured after 20 min of reaction. These concentrations represent the amount after dilution in measuring system. All readings for RSA were performed in triplicate.

Tocopherols. Tocopherol content in buckwheat extracts was analyzed according to AOCS method Ce 8-89 (13). Extracts were separated on a silica column (Phenomenex, Prodigy, 5 µm, 3.2 × 250 mm) held at 28°C using a Shimadzu chromatograph model LC-10A (Columbia, MA). Hexane/2-propanol (99.7:0.3, vol/vol) at a flow rate of 0.8 mL/min was used as mobile phase. For all samples analyzed the injection volume was 40 µL. Components were detected with a fluorescence detector (Hewlett-Packard, HP 1046A, Edmonton, Canada) which was set for excitation at 295 nm and emission at 330 nm. Quantification was carried out in triplicate and external calibration for each individual component utilized.

Determination of phenolic acids. Phenolic acids from buckwheat extracts were isolated using the method described by Tsimidou *et al.* (16). Briefly, the extracts were evaporated to dryness under nitrogen and the residue resuspended in hexane before triple extraction with 30 mL of methanol/water (60:40, vol/vol). Then the solvent was removed from the combined extracts under a stream of nitrogen. The residue was transferred quantitatively with methanol into a volumetric flask (5 mL) which was then filled with this solvent. The chromatographic separation of phenolic compounds was achieved on an Ultrasphere ODS 5 µm column (4.6 mm × 250 mm i.d., Beckman Co., Mississauga, Canada) held at 28°C (17). Water containing 1% acetic acid and methanol (90:10, vol/vol) was used as the mobile phase at flow rate of 1.0 mL/min. Sample volumes of 50 µL were applied with the ultraviolet detector set at 254 nm. Phenolic acids were identified by comparison of their retention data with standards purchased from Sigma (St. Louis, MO). The concentration of individual phenolic acids was calculated from external calibration for each individual component. Quantification of phenolic acids was carried out in triplicate. The results are presented as average of replications.

Determination of flavonoids. Flavonoids from buckwheat extracts were analyzed using the method described by Ohara *et al.* (18). Hexane extracts were extracted three times with 30 mL of methanol/water (70:30, vol/vol) then combined, and the solvent was evaporated to dryness under nitrogen. The residue was transferred quantitatively with methanol/water (70:30, vol/vol) into a volumetric flask (5 mL) that was then filled with this solvent. Other solvent extracts were diluted 1:1 (vol/vol) with methanol/water (70:30, vol/vol). The separation of flavonoid compounds was accomplished on an Ultrasphere ODS 5 µm column (4.6 mm × 250 mm i.d., Beckman Co.) held at 32°C. Water containing 2% acetic acid, acetonitrile, and methanol (70:20:10, vol/vol/vol) was used as the mobile phase at flow rate of 0.85 mL/min. Sample volumes of 50 µL were applied with the detector set at 350 nm. Flavonoid compounds were identified by comparison of their retention data with standards purchased from Sigma. The concentration of individual flavonoid compounds was calculated from external calibrations for each individual component. Three additional peaks were observed during separation; they were treated as unidentified flavonoids and their amounts calculated using rutin calibration. All flavonoids analyses were run in duplicate.

RESULTS AND DISCUSSION

Buckwheat hull (BWH), a by-product of buckwheat groat (BWG), is rarely used, although the seed coat may play an important role in seed protection (6,8). The proportions of BWH and BWG present in analyzed seeds were 20.2 and 79.8%, respectively. BWG was sequentially extracted for 15 h each with HX, DE, EA, AT, and ME (Fig. 1), and the yields are shown in Table 1. The methanol extract contained the highest amount of compounds compared to other solvents. The amount of components extracted by solvents decreased in the following order: ME > HX > AT > EA = DE. These results are in agreement with data reported by Duh *et al.* (19) and Economou *et al.* (20). The former also showed that the yield of extract from peanut hull increased as the polarity of the solvent used increased (19).

A number of papers have reported the presence of components with antioxidant activity in the hulls of rice (18), tamarind (9), navy bean (21), and peanut (19). In this study, however, the methanol extract from buckwheat hulls showed pro-oxidant activity toward canola oil (Fig. 2).

