

## MAFMA Final Report

Project Title: Rapid Analysis to Facilitate Development and Marketing of Wheat with High Antioxidant Activity. MAFMA Project 25-6231-0109-009

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### **Please complete all questions below and attached form**

1. **Objective Summary:** The objective was to evaluate existing methodology for determining antioxidant activity in biological materials, apply them to wheat bran, and define a more rapid method to quantify the antioxidant activity of wheat samples. The method should be suitable for use for wheat breeders or for niche market selection.

2. **Objective Accomplishments:** We have collected a library of over 2000 wheat samples from 2005 and 2006 harvest years. They represent over 47 different cultivars grown in over 60 counties in Kansas during these two years. They were provided with basic seed analysis and location information for each sample from the Kansas Crop Improvement Association. They form the basis of a continuing research program focused on the importance of genetics, growing location, and growing conditions (and their interactions) on antioxidant activity in wheat. A few varieties from this library were selected based on published history of the genetic background, with Madison and Ike examples of high antioxidant wheat, Jagger and Jagalene as intermediate, and Arapahoe as low antioxidant wheats.

### Method Development

This methodology involved two steps, extraction of components possessing antioxidant properties, and the analysis and quantification of these components. Analysis and quantification of relevant compounds was done using both colorimetric and chromatographic methods.

Extraction of Antioxidative Compounds from Bran - Final description of extraction process  
Wheat was milled using a Quadrumat Junior mill. Two mill streams including flour and bran were collected and the bran was sieved (# 40) further to remove the fines and flour particles. The + 40 mesh fraction, which mainly comprised bran, was then further extracted. A sample of wheat bran was defatted using petroleum ether and shaking for 1 h on a wrist-action shaker. The solvent was decanted and air dried. The defatted bran was transferred to a container where liquid nitrogen was introduced to cover the bran to cool and remove oxygen. The nitrogen was allowed to vaporize completely and cold extraction solvent (methanol:acetone:water, 7:7:6) was immediately introduced. The mixture was homogenized for 2 min with the sample holder immersed in ice. The liquid phase was decanted and fresh solvent introduced and the mixture homogenized. The process was repeated for a total of 3 rounds of homogenization/extraction. The rinses were combined and extraction solvent used to dilute to 35 ml. Nitrogen gas was

bubbled into the sample and mixture shaken for 2 hr. The mixture was centrifuged at 5000 g and 5°C for 15 min. The supernatant was decanted off and concentrated to about 15 ml by a vacuum evaporator set at 35°C. The concentrate was then mixed with 30 ml of new solvent (ethyl ether:ethyl acetate, 1:1) in a separatory funnel and shaken vigorously for 30 sec. The mixture was allowed to settle for 1 minute where two phases were formed and collected. The upper phase was stored while the lower phase was reextracted. The process was repeated three more times (5 extractions in total) and thereafter all the upper phases combined and concentrated to  $\approx$  1 ml by vacuum. The concentrate was collected and the flask rinsed thoroughly with  $\approx$  4 mL of methanol. The sample together with the rinse were combined and refrigerated until analyzed.

#### Justification of the extraction process developed

The extraction process developed was based on methods from published literature. Modifications were made to optimize extraction of antioxidant compounds from wheat bran based on the DPPH, total phenolics and orthophenolic test results and HPLC analysis.

In developing the extraction process, evaluation started based on work done by Padula in his PhD Thesis. However, his work was focused on total phenolic acid content. We were also interested in quantifying individual phenolic acids. His method produced adequate total phenolic acid in extracts, but the individual phenolic acids were too low when using HPLC for quantification. To address this concern, two extraction methods, namely Seib's and Pussayanawin's were evaluated. Seib's method was based on the use of homogenizer to reduce particle size of the bran and addition of phenylthiourea (PTU), a polyphenol oxidase inhibitor, to the extraction solvent. Both free phenolics and bound phenolics were recovered. Pussayanawin method was developed on wheat flour, with the use of alpha amylase to digest starch. We showed that more total phenolics and orthophenolics were detected from Seib's method compared to the other two methods. However, even though the use of alpha amylase improved the recovery of total phenolic acids, the amounts of orthophenolics were reduced. The reason for this observation was not clear, but we suspected that amylase products could be interfering with the assay.

