Wild Rice Hull Antioxidants†

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Wild rice hulls (WRH) have not been utilized in any valuable manner. Minnesota WRH have been shown by us to possess antioxidant properties. The methanol extract of hulls showed antioxidant activity when added to ground beef, as evaluated by the content of thiobarbituric acid reactive substances (TBARS). The results of an ammonium thiocyanate assay also showed that some fractions of the hull methanol extract (MeOH:H_2O, 75:25) have strong antioxidant activity. The yield of the evaporated methanol extract was 2.51% of WRH. The crude methanol extract was fractionated according to hydrophobicity. The antioxidant assay revealed that eluates of MeOH:H_2O (50:50, 75:25) and absolute methanol have the strongest antioxidative activity in ground beef, as measured by the content of TBARS. Antioxidants were isolated from the 75:25 eluate and identified by mass spectrometry as 2,3,6-trimethylanisole (anisole); *m*-hydroxybenzaldehyde; 4-hydroxy-3-methoxybenzaldehyde (vanillin); and 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde). Another compound identified, 2,3-dihydrobenzofuran, was a prooxidant.

Keywords: *Wild rice; hulls; natural; antioxidants*

INTRODUCTION

Hundreds of synthetic and natural antioxidants have been developed for food preservation but only synthetic *tert*-butyl-4-hydroxyanisole (BHA) and *tert*-butyl-4-hydroxytoluene (BHT) are used practically (Osawa and Namiki, 1981). Antioxidants prevent rancidity, improve sensory scores, and provide improved consumer acceptance of food products (Salih *et al.*, 1989). Some synthetic antioxidants are suspected as being possible cancer etiologic agents (Addis and Hassel, 1992). It is expected that these antioxidants will be banned or that food companies may decide not to use synthetic antioxidants like BHA to keep the label more appealing to consumers. Most food companies would prefer natural antioxidants to BHA and are interested in novel, new, natural antioxidants. Therefore, it would be tremendously advantageous to identify potent natural antioxidants. On the other hand, tocopherols are widely used as safe antioxidants, but they are not as effective as the synthetic antioxidants and the manufacturing cost is high (Addis and Hassel, 1992).

Based on the production of 5.9 million pounds of wild rice for the year 1992 (Oelke *et al.*, 1992), it is estimated that ~1.9 million pounds of wild rice hulls (WRH) are produced annually in Minnesota. WRH have not been utilized in any valuable manner and thus represent a growing waste problem. We have shown that WRH contain greater antioxidant activity than wild rice kernels (Wu et al., 1994). However, information on the specific antioxidants extracted from the hulls and their mode of action in comparison with the antioxidants extracted from the kernels has not been published. Therefore, this research was undertaken to develop methods to extract antioxidants from WRH, fractionate the extract, evaluate the antioxidant activity of different fractions, identify the chemical components in the fractions with significant antioxidant activity, quantify the identified components, and evaluate the heat stability and flavor effect of these fractions.

MATERIALS AND METHODS

Materials. Wild rice hulls dehulled from grade A wild rice (*Zizania aquatica* L.) were obtained from New Frontier Foods, Inc., Aitken, MN. The BHA (food grade), was obtained from Eastman Chemical Products, Inc., Kingsport, TN. *m*-Hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde (vanillin), and 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde) were obtained from Sigma Chemical Co., St. Louis, MO. 2,3-Dihydrobenzofuran was obtained from Aldrich Chemical Co. Inc., Milwaukee, WI.

Crude Extract of Wild Rice Hulls. A modification of the method of Osawa and Namiki (1981) was used. Each 50 g portion of WRH was extracted overnight with 300 mL of methanol:water (MeOH:H_2O); (75:25). The extract was filtered through Whatman No. 1 filter paper, and the filtrate was evaporated to dryness under reduced pressure on a rotary evaporator (Wheaton Heidolph rotary evaporator Type VV 60; Germany) at 40 °C. Five grams of crude extract was obtained from 200 g of WRH and dissolved in 40 mL of distilled H_2O:ethanol (4:1).