TABLE 1
Yield of Extracts Obtained from Groat

Solvents	Extract yield (% ± SD)[a]
Hexane (HX)	2.56 ± 0.32
Diethyl ether (DE)	0.16 ± 0.06
Ethyl acetate (EA)	0.12 ± 0.08
Acetone (AT)	0.32 ± 0.09
Methanol (ME)	11.86 ± 0.87

[a]Average from triplicate determinations; percentage based on dry matter.

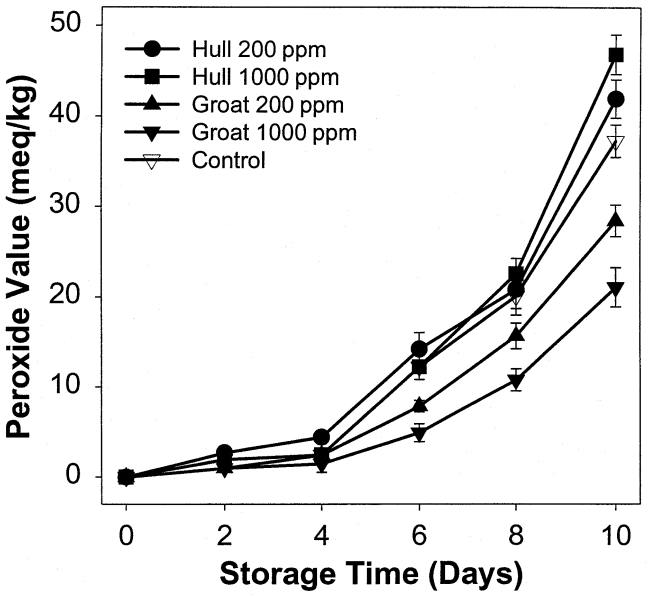

FIG. 2. Antioxidant activity of methanolic extracts produced from buckwheat hulls and groats.

It is evident that pro-oxidant compounds in the methanolic extract showed higher activity than any antioxidants extracted. Pro-oxidant effect increased when the amounts of added extract increased. Watanabe *et al.* (11) found that crude ethanolic extracts from buckwheat hull had slight antioxidant activity. These authors also found that antioxidant activity of fractions obtained from this extract was high, but lower than BHA. Among these fractions they also found one which exhibited pro-oxidative activity.

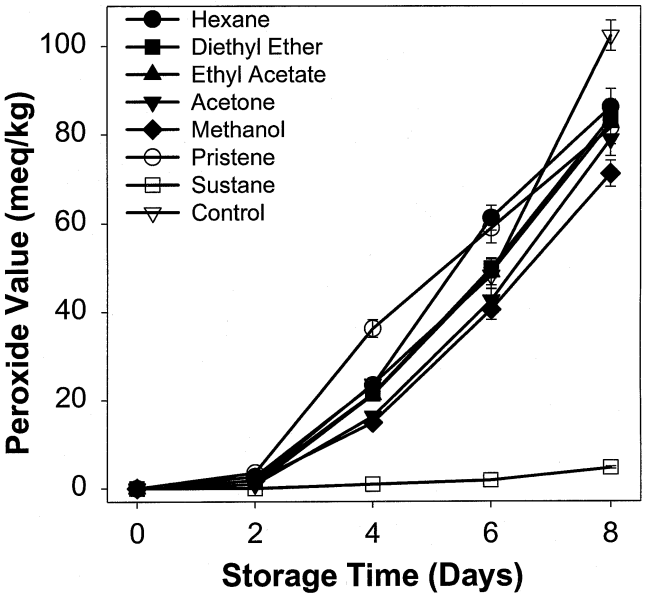

FIG. 3. Oxidation of canola oil during accelerated storage with 200 ppm of buckwheat extracts obtained from groats (for details see Fig. 1 and the Materials and Methods section).

