LINKING CEREAL CHEMISTRY TO NUTRITION: STUDIES OF WHEAT BRAN AND RESISTANT STARCH

by

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B.S., Clemson University, 2007
M.S., Kansas State University, 2009

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
College of Agriculture

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Abstract

Wheat bran is high in dietary fiber. Resistant starch (RS) is considered a source of dietary fiber. Wheat bran and RS have different functional properties and may not have the same nutritional properties. This dissertation covers two areas of importance in cereal chemistry and human nutrition: wheat bran and RS.

Wheat bran chemical and physical influence of nutritional components

Wheat bran has become a hot topic due to positive nutritional correlations, and industrial /humanitarian needs for healthy ingredients. Evolving wheat bran into a demanded product would impact the industry in a positive way. The overall aim of this research was to understand chemical and structural composition, to provide avenues for wheat bran development as a healthy food ingredient. To achieve this goal, antioxidant properties in dry wheat milling fractions were examined, effects of wheat bran particle size on phytochemical extractability and properties were measured, and substrate fermentation was investigated. It was noted that mixed mill streams, such as mill feed, have antioxidant properties (0.78 mg FAE/g; 1.28 mg/g total antioxidant capacity; 75.21% DPPH inhibition; 278.97 µmol $FeSO_4$/g) originating from the bran and germ fractions. Additionally, extraction of reduced particle size whole wheat bran increased measured values for several assays (185.96 µg catechin/g; 36.6 µg/g; 425 µM TE), but did not increase volatile fatty acid production during *in vitro* rumen fermentation over unmilled bran.

RS digestion, glycemic response and human fermentation

In vitro action of enzymes on digestion of maize starches differing in amylose contents were studied. The objectives of this study were to investigate the exact role of α-amylase and amyloglucosidase in determining the digestibility of starch and to understand the mechanism of enzymatic actions on starch granules. Starch digestibility differed (30-60%) without combination of enzymes during *in vitro* assay. Further investigations utilized human glycemic response and fermentation with consumption of a type 3 RS without dietary fiber (AOAC method 991.43). Blood glucose response provided lower postprandial curves (glycemic index value of 21) and breath hydrogen curves displayed low incidences fermentation (40%) with consumption of the type 3 RS, due to the structure of starch and digestion by enzymatic action.

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Major Professor
Yong-Cheng Shi

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Chapter 2: Dr. Jittawan Kubola, Dr. Ron Madl, Dr. Jeff Gwirtz,

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Chapter 5: Dr. Liming Cai

Chapter 6: Dr. Mark Haub, Casey Webber, Dr. Liming Cai, Dr. Lakshmikantha Channaiah

Dedication

This document is in dedication to my sister, Alisa. ☺

Preface

All chapters were written for a specified journal. Therefore, the required journal format was followed for each manuscript, with references following the text and figures in the requisite format for the respective journal; the references follow conclusions in Chapters 1-4, and 6, and the references follow results and discussion in Chapter 5.

For the dissertation, several appendices were included, that may not be available within the final peer-reviewed publication. While this USDA grant led to several studies and publications, six were chosen for this dissertation. The following statements better explain the link of cereal chemistry to nutrition in each chapter:

Chapter 1 – The link of cereal chemistry to nutrition for this chapter was made in the detailed review of information on the various wheat bran layers: epidermis, hypodermis, cross cells, tube cells, testa, nucellar epidermis, and aleurone.
Future studies include utilization of the positive nutritional constituents analyzed to further the use of wheat bran as a functional food ingredient. Current studies within the Carbohydrate Polymers Lab are ongoing of the metabolic possibilities for pentosans and arabinoxylan, as one example.

Chapter 2 – The link of cereal chemistry to nutrition is made in this chapter by an investigation of antioxidants within the wheat kernel and within the dry milling system. This experiment was conducted with as little variability as possible (by limiting the analyzed material) in order to understand the antioxidant availability within various dry milling streams. Identification of high concentrations of antioxidants within low-value mill streams provides applicable use of by-product streams for the wheat industry, and therefore, increased revenue opportunities.
Future studies include examination of antioxidant contributions within multiple mill runs, and those milled from various facilities (to identify differences due to change in processing).

Chapter 3 – The link of cereal chemistry to nutrition is seen in this chapter by determining whether additional grinding could increase nutritional benefit within wheat bran. This technique

is simple, and cost effective, as the technique utilizes equipment already within the dry milling process. Additional grinding is applicable as the use of coarsely or finely milled bran products could alter the projected specifications in a food formulation, if utilizing wheat bran for food health claims.
Future studies include examination of differences in additional mill runs and of products produced by another facility to confirm the current results. Separate analysis of the fractions within whole fractions is also a future aim. Additionally, determination of increased or decreased antioxidative response *in vitro* (cell culture) and *in vivo* utilizing whole wheat bran or wheat bran extract substrates; and determination of wheat bran particle size effect on bile acid binding.

Chapter 4 – The link of cereal chemistry to nutrition is noted in this chapter by the study of rumen fermentation (the first step in ruminant digestion), utilizing live rumen culture *in vitro*. Raw wheat bran can be a food source for beef cattle. This study aids in knowledge of the fate of wheat bran (as the test substrate) for use in feed formulation. By understanding beneficial processing steps, non-advantageous processing can be removed from the system to reduce feed production costs.
Future studies include additional models with rumen fermentation, as well as additional fermentative models with gastric fluids from various sources (pig, synthetic human, etc.).

Chapter 5 – The link of cereal chemistry to nutrition is seen in understanding the mechanism and enzymatic actions during a common *in vitro* (monogastric) starch digestion. The application of this study is in the research area of understanding *in vitro* analyses and starch digestion.
Future studies include applying knowledge of enzyme digestion and synergy to starch for functional food development, as well as additional studies of *in vitro* mechanism on different substrates (wheat, sorghum, etc.) and within additional assays.

Chapter 6 – The link of cereal chemistry to nutrition is most evident in this chapter. The chapter focuses on development of a type 3 resistant starch that is then studied *in vivo*, as the Grain Science department's first *in vivo* trial on human metabolism.
Future studies include additional *in vivo* human response studies funded and conducted by the Grain Science department.

Chapter 1 - Current topics of wheat bran physical and chemical structure

Abstract

In an effort to provide healthier ingredients, researchers are studying staple foods that could better provide satiety and nutritional benefit. Wheat bran is a by-product of conventional milling that contains multiple vitamins, phenolic compounds, and phytochemicals, within a strong fibrous structure. The compounds in wheat bran are well known, but how they come together remains a mystery. Current chemical and structural properties of the multiple layers within wheat bran are often disconnected in published research. This review focuses on wheat bran component structure and functionality throughout the seven layers of the bran, as well as advances in knowledge of potentially health beneficial nonstarch polysaccharides, proteins and various antioxidants. Several studies of wheat bran structural and chemical properties have been reviewed and elucidated to help better understand the ingredient's complex structure and evolve wheat bran as a functional food ingredient.

Introduction

Wheat bran is a by-product of conventional flour milling (Campbell, 2007; Greffeuille et al., 2006; Peyron et al., 2002; Simmons and Meredith, 1979). By-products are seen as secondary within the system and as having little value compared with the main product. In modern processing, by- and/or co-products are utilized when possible to achieve profit gains in tight manufacturing margins (Lee and Stenvert, 1973; Sugden, 2001).

Wheat kernels have three basic sections: endosperm (85%), bran (13%), and germ (2%), in descending total percentage volume (Jacobs and Gallaher, 2004; Osborne and Mendel, 1919).

[Wheat kernel figure is noted in **Appendix A**.] The central endosperm is used as flour, whereas the germ of the kernel contains the highest concentration of lipids, which can be extracted for oil or used as a feed component. When wheat kernels are processed for flour, the lower value components are the bran and the germ (Campbell, 2007).

Wheat bran is mass-produced by roller milling wheat (Campbell, 2007). Roller milling offers a relatively clean separation of the bran and germ from the endosperm (Delcour et al., 1999; Evers and Millar, 2002; Peyron et al., 2002), and the bran is removed with almost all bran layers intact. Instead of a by-product, wheat bran could be considered a co-product, which offers higher value and a greater net gain on the total sale from processing. With a growing population and a high demand for natural foods, widely available co-products should be investigated for optimal utilization.

This review will focus on physical and chemical structure of wheat bran, although wheat bran has more application potential than expressed in this review, such as storage capability (Galliard and Gallagher, 1988), correlation with disease hindrance (Keagy et al., 1988; Wang, Yuan, et al., 2009) and diet/metabolic balance (Slavin and Stewart, 2010; Zacherl et al., 2011; Zoran et al., 1997). The components found in wheat bran are well known, but how they interact and integrate during and after development remains a mystery. This review discusses recent advancements in analytical techniques and chemical analyses that provide answers to wheat bran layer structural unknowns (Anson et al., 2012; Engelsen and Hansen, 2009; Martelli et al., 2010; Parker et al., 2005; P. R. Shewry et al., 2010; Toole et al., 2009; Wang, et al., 2009). We must understand compositional information and identify areas that require additional research to generate attainable queries and understand wheat bran as a functional food.

Wheat bran production

Commercial wheat bran includes the pericarp, seed coats, and aleurone layer along with some remnants of endosperm. Wheat bran produced by conventional milling consists of the outer portions of the kernel with 10–20% of endosperm attached, due to the kernel fractionation method (Chick, 1958). A comprehensive explanation of modern flour milling is available (Campbell, 2007), as well as details about milling conditions, including information on cleaning and adjustments (Sugden, 2001). Briefly, water is added to clean, whole wheat kernels to temper the wheat by softening the crumbly endosperm and toughening the bran. After this process, milling the kernels divides endosperm from bran. Wheat bran yield averages as 21.1–36.5% of the kernel weight (Rakszegi et al., 2008). To optimize flour yield, millers aim to produce large (>2000 μm) bran pieces and minimize *shorts* (broken bran pieces) (Campbell, 2007). Bran thickness in milling is not the only variable in the determination of post-milling particle size (Abecassis, 1993; Peyron et al., 2002). Grinding behavior (speed, equipment, and wear) during milling greatly influences the geometry of the bran pieces (Simmons and Meredith, 1979). Bran particle size in conjunction with biological differences in thickness of the aleurone layer, which can determine the ability of clean endosperm to be removed from the bran, can affect milling efficiency (Crewe and Jones, 1951).

Wheat bran economics

As a staple food in the Western diet, wheat has great importance to society (Von Braun, 2007). Wheat originated in the Fertile Crescent and is cultivated worldwide due to its ease of growth, diverse uses, and long-term storage (Chick, 1958; Lev-Yadun et al., 2000). One bushel of wheat is approximately 60 lb (~27 kg), which provides for approximately 42 lb (~19 kg) of flour and approximately 19 lb (~9 kg) of clean bran. Approximately, two billion bushels of

wheat are produced in the U.S. annually, and account for approximately $5.5 billion (Von Braun, 2007). In May 2012, the average market value for wheat was $7.48 a bushel (USDA Market News, 2012). Per capita, wheat is the most consumed product in the U.S. (Bonjean, 2001; Von Braun, 2007). Americans consume 36% of the domestic-grown wheat crop, export 50%, and use 10% for livestock and 4% for seed (Western Organization of Resource Councils (WORC)., 2002). Approximately 45% of wheat bran is used for animal feed (Gutierrez-Alamo et al., 2008).

Using the current total global production of wheat (Shewry et al., 2012), 600–700 million tonnes of wheat bran are produce annually (~$1.4x10^{12}$ lb/~$2.4\ x10^{10}$ bushels). Annual global growth in wheat consumption is approximately 5% (Von Braun, 2007). Worldwide, wheat consumption is highest in Eastern Europe and Russia, where 30% of calories consumed are from wheat or wheat products; 20% is the global average. Wheat bran can be found in breads (whole grain, stone ground, and specialty), cosmetics, animal feed, and biomass (Kumar et al., 2011).

Wheat bran structure

Wheat bran description by layers

The bran layers/fractions of pericarp, testa, aleurone, and nucellus have been known and reported since one of the first published investigations of wheat bran in the late 1800s (Girard, 1884). The layers that make up bran are generally regarded as the epidermis, hypodermis, cross cells, tube cells, testa, nucellar epidermis, and aleurone. Bran often is referred to in regions: outer, immediate (Jerkovic et al., 2010; T. Nurmi et al., 2012), or inner layers (Barron et al., 2007). Regional composition of bran can be debated, as many layers are incomplete or unrecognizable at maturation. The pericarp often includes the epidermis as well as the longitudinal, cross, and tube cells (Bohm et al., 2002). Alternative terms are aliases for the bran layers, such as perisperm (Raghavan and Olmedilla, 1989) or hyaline layer (Amrein et al., 2003;

Barron et al., 2007; Beaugrand et al., 2004) when referring to the nucellar epidermis. A recent article referred to bran layers by labeling according to the milling method by which they were produced (Nurmi et al., 2012). **Table 1.1** represents the current individual and group names of the wheat bran layers.

Differences in the mechanical properties of bran versus endosperm allow for complete separation, as seen in flour milling (Peyron et al., 2002). Differences in endosperm, aleurone layer, and remaining bran layers make the processing for extracting aleurone cells from the kernel possible (Bohm et al., 2002). A patent is assigned to Bühler AG (Switzerland), wherein the aleurone components are separated from the nonaleurone components in the bran, after removal of bran from endosperm (Bohm et al., 2002). The ability to separate components of the kernel may rely solely on bran layer chemical differences (Anson et al., 2012; Evers and Millar, 2002). In wheat bran, aleurone cells are high in proteins, ferulic acid, and lipids, and are composed of thick nonlignified cell walls (Fulcher and Duke, 2002), whereas the pericarp has thick, lignified cells (Cheng et al., 1987). The bran, as a whole, is generally considered to have large concentrations of branched heteroxylans (with dimers of ferulic acid cross-linking), cellulose and lignin (Fincher et al., 1974; Hemery et al., 2011), even though diversity throughout the layers is known to exist.