The antioxidant heat stability of the crude WRH extract was compared with BHA and controls. The crude extract dry matter was dissolved in 40 mL of distilled H_2O:ethanol (4:1) and divided into three equal aliquots. Two aliquots were heated, one to 100 °C and the other to 60 °C, and a third (control) was not heated. Raw ground beef (15% fat) was mixed with each of the aliquots (at 3 and 6 mL per 100 g of beef; 3 mL extract = 0.375 g of dry matter), and a portion of each mixture was stuffed into 50-mL polystyrene centrifuge tubes that were sealed with screw caps and heated in a water

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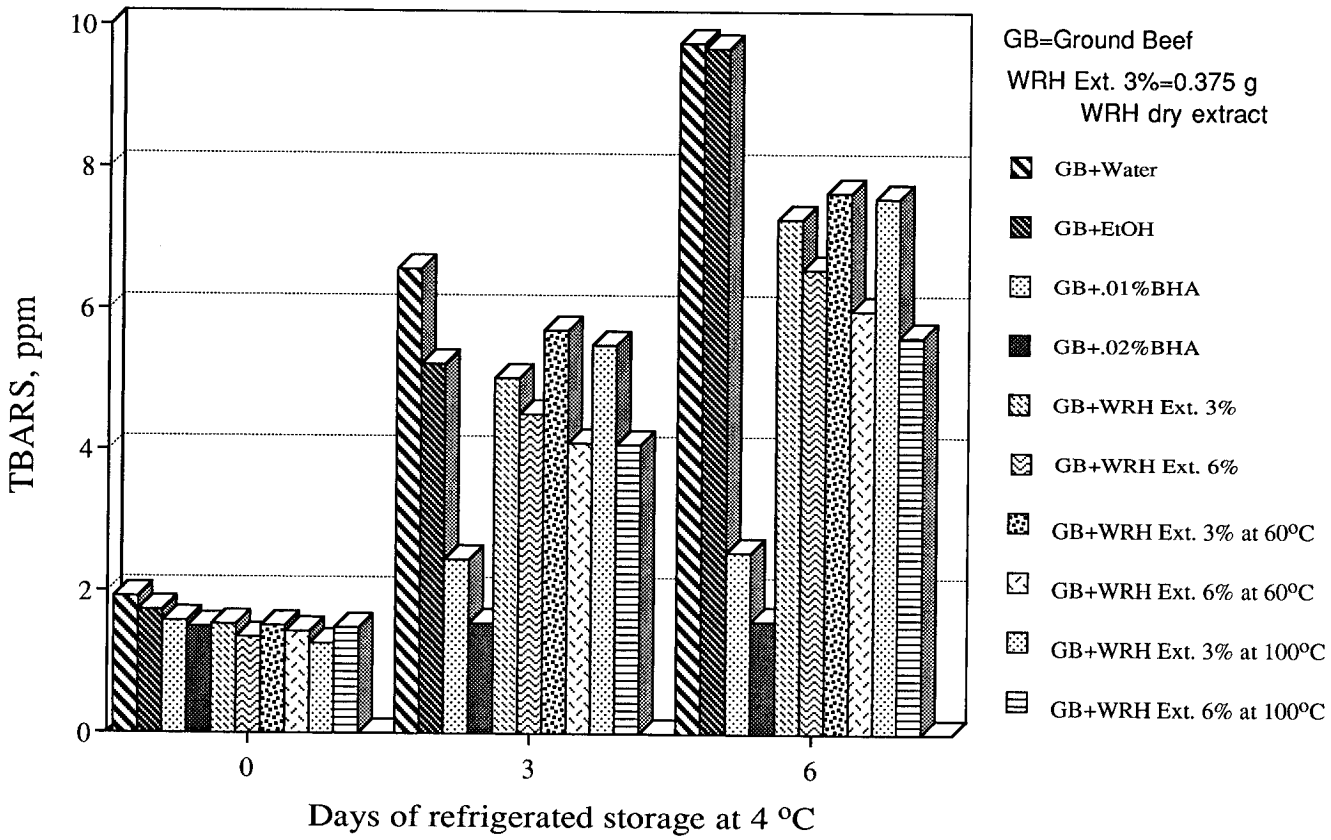

Figure 1. Evaluation of antioxidant activity and heat stability of crude WRH extract over 0, 3, and 6 days of refrigerated storage at 4 °C.