In contrast, extracts prepared from buckwheat groats exhibited antioxidant activity at the concentrations analyzed (Fig. 2). The BWG extracts acquired using different solvents showed increased antioxidant activities when the polarity of solvent applied increased (Fig. 3). The extracts obtained using methanol and acetone tended to have stronger antioxidant activity to canola oil during storage than tocopherols (Pristene), but were less effective than TBHQ (Sustane 20A). Of the five solvents used, the ME extract exhibited the strongest antioxidant activity. At the end of storage time extracts produced using HX, DE, and EA showed similar antioxidant activity to tocopherols. Based on protection of canola oil during storage, the following order of decreasing antioxidant activity can be proposed: ME > AT > DE = EA > HX. Canola oil contains endogenous antioxidants such as tocopherols, and interaction with some components of extracts can be expected. The highest antioxidative activity of ME and EA extracts can be partially attributed to the synergistic activity of phenolic compounds toward tocopherols, enhancing their activity (2). Many researchers found that methanolic extracts contained the most effective antioxidants when produced from peanut (6,21), peabean (5), and ajowan (7) hulls. Some of the components with antioxidant activity were identified as flavonoids such as luteolin in ME extract from peanut and other hulls (5–7,21). These researchers also found that methanol extracts exhibited the strongest antioxidant activity when prepared from the plants of *Labiatae* family and peanut hulls. Methanol appeared to be the best solvent for extracting compounds such as phenolics, flavonoids, and other polar material (22). Toda *et al.* (23) reported that methanol extracts prepared from herbs showed the strongest antioxidant activity and identified active components—quinones. In contrast, Zhang *et al.* (24) reported that the DE extract produced from *Salvia miltiorriza* roots had very strong antioxidant activity. However, these researchers did not use methanol as extractant and did not compare to synthetic antioxidants. These authors identified the active components in this extract as quinones. Kim *et al.* (22) reported that the antioxidant activity of extracts produced from herbs was dependent on the type of herb rather than the solvent used.

Elimination of DPPH radicals is used to indicate the presence of hydrogen donors in a reaction system. Ionized DPPH produces a color which changes when radicals are removed from the system and is measured by diminishing absorption at 515 nm over time (25,26). As shown in Figure 4, the absorption of ionized DPPH solution changed when extracts or antioxidants were added. ME, AT, and EA extracts rapidly scavenged DPPH radicals and reached a plateau in less than 15 min, whereas DE and HX extracts reacted very slowly with only small scavenging activity. Brand-Williams *et al.* (25) reported that the different reaction kinetics for DPPH radicals depended on the nature of the antioxidant. They also concluded that ascorbic acid showed rapid scavenging activity and α-tocopherol showed intermediate activity, whereas some phenolic compounds had slow kinetics of scavenging. In the present study, extracts reached a steady state in less than 20–60 min, after which the scavenging kinetics slowed down. It is interesting to note that citric acid, which has been used in oils as a synergist to antioxidants and chelator for metals (3), did not react with DPPH radicals; this component is not a hydrogen donor. Tocopherols and TBHQ, however, reacted rapidly with DPPH radicals with a plateau reached in less than 5 min. Brand-Williams *et al.* (25) found that tocopherol was a very effective radical scavenger/hydrogen donor,

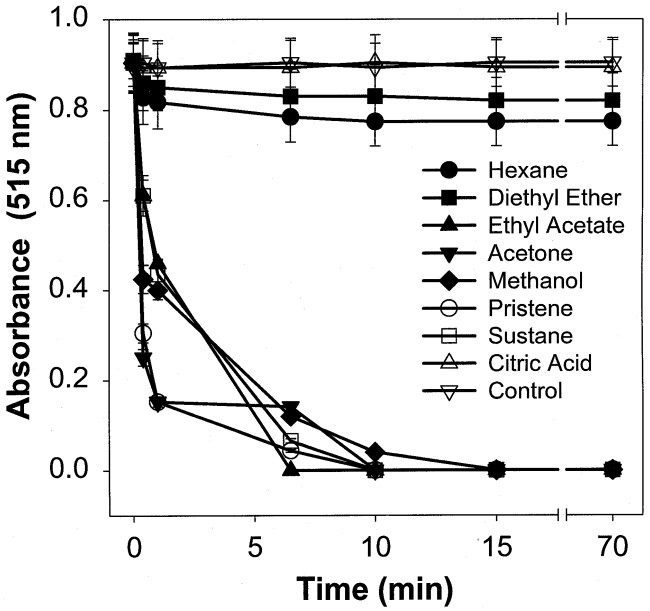

FIG. 4. Radical scavenging activity of 200 ppm of buckwheat groat extracts using 2,2-diphenyl-2-picrylhydrazyl (DPPH) radicals (for details see Fig. 1 and the Materials and Methods section).

