Antioxidant Activity of Durum Wheat Bran[†]

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Freeze-dried extract from durum wheat bran exhibited stronger antioxidant activity than extracts from other milling fractions. At 9 h under active oxygen method (AOM) conditions, peroxide value (PV) determinations showed that oil with the bran extract had a PV of 38.0 Mequiv/kg while the control oil had a PV of 129.0 Mequiv/kg. Extracts from the bran fractions of six durum wheat varieties had similar antioxidant activities in soy oil (PV 37.6–42.0 Mequiv/kg). However, Tenox butylated hydroxyanisole–butylated hydroxytoluene (BHA–BHT) mixture and Rosemary-AR were significantly stronger antioxidants ($P < 0.005$) than the extract from durum wheat bran at all the levels studied. High-performance liquid chromatographic and thin-layer chromatographic analyses of the durum wheat bran extract revealed the presence of protocatechuic, *p*-hydroxybenzoic, gentisic, caffeic, vanillic, chlorogenic, syringic, *p*-coumaric, and ferulic acids. Among the identified free phenolics, ferulic, vanillic, and *p*-coumaric acids were present in the highest amounts. The phenolic acids also appear to be partially responsible for the antioxidant activity of the extract.

INTRODUCTION

Durum wheat (*Triticum durum*), predominantly grown in the United States, in North Dakota, and Canada, is the wheat of choice for the manufacture of the highest quality pasta products such as macaroni and spaghetti. During the milling of wheat, only the endosperm portion is ground to produce semolina while the bran and shorts are treated as byproducts. To add value to the durum wheat crop, the bran has been used for animal feeds or as fiber supplements in bread or muffins.

Results of toxicological and nutritional studies which link some synthetic antioxidants to cancer and other diseases have forced regulatory agencies to impose severe restrictions on their use in human foods. Consumer preference also had led to increased interest in natural antioxidants by food manufacturers. Most of the synthetic antioxidants available commercially for food use have phenolic structures which are essential for their antioxidant activities. Dugan (1980) reported that natural antioxidants found in many plant materials also commonly include an aromatic ring as part of their molecular structure. He stated that these may be associated with a variety of cyclic ring structures and possess one or more hydroxyl groups to provide a labile hydrogen and a basis for free radical formation. He concluded that the latter may be a transcient component of the antioxidant functioning mechanism, or in some cases, it may be the actual antioxidant. Several other workers have reported extracts from plant sources possessing very strong antioxidant activity. Chang et al. (1977) found that purified extracts from rosemary inhibited reversion in soybean oil and the formation of peroxides in potato chips. Pratt (1972) reported the existence of natural antioxidants in soybeans. In 1983, Nestle S.A. patented a process for extracting antioxidants from rosemary, sage, and parsley. Extract from rosemary (Rosemary-AR) has since been commercialized after receiving approval from the U.S. Food and Drug Administration (FDA) for direct food application. Onyeneho (1990) and Onyeneho and Hettiarachchy (1991) reported that extract from navy bean hull was an effective antioxidant in vegetable oils.

Reports which indicate that phenolic compounds are concentrated in the aleurone and bran portions of cereal kernels include that of Pussayanawin and Wetzel (1987). They reported a high concentration of phenolic acids and especially ferulic acid in the bran of durum wheat. Similar observations were made by Fulcher et al. (1972). Fulcher et al. (1979) and Ramarathnam et al. (1988) demonstrated that phenolic compounds were concentrated in wheat and rice brans, respectively.

High-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) have found wide applications in the separation and identification of phenolic compounds in plant extracts. Effective uses of these techniques in phenolic compound analyses have been reported by Mueller-Harvey et al. (1982), Pussayanawin and Wetzel (1987), McMurrough et al. (1984), and Rotson and Kissinger (1982). The retention data of phenolic acids separated by HPLC have also been compiled by Banwart et al. (1985).

The objectives of this study were to (1) prepare and test freeze-dried extract from durum wheat bran for antioxidant activity and (2) separate and identify the phenolic compounds in the freeze-dried extract.