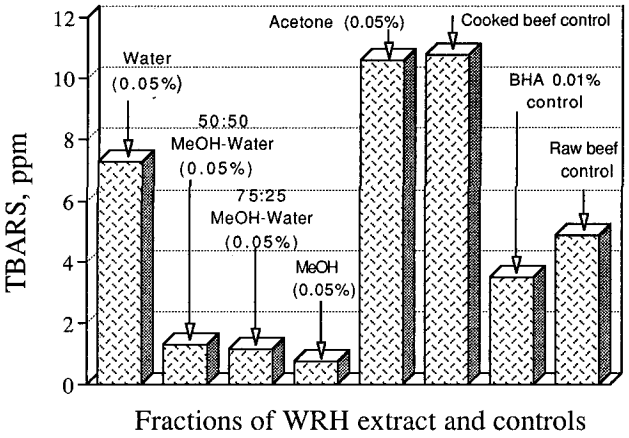

Figure 2. Effect of different fractions of WRH extract on TBARS values of cooked ground beef after 6 days of refrigerated storage at 4 °C.

bath to 72 °C for 1 h. Seven panelists experienced in meat flavor sensory evaluation scored the cooked beef patties for off-flavors. A triangle test was used for the sensory evaluation (Larmond, 1977).

Extraction and Fractionation of Wild Rice Hulls. The WRH were pulverized with a mill (All-Grain Flour Mill, model B-Sox; All-Grain Co., Tremonton, UT). A 200-g portion of pulverized WRH was extracted with 1200 mL of methanol overnight, the extract was filtered through Whatman No. 1 filter paper, and the filtrate was evaporated to dryness under reduced pressure on a rotary evaporator at 40 °C. The extract (5.02 g) was dissolved in MeOH:distilled H_2O (50:50), fractionated on a Bio-Beads SM-2, 100−200 mesh column (25 × 350 mm; Bio-Rad Laboratories, Richmond, CA), and eluted in a stepwise manner with deionized distilled water, MeOH:H_2O (50:50), MeOH:H_2O (75:25), and MeOH. The remaining residue was washed with acetone. The separated fractions were evaporated to dryness under reduced pressure, weighed to determine the yield, and redissolved in ethanol for further analysis.

High-Performance Liquid Chromatography (HPLC). The MeOH:H_2O 50:50 and 75:25 eluates were fractionated by HPLC to individual components. Both MeOH:H_2O (50:50, 75:25) WRH extract fractions were analyzed by a Star 9010 Varian HPLC (Varian Associates, Sugar Land, TX) equipped with an analytical HS reversed-phase C18 (4.6 mm [i.d.] × 250 mm) column with 5-μm diameter particle size, a semipreparatory HS reversed-phase C18 (10 mm ([i.d.] × 250 mm) column with 5-μm diameter particle size, and a high-performance guard column of the same packing material (Vydac, Hesperia, CA). The detector was a Star 9050 variable wavelength UV−vis spectrophotometer (Varian Associates, Sugar Land, TX) set at 280 nm. The MeOH:H_2O (50:50) fraction was eluted with a linear gradient (flow rate, 1 mL/min) ranging from H_2O to 80:20 MeOH:H_2O over 70 min. The MeOH:H_2O (75:25) fraction was eluted under similar conditions. Separated peaks representing MeOH:H_2O (50:50) subfractions were 50:50 I, 50:50 II, 50:50 III, and 50:50 IV, and subfractions representing MeOH:H_2O (75:25) were 75:25 I, 75:25 II, and 75:25 III (Figure 3). All peaks were collected from several injections of the semipreparative column.