Seib's method was then selected for further work. In addition, it was realized that the bran to be used should be as clean as possible - devoid of starchy material that would dilute the sample. For this reason milling was done on the Quadrumat Junior mill and the material sieved appropriately to remove fines. This precluded the need to use alpha amylase as a step in the extraction process. To improve the extraction process further, utilization of liquid nitrogen to freeze the bran before homogenizing was also incorporated into the protocol at this stage. Through the freezing step, it was envisaged that the extraction process would be facilitated by increasing the brittleness of the bran resulting in more severe shredding of the bran during the homogenization step.

The method was refined further by incorporating steps from the Sasowlki procedure that enhanced free phenolic levels by separating the extracts into the free and the esterified fraction through liquid-liquid extraction. This was followed by deesterification through saponification, as well as bubbling the solution with nitrogen gas during the saponification step. We tested the effectiveness of these procedures on one of the cultivars (Arapahoe) and results showed that bubbling with nitrogen gas resulted in better results. The liquid-liquid extraction step with a mixture of ether and ethyl acetate also improved the orthophenolic and total phenolics recovery. Liquid-liquid extraction step also cleaned up the sample resulting in clearer chromatograms when identifying the phenolics on HPLC. Homogenizing the mixture 6 times for 2 min did not show significant difference from 3 times for 2 min. The latter was then retained for further work.

The next step was to test the contribution of phenylthiourea (PTU, polyphenol oxidase inhibitor) in the extraction procedure, the extraction pH, as well as determining the best extraction solvent. We observed that PTU reacted with the chromophore, contributing to the blue color characteristic for total phenolics and therefore resulting in overestimation of phenolic concentration. Results from colorimetric tests, total phenolic and orthophenolics, showed varied results, with differences across the cultivars, pH, and solvents. Higher amounts of total free phenolics and orthophenolics were obtained when PTU was applied to the solvent. Methanol-acetone-water solvent gave higher total free phenolics compared to acidified methanol for the three wheat cultivars. In general, there was a reduction in the phenolic content of the extract as the pH increased from 2 to 7. For the above reasons, pH 2 and methanol-acetone-water without PTU were selected as the preferred solvent in the extraction protocol.

Even though the values generated by both HPLC analysis and the colorimetric methods, including DPPH test, increased when the saponification step was included, it was noted that there was poor repeatability of data. The saponification step involved treating the extract with 2 N NaOH for 1 h while bubbling the mixture with nitrogen gas at room temperature and acidifying the mixture down to pH 2 with 6 N HCl, in an ice bath. It is not clear why repeatability was poor even when the saponification conditions were carefully controlled. In addition, the step was cumbersome and time consuming. For that reason this step was omitted. However, omission of the saponification step, resulted in poor detectability of phenolics using HPLC due to the low levels present. For that reason, the extracts to be subjected to HPLC analysis were concentrated five fold by using an evaporator after the liquid-liquid extraction step.

Attempts were also made to determine which milling fraction would be the best starting material for the extraction process. Four cultivars were milled using Quadrumat Junior mill to yield 2 fractions, namely bran and flour. The bran fraction was sieved through a # 40 Fisher sieve to yield the “overs” and the “throughs”. The three milling fractions were extracted using methanol-acetone-water solvent and the extracts colorimetrically analyzed for total phenolics and orthophenolics. Results showed differences across the cultivars and the milling fractions. As was expected, orthophenolics and total phenolics were low in flour and highest in the bran overs.

#### Analytical Methods Used

Total Phenolic acids were determined by colorimetric assay.

Folin-Ciocalteu reagent was used to generate color. Ferulic acid was used as standard.

Ortho-phenolic acids were also determined colorimetrically.

Complexation with molybdate generates color to measure. Caffeic acid is used as a standard.

Free radical scavenging capacity was measured using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) as the chromophore. Antioxidant in the sample reacts with DPPH and remaining DPPH is quantified colorimetrically.