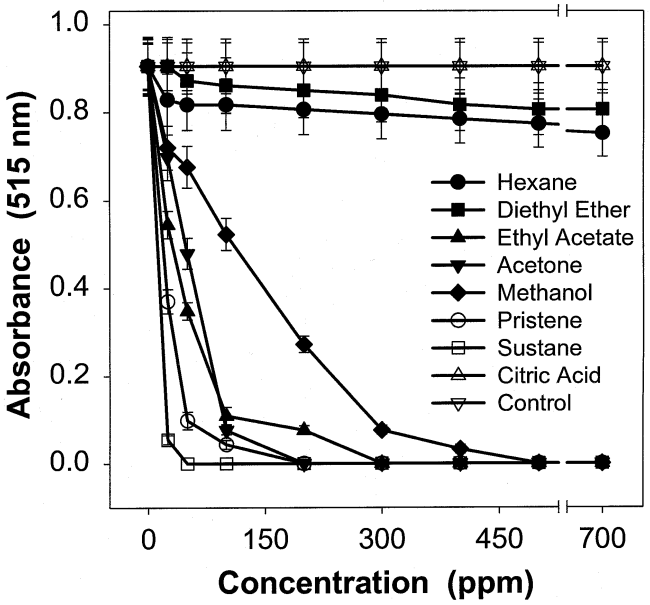

FIG. 5. Effect of the amounts of buckwheat groat extracts on scavenging activity of DPPH radicals (see Fig. 1 and the Materials and Methods section). See Figure 4 for abbreviation.

whereas monophenolics such as coumaric and vanillic acids reacted poorly with DPPH radicals. Polyphenolics and compounds with a sterically hindered hydroxyl group, such as TBHQ, showed much better scavenging activity than their mono derivatives (25).

Scavenging activity of all extracts analyzed in this study increased when the amounts added increased (Fig. 5). In this experiment time was constant—20 min—for all extracts and commercial antioxidants, while concentration of hydrogen donors/radical scavengers added was different. ME, AT, and EA extracts again proved to be the most effective DPPH radical scavengers, while DE and HX extracts were the least effective. Extracts isolated with polar solvents, such as methanol, contained higher amounts of components which act as hydrogen donors than extracts isolated with nonpolar solvents, such as hexane. These extracts also showed stronger antioxidant activity during canola oil storage compared to extracts isolated with nonpolar solvents (Fig. 3). The RSA of extracts, based on reactivity with DPPH radicals, increased in the following order: EA = AT > ME > HX > DE. This order differed with respect to antioxidant activity, as discussed previously in the canola oil storage. These results suggest that evaluation of antioxidant activity based solely on measurement of radical-scavenging effectiveness—hydrogen donors—using DPPH radicals can produce a different picture because this methodology measures only one aspect of antioxidant activity (25,26). In addition, if the test is performed in polar medium the effectiveness of polar antioxidants can be different, as described by the "polar paradox" which states that lipophilic antioxidants are more effective in polar media, whereas polar antioxidants are more active in lipophilic media (27).

TABLE 2
Composition of Buckwheat Extracts[a]

	Solvent (ppm)				
	HX[b]	DE	EA	AT	ME
Phenolic acids					
Caffeic			112.5	561.7	852.4
o-Coumaric			272.3	565.6	492.7
p-Coumaric					229.6
Ferulic			59.9	328.3	230.0
Gallic	5.9	16.0	40.6	178.8	263.3
p-Hydroxybenzoic	3.8	24.7	82.1	45.8	128.4
Syringic			10.5	145.0	17.2
Vanillic	3.9			160.4	2938.0
Subtotal	13.6	40.7	577.9	1985.6	5151.6
Flavonoids					
Rutin			14.1	76.3	458.4
Quercetin					32.7
Quercitrin					12.5
Others[c]			18.2	63.3	254.8
Subtotal			32.3	139.6	758.4
Tocopherols					
α	380.1				
β	16.5				
γ	105.6				
δ	36.4				
Subtotal	538.6				
Total	552.2	40.7	610.2	2125.2	5910.0

[a]Averages from triplicate analysis and Average Relative Standard Deviation were: phenolics, 8.56%; flavonoids, 8.21%; tocopherols, 6.32%.
[b]Solvents used for extraction: HX, hexane; DE, diethyl ether; EA, ethyl acetate; AT, acetone; ME, methanol.
[c]Unidentified peaks quantified as rutin.