Antioxidant Activity Determination. Antioxidant activity of WRH extract fractions was evaluated by thiobarbituric acid reactive substances (TBARS) according to the extraction TBARS method of Salih *et al.* (1987), with ground beef (15% fat) as a model system. An ammonium thiocyanate assay, with a linoleic acid model system (Ramarathnam *et al.*, 1988), was used with some modifications for each of the individual components of the MeOH:H_2O (75:25, 50:50) subfractions. A fixed amount (250 μg) of each sample was added to a solution mixture composed of 0.13 mL of linoleic acid, 0.13 mL of Tween 20, 10 mL of 0.02 M phosphate buffer, and 10 mL of 30% ethanol. The total volume was adjusted to 25 mL with distilled water. The mixed solution was kept in a conical flask at 40 °C. The absorbance was determined at 507 nm daily for a period of 10 days. Two milliliters of the incubated solution,

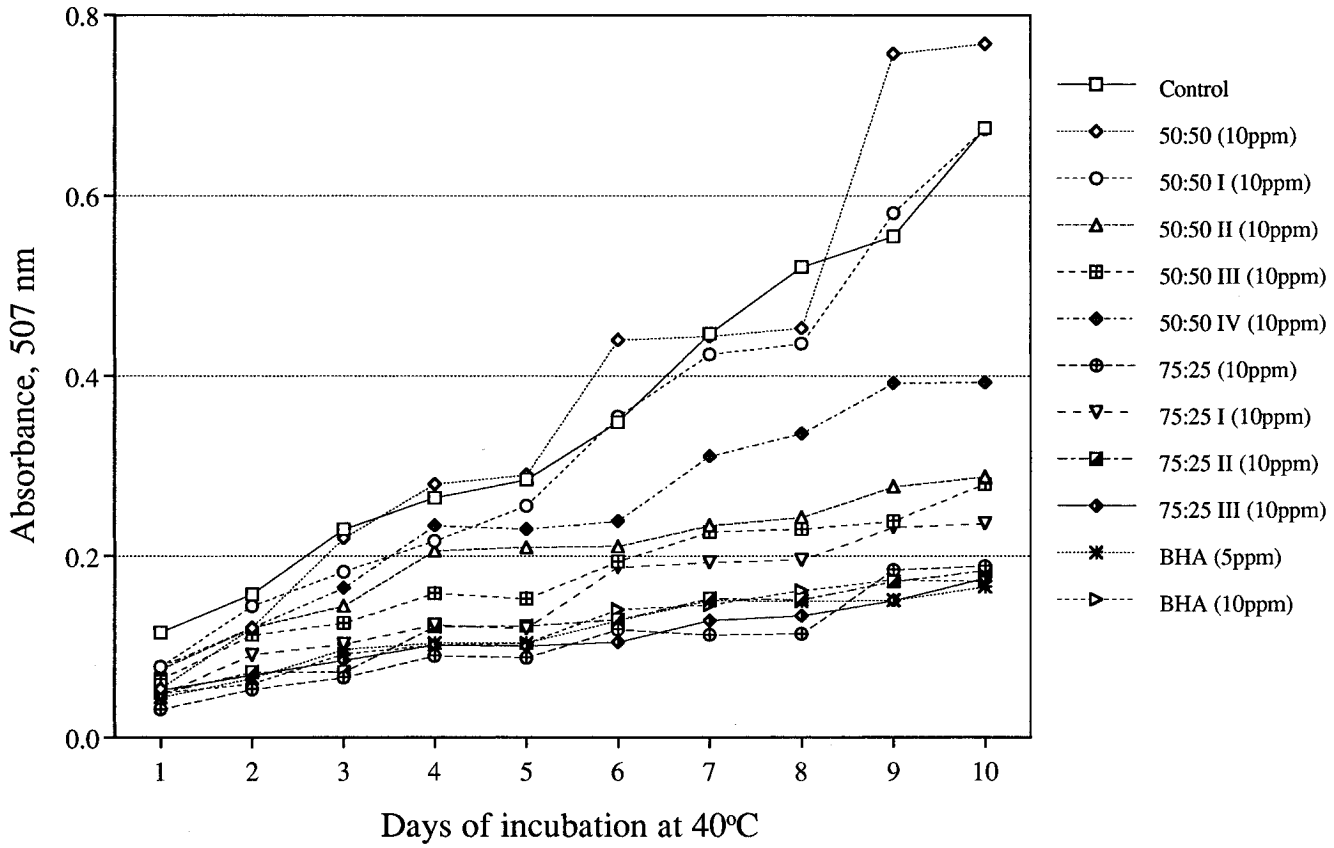

Figure 3. Antioxidant activity of WRC fractions and subfractions, determined by an oil-based ammonium thiocyanate assay over 10 days of incubation.

7.8 mL of methanol, 0.1 mL of ammonium thiocyanate solution, and 0.1 mL of ferrous chloride were mixed together, and the absorbance value was determined against methanol contained in a reference cell. Standards, 2,3-dihydrobenzofuran, *m*-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde (vanillin), and 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde), were also evaluated for their antioxidant activities according to the extraction TBARS method of Salih *et al.* (1987). Freshly ground beef (20% fat) was divided into 100-g portions, and each portion was mixed with one of the above standards, composite sample of the above standards, BHA, and ground beef (control). Each treatment was dissolved in 5 mL of ethanol to make a final concentration of 0.1 and 0.5% of the ground beef on fat percentage basis. Fifty grams of each of the treated ground beef samples were tightly packed into 50-mL polystyrene centrifuge tubes, and the tubes were sealed with a screw cap, heated in a waterbath to 72 °C internal temperature, and