MATERIALS AND METHODS

Materials. Durum wheat (*Triticum durum*) varieties Lloyd, Monroe, Rugby, Stockholm, Reinville, and Vic were purchased from Sinner Seed Farm, Casselton, ND. Pure expeller-pressed soy oil was purchased from Hain Pure Foods Inc., Los Angeles, CA. Tenox (butylated hydroxyanisole–butylated hydroxytoluene mixture composed of 20% BHA, 20% BHT, and 60% corn oil) was obtained from Eastman Kodak Co., Kingsport, TN, while Rosemary-AR was obtained from Culinar Corp., Fjalkinge, Sweden. Standard phenolic acids were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were of analytical grade.

Sample Preparation. Wheat samples (50 lbs of each) were cleaned, tempered, and fed into the Buhler MLU 202 Experimental mill and milled using roll settings and sieve dressings usually employed for milling hard red spring wheat (Figure 1).

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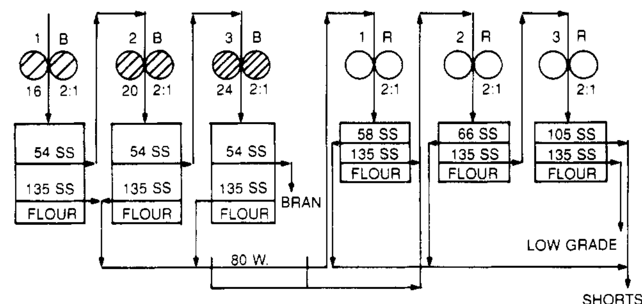

Figure 1. Flow diagram of the continuous pneumatic Buhler MLU 202 Experimental mill. B = break rolls, R = reduction rolls, SS = stainless steel scalping sieves, W = stainless steel wire.

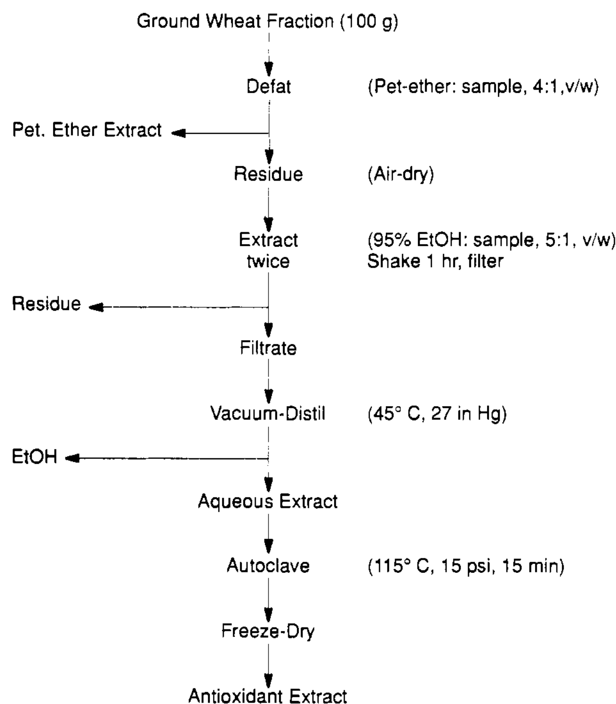

Figure 2. Antioxidant extract preparation.

The following milling fractions were collected: bran, head shorts, tail shorts, low-quality flour, and low-grade flour. The bran, tail shorts, and head shorts were further ground in a Wiley mill with a 0.5-mm screen. Each fraction (250 g) was defatted by shaking twice with 4 volumes of petroleum ether in an Eberbach shaker for 1 h. After filtration, the residue was dried under a hood at room temperature until all traces of petroleum ether had evaporated.

Antioxidant Extract Preparation. One hundred grams of each defatted fraction was used to prepare the antioxidant extract as outlined in Figure 2. The freeze-dried extract was stored in a sealed container at 5 °C until used. This procedure was utilized to prepare antioxidant extracts from the bran fractions of the six durum wheat varieties.

Antioxidant Activity Testing. To determine the effectiveness of the freeze-dried extracts as antioxidants, 0.05 g of each extract was reconstituted in 5.0 mL of absolute alcohol in a 100-mL beaker. Pure expeller-pressed soy oil (50.0 g) was added to the extract solution and homogenized in an ice bath with a Braunsonic 2000 sonicator. The resulting emulsion (20.0 g) was added to aeration tubes in duplicate and analyzed by the active oxygen method (AOM) procedure (AOCS method Cd 12-57). Duplicate 1.0-g oil samples were removed at 9 h and peroxide values were determined (AOCS method Cd 8-53). Based upon the results of preliminary tests, extracts from the bran fraction of each of the six durum wheat varieties were added to soy oil (0.05%) and analyzed for antioxidant activity by the AOM procedure and compared with the control sample and the synergistic effect of BHA–BHT mixture. Dose response studies of the extract from the bran of Vic variety, Tenox (BHA–BHT) mixture, and Rosemary-AR were conducted using 100, 200, 500, and 1000 ppm levels.