Characterization of extract components. Eight phenolic acids were identified in solvent extracts from BWG as shown in Table 2. The amount of total phenolic components increased when polarity of the extracting solvent was higher, indicating that nonpolar solvents were ineffective in extracting phenolic acids. As expected, hexane extracted all tocopherols owing to their good solubility in this solvent, but only a small amount of phenolics was detected—2% of total amount of compounds analyzed. Polar solvents extracted phenolics and flavonoids with different efficiencies where the most polar solvents used, AT and ME, extracted almost four and ten times more of these components than EA, respectively. This was confirmed recently by Tian and White (28) who reported that nonpolar solvents were unsuitable for extracting phenolics from oats. Gallic and *p*-hydroxybenzoic acids were present in all extracts, while *p*-coumaric acid was only found in the ME extract. The major phenolic acids detected in the AT extract were caffeic, *o*-coumaric, and ferulic acids. These were different from the major phenolics found in the ME extract, which were caffeic, *o*-coumaric, and vanillic acids. Durkee (12) detected *p*-hydroxybenzoic, syringic, and vanillic acids as soluble constituents in the aleurone fraction of buckwheat bran, whereas *p*-coumaric, *p*-hydroxybenzoic, and vanillic acids were found in buckwheat flour. The total content of phenolic acids in extracts decreased in following order: ME > AT > EA > DE > HX. This order parallelled the antioxidant activity of BWG extracts analyzed during accelerated storage of canola oil. The effect of buckwheat extracts on the oxidative stability of canola oil can be interpreted as the combined action of endogenous antioxidants and stimulants added with extracts (2). However, when acetone and methanol extracts, which contain mainly phenolic compounds, are added, important antioxidant activity of these components can be expected as well as synergistic activity with tocopherols. Dziedzic and Hudson (29) noted that the antioxidant activity of phenolic acids and their esters were influenced by the number and position of hydroxy groups in the molecule, which could be strengthened further by steric hindrance. Hydroxylated cinnamic acids were more effective than their corresponding benzoic acid counterparts. In addition, it is known that flavonol and phenolic compounds have a tendency to chelate metals at their 3-hydroxy-4-keto and/or 5-hydroxy-4-keto groups and make them less active as catalysts (2).

The major tocopherol in buckwheat was the α-isomer, which constituted up to 70.5% of the total tocopherols. The remaining, β, γ, and δ tocopherols accounted for 3.1, 19.6, and 6.8% of the total, respectively (Table 2). A similar distri-

bution of tocopherols was reported in wheat and barley, although the total amount in buckwheat was similar to that found in oats (30–32). The level of tocopherols in buckwheat, however, was two and three times lower than in wheat and corn, respectively. The amounts of tocopherols in buckwheat were three- and sevenfold higher than reported for barley and rice, respectively (33,34).

Rutin was found to be the major flavonoid present in the extracts isolated with polar solvents; however, the highest amount was observed in methanol. Quercetin and quercitrin, which are degradation products of rutin, were only observed in methanol extract (Table 2). A similar level of rutin was reported in Japanese buckwheat and soba noodles (18) and in Canadian buckwheat varieties (10). The effectiveness of flavonoids as antioxidants was evaluated and some of them can have very high activity and act as synergists with other components (2).

In conclusion, evaluation of antioxidant activity based solely on the RSA of DPPH radicals only describes part of antioxidant behavior and should be used with other antioxidant activity tests. Nevertheless, radical scavenging can be a significant method for evaluating the effectiveness of an antioxidant due to the very short evaluation time and high reliability (25). Buckwheat groat extracts showed antioxidant activity during storage of canola oil, as well as strong RSA. The activity of individual components found in buckwheat extracts requires further study using different food systems.

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