held for 1 h. The tubes were removed from the waterbath, cooled to room temperature for 30 min, and mixed thoroughly before testing to eliminate variations within the sample. The TBARS test was conducted within 1 h (0-day) in duplicate on a portion of each treatment. The other portion was tested after 3 days of refrigerated storage at 4 °C.

Gas Chromatography−Mass Spectrometry. The individual components that demonstrated significant antioxidant activity were identified by gas chromatography−mass spectrometry (GC-MS). The analyses were performed on a Kratos MS-25 GC/MS (Kratos Analytical, Ramsey, NJ). A DB-1 fused silica column (30 m × 0.25 mm, i.d.) with a 0.25-μm film thickness (J&W Scientific, Folsom, CA) was temperature programmed from 60 to 260 °C at 10 °C/min, and helium was used as the carrier gas. All spectra were obtained with an ionization potential of 70 eV.

Statistical Analysis. The results of the determinations of antioxidant activity and heat stability of WRH crude extract were statistically analyzed by two-way analysis of variance (ANOVA). The data were grouped by storage and treatments; the dependent variable was TBARS values. The Tukey HSD test was used for multicomparisons. The antioxidant activity of purified WRH fractions was analyzed by one-way ANOVA. Dunnett's test was used to compare effect of different fractions to the control. The results of antioxidant activity of the MeOH:H_2O (75:25) and MeOH:H_2O (50:50) fractions and their individual subfractions were statistically analyzed by two-way ANOVA. The data were grouped by incubation time and treatments; the dependent variable was the absorbance. The Tukey HSD test was used for multicomparison. The results of antioxidant activity of the standards and controls that correspond to the components isolated by the GC-MS from 75:25 fraction were statistically analyzed by three-way ANOVA. The data were grouped by storage, treatments, and level of application; the dependent variable was the TBARS values. The Tukey HSD test was used for multicomparisons. Systat (Systat, Inc., 1992) was used for statistical analyses.