Studying the individual layers of wheat bran is difficult because individual layer sections are difficult to obtain (Martelli et al., 2010; Parker et al., 2005). A dissection of mature wheat bran layers was performed after overnight hydration at 4°C (Parker et al., 2005). The kernel cheek regions were scraped from the aleurone layer, and all peripheral layers were removed with a hypodermic needle. This portion accounted for the total bran sample (Parker et al., 2005). The outer pericarp was removed from the soaked grain with forceps, and the aleurone layer was

dissected from the shell. The cross cells (brown in hue) were dissected from the outside of the remaining layers. After cross cells were removed, the testa and the nucellar epidermis remained. The researchers obtained five layers from mature bran. They achieved partial separation of mature layers, with the exception of separation of the testa from the nucellar epidermis layer (Martelli et al., 2010; Parker et al., 2005). A full separation of testa from the nucellar epidermis layer of mature wheat has not been reported with evidence of full separation. Few reviews and in-depth descriptions are provided on individual bran layers. The most recent detailed bran layer description for cereal grains was reviewed by Evers and Millar (2002). While, informative and novel, the 2002 review was on all cereal grains, not just wheat bran. Therefore, a separate thorough description on wheat bran is needed. A description of each wheat bran layer is available in **Table 1.2**. The table describes each layer by name, the origin of the layer prior to maturation, whether the layer can be distinguished in a mature kernel, macro and micro components, characteristics and important distinctive traits. The diversity of each layer is defined in the table, and table data are from multiple references, provided only if authors referred to the layer by name.

The cross section of wheat bran

The reported cross-section descriptions of bran and bran components vary. In **Figure 1.1A**, the cross-section (Toole et al., 2009) portrays a bran layer that is 125–155 µm thick, which was extrapolated from the original figure, based on the scale dimensions provided by the authors. With electron microscopy, mature bran layers appear only three cells thick (DuPont and Selvendran, 1987), but seven layers are present. A visual representation of the layers, **Figure 1.1B**, adapted from Benamrouche (2002), provides an autofluorescence of hydrated wheat bran layers and portrays the width of this bran section as over 500 µm. **Figure 1.1C**, a UV

autofluorescence photograph, is modified from the original image and labels each layer (Parker et al., 2005). The scale bar on **Figure 1.1C** constitutes 10 µm, reporting a wheat bran layer of 40 µm. Alternate cross-sections and widths are represented in other publications whose images are not shown in this manuscript (Dexter and Wood, 1996; Jerkovic et al., 2010; Martelli et al., 2010).

Variation in reported dimensions may derive mainly from sample preparation and dissection technique. Different descriptions and dimensions are probably due to types of wheat, age of wheat, and/or moisture content (Antoine et al., 2004; Gebruers et al., 2008; Greffeuille et al., 2006). Structural differences vary between varieties (Gebruers et al., 2008), and variability in bran size is greatly dependent on the genetic origins of the cultivar (Bonjean, 2001). Spelt wheat seems to have the thickest bran for its size, and had the highest bran content of all the samples in the HEALTHGRAIN diversity screening (Gebruers et al., 2008). Regardless of the amount found from variety to variety, the bran is always considered the outer most portions.

Recent structural understanding of wheat bran

The structure of wheat bran corresponds with its natural function: to protect the kernel. Water does not easily penetrate the mature kernel (Evers and Reed, 1988; Evers and Millar, 2002), so extended tempering of bulk grain stores is necessary prior to flour milling. Tempering is utilized to soften the endosperm and toughen the bran for conventional flour milling (Glenn and Johnston, 1992). Water absorption in the kernel is heterogeneous (Becker, 1960) and enters quickly at the micropyle, a hole at the junction of the testa (Evers and Millar, 2002). The structure of bran is strong, and small flaws are not easily made in the bran under stress (Peyron et al., 2002). In processing, the bran layer is usually removed as one component with some endosperm attached, but processes have been developed to remove certain bran layers from the

kernel and leave the inner bran portions attached to the kernel (Dexter and Wood, 1996; Tkac, 1990).

Studies on wheat's outer layers have shown that breaking the bran layers into three sections: aleurone, intermediate, and pericarp, results in fractions with different properties. Confocally (Antoine et al., 2003) and spectroscopically (Wetzel, 2009), these three sections have different patterns and chemical vibrations, respectively, that distinguish them from one another. A mechanical study showed the physical strength of the intermediate layer; its resistance to rupture (longitudinally and radially) was higher than the pericarp and aleurone layer combined (Antoine et al., 2003).

Investigations of aleurone cells are abundant due to their distinct differentiation from the remaining bran layers. Minerals are concentrated in the aleurone cells of mature wheat, but the kernel as an embryo has a more diverse distribution (Ozturk et al., 2006; Pomeranz, 1988). The aleurone layer contributes significantly to whole wheat's dietary fiber content (Stewart and Slavin, 2009). Aleurone cells resist digestion, but are digested to a greater extent than the wheat bran outer layers (Amrein et al., 2003). Wheat bran also resists fermentation by human fecal bacteria (Slavin, 2000). When insoluble fiber is fermented by intestinal microflora, bound antioxidants can be released (Slavin, 2000; Slavin, 2003). After 8 hours of exposure to intestinal microflora, bran particles are approximately their original size, and the aleurone cells are still recognizable cubicle cells. Researchers have assumed that the aleurone cell walls (noted to contain less ferulic acid) were the first to be degraded by colonic bacteria and that the amount of ferulic acid is important to the fermentability of aleurone cells and wheat bran (Amrein et al., 2003; Klepacka and Fornal, 2006). Digestion of different bran layers within the human gastrointestinal tract merits further investigation and has not been well documented

Chemical components of wheat bran

The compositional analysis of bran was well established in late 20th-century publications (Kyriazakis and Emmans, 1995; Soest, 1984; P. J. Van Soest et al., 1991). The bran layers are chemically comprised of approximately: arabinoxylan (AX) (38%) > protein (25%) > cellulose (16%) > lignin (6.6%) (Brillouet and Mercier, 1981; Brillouet et al., 1982; DuPont and Selvendran, 1987). Average bran composition from commercial milling is noted in **Table 1.3**. While lipids are available throughout the kernel, the majority of wheat lipids are located within the germ. In addition, bran contains multiple micronutrients and phytochemicals (Evers and Millar, 2002; Pomeranz, 1988) with phenolic compounds (3.3–3.9 gallic acid equivalents per gram), phytosterols [1790–2140 μg/g (Nurmi et al., 2008; Nurmi et al., 2012; Piironen et al., 2002; Piironen et al., 2008)], and carotenoids [0.50−1.80 μg/g lutein (Zhou et al., 2004)] in high concentration. The essential vitamins found in wheat bran per 100 g bran include: biotin (0.048 mg), thiamin (0.54 mg), riboflavin (0.39–0.75 mg), pantothenic acid (2.2–3.9 mg), vitamin E (1.4 mg), niacin (14–18 mg), vitamin B6 (1–1.3 mg), and folate (79–200 μg) (Anson et al., 2012; Ball, 2006). A recent review of the bioactive potential of wheat bran by processing provides details about the chemical components within the bran, their development in the kernel, and their purpose (Anson et al., 2012).

Advances in wheat protein investigations

Wheat bran contains the majority of protein found in the kernel (Dong et al., 1987; Pedersen and Lindberg, 2010). Wheat bran protein content is now known to be lower than original reports (Girard, 1884; Osborne and Mendel, 1919), with protein concentrations of 10.2–13.8% (Amrein et al., 2003; Van Soest, 1994; Van Soest et al., 1991). Protein levels in wheat are generally higher those that of other cereal grains (Jerkovic et al., 2010; Sauvant, 2004). During

wheat development, the overall protein content can change based on growing conditions (Jerkovic et al., 2010). Lysine and tryptophan are the most abundant wheat bran amino acids (Dong et al., 1987; Pedersen and Lindberg, 2010). A quantitative amino acid profile for wheat bran is available from Pedersen and Lindberg (2010).

The location of all enzymes (proteins) present in the bran portion of mature wheat was determined using proteomics (Jerkovic et al., 2010). Proteomic studies are intensive chemical and/or molecular analyses focusing on protein and their constituents (Laubin et al., 2008; Oda et al., 2006). Using immunofluorescence microscopy, researchers determined that oxalate oxidase, peroxidase, and polyphenol oxidase reside in the outer layers, xylanase inhibitor protein I in the intermediate fraction, and pathogenesis-related protein 4 in the aleurone layer. All proteins found in the outer portion (epidermis and hypodermis layers) function to protect the kernel from degradation (Jerkovic et al., 2010). A diverse protein profile is present in the intermediate (cross cells, tube cells, testa, and nucellar epidermis layers) fraction (Jerkovic et al., 2010), and the inner layer (the aleurone cells) is rich in antioxidants and defense enzymes, as well as storage protein 7s globulin, which is specific to this layer (Jerkovic et al., 2010). Details of the enzymes in the outer layer, inner layer, and water-soluble bran portion are available in the original publication (Jerkovic et al., 2010).

Advances in knowledge of vitamins, minerals, and antioxidants matrices in wheat bran

Engelsen (2009) determined the distribution of the eight variants of vitamin E within milling fractions. The majority of vitamin E is found in the germ portion of the kernel, with bran containing the second-highest amount. Tocopherols are highly concentrated in the germ portion but are more distributed within the grain, with a slightly greater content in the finer shattering

bran portions (**Table 1.4**) (Engelsen and Hansen, 2009). *In vivo*, the variants of vitamin E are absorbed by the same mechanism, until uptake at the liver (Traber and Atkinson, 2007). Only α-tocopherol contributes to serum levels of vitamin E due to a highly specific lipid-soluble vitamin receptor mechanism in the liver. α-tocopherol is taken up by α-tocopherol transfer protein (αTTP), whereas the other forms are broken down by enzymatic action (Takada and Suzuki, 2010; Traber and Atkinson, 2007). As certain variants possess enhanced nutritive value, Engelsen and Hansen (2009) also calculated α-tocopherol equivalents, for a better representation of bioavailability. The study concluded that reduction of wheat bran particle size significantly increases the available α-tocopherol portion (Engelsen and Hansen, 2009). This phenomenon could be true for other minerals or vitamins in bran (Engelsen and Hansen, 2009; Kahlon et al., 1986), but it has not been investigated.

With identification of the high portion of antioxidants comparatively (to other antioxidant containing foods) found in wheat (Okarter and Liu, 2010; Qu et al., 2005), wheat antioxidants have become a subject of intensive investigation. Phenolic compounds are the main antioxidant found in wheat (Alvarez-Jubete et al., 2010; Barron et al., 2007; Jonnala et al., 2010; Klepacka and Fornal, 2006; Mattila et al., 2005; Menga et al., 2009; Parker et al., 2005; Stalikas, 2007; Verma et al., 2009). From the same kernel, phenolic compounds in bran are 10–20 times higher than in endosperm (Klepacka and Fornal, 2006; Mattila et al., 2005). Ferulic acid is the dominant phenolic acid in the bran (Abdel-Aal, 2001; Klepacka and Fornal, 2006), and is in conjunction with AX, which is further discussed in the section of ***Progress in the knowledge of polysaccharides***. Phenolic compounds are predominantly found in the outer layers of wheat bran as a structural component of the cell wall, providing UV radiative and pathogenic element protection (Parker et al., 2005; Stalikas, 2007). Phenolic content and availability are affected by

environment, farming, mutation, and processing (Gélinas and McKinnon, 2006; O″zer et al., 2006; Yu et al., 2003).

Multiple methods exist to measure the phenolic contents in wheat. **Table 1.5** depicts the variety of phenolic extractions that have been published. After compound extraction from the solid sample, a total phenolic content (TPC) assay can be quantified on a spectrophotometer. TPC is determined by a Folin Ciocolteau reagent assay on extracted compounds in solution and spectrometrically read at 760–765 nm. Folin-Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate and reacts with any reducing substance to measure the total reducing capacity of a sample. The intensity of the color, determined by the mixture of reagent and sample, represents the amount of the sample required to inhibit the oxidation of the reagent. The TPC assay by colorimetric analysis is relatively uniform in many lab groups (Adom and Liu, 2002; Singleton et al., 1999; Verma et al., 2008). Methods to extract phenolic acids (whether free, bound, or total) vary between research groups, and can inhibit sound comparisons between groups. Some groups begin by extracting lipids from the sample with a hexane (Kim et al., 2006; Kim et al., 2011; Zhu et al., 2010) or petroleum ether (Onyeneho and Hettiarachchy, 1992). To some extent, lipid-soluble vitamins could be removed by this treatment, depending on the method, force, and particle size (Engelsen and Hansen, 2009). Some researchers begin a phytochemical and/or phenolic acid extraction (bound or total) with alkali (Adom and Liu, 2002; Adom et al., 2005; Butsat et al., 2009; Okarter et al., 2010; Siebenhandl et al., 2007), and others begin with alcohol (Abdel-Aal and Hucl, 2003; Butsat and Siriamornpun, 2010; Verma et al., 2009; Zhou and Yu, 2004). Free phenolic compounds in plant tissue can be extracted with organic solvents (Adom and Liu, 2002; Butsat et al., 2009; Okarter et al., 2010; Zielinski and Kozlowska, 2000), but the bound portion remains intact. Some research groups adjust moisture

prior to extraction (Verma et al., 2008), and others employ ultrasonic baths (Wang et al., 2008), because recovery with most extraction methods is relatively low (Liyana-Pathirana and Shahidi, 2006). Results vary when multiple extraction methods are tested simultaneously (Zhou and Yu, 2004), and a consensus on the proper technique to quantify these compounds for referencing and comparison across publications is needed. Selective extraction is recommended, because subsequent selective analysis or extraction of desired compounds does not minimize interference of other compounds that coexist or reduce contamination (Jones and Kingkorn, 2006).

Many studies have evaluated the variability of different nutrients in wheat varieties (Gutierrez-Alamo et al., 2008). For more details, an extensive study of the phenolic acid composition and antioxidant capacity of hydrolyzed wheat bran fractions was completed by Verma et al. (2009) and chemical/compositional wheat cultivar comparisons were conducted as part of the HEALTHGRAIN project (Andersson et al., 2008; Nurmi et al., 2008; Piironen et al., 2008; Poutanen et al., 2008; Rakszegi et al., 2008; Ward et al., 2008). In 2005, a project entitled the HEALTHGRAIN project began in the European Union under the sixth Framework Program, project code FOOD-CT-2005-514008 (Poutanen et al., 2008). One hundred and fifty wheat lines were screened for bran yield, fiber composition, gluten contents, phytochemicals, protein contents, and various technical aspects; comparing between varieties, from different origins, all grown in the same location (Rakszegi et al., 2008).