Determination of Free Phenolic Acids. *Sample Preparation.* Freeze-dried extract of the bran fraction (0.01 g) was dissolved in 5 mL of distilled water, and the solution was prefiltered through Whatman No. 4 filter paper. The filtrate was passed through an ultra-sep C_{18} extraction cartridge (Phenomenex Co., Torrance, CA) and rinsed with 2.0 mL of distilled water, and the adsorbed free phenolics were desorbed from the column with 2.0 mL of HPLC-grade MeOH and filtered through a nylon-66 filter (0.45-μm pore size). Standard phenolic acids were prepared in methanol at a concentration of 2.0 mg mL^{-1}.

Apparatus. High performance liquid chromatography (HPLC) was performed with a Hewlett-Packard 1090L liquid chromatograph equipped with an autosampler and a diode-array detector. The analytical column was a Beckman Ultrasphere ODS C_{18}, 250 mm × 4.6-mm i.d., with a 5 μm material. The precolumn was a 20-mm × 4.6-mm-i.d. tube with the same packing material.

Analysis. The detector was programmed to measure between 280 and 354 nm with an optical bandwidth of 4 nm. Absorption at 550- and 4-nm bandwidth was employed as the reference wavelength. Samples (10 μL) of phenolic acid standards were chromatographed singly and in a mixture. The same volume of the bran extract was chromatographed under the same conditions. Two solvents constituted the mobile phase: methanol and ammonium acetate buffer, pH 5.4 (12:88, v/v) at a flow rate of 1 mL min^{-1} and ambient temperature. The buffer was prepared by adjusting the pH of 0.01 N ammonium acetate solution to 5.4 with glacial acetic acid. Peak retention times and areas were monitored and computed automatically by an HP 3396A integrator. Peak identification was conducted by cochromatographing the suspected peak eluting from the sample with the corresponding standard and comparing respective retention times. Fractions of the eluted peaks were collected and concentrated under nitrogen to about 5 mL and used for antioxidant activity testing on TLC plates.

Quantitative Analysis of Free Phenolics. Solutions of phenolic acid standards prepared in MeOH were analyzed directly by HPLC, and plots of weight vs peak area were found to be linear for each standard in the range of 0.05–4.0 mg. A calibration mixture was used to calculate the response factors by dividing the known weight of a phenolic acid standard by its corresponding peak area. Free phenolic acids in the extract were quantified from peak areas from the calculated response factors.

Thin-Layer Chromatography (TLC). Solutions of durum wheat bran extract (50 μL), as prepared for HPLC analysis, fractions collected by HPLC separation (50 μL), and standard phenolic acid solutions (5 μL) were spotted on duplicate KC_{18}-reversed phase TLC plates, 20 cm × 20 cm (Whatman) and on duplicate silica gel 60F-254 plates, 20 cm × 20 cm (Merck), all with 200-μm layer thickness. After drying the plates with a hot-air hair dryer, they were developed with a mixture of chloroform–ethyl acetate–formic acid mixture (5:4:1, v/v/v), dried, and observed under UV light. One set of dried plates was uniformly sprayed with Folin–Ciocalteu's reagent–water mixture (1:2, v/v) and dried. The second set of plates was uniformly sprayed first with a 20% solution of sodium carbonate, dried, and sprayed with Folin–Ciocalteu's reagent, and dried. All the plates were again observed under UV light to locate faintly-stained spots. The R_f values of the standards were compared with those of the sample and fractions and used tentatively to identify the phenolic acids in the extract.