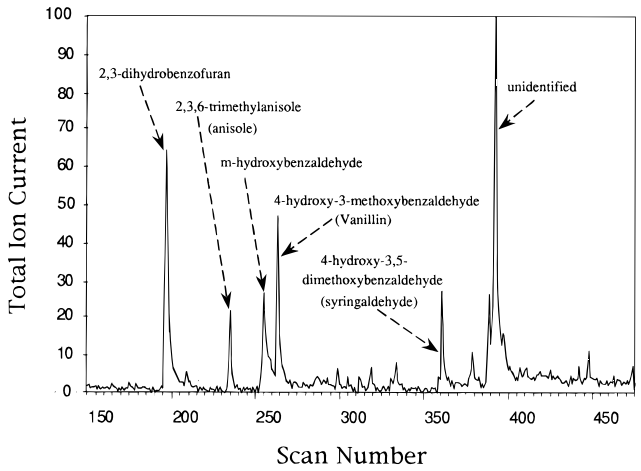

Figure 4. GC-MS chromatogram of WRH fraction MeOH:H_2O (75:25).

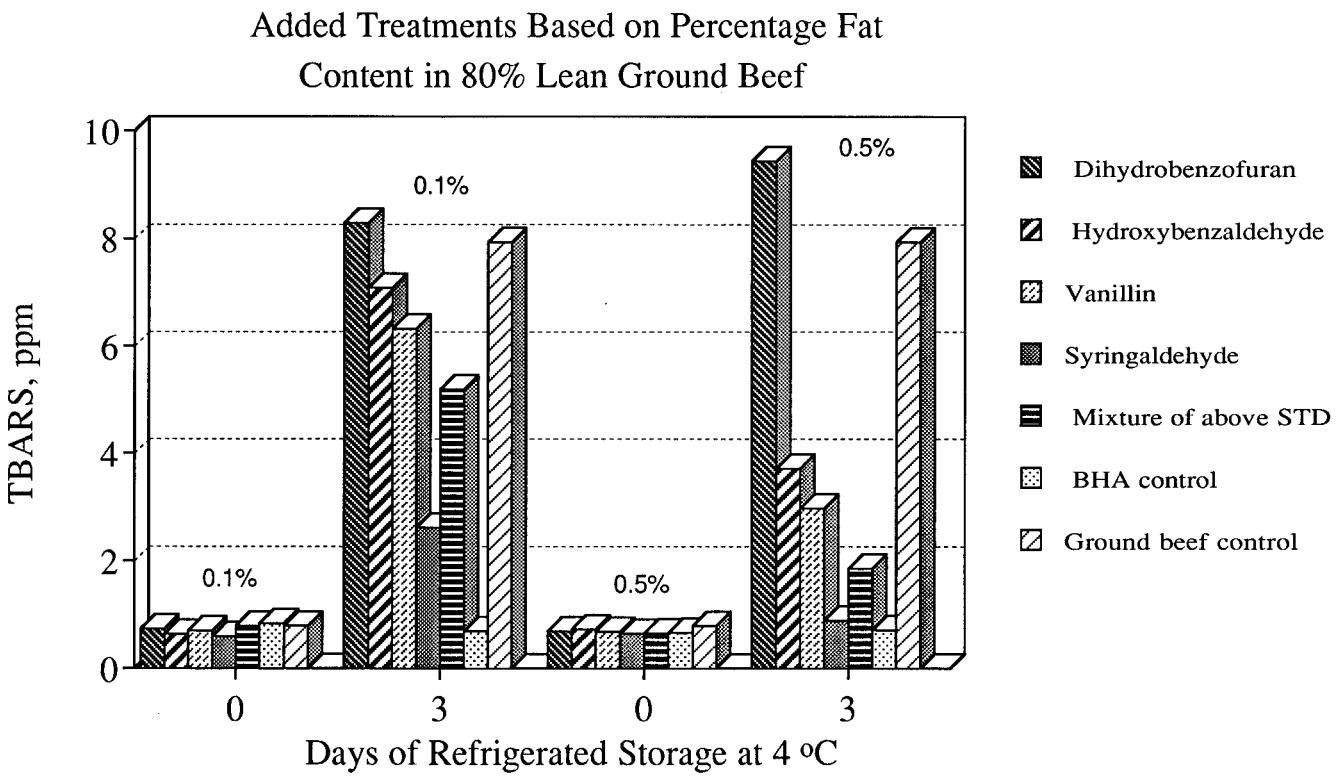

Figure 5. Antioxidant activity of standards corresponding to compounds identified from WRH at two levels of concentration over 0 and 3 days of refrigerated storage as determined by TBARS.