Phenolic compounds, vitamins, and remaining antioxidants are thought to reside in the same matrix. In free and bound form, many wheat bran antioxidants cannot be digested in the human gastrointestinal tract in their complex form (Anson et al., 2009; Anson et al., 2011; Menga et al., 2009; O˝zer et al., 2006). Some bound components in bran can be released through different processing techniques and digestions (Stalikas, 2007; Thanh and Nout, 2002). The majority of

antioxidant compounds are bound in ester linkages to the abundant polysaccaharides in the cell wall and present as lignin, therefore rendering them unavailable (Stalikas, 2007). Lignin is an organic polymer that fills spaces in the cell wall between cellulose and hemicellulose, accounting for 3–7% of the bran. Lignan, a bound antioxidant in wheat bran, is a dietary phytoestrogen that is bound until released and is reported as 2.6–3% of total bran (National Research Council (US). Subcommittee on Feed Composition, 1982; Robertson and Eastwood, 1981). Secoisolariciresinol diglucoside is the major lignan compound found in wheat bran, it can be extracted with methanol, and the metabolic components derived from wheat bran secoisolariciresinol diglucosides are involved in antitumor activities in colon cancer SW480 cells when unbound (Qu et al., 2005). Bound components in bran can be released by different processing techniques (Stalikas, 2007; Thanh and Nout, 2002) and reactions (Slavin, 2000; Slavin, 2003).

Of all wheat bran layers, the aleurone layer has the highest concentration of phenolic acids and antioxidant activity (Zhou and Yu, 2004; Zhou et al., 2004), but increased efforts are needed to decipher which layer contains the most bioavailable antioxidants. The antioxidants present in a sample and antioxidant activity are not the same value. To be biologically available in the human digestive system, an antioxidant must first arrive to a point of absorption, be unbound for absorption, and not be inhibited by molecules around the site of absorption. When unavailable antioxidants are present, the nutritional value depends on the amount released during any part of the digestive process that is absorbed and utilized

Multiple compounds within wheat bran exude antioxidant properties (Lachman et al., 2012) and the chemical diversity of antioxidants makes it difficult to separate and quantify individual antioxidants (Ou et al., 2002). No single measure of antioxidant concentration can express the ability, activity and capacity of antioxidants present. In addition, diverse antioxidant

compounds have the ability to act *in vivo* via multiple mechanisms (Pellegrini et al., 2003), thus multiple *in vitro* antioxidant models are available to investigate 'total antioxidant' properties.

Chemically distinct antioxidant quantification methods between an oxidant and a free radical are mechanistically based on either electron or hydrogen atom transfer, and it is necessary to evaluate whether different methods can provide comparable antioxidant values for the same sample (Ou et al., 2002). Antioxidants can be measured *in vitro* by 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS+), ferric ion reducing antioxidant power (FRAP) assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity, oxygen radical absorbance capacity (ORAC), Trolox equivalent antioxidant capacity (TEAC) or TPC assay (Herald et al., 2012; Roy et al., 2010; Thaipong et al., 2006). ABTS+, DPPH and TEAC utilize single electron transfer measurement to determine antioxidant reducing capacity (Roy et al., 2010; Thaipong et al., 2006). FRAP measures antioxidant power by reducing power of the electron donating antioxidants present in the extract (Benzie and Strain, 1999; Ou et al., 2002; Thaipong et al., 2006). ORAC measures antioxidant properties by hydrogen atom transfer, assessing antioxidant donating capacity (Huang et al., 2005; Roy et al., 2010). ORAC is noted for its representation of measuring antioxidant capacities as they would appear *in vivo*, but the mechanism is unconfirmed. ORAC is also thought to be related to FRAP and DPPH, although it is not linear (Thaipong et al., 2006). Multiple methods are often employed to investigate 'total antioxidant' response, as there is difficulty in separating and quantifying individual antioxidants due to the chemical diversity of antioxidants (Ou et al., 2002). Antioxidant properties can be measured by high-performance liquid chromatography or spectrophotometry (Alvarez-Jubete et al., 2010; Alvarez-Jubete et al., 2010; Herald et al., 2012; Kubola and Siriamornpun, 2008).

Progress in the knowledge of non-starch polysaccharides

Major non-starch polysaccharides in wheat bran include AX (70%), cellulose (24%), and beta 1–4 glucan (6%) (Brillouet and Mercier, 1981; Selvendran, 1987). Small amounts of glucomannan (Gruppen et al., 1989), arabinogalactan (Fincher et al., 1974), xyloglucan (DuPont and Selvendran, 1987) have also been reported. A significant number of investigations are on examining AX in breadmaking functional properties (Courtin et al., 1999; van Craeyveld et al., 2009), AX as a food additive (Izydorczyk and Biliaderis, 1995), and promotion of AX with several health claims (Cao et al., 2011; Courtin et al., 2008; Swennen et al., 2006). Current research investigations are on AX and its constituents include defining its origin, uses, and quantification within grains (Cao et al., 2011; Courtin et al., 2008; Kiszonas et al., 2012; Mandalari et al., 2005; Pollet et al., 2012; Swennen et al., 2006; Toole et al., 2009), as well as best methods for extraction (Delcour et al., 1999; Mandalari et al., 2005; Marinkovic and Estrine, 2010; Swennen et al., 2006; van Craeyveld et al., 2009). Therefore, current explorations of AX will be discussed. In wheat, the main hemicellulose portion is AX. AX is a feruloylated oligosaccharide, where the xylan and phenolic acid matrices coalesce, but a percentage can be isolated by mild acid hydrolysis (Wang, et al., 2009). Secondary plant cell wall digestion by enzymes is limited, and 35% of cell wall xylan is able to be released after enzymatic digestion of wheat bran (Lequart et al., 1999). AX is fully developed 20 days post-anthesis and shows no significant change afterward (Beaugrand et al., 2004). The exact arrangement of AX is unknown, but AX is theorized to coat or cross cellulose microfibrils (Carpita, 1996; Mitchell et al., 2007), in which case primary cell walls, cellulose, and hemicellulose may act as a network embedded in the protein matrix and make AX and cellulose part of the matrix themselves (Mitchell et al., 2007). Xylan concentrations are ~5% of the primary cell wall in dicots as opposed to 20% in the seeds of grasses (McNeil et al., 1984). The constituents of the pericarp average 660 g AX/kg and

320 g cellulose/kg. In the aleurone cells, small contents of beta glucan (310 g/kg) and AX (650 g/kg) are present (Bacic and Stone, 1981; Selvendran, 1984; Selvendran, 1987). Genes that are responsible for encoding AX are under investigation (Mitchell et al., 2007).

Great variation is possible for phytochemicals in wheat. AX is affected by temperature variation and environmental conditions (Finnie et al., 2006; Toole et al., 2010). The arabinose to xylan ratio (A/X) is slightly lowered in cooler/wetter growth environments, and esterification of the endosperm cell walls with ferulic acid is lowered under hot/dry conditions (Toole et al., 2010). With respects to the milling yields, soluble AX in white flour can vary by 4.7-fold from genetic and environmental differences (Shewry et al., 2010).

Advanced analytical techniques now elucidate once unknown biological factors. Using Fourier transform infrared (FT-IR) spectroscopic analysis on feruloylated oligosaccharides, researchers noted that a band at 1731 cm^{-1} is due to the ferulic acid ester group on the oligosaccharides, whereas conjugated double bonds are at 1253 cm^{-1}, and vibration of the phenyl ring is found at 1596 cm^{-1}. The band representing xylooligosaccharides is at 1042 cm^{-1}, with 897 cm^{-1} representing beta glycosidic linkages between glucose units. The band at 811 cm^{-1} was proposed to be from the furanose derivatives of the beta glycosidic linkages (Wang, et al., 2009). Using a Perkin-Elmer (Shelton, CT) Spectrum Spotlight IR microscope optically interfaced to a SpectrumOne spectrometer in the Kansas State University Microbeam Molecular Spectroscopy Laboratory (Manhattan, KS), wheat bran spectra were collected in an attempt to view the bands coordinating with feruloylated oligosaccharides. Using the spectrum software supplied with the imaging system (Spotlight 7.0) and OMNIC (both from Thermo-Fisher, Madison, WI), an average spectrum was processed in absorbance to label the previously identified bands (Wang, et al., 2009) within the fingerprint region (Wetzel and Brewer, 2010). In **Figure 1.2**, FT-IR

spectroscopic wheat bran mapping to obtain multiple spectra noted the band at 1730 cm^{-1} due to the ferulic acid ester group on the oligosaccharides, with conjugated double bonds at 1252 cm^{-1} and vibration of the phenyl ring around 1596 cm^{-1}. In the previous study, bands for xylooligosaccharides were noted at 1042 cm^{-1} and 897 cm^{-1}; however, the proposed band at 811 cm^{-1} from furanose derivatives of the beta glycosidic linkages was not detected in **Figure 1.2**. Absence of this band may be due to the range of instrument detection, the concentration of furanose derivatives of the beta glycosidic linkages within the sample, or diminished vibration from the calculation of an average spectrum. The identification of bands in molecular spectroscopy aids in the use of FT-IR in grain research (Wetzel and Brewer, 2010).

Analysis of FT-IR spectra determined that the shoulder height in the spectrum at 1,075 cm^{-1} reflected the extent of branching of the AX structure (Toole et al., 2009). For analytical comparison, the ratio of the 1,075 cm^{-1} shoulder to the major peak at 1,041 cm^{-1} was calculated. Martelli et al. (2010) collected attenuated total reflectance FT-IR spectra from specific layers within the bran layer (the aleurone layer and the nucellar epidermis) and noted that a band at 1740 cm^{-1} was present only in the aleurone spectra, and not in the nucellar epidermis spectra. A band at 1740 cm^{-1} represents a carbonyl vibration and is associated with lipid content in molecular spectroscopy (Brewer et al., 2012; Wetzel and Brewer, 2010).

AX doublets (1,024–1,151 cm^{-1}) were calculated using Raman spectroscopy from the second derivative spectra (and multiplied by -1) (Toole et al., 2009). The A/X ratio was calculated from the intensities of the peaks at 494 and 570 cm^{-1}. Raman provides the best spatial resolution for this experiment because FT-IR microspectroscopy provides limited information on AX structure, making it difficult to determine changes with weak absorbance and overlap of cell wall compounds. Even with Raman, distinguishing monomeric and dimeric forms of bound

ferulic acid is not possible (Toole et al., 2009). Monomeric and dimeric forms of bound ferulic acids are in need of further research and understanding.

More traditional analytical methods can be used to quantify xylan constituents. Quantification of AX content and A/X can be determined by gas chromatography of alditol acetates (Courtin et al., 2000) and ferulic compounds (Abdel-Aal, 2001). Further investigation of the chemical components and their arrangement in space is needed. The chemical components in a mature kernel cell wall can be assorted at a supramolecular level, making analytical separation with high-tech instrumentation difficult (Autio, 2001).

Conclusions

Many advances in basic wheat bran knowledge have been elucidated in the past 20 years. Wheat bran has become a hot topic due to its positive nutritional correlations, industrial and humanitarian needs for healthy ingredients, and availability within the industry. The knowledge reviewed in this article was collected to enhance the understanding of and to stimulate readers to pursue additional experiments with the diverse chemistry and structure of wheat bran. Developing wheat bran as an ingredient and higher demand product would positively affect the wheat industry.

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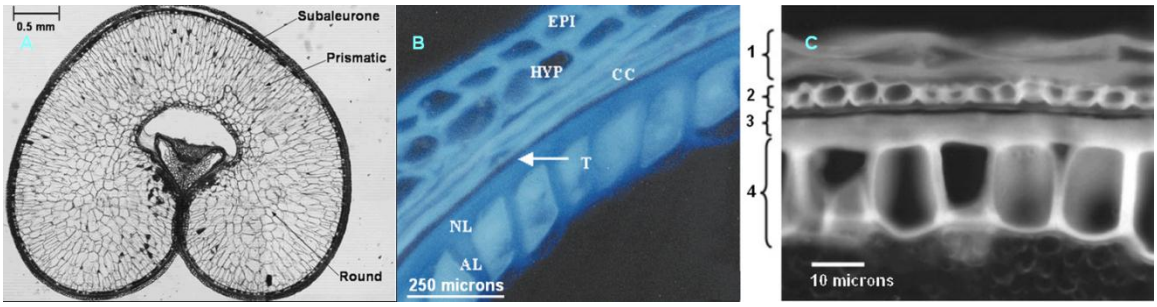

Figure 1.1 Cross sections of wheat

A: Photomicrograph of a wheat kernel cross-section (Toole et al., 2009). B: an autofluorescence image of hydrated wheat bran layers. AL: aleurone layer, NL: nucellus, T: testa, CC: cross cells, EPI: epidermis, HYP: hypodermis. C: UV autofluorescence photograph showing: 1) outer layer, 2) cross cells, 3) testa and nucellar epidermis, and 4) aleurone layer; modified (Parker et al., 2005). Sizing bar is included in each image.

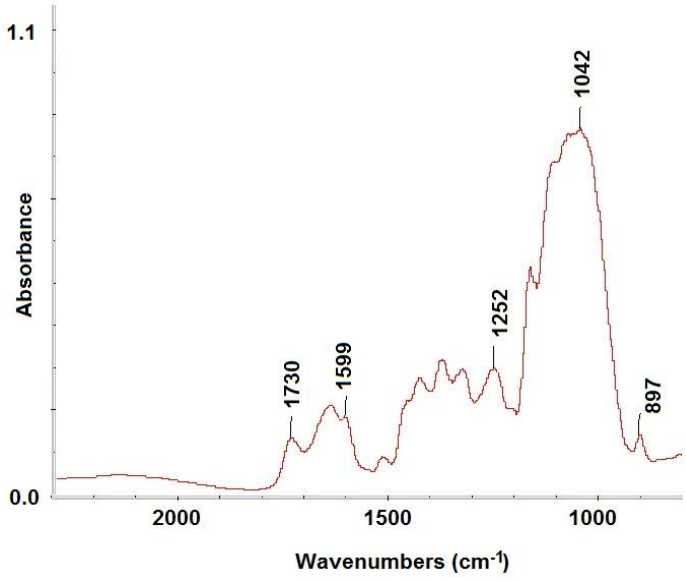

Figure 1.2. Average spectrum of wheat bran via FT-IR microscopic analysis of wheat bran.