Antioxidant Activity of Free Phenolics. Duplicate silica gel TLC plates were separately spotted with standard phenolic acid solutions (5 μL), solution of durum wheat bran extract as previously prepared (50 μL), and concentrated HPLC fractions (50 μL). The plates were developed in chloroform–ethyl acetate–formic acid solution (5:4:1, v/v/v), dried, and observed under UV light. The locations of the phenolics were marked under UV light. A carotene spray solution was prepared (6 mg of β-carotene in 30 mL of chloroform), mixed, and added to linoleic acid–ethanol solution (2 mL of purified linoleic acid in 60 mL of 95% ethanol). One set of plates were uniformly sprayed with this carotene solution, and the plates were exposed to daylight for

Table I. Yield and Peroxide Values of Oil Treated with Freeze-Dried Extracts of Durum Wheat Milling Fractions (Vic Variety)

milling fraction	yield of extract,[a] %	peroxide value[b] 9-h AOM, Mequiv/kg
head shorts (HS)	12.1 ± 0.5	49.0
tail shorts (TS)	8.9 ± 1	59.0
low-quality flour (LQF)	5.8 ± 1	64.0
low-grade fluor (LGF)	4.9 ± 2	66.0
bran	9.7 ± 1	38.0
control (oil, no additive)		129.0
Tenox (BHA–BHT)		23.0

[a] Values are means of duplicate determinations. [b] Values are means of duplicate determinations, 0.05% of extract of Tenox based on weight of oil.

Table II. Yield and Peroxide Values of Oil Treated with Freeze-Dried Antioxidant Extracts of Durum Wheat Bran

durum wheat variety	yield,[a] %	peroxide value[b] 9-h AOM, Mequiv/kg
Lloyd	9.4	42.0
Monroe	9.2	39.6
Reinville	9.0	40.1
Rugby	8.8	38.9
Stockholm	8.5	39.8
Vic	9.3	37.6
control (oil, no additive)		129.0
Tenox (BHA–BHT)		22.0

[a] Values are means of duplicate determinations. [b] Values are means of duplicate determinations, 0.05% of additive based on weight of oil.

about 4 h when the background color was bleached. Spots in which yellow color persisted possessed antioxidant activity, and the intensity of color was an indication of the degree of activity (Pratt, 1980). A second set of plates were dotted with a solution of pure linoleic acid (10% linoleic acid in petroleum ether) at the marked positions of the phenolic acids and heated at 65 °C overnight. White spots corresponding to the yellow spots were observed, which also indicated antioxidant activity (Kramer, 1985).

Statistical Analysis. The data obtained were analyzed by the general linear models (GLM) program for analysis of variance and regression estimation (SAS Institute, 1985).

RESULTS AND DISCUSSION

Yield of Antioxidant Extracts. The yields of the freeze-dried antioxidant extracts from the various milling fractions (expressed as percent by weight of starting raw material) are shown in Table I. The head shorts (HS), bran, and the tail shorts (TS) gave greater amounts of freeze-dried extracts than the flour fractions. Table II lists the yields of extracts from the bran fraction of six durum wheat varieties. The varieties Lloyd, Vic, Monroe, and Reinville had slightly greater yields than Rugby and Stockholm, but the differences were not significant ($P >$ 0.05).

Antioxidant Activity of Extracts. Freeze-dried extracts from the various milling fractions were evaluated for antioxidant activity by the active oxygen method (AOM). Peroxide values (PV) determined at completion of analysis were used as indicators of the level of resistance to oxidative randicity of the treated and untreated oil samples. Table I lists the PVs of oil treated with 0.05% of the extract and 0.05% of Tenox (BHA–BHT) mixture. All the extracts from the various fractions exhibited strong antioxidant activity but to varying degrees when compared to the control (oil without additive) with a PV of 129.0 Mequiv/kg. Extract from the bran fraction (PV 38 Mequiv/kg) appeared to possess greater antioxidant activity ($P < 0.05$) than those of other fractions. However, the activity of BHA–BHT mixture was significantly greater ($P < 0.005$) than that of the bran extract.

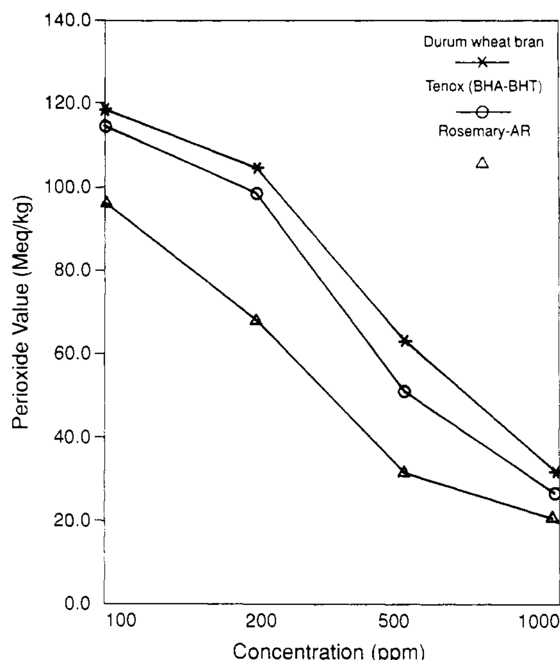

Figure 3. Effect of concentration of durum wheat (Vic) bran extract and other antioxidants on the peroxide value of soy oil at 9-h AOM.