RESULTS AND DISCUSSION

Crude Wild Rice Hull Extract Antioxidant Activity. The results of TBARS analysis of crude WRH extract on ground beef are shown in Figure 1. There were significant differences ($p < 0.05$) between the three storage times for all treatments except for BHA at 0.02% concentration. All treatments of the same concentration had significantly ($p < 0.05$) lower TBARS values when compared with controls for 3 and 6 days of refrigerated storage. Treatments with crude extracts exposed to 60 and 100 °C were not significantly different ($p > 0.05$) from the corresponding treatments with crude extracts not exposed to heat. The results of this study also demonstrated that the antioxidant activity of crude WRH extract is heat stable, which is a desirable property. The preliminary sensory evaluation test showed that there was no significant abnormal flavor originating from the addition of crude WRH extract to ground beef patties ($p < 0.01$). Only one out of seven panelists suspected the presence of different flavor in one sample. The antioxidant activity of the crude WRH extract was less than that of BHA. This difference may be due to a prooxidant antagonistic effect of the water-soluble fraction in the crude extract that might have counteracted part of the antioxidant activity of the methanol-soluble fractions.

Purified Wild Rice Hull Extract Antioxidant Activity. There was significant antioxidant activity ($p < 0.05$) shown by each of three fractions of the WRH extract [i.e., the MeOH:H_2O (50:50, 75:25) and MeOH fractions]. However, two fractions, eluted by water and acetone, did not have any antioxidant activity. The antioxidant activity shown by the first three fractions was after 3 and 6 days of refrigerated storage of meat at 4 °C (Figure 2). The highly significant antioxidant activity ($p < 0.01$) for each of the individual subfractions of MeOH:H_2O (75:25) of the WRH extract is shown in Figure 3. However, only the 50:50 II and 50:50 IV individual subfractions of MeOH:H_2O (50:50) revealed moderate antioxidant activity ($p < 0.05$). As a composite, the subfractions of the MeOH:H_2O (75:25) fraction activity were as powerful as BHA when the linoleic acid model system was used for the ammonium thiocyanate assay.

The chromatogram in Figure 4 shows the components isolated by GC-MS from the 75:25 fraction. These components have been compared with the corresponding standards for the purpose of confirming the identification and in each case have been found to have identical mass spectra. The standards, *m*-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde (vanillin), and 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde), showed strong antioxidant activity when added at 0.1 and 0.5%, based on percentage of fat content in 80% lean ground beef. However, 2,3-dihydrobenzofuran showed a prooxidant activity (Figure 5). The antioxidants showed much higher activity at 0.5% than at 0.1% concentration.

The yield of the evaporated methanol extract was 2.51% of WRH. The fraction MeOH:H_2O (75:25) constituted 20% of the total WRH methanol extract and was equivalent to 0.5% of the WRH on a weight basis. When this fraction was analyzed by MS for the individual components, it was found to contain 21.65% dihydrobenzofuran, 6.03% anisole, 11.19% hydroxybenzaldehyde, 16.85% vanillin, 7.87% syringaldehyde, and 36.41% of an unidentified compound. These values were reported as relative peak areas. Among these components, anisole and vanillin are known antioxidants that have a pleasant flavor. Vanillin flavor was predominant in the MeOH:H_2O (75:25) fraction, and syringaldehyde showed the strongest antioxidant activity.

We anticipate a potential economic benefit of natural WRH antioxidants. These antioxidants will prevent

rancidity and may improve sensory scores and provide improved consumer acceptance of meat and/or oil products. Addition of WRH natural antioxidants to convenience foods that are subjected to extensive processing, such as precooking, freezing, and long periods of storage, will definitely reduce the development of rancidity and improve the flavor. In addition, there are believed to be health benefits associated with the consumption of antioxidant-enriched foods (Addis and Hassel, 1992).

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