Band numbers represent molecule bands identified in on feruloylated oligosaccharides by (Wang, et al., 2009). In the wheat bran spectrum, the following partially contributed to the band intensity at each point: 1730 cm^{-1} - ferulic acid ester groups, 1599 cm^{-1} - phenyl rings, 1252 cm^{-1} - conjugated double bonds, 1042 cm^{-1} - xylooligosaccharides, and 897 cm^{-1} - beta glycosidic linkages.

Table 1.1. Layers of wheat bran and their function.

Region	Layers often included	Function to the wheat kernel
Aleurone	Aleurone, aleurone cells with subaleurone (peripheral cells)	Major AX and phytochemical storage layer. Transfers nutrients to endosperm before maturation (T. Evers and Millar, 2002).
Inner layer	Nucellar epidermis, aleurone	Contains the largest portion of AX and nutrients (T. Evers and Millar, 2002).
Intermediate	Cross cells, tube cells, testa, nucellar epidermis	Diverse protein composition (Jerkovic et al., 2010).
Nucellar epidermis	Nucellar, hyaline layer, perisperm, seed coat	During maturation, this layer is the perisperm, a storage tissue (Raghavan and Olmedilla, 1989).
Outer layer	Epidermis, hypodermis	Protective layer, approximately 15-30 µm thick (Barron et al., 2007); does not prevent water from penetrating (T. Evers and Millar, 2002).
Pericarp	Outer: Epidermis, hypodermis; Inter: longitudinal cells, cross and tube cells	Protective layer, approximately 30-50 µm in length (Moss, 1977); composed of empty cells due to cytoplasmic degeneration of cross and tube cells (Morrison, 1976).
Testa	Seed coat, carpel wall, spermoderm	Protective, water proof layer (T. Evers and Millar, 2002), approximately 6 µm thick (Moss, 1977).

Table 1.2. Compiled description of each layer of wheat bran.

Layer*	Origin	Notable in the mature kernel	Macro components (Protein, starch, lipid, cellulose, hemicellulose)	Micro components (chemicals, vitamins)	Notably physical characteristics	Research highlights
Aleurone	Present in the embryo (T. Evers and Millar, 2002).	Yes	High levels of protein: 13.4-16.2% (Amrein et al., 2003; Lee and Stenvert, 1973), as mostly lysine and arginine (Moore et al., 2005). Once believed to contain the largest lipid content in bran (Amrein et al., 2003; Lee and Stenvert, 1973), but recently reported to contain only small levels of lipids (Moore et al., 2005). 5–8% cellulose (Amrein et al., 2003). Contains 25% of AX in the bran (Benamrouche et al., 2002). Contains traces of glucomannan and arabinogalactan (Fincher et al., 1974; Gruppen et al., 1989).	High concentration of inhomogeneous distributed ferulic acid (Saadi et al., 1998). Location of delphinidin 3-glucoside, the anthocyaninic compound in blue wheat (Abdel-Aal et al., 2006). Contains 60% of all minerals found in the kernel (Amrein et al., 2003). 5.1–5.4% phytate and 47.9–53.4% fiber (Amrein et al., 2003; Bacic and Stone, 1981). Thiamin is confined to this layer (Ball, 2006). Lowest concentration of uronic acid (Parker et al., 2005). 80% of niacin, 60% of pyridoxine, and 60% of the total minerals found in the kernel (Bacic and Stone, 1981; Stewart and Slavin, 2009).	Each cell is 40–50 µm diameter (Martelli et al., 2010), and up to 55 X 100 µm (Barron et al., 2007; Martelli et al., 2010). Cubic cells with 6–8 µm thick cell walls (T. Evers and Millar, 2002; Stevens et al., 1988). 50% of the bran (Anson et al., 2012; Bacic and Stone, 1981) 6.7% (volume) of the wheat kernel (T. Evers and Millar, 2002). One of the two remaining cells from grain development, present within 8 days post-anthesis (Morrison, 1975). Contains high levels of fiber (Moore et al., 2005).	AX matrix greatly affects the rate of water adsorption in the aleurone (Lee and Stenvert, 1973) (not reported in reference to the kernel). The composition (protein, etc.) causes this layer to restrict water absorption into the endosperm (Hook et al., 1982). Weakest portion is at the junction of two cells (Amrein et al., 2003). Phenolic acids and beta glucans are higher in the aleurone layer (Antoine et al., 2003; Antoine et al., 2004). Contains no peroxidase, polyphenol oxidase, or oxalate oxidase (Jerkovic et al., 2010).
Cross cells	From the carpel wall (Parker et al., 2005)	Yes	Starch is present only in the early	High concentration of dehydro-	Considered a fruit coat.	Thought to not autolyse in

			days post-anthesis, where heterodistributed amyloplasts have starch deposits (Morrison, 1976).	diferulates (Antoine et al., 2003; Parker et al., 2005). Highest concentration of uronic acid (Parker et al., 2005). Contains significant quantities (in comparison to the other layers) of vanillin, vanillic acid, *p*-hydro-xybenzoic acid, and *p*-coumaric acid (Parker et al., 2005).	Supported to the cuticle of the testa (T. Evers and Millar, 2002). A complete cell layer around the kernel, unlike the tube cells (Morrison, 1975).	maruation (Parker et al., 2005). 21 days post-anthesis, there is no starch within (Morrison, 1976). Contains chloroplasts, with active amylopast post-anthesis (Morrison, 1976).
Epidermis	From the carpel wall (Parker et al., 2005).	Yes	Mostly cellulose (Lee and Stenvert, 1973).	High concentration of dehydrodiferulates (Antoine et al., 2003; Parker et al., 2005).	Considered a fruit coat. Cells are elongated in the direction of the embryonic axis (T. Evers and Millar, 2002).	Resistant to endoxylanase treatment (Benamrouche et al., 2002).
Hypodermis	From the carpel wall (Parker et al., 2005).	No		High concentration of dehydrodiferulates (Antoine et al., 2003; Parker et al., 2005).	Considered a fruit coat. Cells are elongated in the direction of the embryonic axis (T. Evers and Millar, 2002).	Resistant to endoxylanase treatment (Benamrouche et al., 2002).
Nucellar epidermis	Present in the embryo. Derived from or near the chalaza (T. Evers and Millar, 2002).	No	Contains a high portion of the protein found in bran (Lee and Stenvert, 1973). May contain largest lipid content in bran (Lee and Stenvert, 1973). Rich in cellulose (Autio, 2001). Contains 25% of		Has pigment. Mass of this tissue is present in the crease (T. Evers and Millar, 2002). Cuticular on the outer surface (A. D. Evers and Reed, 1988; Morrison, 1975). The outer cuticle is 1–1.5 µm thick	Possibly the least permeable to water (Morrison, 1976). Source tissue degenerates by programmed cell death and compacts the tissue area in maturation (Domínguez et al., 2001).

			AX in the bran (Benamrouche et al., 2002) with high amounts of monomer ferulic acid and low amount of dimers (few crosslinks) (Barron et al., 2007).		and visible 7 days post-anthesis and-compressed within 3–4 weeks (Morrison, 1976; Ugalde and Jenner, 1990). Thinner than the testa (T. Evers and Millar, 2002).	Could not be studied by synchrontron FT-IR analysis due to insufficient spatial resolution (Jamme et al., 2008).
Testa	From the rough protective layer of the carpel and the chalaza (T. Evers and Millar, 2002).	No	Contains 2% of AX in the bran (Benamrouche et al., 2002). Rich in lignin (Landberg et al., 2008)	Alkylresorcinols are specific to this layer (Landberg et al., 2008).	Has pigment; structure discontinues in the crease (T. Evers and Millar, 2002). Hypothesized to be the thinnest mature layer: 5–8 µm (Barron et al., 2007; Moss, 1977); however, has been reported as thicker than the nucellar epidermis (T. Evers and Millar, 2002). Two layers: a pigment layer and a waxy cuticular layer. They are flattened together from the expanding endosperm in maturation (A. D. Evers and Reed, 1988) The inner most pericarp tissue (A. D. Evers and Reed, 1988)	Hydrophobic tissue (Landberg et al., 2008); possibly the least permeable to water (T. Evers and Millar, 2002; Hinton, 1955). Strong correlation is found between the degree of red pigment found in this portion and a resistance to preharvest sprouting (T. Evers and Millar, 2002). Takes 1 hour for water to penetrate this layer (Hook et al., 1982).

				Cuticular on the outer surface (A. D. Evers and Reed, 1988; Morrison, 1975)	
Tube cells	From the carpel wall (Parker et al., 2005).	Yes	Free of starch prior to maturation, just before the cross cells are free of starch deposits (Morrison, 1976).	Considered a fruit coat; with 180 µm cells at the longest diameter (Morrison, 1976). Fragmentary nature; discontinuously lying on the testa (T. Evers and Millar, 2002; Morrison, 1975).	Contains chloroplasts, with active amylopast post-anthesis (Morrison, 1976).

*The bran layers include: the epidermis, hypodermis, cross cells, tube cells, testa, nucellar epidermis, and aleurone. Data include durum, spring, and winter wheats.

Table 1.3. Major constituents of wheat bran produced by commercial flour milling by percentage.

Constituent	Percentage	Reference
Nonstarch polysaccharides (26% AX)	41–60	(Amrein et al., 2003)
Starch	10–20	(Maes and Delcour, 2001)
Protein	15–20	(Maes and Delcour, 2001)

Table 1.4. Roller milled sample derivations of tocopherols, adapted from Engelsen and Hansen (2009).

All numerical values are reported as μg/g.

Roller milled	α- T*	β- T*	α- T3*	β- T3*	α- TE**	Total tocols
Coarse bran	5.0±1.1	3.3±0.4	6.5±0.1	25.3±1.3	0.010	40.1
Fine bran	29.5±1.8	12.8±0.1	9.5±0.7	31.0±0.4	0.440	82.8
Germ	195.2±2.4	71.3±0.6	2.8±0.7	17.2±0.9	0.232	286.5
Wheat flour	7.8±0.1	4.6±0.1	3.5±0.1	21.9±0.2	0.012	37.8

*values determined for α-T, β-T, α-T3, and β-T3 are α-tocopherol, β-tocopherol, α-tocotrienol, and β-tocotrienol, respectively.

** where α-TE = [(α-T × 1.0) + (β-T × 0.5) + (α-T3 × 0.3) + (β-T3 × 0.05)]

Table 1.5. Phenolic acid extractions in publications.

Group*	Multi extraction	Sample size	Minimum time to complete method	Extraction solvents	Approx. centrifuge speed	Stir speed	Filter	Nitrogen gas	Dissolve solvent	Special requirements
Alvarez-Jubete (Alvarez-Jubete et al., 2010)	No	1.25 g	1 hour	Methanol	2,000 rpm	12000 rpm	Yes	No	None	Homogenize sample before extraction.
Beta (Beta et al., 1999; Hirawan et al., 2010; Li, Pickard, and Beta, 2007a; Li, Pickard, and Beta, 2007b)	No	Raw material – 200 mg or 1 g Food product – 4 g	2-4 hours	100% methanol 36.5–38% methanol:hydrochloric acid (99:1, v/v)	10,000 rpm at 5°C 7,800 *g* (Hirawan et al., 2010; Li, Pickard, and Beta, 2007a) 3,000 rpm (Beta et al., 2005; Menga et al., 2009)	300 rpm	No	No	None	Use rotary shaker during extraction.
Chibbar (Verma et al., 2008; Verma et al., 2009)	Yes	1 g	5 hours	Chilled ethanol/ water (80:20) Water 2N sodium hydroxide 2N hydrochloric acid Ethyl acetate Hexane Ethanol/water	2500 *g* 1000 *g*	2000 rpm	No	Continuous (for bound)	Water Methanol	Temper starting material to 15% moisture. Method from previous publication (Adom et al., 2003), with use of chilled solvent and room temperature extractions.

Fulcher (Gélinas and McKinnon, 2006; McKeehen et al., 1999; Sen et al., 1991)	Yes	200 mg (dw)	4 hours	Water Ice-cold 6N hydrochloric acid 4N sodium hydroxide Ethyl acetate	3000 *g*	Wrist-action shaker, speed not specified	No	Yes	None	Use of a separatory funnel. Pellet is diluted with distilled H_2O, vortexed, centrifuged, and all supernatants pooled.
Hartley (Hartley and Morrison III, 1991; Parker et al., 2005)	No	20 mg	Over 24 hours	0.1molL^{-1} sodium hydroxide (oxygen-free) 50% methanol 12 molL^{-1} hydrochloric acid Ethyl acetate	None	None	Yes	Yes	None	Repeated extraction with pooled supernatants.
Krygier (Krygier et al., 1982)	Yes	1 g	6 hours	Hexane Sodium hydroxide Ether-ethyl acetate 70% methanol-70% acetone (1:1) 6N Hydrochloric acid Diethyl ether-ethyl acetate (1:1)	Not specified	Yes	No	No	None	Some extracts are defatted again with hexane
Liu method A (Adom et al., 2005)	No	1 g	2 hours	Hexane 2M hydrochloric acid 2M sodium hydroxide Ethyl acetate	None	None	No	Continuous	Methanol	Room temperature extraction, in which samples are extracted five times.
Liu method B (Adom and Liu, 2002; Adom et al., 2003; Adom et al., 2005; Butsat et al., 2009; Okarter et al., 2010)	Yes	1 g	3 hours	Hexane Ethanol/water 2M hydrochloric acid 2M sodium hydroxide Ethyl acetate	None	None	No	Continuous	Methanol	Room temperature extraction, in which samples are extracted five times.