Based upon the result obtained above, extracts from the bran fractions of six durum wheat varieties were analyzed for antioxidant activities by the AOM procedure using soy oil as substrate. The PVs of oil samples treated with the bran extracts from the various durum wheats are listed in Table II. Results showed no significant differences among the extracts ($P > 0.05$). As was observed with the extracts from the milling fractions, the activity of BHA–BHT was greater than that of any bran extract.

Freeze-dried extract from the bran of Vic variety was selected and used for a dose–response study. Concentrations (based on the weight of oil) of 100, 200, 500, and 1000 parts per million (ppm) of durum bran extract, Tenox (BHA–BHT), and Rosemary-AR were added to oil and analyzed by the AOM procedure. The effect of concentration of these additives on the PV of soy oil after 9 h under AOM conditions is shown in Figure 3. Increasing the concentration of the additives led to increased antioxidant activity (decrease in PV) at the levels studied. Again, at all the concentrations investigated, both Rosemary-AR and BHA–BHT appeared to be stronger ($P <$ 0.005) antioxidants than the extract from durum wheat bran.

Phenolic Acid Composition of Durum Bran Extract. Figure 4 illustrates the high-performance liquid chromatograms of both the standard mixture of phenolic acids (a) and the phenolic acids present in the freeze-dried extract of durum wheat bran (b). The retention times (t_R) of individual phenolic acids are listed in Table III. Gallic, salicylic, and cinnamic acids were not detected under the conditions of the experiment. Ferulic, syringic, gentisic, and caffeic acids appeared to be the major phenolic acids in durum wheat bran. All the peaks in the extract were eluted before 30 min at a flow rate of 1 mL min^{-1}. Spiking the retention times under the same analytical conditions with the corresponding authentic phenolic acid standards was also used to tentatively identify the phenolic acids.

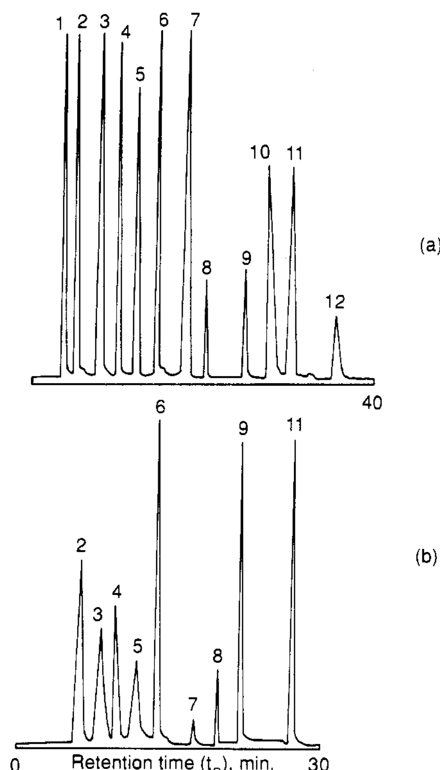

Figure 4. HPLC profiles of phenolic acids in (a) mixed standards and (b) durum wheat bran extract. Chromatographic conditions are in the text. Peak numbers correspond to the phenolic acids in Table III.

Table III. Retention Times (t_R) and hR_f Values of Phenolic Acids Separated on a Beckman Ultrasphere ODS C_{18} Column

peak	phenolic compd	t_R, min	hR_f[a]
1	gallic acid	3.03	50.0
2	protocatechuic acid	3.79	66.0
3	*p*-hydroxybenzoic acid	4.19	75.0
4	gentisic acid	5.69	72.0
5	caffeic acid	6.93	66.0
6	vanillic acid	8.12	78.0
7	chlorogenic acid	9.23	22.0
8	syringic acid	11.96	73.0
9	*p*-coumaric acid	16.15	76.0
10	salicyclic acid	20.21	80.0
11	ferulic acid	23.26	79.0
12	*t*-cinnamic acid	31.05	81.0

[a] $hR_f = R_f \times 100$.