#### Chromatographic Analysis of Individual Phenolic Acids:

Primary phenolic acid standards used included Vanillic, Caffeic, Syringic, Coumaric, Ferulic, and Chlorogenic Acids. Ferulic, Syringic, and Caffeic were most prevalent.

A stepwise gradient elution protocol composed of two solvent system - Solvent A, 2% acetic acid in water and solvent B, 100% acetonitrile, was used in a C-18 column.

Lignan determination by HPLC:

The active compound from lignan is Secoisolariciresinol diglucoside (SDG), which contains a dimer of ferulic acid. In the gut, it is hydrolyzed to an enterodiol, and then oxidized to an enterolactone.

A straight gradient elution was also utilized composed of two solvent system - 10% acetonitrile in 5 mM phosphate in solvent A and 100% acetonitrile in solvent B in a C-18 column.

#### Overall Conclusion

The extraction method developed appears to extract the antioxidant components from wheat bran. Analysis of this extract discriminated between different varieties of wheat after analysis by both colorimetric and HPLC methods based on their antioxidant activity. The resulting wet chemistry method is specifically designed to detect the primary components of wheat most likely responsible for cancer suppressing benefits. It has been optimized for use with wheat and will be suitable as a reference method.

However, it still takes a day for extraction and isolation of the active components and another half day for quantification of the target compounds. NIR screening work at Colorado State University has not proven fruitful. These results have prompted our return to a tissue culture method initially used to qualitatively distinguish between wheat cultivars. While still requiring a couple of days for a test, many, many samples can be tested within that period of time. The tissue culture method requires only a sample grinding preparation step (eliminating the lengthy, tedious extraction process), addition to the cells in media, a growth period, and a viable cell counting or other quantification step. This test method is showing promise as an effective screening tool. Our laboratory is currently refining test parameters to optimize reproducibility and minimize time requirements.

#### Extenuating circumstances

Our biggest issue managing the project was keeping qualified personnel on the project. Two post-Docs worked on the project to get it started, but then both for personal or family reasons. Finding and training chemists on the analytical techniques necessary for the testing took time and delayed progress.

#### 3. Unexpected findings, if any

We expected NIR to show potential as a rapid screening test, but it didn't correlate well with the antioxidant levels.

Once we reexamined the tissue culture testing methods, we were surprised at the degree to which it could discriminate between levels of antioxidant activity. We are confident that it can be used as a screening tool once precision of the method is improved.

#### 4. Practical impacts of research efforts

a. Short Term Impacts – I have presented earlier antioxidant research on many occasions. It has stimulated interest among several companies (two milling companies and a farmer-based LLC), universities (three), and even international groups (two plant breeding companies). They have sent me samples for testing. Once the screening test is finalized, it should generate a good deal of interest for ongoing testing from breeders and entrepreneurial companies.

b. Long Term Impacts – With the current interest in whole grains and the nutritional benefits from grains, enhancing specific properties, such as antioxidants, should become a goal. Both wheat breeders and food processors with an interest in marketing to the healthy foods niche will be interested in how antioxidant levels can be enhanced and quantified in their products.

My interest will be to continue to provide testing support to wheat breeders and other interested industry groups. I also have interest in monitoring the fate of antioxidants in the processing of foods. Finally, I have interest in better understanding the genetic triggers that control the level of antioxidants in plants to provide a more consistent product to the market.

5. If you are also making reports to other funding agencies in the course of this research work, please include a copy of that report. N/A

6. If any publications resulted from the research, a copy must be included. Please note we were notified by the USDA/CSREES National Program Leader for the Midwest Advance Food Manufacturing Alliance (MAFMA) that all publications resulting from research that was funded by MAFMA must include the following wording “The project was supported by the USDA Cooperative State Research, Education and Extension Service, special research grant number 2005-34328-16024.”

I was invited to summarize the body of work we've completed in grain antioxidants, diet and cancer suppression at the Cereals & Europe conference earlier in May, 2007. The Europeans are very interested in this area, as shown on the [www.Healthgrain.org](http://www.Healthgrain.org) website. I was asked to write a 4 page short paper describing the presentation for inclusion in a Proceedings book. My most recent draft that will be submitted for publication is attached.

RLM  
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