Onyeneho (Onyeneho and Hettiarachchy, 1992)	No	100 g	2 hours	Petroleum ether 95% ethyl alcohol	None	None	Yes	No	None	Air dry, vacuum distill, defat with petroleum ether, autoclave, and freeze dry
Matilla (Anson et al., 2009; Mattila and Kumpulainen, 2002; Mattila et al., 2005)	no	0.1–0.5 g	20 hours	Cold diethyl ether and ethyl acetate (1:1) Methanol containing 2 g/L butylated hydroxyanisole (BHA) Concentrated and 10% acetic acid (85:15) hydrochloric acid	None	Not specified	Yes	Not specified – air drying	Water	Ultrasonication using cold solvent.
Tsao (Kim et al., 2006)	Yes	200 g	9 hours	Hexane/water (4:1) Methanol/water (80:20) Acidified water (pH 2 with hydrochloric acid) Ethyl ether 2M sodium hydroxide 6M hydrochloric acid	None	None	Whatman no. 1	No	Methanol	Defat sample. Use of rotary evaporation.
Yu (Yu et al., 2002; Yu et al., 2003; Zhou and Yu, 2004; Zhou et al., 2004)	No	2–4 g	2–3 hours – with Soxhlet 15 hours – not under pressure	Absolute ethanol 50% acetone (v/v) 70% ethanol (v/v) 70% methanol (v/v)	None	None	No	Under nitrogen	Dimethyl sulfoxide (or) benzene	Soxhlet extraction. Use of rotary evaporation. Extract sample in darkness. Vacuum removal of excess solvent.
Zhu (Kim et al., 2011; Zhu et al., 2010)	No	5 g	6 hours	Hexane Water 30% ethanol/water (v/v) 50% ethanol/water (v/v) 70% ethanol/water (v/v) 100% ethanol	10,000 rpm at 4°C	Gentile	No	No	None	Defat sample. Concentrate under vacuum at 50°C and freeze-dry.

*If the principal investigator could not be determined, the first author's name was used to title the method.

Chapter 2 - Antioxidant contribution within fractions and mill streams produced from the same wheat kernels

Abstract

Mature wheat kernels contain three main parts: endosperm, bran, and germ. Flour milling results in multiple streams that are chemically different; however, distribution of antioxidants and phenolic compounds in each stream has not been well documented. In this study, antioxidant activities of each mill stream were evaluated employing diphenylpicrylhydrazyl (DPPH) radical-scavenging activity, ferric reducing/antioxidant power (FRAP), and total antioxidant capacity assays and determined phenolic compounds (total, flavonoid, and anthocyanin contents) in each fraction. Samples included the different parts of wheat (bran, flour, and germ) and wheat milling streams (mill feed, red dog, shorts, and whole ground wheat). Significant differences were observed in phenolic concentrations between fractions of bran, flour, and germ milled from the same kernels. Post analysis of multiple antioxidant techniques, germ accounted for the majority of antioxidant properties, whereas bran contained a substantial portion of phenolic compounds and anthocyanins. Mill feed was comparatively high in phenolic acids (0.78 mg FAE/g), total antioxidant capacity (1.28 mg/g), and antioxidant activity (75.21% DPPH inhibition and 278.97 µmol $FeSO_4$/g). The investigated mill streams could provide avenues for future human consumption of traditional by-products from flour milling.

Introduction

Wheat (*Triticum aestivum L.*) is a staple of the Western diet. Wheat originated in the Fertile Crescent and is cultivated worldwide due to its ease of growth, diverse uses, and long-term storage stability (Chick, 1958). A wheat kernel is composed of approximately 85%

endosperm, 13% bran, and 2% germ (Sugden, 2001). Kernel endosperm is used as flour, whereas kernel germ contains high concentrations of lipids that are extracted for oil or processed as higher value feed components (Zhu et al., 2010). Americans consume 36% of the domestic wheat crop, 50% is exported, and 10% and 4% go to livestock and seeds, respectively (Western Organization of Resource Councils (WORC)., 2002). The annual total global production of wheat is 600–700 million tonnes (Shewry et al., 2012).

Milling divides bran from endosperm. To optimize flour yield, millers aim to produce large bran pieces (bigger than 2000 μm) and a minimum amount of shorts (broken bran pieces) (Sugden, 2001). Red dog (high bran flour) (Martin et al., 2007) and mill feed (low quality, variable, highly contaminated bran mixture) (Sugden, 2001) are produced during milling and sold as feed products. Due to differing compositions and functions of bran, germ, and endosperm from the kernel, all fractions in milling are chemically different (Wetzel et al., 2010).

Antioxidants are found in high concentration, comparative to several staples in the Western diet (Qu et al., 2005). [**Appendix B** list the chemical structures of all antioxidants measured within this dissertation.] Prior investigations have shown that bran and germ serve as possible sources for antioxidants with potential health benefits (Liyana-Pathirana and Shahidi, 2006). Phenolic compounds are the main antioxidant components found in wheat (Mattila et al., 2005; Parker et al., 2005; Verma et al., 2008; Jonnala et al., 2010), and ferulic acid is the dominant phenolic acid in hard red winter wheat (Manach et al., 2004; Klepacka and Fornal, 2006; Verma et al., 2008). The ferulic acid content of wheat is 0.8–2 g/kg dry weight (Lempereur et al., 1997) and is always higher in bran than in flour from the same kernel by 10–20 times (Klepacka and Fornal, 2006). Phenolic compounds are predominantly found in the outer layers of wheat bran as a structural component of the cell wall and provide protection from natural

elements, pathogenic organisms, and ultraviolet rays (Mattila et al., 2005; Parker et al., 2005; Stalikas, 2007). Measurable phytochemical content is affected by environment, farming, mutation, and processing (Yu et al., 2003; Gélinas and McKinnon, 2006; Verma et al., 2008). Present in free and bound forms, many wheat bran antioxidants cannot be digested in the human gastrointestinal tract in their complex (bound) form (Anson et al., 2009; Menga et al., 2009; Anson et al., 2011). The majority of phenolic compounds are bound in ester linkages to abundant polysaccharides in the cell wall and are therefore unavailable for digestion (Stalikas, 2007). Bound components in bran can be released by different processing techniques (Thanh and Nout, 2002; Stalikas, 2007; Anson et al., 2009). Wheat bran also contains lignin, a complex organic polymer that fills spaces in the cell wall between cellulose and hemicellulose, and lignan, a bound antioxidant in wheat bran that is 2.6–3% of total bran (Robertson and Eastwood, 1981; National Research Council (US). Subcommittee on Feed Composition, 1982). Lignan can be extracted with methanol, and when derived from wheat bran is involved in antitumor activities in colon cancer SW480 cells (Qu et al., 2005).

Many studies have evaluated the variability of nutrients in wheat cultivars (Gutierrez-Alamo et al., 2008; Rakszegi et al., 2008; Verma et al., 2008; Lv et al., 2012), but antioxidants and phenolic compounds distributed throughout the mill stream have not been well documented. Previous studies investigated antioxidative properties of bran and germ together as one sample (Adom et al., 2003; Adom et al., 2005), antioxidative properties of bran, flour, and grain (Vaher et al., 2010) or antioxidative properties of select output streams (Liyana-Pathirana and Shahidi, 2006; Liyana-Pathirana and Shahidi, 2007). It was theorized that differences in antioxidant properties would be observed between mill fractions. In this study, components of the wheat kernel were separated into different fractions and mill streams produced by dry-milling, and

subsequently analyzed for concentration of phenolic compounds and antioxidant activity in each fraction to determine their variation.

Materials and methods

Wheat and milled wheat fractions

Kansas hard red winter wheat (Likes et al., 2007) from the 2010 crop year, tempered to 16% moisture, was milled (70% extraction) in the Kansas State University (KSU) Shellenberger Hall industrial-scale milling system (Manhattan, KS). The milling system has been previously described (Pussayanawin et al., 1988; Mahroof et al., 2003; Likes et al., 2007). Bran, flour, germ, mill feed, red dog, and shorts were collected from one mill run in the industrial milling system by adjusting mill settings for optimum percentage of the desired fraction. In addition, whole wheat kernels that were collected from the mill run after cleaning were ground on a Burr Mill (Falling Number grinder, Perten Instruments, Springfield, IL) to have less than 3% overs on a 150 µm screen to represent a whole ground wheat sample. Photographs of each fraction were taken for reference, and each wheat sample is depicted in **Figure 2.1**. Prior to any chemical assay, all samples were ground to pass through a 150 µm screen. All chemicals, reagents, and standards were ACS grade. Ascorbic and phenolic acid standards were purchased from Sigma-Aldrich, Inc. (St. Louis, MO).

Soluble and bound phenolic acids

A two-part extraction for soluble and bound phenolic acids was performed for each sample as previously described (Adom et al., 2003). Each sample (1.000 g) was extracted for 10 min with 10 ml 80% methanol (v/v) at 25 °C under constant stirring. After 10 min, the extract was removed and the extraction procedure was repeated twice on the residual pellet. Extracts were pooled and evaporated under continuous nitrogen gas. Each extracted sample was

lyophilized and weights recorded prior to dissolving in 5 ml methanol. Determination of bound phenolic acids was conducted as previously described (Adom et al., 2005). The above pellet was hydrolyzed with 2 M sodium hydroxide at 25 °C for 1 h under continuous nitrogen gas. The extraction was neutralized with 2 M hydrochloric acid and extracted with pure hexane. After hexane removal, the hydrolysis was extracted five times with ethyl acetate. Ethyl acetate extracts were pooled together and evaporated to dryness under continuous nitrogen gas. Dried extracts were dissolved in 10 ml methanol and stored at -20°C until use. Determination of total phenolic content (TPC) in each fraction was conducted as previously described (Dewanto et al., 2002). The reduction of Folin−Ciocalteu reagent in the presence of phenolates was measured spectrometrically on a Perkin-Elmer Lambda 800 UV–Vis spectrophotometer (Perkin-Elmer, Inc., Waltham, MA). Using ferulic acid as the standard, TPC was expressed as ferulic acid equivalents (FAE) per gram of mill fraction. A ferulic acid standard solution or extract sample (125 µl) was added to 0.5 ml deionized water and 125 µl Folin−Ciocalteu reagent in a test tube and vortex-mixed. Samples were allowed to stand for 6 min. Subsequently, 1.25 ml 7% sodium carbonate and deionized water were added to adjust final volume to 3 ml. After 90 min at 25 °C, absorbance was measured at 760 nm against the blank and compared with the known standards for quantification.

Phytochemical extraction

Phytochemicals were extracted from each sample as previously described (Hentschel et al., 2002; Adom et al., 2005). Each sample (600 mg) was weighed with 60 mg magnesium carbonate in a loosely closed screw-capped test tube. Solids were blended prior to a rapid extraction with 2 ml 1:1 (v/v) methanol/tetrahydrofuran mixture in a water bath at 75 °C for 5 min. Extracts were cooled and centrifuged at 2,500 g for 5 min, and the organic phase was

removed. The pellet was extracted two additional times with methanol/tetrahydrofuran (2 ml) in a water bath at 75 °C for 5 min then cooled and centrifuged at 2,500 g for 5 min. Pooled organic phases were dried with 1 g anhydrous sodium sulfate and evaporated under continuous nitrogen gas at 35 °C. Residues were dissolved in 1 ml methanol/tetrahydrofuran, stored at -20 °C, and analyzed within 2 weeks.

Total flavonoid content

Determination of total flavonoid content in each fraction was conducted as previously described (Liu et al., 2002). Extracts from the phytochemical extraction (0.25 ml) were mixed with 1.25 ml distilled water in a test tube. After, 75 µl of 5% sodium nitrite solution was added, test tubes were held at 25 °C for 6 min., 150 µl 10% aluminum chloride was added in each test tube, and test tubes were held at 25 °C for 5 min. Subsequently, 0.5 ml 1 M sodium hydroxide was added and solutions were brought up to 2.5 ml with distilled water, then mixed. Samples were immediately measured against a blank at 510 nm on a spectrophotometer. Flavonoid content was calculated as microgram of catechin equivalent (CE) per gram of sample (µg/g CE) against a standard curve of catechin (Liu et al., 2002).

Total anthocyanin content

Extraction and determination of anthocyanin content in each milling fraction were conducted as previously described (Abdel-Aal and Hucl, 1999; Abdel-Aal and Hucl, 2003). Samples (3.000 g each) were extracted twice by turbulent-mixing with 24 ml acidified methanol [1 N hydrochloric acid (85:15, v/v)] for 30 min. Apparent pH was adjusted to 1.0 before timing and rechecked after 15 and 30 min of extraction. Extracts were centrifuged at 21,000 g (4 °C) for 20 min and refrigerated for 2 d to precipitate. Again, extracts were centrifuged 21,000 g (4 °C) for 20 min and concentrated to 2 ml under continuous nitrogen. For total anthocyanin content

determination, the concentrated supernatant was poured into a 50-ml volumetric flask and made up to volume with acidified methanol. Absorbance was measured on a spectrophotometer at 535 nm, and anthocyanin content calculated as micrograms per gram of the sample according to the original method (Abdel-Aal and Hucl, 1999).

Total antioxidant capacity

Determination of total antioxidant capacity in each fraction was conducted as previously reported (Adom and Liu, 2002). In one test tube, 0.3 ml from the soluble/bound phenolic extraction and 3 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) were incubated at 95 °C for 90 min. After the mixtures cooled to 25 °C, absorbance of the solutions were read at 695 nm against a blank on spectrophotometer and calculated against a reference of the total antioxidant capacity of ascorbic acid. Total antioxidant capacity is expressed as milligram equivalents to ascorbic acid per gram wheat fraction.

Diphenylpicrylhydrazyl (DPPH) assay

Determination of DPPH radical absorbance in each fraction was conducted as previously reported (Yu et al., 2002). DPPH reagent (Liyana-Pathirana and Shahidi, 2006) was prepared the day of analysis. The reagent was composed of DPPH (0.004%) in methanol. In each test tube, 1.9 ml DPPH reagent and 100 µl extract from the soluble/bound phenolic extraction were mixed, and tubes were kept in a dark room to react. After 30 min, absorbance was tested at 517 nm on a spectrophotometer. IC_{50} value was used to calculate DPPH value and was defined as the concentration of the sample necessary to have 50% inhibition as determined with interpolated linear regression (Qingming et al., 2010), where a lower IC_{50} value was associated with a higher radical scavenging activity. All DPPH values are reported as '% inhibition.'

Ferric ion reducing antioxidant power (FRAP) assay

Determination of FRAP for each extract was conducted as previously reported (Yu et al., 2003). FRAP reagent was prepared the day of analysis and kept in a water bath at 37 °C when not in use, up to 3 h. Detailed preparation of FRAP reagent has been previously reported (Benzie and Strain, 1999). In short, acetate buffer 300 mM pH 3.6, was added to 2, 4, 6-tripyridyl- s-triazine (10 mM in 40mM HCl) and $FeCl_3$ $6H_2O$ (20 mM) in a ratio of 10:1:1 and called the FRAP reagent. To determine FRAP of each sample, 1.8 ml FRAP reagent, 300 µl extract from the soluble/bound phenolic extraction, and 180 µl distilled water were combined in one test tube and incubated at 37 °C for 4 min. Absorbance was measured at 593 nm on a spectrophotometer and reported in micromole ferrous sulfate ($FeSO_4$) per gram defatted material.