Thin-layer chromatography (TLC) was also used to separate and identify the phenolic acids in the extracts. Silica gel F254 precoated TLC plates gave better separations of the phenolic acids in both the extract and in the mixture of standards than the K_{C18}-reversed phase plates. The hR_f values of individual phenolic acids are given in Table III. Only a few spots were observed under UV light. However, when the plates were first sprayed with a 20% solution of Na_2CO_3, dried, and finally sprayed with Folin–Ciocalteu's reagent–H_2O (1:2, v/v), the phenolic acids appeared as blue spots on a white background (Ragazzi and Veronese, 1973). However, salicylic acid's spot was not visible after the sprays, but appeared as bright fluorescent blue spot when the sprayed plates were examined under UV light. Spots from the extract were matched with spots from the standard phenolic acids and used for identification. Thin-layer chromatographic results and HPLC data were compared and used to identify the individual phenolic acids, since the same extract preparation was used in both HPLC and TLC analyses.

Table IV. Free Phenolic Acid Composition of Durum Wheat Bran Extract

phenolic acid	content,[a] mg/100 g of extract
protocatechuic acid (PA)	226.0
p-hydroxybenzoic acid (pBA)	124.0
gentisic acid (GA)	108.0
caffeic acid (CaA)	116.0
vanillic acid (VA)	637.0
chlorogenic acid (CA)	84.0
syringic acid (SA)	130.0
p-coumaric acid (pCA)	580.0
ferulic acid (FA)	764.0

[a] Values are means of duplicate determinations.

Table V. Peroxide Values of Oil Treated with Standard Phenolic Acids, Bran Extract, and Simulated Extract by 9-h AOM

additive	PV,[a] Mequiv/kg
control (oil, no additive)	131.0
protocatechuic acid (PA)	39.0
p-hydroxybenzoic acid (pBA)	48.0
gentisic acid (GA)	62.0
caffeic acid (CaA)	44.0
vanillic acid (VA)	68.0
chlorogenic acid (CA)	41.0
syringic acid (SA)	81.0
p-coumaric acid (pCA)	84.0
ferulic acid (FA)	66.0
durum wheat bran extract	37.0
simulated extract	46.0

[a] Values are means of duplicate determinations.

Table IV shows the quantitative characteristics of free phenolic acid composition in the freeze-dried extract of durum wheat bran as determined by HPLC. Ferulic, vanillic, and *p*-coumaric acids were the predominant free phenolics, while chlorogenic acid appeared to occur in the least among the identified compounds. From the percentage of the various phenolics, the extract was simulated using authentic standards in the correct proportions and tested in soy oil for antioxidant activity by the AOM procedure. Individual phenolic acid standards were similarly analyzed. The results obtained (PVs) indicated that the phenolic acids varied in antioxidant activity (Table V). The order of decreasing antioxidant activity was PA > CA > CaA > pBA > GA > FA > VA > SA > pCA. The intensity of yellow color on the TLC plates sprayed with carotene mixture and that of whiteness of the linoleic acid spots followed similar order. However, the color differences among GA, VA, and FA were not easily discernible while the colors of pCA and SA were faint, indicating weak antioxidant activities.

The simulated extract was less active (PV 46.0) than the bran extract (PV 37.0). The results obtained by the procedure described in the present work show that the phenolic acids, acting together, appear to be the major antioxidant-active components of the durum wheat bran extract. Other substances, yet to be determined, appear to be acting synergistically with the phenolics to produce stronger antioxidant activity of durum wheat bran extract.

Evidence has been presented here which indicates that extract from durum wheat bran could be an alternative natural antioxidant. Further improvement in the extraction procedure could result in an extract with antioxidant property equal to or greater than those of existing synthetic antioxidants. Although it appeared that the phenolic compounds may be mostly responsible for the antioxidant activity, complete elucidation of the active components in the extract will be carried out in future studies.

ACKNOWLEDGMENT

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Registry No. PA, 99-50-3; PBA, 99-96-7; GA, 490-79-9; CaA, 331-39-5; VA, 121-34-6; CA, 327-97-9; SA, 530-57-4; PCA, 7400-08-0; FA, 1135-24-6.