Statistical analysis

All tests were performed in triplicate, unless otherwise noted. Means and standard deviations were calculated for all analyses. Significance of differences between groups were compared using column analysis of one-way ANOVA with Tukey's post hoc test at a significance level of α: 0.05 (GraphPad, GraphPad Software Inc, La Jolla, CA). P values (two-tailed) of less than 0.05 were considered to be a sign of statistical significance. N is listed where n equals the number of replicate assays. Subreplicates ranged (2-3) per assay.

Results

TPC of wheat fractions

Extraction yield of soluble phenolic acids refers to free and conjugated phenolic acids extracted with 80% methanol, whereas that of bound phenolic acids refers to alkaline-hydrolyzed extract; with all TPC results provided in **Table 2.1**. The highest soluble TPC concentration was observed in germ extract (2.91 mg FAE/g of defatted material), and the lowest was determined in

flour extract (0.13 mg FAE/g of defatted material). The order of TPC for soluble extracts was: germ > whole ground wheat > mill feed > bran > red dog> shorts> flour. Similarly, bound extract TPC of wheat fractions was highest in germ (0.92 mg FAE/g of defatted material), and the lowest TPC was found in flour (0.07 mg FAE/g of defatted material). The order of TPC for bound extracts was: germ > mill feed > whole ground wheat > bran > red dog > shorts > flour.

Flavonoid concentration in wheat fractions

Total flavonoid content is reported in µg/g of extracted material for each wheat fraction in **Table 2.2**. Mill feed had a significantly higher concentration of flavonoid compounds (360.68 µg/g CE), whereas flour was significantly lower (192.85 µg/g CE) than other samples. The measured flavonoid content for all wheat fractions was mill feed > shorts > red dog > whole ground wheat > bran > germ > flour. All samples were significantly different from all other samples.

Anthocyanin concentration in wheat fractions

Anthocyanin content is reported in µg/g of extracted material for each wheat fraction in **Table 2.2**. Bran had the highest anthocyanin content (72.70 µg/g), followed by red dog (43.7 µg/g). The measured anthocyanin content by wheat fraction was in the following order: bran > red dog > mill feed > shorts ~germ > whole ground wheat > flour. Germ and shorts anthocyanin contents did not differ significantly.

Total antioxidant capacity of wheat fractions

Bound and soluble extracts followed the same order (**Table 2.3**). On average, total antioxidant capacity in soluble extracts were at least two times higher than in bound extracts from the same material. The order of total antioxidant capacity was: germ > mill feed > bran > whole ground wheat > red dog ~ shorts> flour. Germ extracts had a significantly higher total

antioxidant capacity, whereas flour was significantly lower in both cases, comparatively to other samples.

DPPH radical scavenging activity of wheat fractions

All wheat fraction extracts showed DPPH scavenging activities in concentration measured by IC_{50} value (**Table 2.3**). As the standard, ascorbic acid was measured at 96% DPPH inhibition with this sample set. Soluble and bound germ extracts displayed significantly higher DPPH values than other fractions. The order of ability to scavenge DPPH radicals by wheat fractions was: germ > mill feed > red dog > bran > shorts > whole ground wheat > flour for soluble extracts; and, germ > mill feed > bran > shorts > whole ground wheat > red dog > flour for bound extracts. Germ, mill feed, and red dog demonstrated the highest abilities to scavenge DPPH radicals.

FRAP of wheat fractions

Antioxidant activity by FRAP assay is reported in **Table 2.3**. Mill feed and red dog had similar soluble portions; however, the remaining samples had significantly different abilities to reduce iron. Germ-soluble extracts (1006.12 μmol of $FeSO_4$/g defatted material) had significantly higher FRAP values than other fractions, whereas flour extracts had significantly lower FRAP values (3.36 μmol of $FeSO_4$/g defatted material). For wheat fraction soluble extracts, ability to reduce Fe^{3+} to Fe^{2+} was in the order of germ > red dog ~ mill feed > bran > shorts > whole ground wheat > flour. For bound extracts from different fractions of wheat, germ extracts had significantly higher FRAP values (280.62 μmol of $FeSO_4$/g defatted material) than other fractions, and extracts from flour were lowest (15.74 μmol of $FeSO_4$/g defatted material). For all samples, ability of bound extracts to reduce Fe^{3+} to Fe^{2+} was in the order of germ > mill feed > bran > shorts > whole ground wheat > red dog > flour.

Discussion

On average, and using the current method, soluble TPC was 2–5 times higher than bound TPC in all fractions, as was consistent with a previous report of other cereals (Weidner et al., 2001). Germ extract contained the largest concentration of phenolic compounds, with mill feed and whole ground wheat also containing relatively high concentrations. The germ of the kernel contains the highest portion of oils and antioxidants (Zhu et al., 2010). Mill feed and whole ground wheat both contain germ. Flour, which contains little germ, had minor detectable phenolic compounds and was found to have low levels of phenolic compounds in previous research (Adom and Liu, 2002; Adom et al., 2003).

For all samples, bound and soluble results were similar in order, with TPC results being similar to previous reports (Liyana-Pathirana and Shahidi, 2006; Vaher et al., 2010) due to use of the same type of extraction procedure; however, the current study found whole ground wheat to contain relatively higher (Liyana-Pathirana and Shahidi, 2006), and bran relatively lower (Vaher et al., 2010) phenolic compounds. The difference in values between studies may be attributed to milling techniques used, source of wheat, and variations during extraction. A Folin-Ciocalteu reagent assay is not specific to phenolic acids and can also react with sugars and peptides, and many of these compounds are soluble in aqueous solutions (Singleton et al., 1999). It is suspected that these compounds partly account for the soluble extract quantities and would vary between materials. The bound extraction used in this experiment utilized a low temperature and a relatively short extraction time, which may be incomplete. Previous studies have also suspected that this method could leave additional bound phenolic compounds within the extracted material (Adom et al., 2003). Dominant phenolic compounds in hard wheat were individually identified in

previous research performed at KSU (Jonnala et al., 2010); therefore, clarification of total antioxidant distributions throughout milling fractions was the focus of the current experiments, and individual phenolic compounds were not calculated and reported in this experiment. Phenolic compounds may contribute directly to antioxidant action (Awika et al., 2003); consequently, it is necessary to investigate TPC when measuring antioxidant properties.

Total flavonoid content reflects the available polyphenol population in the sample and measures several C6-C3-C6 compounds. Extract concentrations were measured with respect to catechin, a major plant secondary metabolite, and the current results were similar to previous research on the wheat kernel (Feng and McDonald, 1989). Mixed mill stream samples (whole ground wheat, shorts, mill feed, red dog) were high in flavonoid concentrations compared with separated fractions (bran, flour, germ). Flavonoids are a type of phenolic compound that provide pigment, germ flavonoids are highly concentrated, and flour flavonoids are low in concentration and dispersed in red hard winter wheat, most likely as phlobaphene or proanthocyanidin, which are derivatives of catechin-tannin (Miyamoto and Everson, 1958). Only flavones and flavonols are well resolved in assays using aluminum chloride (Martos et al., 1997), and including TPC in this study (which utilizes a different extraction) accounted for all flavonoid compounds (including flavanones and flavanonols) that many not have been measured in the total flavonoid content assay. Flavonoid differentiation is made by number and nature of substituent groups attached to the rings, which are accurately measured, individually with mass spectrometry (Cavaliere et al., 2005).

Some components (germ and mill feed) were high in flavonoid content, but not in chemical compounds determined by the total anthocyanin assay, which means that the 3-phenyl-1,4-benzopyrone structures in these milling fraction extracts were present to represent phenol

groups, but were distinct by degree of unsaturation and oxidation. Anthocyanin concentrations differed from flavonoid concentrations in whole ground wheat and mill feed for the same reason. Anthocyanins are flavonoids that are concentrated in the outside layers of the kernel (Abdel-Aal and Hucl, 1999; Abdel-Aal and Hucl, 2003; Abdel-Aal et al., 2006) and synthesized through a flavonoid biosynthetic pathway (Warner et al., 2000). As anthocyanidin derivatives, these flavonoids are differentiated by R- groups on the isoflavan structure; therefore, total anthocyanin concentration reflects the polyphenol population in a sample as the portion of anthocyanidins with glucosidic compounds (Cavaliere et al., 2005). When measuring anthocyanins, their color is dependent on pH flavylium to hemiketal transformations within the chemical structure; therefore, pH assays are reliable in measuring anthocyanins (Giusti and Wrolstad, 2001; Al Farsi et al., 2005). As observed in the current analysis, anthocyanins were more concentrated in bran containing fractions with high distributions of fine particles.

Antioxidants are distinguished as multiple compounds (Lachman et al., 2012). No single antioxidant measurement can express ability, activity, and capacity of antioxidants present in solution. Antioxidants have the ability to act *in vivo* via multiple mechanisms (Pellegrini et al., 2003); consequently, multiple methods were chosen to investigate antioxidant distribution within milling fractions. Mill feed and whole ground wheat had approximately the same TPC, but different soluble extract contributions to total antioxidant capacity. Total antioxidant capacity quantifies by cumulative capacity to scavenge free radicals (Pellegrini et al., 2003). In flour milling, bran and germ usually are collected and flattened in the same fraction; however when separated, significant differences were observed in the antioxidant capacity of bran and germ extracts. The concentration or ratio of each fraction could affect the antioxidant quantities in mill streams operated under differing processing conditions.

The ability to act as donors of hydrogen atoms in DPPH radical transformation to a reduced form proved to be distinctive when measuring antioxidants within mill streams. DPPH assay measures single electron transfer to determine antioxidant reducing capacity (Huang et al., 2005). Although the seven samples analyzed in the current study are composed of different ratios of the three components of the kernel, each mill stream had significantly different values. When using percent inhibition, higher inhibition demonstrations stronger antioxidant activity (Butsat et al., 2009; Butsat and Siriamornpun, 2010), as measured in all germ fractions. Bran, shorts, and red dog had no significant difference in total antioxidant capacity, but differed when measuring DPPH inhibition. Differences may be due to the DPPH assay reaction depending on the structural conformation of the antioxidants being examined (Mensor et al., 2001; Mielnik et al., 2003). It is assumed that structural changes may have occurred with grinding/processing to time of analysis. Additionally, the chemical composition of bran, endosperm and germ are known to differ (Wetzel, 2009), which includes micronutrients.

A previous study investigated DPPH of milling fractions (Liyana-Pathirana and Shahidi, 2006). Common fractions used in this study and the previous publication showed some differences. Shorts were lower than bran in the current study, and the two samples were always statistically different in the soluble fraction (**Table 2.3**). Differences in DPPH results from results in previous studies may be due to type of wheat and milling methods. Wheat bran yield can average 21.1–36.5% of the kernel weight (Rakszegi et al., 2008), and chemical composition can vary between cultivar (Andersson et al., 2008; Shewry et al., 2010). The DPPH radical method could reflect actual capacity of the extract in transferring electrons or hydrogen atoms (Pérez-Jiménez et al., 2008), and the current results showed distinctive DPPH inhibition for different milling streams.

Fractions containing increased fines (<150 µm) before grinding, due to endosperm concentration (flour, whole ground wheat, red dog) had significantly lower bound extract antioxidant power, as determined by FRAP assay. Increased surface area to mass may have enabled easier extraction with soluble solvents (Zhou and Yu, 2004) with reduction of all sample particle sizes to <150 µm. Red dog, shorts, and bran are all samples high in fractured bran pieces. If extractability could be swayed by particle composition and size due to exposed surface during hydrolysis, it is possible that any geometric difference between bran and bran-containing samples could enhance from particle size differences. Grinding behavior (speed, equipment, and wear) during milling greatly influences the geometry of bran pieces (Simmons and Meredith, 1979). With particle size reduction, all samples would mill differently according to composition of the sample and friction produced during grinding; therefore causing a source of variability.

Micronization of food has shown that particle size affects various aspects of structure, surface area, and functional properties of the particles (Chen et al., 2006; Chau et al., 2007; Wu et al., 2007; Hemery et al., 2011). Whole ground wheat represented the whole kernel in correct proportion and had large variation between samples in this assay. This result could be due to the heterogeneous particle size of this sample caused by processing the kernels on reduction rolls. Whole grain, in which all portions of the kernel are ground separately and recombined, compared with whole wheat, where all portions are ground together with no waste or by-product stream, can be finer in texture due to processing. Whole wheat is more heterogeneous than the milled endosperm portion of the kernel (Manthey and Schorno, 2002). Previous researchers have noted that FRAP activity correlated with TPC of brewers' spent grain extracts (McCarthy et al., 2012), but only germ, bran, and flour always displayed TPC and FRAP in the same order. All mixed milling streams varied for TPC and FRAP assays. Overall, FRAP was greatly influenced by the

physical composition of the samples. FRAP assay is a measurement of antioxidant power, and the ability to reduce Fe^{3+} to Fe^{2+} could provide positive effects *in vivo*.

Conclusions

Antioxidant activity and phenolic content varied between bran, germ, flour, and various mill streams. In addition, antioxidants did not have the same distribution pattern throughout the flour milling fractions. Germ extract was high in antioxidant ability and phenolic acids, which had the highest value in all antioxidant measurements and TPC. Bran-containing fractions were high in anthocyanins, and flour extracts had the lowest concentrations in all phenolic compounds and antioxidant responses, which concur with previous research. The use of the same wheat kernels was implemented for optimum fraction comparison and may not be representative of all hard wheat cultivar. Although mill feed showed high concentrations of antioxidant activities in the current experiment, note that milling products vary in composition when produced from different mills, kernels, and operational conditions; therefore, analysis from multiple mills and wheat cultivars should be compared in future studies. Prospective research includes utilization of by-product streams for higher profit uses and investigation of antioxidant activity deterioration post-processing in products for human consumption.

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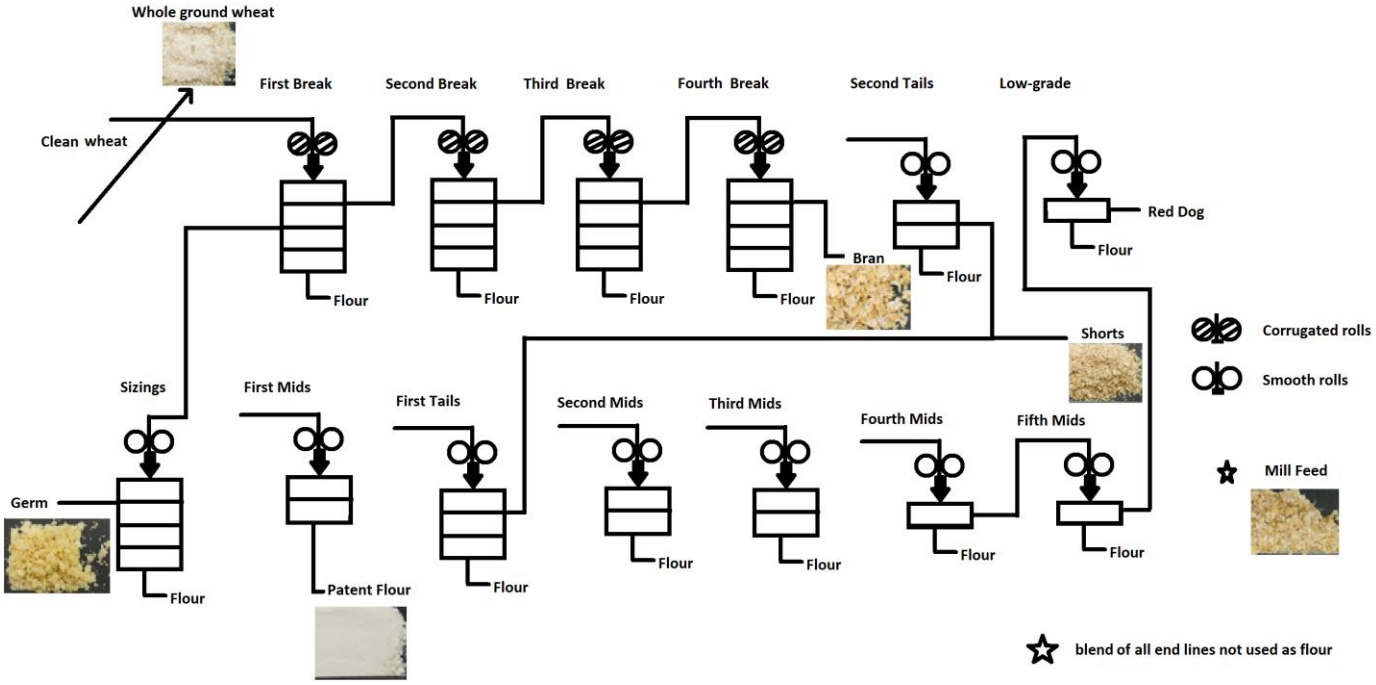

Figure 2.1. Sample description and image for wheat fractions derived from the same wheat kernels and milling operation.

Description of samples:

Bran: Clean bran from milling

Flour: Patent flour from milling

Germ: Cleaned, flatted germ pieces

Mill feed: Milling by product, collected from various steams; sent to animal feed lots

Red dog: Low grade/high ash flour from milling

Shorts: Broken bran pieces

Whole-ground wheat: Whole kernels ground on a burr mill

Table 2.1. Total phenolic acid content of wheat fractions milled from the same wheat kernels and milling operation.

Wheat fraction	Total phenolic compounds[a,b] (mg FAE/g of defatted material)	
	Soluble	Bound
Bran	0.64±0.04c	0.19±0.02a
Flour	0.13±0.01d	0.07±0.01a
Germ	2.91±0.42a	0.92±0.02a
Mill feed	0.78±0.11bc	0.31±0.08a
Red dog	0.62±0.03c	0.16±0.01a
Shorts	0.61±0.01c	0.18±0.01a
Whole ground wheat	0.90±0.07b	0.20±0.88a

[a] Column data with like letters are not significantly different (P>0.05); n=3.
[b] The total phenolic contents are expressed as ferulic acid equivalents (FAE).

Table 2.2. Flavonoid and anthocyanin concentrations in wheat fractions milled from the same wheat kernels and milling operation.

	Total contents	
Wheat fraction	Flavonoid (µg/g CE)[a,b]	Anthocyanin (µg/g)[a]
Bran	293.80±4.19d	72.7±0.8a
Flour	192.85±0.58f	1.3±0.1e
Germ	283.47±0.64e	22.4±1.0c
Mill feed	368.68±4.66a	24.1±0.5c
Red dog	321.71±2.92bc	43.7±0.8b
Shorts	327.80±1.19b	22.6±0.4c
Whole ground wheat	314.46±2.21c	4.5±0.3d

[a] Column data with like letters are not significantly different (P>0.05); n=3.

[b] Total flavonoid contents are expressed as catechin equivalent (CE).

Table 2.3. Antioxidant activities of wheat fractions milled from the same wheat kernels and milling operation.

Wheat fraction	Antioxidant activities[a]					
	Total antioxidant capacity (equivalent to ascorbic acid [mg/g])		DPPH (%Inhibition)		FRAP (µmol $FeSO_4$/g of defatted material)	
	Soluble	Bound	Soluble	Bound	Soluble	Bound
Bran	0.87±0.10c	0.29±0.04bc	52.39±0.16d	19.94±0.54c	245.79±0.25c	87.48±0.09c
Flour	0.15±0.01d	0.06±0.00d	12.85±0.32g	6.03±2.29d	3.36±0.01f	15.74±0.02g
Germ	4.66±0.12a	0.74±0.09a	88.00±0.21a	51.66±2.74a	1006.12±1.01a	280.62±0.03a
Mill feed	1.28±0.09b	0.37±0.01b	75.21±0.27b	38.39±0.67b	278.97±0.28b	231.03±0.23b
Red dog	0.80±0.01c	0.23±0.01c	64.07±0.71c	8.39±0.63d	279.18±0.28b	42.92±0.04f
Shorts	0.79±0.10c	0.28±0.06c	44.48±0.21e	18.60±0.47c	166.65±0.17d	70.86±0.07d
Whole ground wheat	0.80±0.06c	0.41±0.01b	40.39±0.39f	16.29±0.56c	125.60±2.64e	50.24±0.05e

[a] Column data with like letters are not significantly different (P>0.05); n=3.

Chapter 3 - Wheat bran particle size influence on phytochemical extractability and antioxidant properties

Abstract

Wheat bran contains many phytochemicals that are easily extractable. It is unknown if particle size plays a role in extracting health promoting compounds in wheat bran. The distribution of antioxidant and phenolic compounds with particle size reduction has not been well documented. In this study, unmilled whole bran (coarse treatment) was compared to whole bran milled to medium and fine treatments from the same wheat bran. Antioxidant properties (capacity, ability, power), phenolic compounds (phenolic acids, flavonoids, anthocyanins) and carotenoids were measured and compared. The ability of whole bran fractions of differing particle size distributions to scavenge free radicals was assessed using four *in vitro* models, namely, diphenylpicrylhydrazyl (DPPH) radical-scavenging activity, ferric reducing/antioxidant power assay, oxygen radical absorbance capacity (ORAC), and total antioxidant capacity. Significant differences phytochemical concentrations and antioxidant properties were observed between whole bran fractions of reduced particle size distribution for some assays. The coarse treatment exhibited significantly higher antioxidant properties compared to the fine treatment; except for the ORAC value, in which coarse was significantly lower. The coarse treatment was comparatively higher in phenolic acids (0.67 mg FAE/g), antioxidant capacity (0.79 mg/g), and antioxidant activities (55.29% DPPH inhibition and 165.32 µmol $FeSO_4$/g) than bran milled to the finer treatment. The fine treatment was significantly higher in flavonoid (206.74 µg catechin/g), anthocyanin (63.0 µg/g), and carotenoid contents (beta carotene, 14.25 µg/100g; zeaxanthin, 35.21 µg/100g; lutein 174.59 µg/100g) as compared to the coarse treatment. An

increase of surface area to mass increased the ORAC value by over 80%. With reduction in particle size distribution, there was a significant increase in extracted anthocyanins, bound total antioxidant capacity, carotenoids and ORAC value. Particle size does effect the extraction of phytochemicals.

Introduction

Wheat flour milling separates the endosperm from bran to produce flour (Sugden, 2001). Wheat bran is a by-product of conventional milling that contains hemicellulose, protein, cellulose, and micronutrients at relatively high concentrations (Pomeranz, 1988, Evers & Millar, 2002). The multiple layers of wheat bran are chemically composed of arabinoxylan (38%) > protein (25%) > cellulose (16%) > lignin (6.6%) (Brillouet & Mercier, 1981, Brillouet et al., 1982, DuPont & Selvendran, 1987). Bran composition from commercial flour milling contains 41-60% nonstarch polysaccharides (26% are arabinoxylans) (Amrein et al., 2003), 15-20% protein and 10-20% residual starch (Maes & Delcour, 2001).

Phytochemicals are bioactive plant compounds produced in edible plants (Okarter et al., 2010). Whole grain foods offer a wide variety of phytochemicals that are proposed to be responsible for the health benefits of whole grain consumption. Wheat bran has many health benefits and health promoting compounds such as phenolic acids, flavonoids and carotenoids (Anderson, Smith & Gustafson, 1994, Muir et al., 2004). Several subclasses exist within the numerous chemical compounds that represent the phytochemicals found in wheat (alkylresorcinols, phenolic acids, etc.). For example, anthocyanins are a type of flavonoid, while flavonoids are a type of water soluble phenol found in plants. Phytochemicals are important sources of exogenous antioxidants in the diet (Ou et al., 2002). [Several basic chemical structures of antioxidant phytochemicals found within wheat are drawn in **Appendix 2**.]

Wheat bran contains several phytochemicals that could be absorbed during digestion, yet, due to structure and transit time in the human gastrointestinal tract, are unavailable (Anson et al., 2010). After mastication, wheat bran particle size is scarcely altered, nor greatly digested prior to the large intestine. Mostly intact, wheat bran travels to the distal colon, where it is fermented (Brownlee, 2011); therefore, initial particle size is important. Based on this research, a reduction in particle size may increase the proportion of available phytochemicals in wheat bran.

The concentration and extractability phytochemicals in relation to exposed surface area is not well documented. Previous studies on bran particle size have examined bran not as whole, but fractionated to two or more parts from the same stock material (Zhang & Moore, 1997, Hassan, Alkareem & Mustafa, 2008) or in combination with germ, due to their common combination in commercial milling (Adom, Sorrells & Liu, 2005). Multiple studies have investigated bran particle size effects on digestion, noting that reduced particle size usually coincides with a decrease in total stool water (Brownlee, 2011), where only course to medium bran particle sizes are recommended for increased fecal production or rate (Brodribb & Groves, 1978). However, few studies on the bioavailability of biochemical components and effects of particle size distribution have been reported. Investigations of wheat bran antioxidant properties with reduction by ball milling has been reported using one reduced particle size dietary fiber derived from wheat bran (Zhu et al., 2010), while variation in tocopherols and tocotrienols with reduction in particle size has also been observed (Engelsen & Hansen, 2009). In addition, it is thought that processing may release bound phytochemicals from grains (Fulcher & Duke, 2002). Such research leads to questioning whether additional phytochemicals, such as antioxidants, are more extractable with a reduction in particle size. The objective of this research was to determine if particle size distribution of whole wheat bran affects phytochemical extractability and

antioxidant properties as determined by *in vitro* testing. No single measure of antioxidant concentration can express the ability, activity and capacity of antioxidants present, as the chemical diversity of antioxidants makes it difficult to separate and quantify individual antioxidants (Ou et al., 2002). Several commonly used 'total antioxidant' methods [DPPH, Ferric reducing antioxidant power (FRAP) assay, oxygen radical absorbance capacity (ORAC), and total antioxidant capacity] (Roy et al., 2010) were utilized to determine wheat bran ability to scavenge free radicals and reactive oxygen species using *in vitro* antioxidant models. A reduction in particle size may increase the proportion of available phytochemicals in wheat bran.

Materials and methods

Wheat bran samples

Kansas hard red winter wheat (Likes et al., 2007) from the 2010 crop year was conditioned to 16% moisture and milled using the Hal Ross Mill (Kansas State University, Manhattan, KS) at a 72% extraction rate (5.2% ash). The milling system used has been previously described (Likes et al., 2007); and 72% is a normal extraction for straight grade flour (Lamsal et al., 2008). All wheat bran was collected from one outlet after the purifier, during one mill run. Unmilled, whole wheat bran acted as the control and is referred to throughout as 'the coarse treatment'. The coarse treatment was collected, kept in tinted, air-tight containers and stored at 4°C for no more than six months prior to analysis. A portion of the coarse treatment was used for particle size profiling to provide a reference for processing two additional treatments and other analyses. The remainder of the coarse treatment was divided in half to make-up the two treatments. For preparation of the two treatments, a corrugated (20/22 corrugation per square inch; 2.5:1 differential) Ross experimental roller mill (serial # 915, size 9x6; Oklahoma City, OK) was employed (Experimental Milling Lab, Manhattan, KS) with an

experimental gap size, described as follows. The first treatment, defined and referred to throughout as the "fine treatment", was milled to the finest whole wheat bran particle size distribution achievable. The gap is defined as when the rolls were adjusted to just above zero gap, where the corrugated rollers were touching (as noted by sound), but not stopping the rotation of the rolls. Once the rolls were adjusted, the treatment was milled via three passes. Three passes was noted to be efficient with the set gap; and three passes were incorporated to reduce the wheat bran to the desired size, without damaging the product or equipment, and without applying too much energy/heat to the bran. Based on the particle profile, the second treatment was milled so the bran particle size was in between the particle size distribution of the coarse and fine treatments. This treatment was designated as 'the medium treatment', and later analyzed and defined by sieving (**Table 3.1**). The medium treatment was prepared by increasing the gap between the rolls slightly so that the medium treatment visually differed from the coarse and fine treatments. The medium treatment was also milled via three passes through the same Ross experimental mill. The resulting bran samples are described in **Figure 3.1**. [**Appendix C** provides the particle size reduction milling schematic.]

Chemicals

All chemicals, reagents, and standards were ACS or HPLC grade. Ascorbic and phenolic acid standards were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Carotenoid standards were obtained from DSM (DSM Nutritional Products, Boulder, CO).

Particle size determination

All whole wheat bran samples were sieved on a standard Tyler Rotap sieve shaker (W. S. Tyler, Mentor, Ohio). To determine whole wheat bran size distribution from milling, coarse treatments were sieved through 900, 750, 500, and 355 µm mesh screens. To determine whole

wheat bran size distribution from grinding, the fine treatments were sieved through 355, 200, 150, and 100 µm mesh screens. Medium treatments were sieved through 1041, 500, 355, and 240 µm mesh screens. The pan is noted as any material that passed through all sieve mesh dimensions utilized.

Soluble and bound phenolic acids

A two-part extraction for soluble and bound phenolic acids was performed for each sample as previously described (Adom, Sorrells & Liu, 2003). Each sample (1.000 g) was extracted for 10 min with 10 ml 80% methanol (v/v) at 25 °C under constant stirring. After 10 min, the extract was removed and the extraction procedure was repeated twice on the residual pellet. Extracts were pooled and evaporated under continuous nitrogen gas. Each extracted sample was lyophilized and weights recorded prior to dissolving in 5 ml methanol. Determination of bound phenolic acids was conducted as previously described (Adom, Sorrells & Liu, 2005). The above pellet was hydrolyzed with 2 M sodium hydroxide at 25 °C for 1 h under continuous nitrogen gas. The extraction was neutralized with 2 M hydrochloric acid and extracted with pure hexane. After hexane removal, the hydrolysis was extracted five times with ethyl acetate. Ethyl acetate extracts were pooled together and evaporated to dryness under continuous nitrogen gas. Dried extracts were dissolved in 10 ml methanol and stored at -20°C until use. Determination of total phenolic content (TPC) in each fraction was conducted as previously described (Dewanto et al., 2002). The reduction of Folin−Ciocalteu reagent in the presence of phenolates was measured spectrometrically on a Perkin-Elmer Lambda 800 UV–Vis spectrophotometer (Perkin-Elmer, Inc., Waltham, MA). Using ferulic acid as the standard, TPC was expressed as ferulic acid equivalents (FAE) per gram of mill fraction. A ferulic acid standard solution or extract sample (125 µl) was added to 0.5 ml deionized water and 125 µl

Folin−Ciocalteu reagent in a test tube and vortex-mixed. Samples were allowed to stand for 6 min. Subsequently, 1.25 ml 7% sodium carbonate and deionized water were added to adjust final volume to 3 ml. After 90 min at 25 °C, absorbance was measured at 760 nm against the blank and compared with the known standards for quantification. [**Appendix D** lists instructions to perform all antioxidant property assays utilized, as written in the author's lab notebook, as well as the theorized mechanism of each test.]

Phytochemical extraction

Phytochemicals were extracted from each sample as previously described (Hentschel et al., 2002, Adom, Sorrells & Liu, 2005). Each sample (600 mg) was weighed with 60 mg magnesium carbonate in a loosely closed screw-capped test tube. Solids were blended prior to a rapid extraction with 2 ml 1:1 (v/v) methanol/tetrahydrofuran mixture in a water bath at 75 °C for 5 min. Extracts were cooled and centrifuged at 2,500 g for 5 min, and the organic phase was removed. The pellet was extracted two additional times with methanol/tetrahydrofuran (2 ml) in a water bath at 75 °C for 5 min then cooled and centrifuged at 2,500 g for 5 min. Pooled organic phases were dried with 1 g anhydrous sodium sulfate and evaporated under continuous nitrogen gas at 35 °C. Residues were dissolved in 1 ml methanol/tetrahydrofuran, stored at -20 °C, and analyzed within 2 weeks.

Total flavonoid content

Determination of total flavonoid content in each fraction was conducted as previously described (Liu et al., 2002). Extracts from the phytochemical extraction (0.25 ml) were mixed with 1.25 ml distilled water in a test tube. After, 75 µl of 5% sodium nitrite solution was added, test tubes were held at 25 °C for 6 min., 150 µl 10% aluminum chloride was added in each test tube, and test tubes were held at 25 °C for 5 min. Subsequently, 0.5 ml 1 M sodium hydroxide

was added and solutions were brought up to 2.5 ml with distilled water, then mixed. Samples were immediately measured against a blank at 510 nm on a spectrophotometer. Flavonoid content was calculated as microgram of catechin equivalent (CE) per gram of sample (µg/g CE) against a standard curve of catechin (Liu et al., 2002).

Total anthocyanin content

Extraction and determination of anthocyanin content in each milling fraction were conducted as previously described (Abdel-Aal & Hucl, 1999, Abdel-Aal & Hucl, 2003). Samples (3.000 g each) were extracted twice by turbulent-mixing with 24 ml acidified methanol [1 N hydrochloric acid (85:15, v/v)] for 30 min. Apparent pH was adjusted to 1.0 before timing and rechecked after 15 and 30 min of extraction. Extracts were centrifuged at 21,000 g (4 °C) for 20 min and refrigerated for 2 d to precipitate. Again, extracts were centrifuged 21,000 g (4 °C) for 20 min and concentrated to 2 ml under continuous nitrogen. For total anthocyanin content determination, the concentrated supernatant was poured into a 50-ml volumetric flask and made up to volume with acidified methanol. Absorbance was measured on a spectrophotometer at 535 nm, and anthocyanin content calculated as micrograms per gram of the sample according to the original method (Abdel-Aal & Hucl, 1999).

Diphenylpicrylhydrazyl (DPPH) assay

Determination of DPPH radical absorbance in each fraction was conducted as previously reported (Yu et al., 2002). DPPH reagent (Liyana-Pathirana & Shahidi, 2006) was prepared the day of analysis. The reagent was composed of DPPH (0.004%) in methanol. In each test tube, 1.9 ml DPPH reagent and 100 µl extract from the soluble/bound phenolic extraction were mixed, and tubes were kept in a dark room to react. After 30 min, absorbance was tested at 517 nm on a spectrophotometer. IC_{50} value was used to calculate DPPH value and was defined as the

concentration of the sample necessary to have 50% inhibition as determined with interpolated linear regression (Qingming et al., 2010), where a lower IC_{50} value was associated with a higher radical scavenging activity. All DPPH values are reported as '% inhibition.'

Ferric ion reducing antioxidant power (FRAP) assay

Determination of FRAP for each extract was conducted as previously reported (Yu et al., 2003). FRAP reagent was prepared the day of analysis and kept in a water bath at 37 °C when not in use, up to 3 h. Detailed preparation of FRAP reagent has been previously reported (Benzie & Strain, 1999). In short, acetate buffer 300 mM pH 3.6, was added to 2, 4, 6-tripyridyl- s-triazine (10 mM in 40mM HCl) and $FeCl_3\ 6H_2O$ (20 mM) in a ratio of 10:1:1 and called the FRAP reagent. To determine FRAP of each sample, 1.8 ml FRAP reagent, 300 µl extract from the soluble/bound phenolic extraction, and 180 µl distilled water were combined in one test tube and incubated at 37 °C for 4 min. Absorbance was measured at 593 nm on a spectrophotometer and reported in micromole ferrous sulfate ($FeSO_4$) per gram defatted material.

Oxygen radical absorbance capacity (ORAC)

Determination of ORAC value for each extract was conducted as previously reported (Ou et al., 2002), with modification to the extraction time (increased to 1 h) and stirring equipment. Each whole bran sample (500 mg) was added to 20 ml hexane:dichloromethane (1:1) to extract lipophilic antioxidant constituents. Mixtures were stirred with magnetic stirring bars to turbulence at 25 °C for 1 h, under nitrogen gas flush. Extracts were removed and evaporated at 25 °C under nitrogen gas flush to dryness, and stored at -20°C until analysis. Lipophilic extracts were solubilized with 1 ml methanol prior to analysis. To extract hydrophilic antioxidant constituents, bran previously extracted for lipophilic compounds was mixed to turbulence at 25 °C for 1 h with acetone:water (70:30), under nitrogen gas flush. Hydrophilic antioxidant samples

were centrifuged at 12,100 g for 15 min, and stored at -20°C until analysis. Both extracts (hydrophilic and lipophilic) were analyzed according to a previously reported method utilizing a Synergy 2 microplate reader equipped with Gen5TM data analysis software (Biotek Instruments Inc., Winooski, VT, USA), and reported as Trolox equivalients (TE) micromole per gram of extract (Ou et al., 2002, Awika et al., 2003b).

Total antioxidant capacity

Determination of total antioxidant capacity in each fraction was conducted as previously reported (Adom & Liu, 2002). In one test tube, 0.3 ml from the soluble/bound phenolic extraction and 3 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) were incubated at 95 °C for 90 min. After the mixtures cooled to 25 °C, absorbance of the solutions were read at 695 nm against a blank on spectrophotometer and calculated against a reference of the total antioxidant capacity of ascorbic acid.

Carotenoid Analysis

Determination of carotenoid content in each fraction was conducted as previously described (Adom, Sorrells & Liu, 2003), with modifications to the system used and interdiameter of the column. An Agilent 1200 HPLC system was used with a C-30 column (250 × 4.6 mm, 5 µm column, Waters Corp., Milford, MA). The mobile phase was composed of solvent A: methanol/water (95:5, v/v), and solvent B: pure methyl *tert*-butyl ether; as 75% solvent A and 25% solvent B. The mobile phase had a constant flow rate of 1.0 ml/min, and a measured pH of 6. Analyte detection, from a 10 µl injection volume, was measured at 450 nm, under constant temperature (30°C). Beta carotene, lutein and zeaxanthin were measured from fine, medium, and coarse treatment phytochemical extracts against commercial standards of the respective carotenoid (reported as µg/100g).

Statistical analysis

All tests were performed in triplicate, unless otherwise noted. Means and standard deviations were calculated for all analyses. Significance of differences between groups were compared using column analysis of one-way ANOVA with Tukey's post hoc test at a significance level of α: 0.05 (GraphPad, GraphPad Software Inc, La Jolla, CA). P values (two-tailed) of less than 0.05 were considered to be a sign of statistical significance. N is listed where n equals the number of replicate assays. Subreplicates ranged (2-3) per assay.

Results

Particle size distributions

All samples were sieved to determine particle size distribution, and volume fractions from sieving are noted in **Table 3.1**. Sieving represented the milled ratio of desired bran fraction to overs after milling with an experimental gap. The particle size distribution by sieving the coarse, medium and fine treatments were rounded to the nearest percent, therefore, some total values are above 100%, but accurately display the fractions within the sample. Sieving determined over 90% of particles in the coarse treatment were greater than 900 µm.

TPC extraction

The results of TPC extraction are found in **Table 3.2,** expressed as milligrams FAE per gram of bran. The order of soluble TPC for each treatment was follows: coarse > fine > medium. The extraction yield of soluble phenolic acids refers to free and conjugated phenolic acids extracted with 80% methanol, whereas that of bound phenolic acids refers to alkaline-hydrolyzed extract expressed as dry weight of crude solid material extracted per gram of bran. The extraction yields of soluble extracts showed significant differences among fractions. The highest

concentration of soluble TPC was observed in the coarse treatment (0.56 mg FAE/g of defatted bran). Bound TPC was lowest in the fine treatment (0.08 mg FAE/g of defatted bran), however, coarse, medium and fine treatment bound extracts did not significantly differ. An increase in TPC was observed with some reduction in particle size, however fine to medium treatments roller milled from the coarse treatment had no significant difference *in vitro* by TPC. On average, soluble TPC was 3-7 times higher than bound TPC in all fractions.

Flavonoid concentrations

Total flavonoid content reflects the available polyphenol population in the sample. Values are reported as µg/g of extracted material, measured from the phytochemical extracts of each sample, and calculated against catechin for each bran size distribution in **Table 3.2**. The order of flavonoid content was determined as fine > coarse ≈ medium, for all samples. The highest flavonoid concentration was observed in the fine treatment (206.74 µg/g). The medium treatment did not significantly differ from the coarse treatment.

Anthocyanin concentrations

The total anthocyanin concentration reflects the available polyphenol population in the sample extracted with 85:15, v/v ethanol:hydrochloric acid (1N) based on a previously published method (Abdel-Aal & Hucl, 2003). Anthocyanin contents (**Table 3.2**) are reported in µg/g of extracted bran, for each treatment. Anthocyanin concentration by whole bran composition was in the order as follows: fine > medium > coarse, for all samples.

DPPH radical- scavenging activity

All bran extracts showed DPPH scavenging activities in concentration. DPPH scavenging activities of the treatments are reported in **Table 3.3**. The ability to scavenge DPPH radicals by bran composition for soluble and bound extracts was in the order of: medium > coarse > fine. As

the standard, ascorbic acid (Hatano, Takagi, Ito, & Yoshida, 1997) was measured at 96% DPPH inhibition with this sample set.

FRAP assay

The antioxidant power of bran extracts was evaluated by FRAP assay, as shown in **Table 3.3**. The ability to reduce Fe^{3+} to Fe^{2+} for all treatments was in the order of: coarse > fine > medium, for all soluble extracts. Coarse treatment soluble extracts had significantly higher FRAP values than other samples and extract from the fine treatment had the lowest FRAP value (32.06 μmol of $FeSO_4$/g defatted bran). FRAP values for bound extracts were as follows: medium > fine > coarse. The antioxidant power of the bound fraction from the medium treatment (85.64 μmol of $FeSO_4$/g defatted bran) was three times greater than the fine and coarse treatments. Fine and coarse treatment bound extracts but did not significantly differ.

ORAC assay

ORAC assay was reported for all treatments as TE within the extract in **Table 3.3**. For all hydrophilic and lipophilic extracts, ORAC values were on the order of fine ≈ medium > coarse. The fine and medium treatment extracts were not significantly different. TE of reduced particle size whole bran samples were significantly higher than the coarse treatment.

Total antioxidant capacities

Total antioxidant capacity values, expressed as equivalents to ascorbic acid (mg/g) per gram of bran, are shown in **Table 3.3**. The order of total antioxidant capacity for the soluble extraction was in the order of coarse > fine ≈ medium, with the fine and medium treatments determined as not significantly different. The highest total antioxidant capacity was observed in the coarse treatment (0.56 mg/g of defatted bran). The order of total antioxidant capacity for the

bound extraction was in the order of fine > medium ≈ coarse, with the medium and coarse treatments determined as not significantly different. Bound total antioxidant capacity was highest in the fine treatment (0.30 mg/g of defatted bran).

Carotenoid concentration

Carotenoid concentrations are reported as micrograms of the respective carotenoid per hundred grams of extracted bran for each sample in **Table 3.4**. All bran samples had detectable levels of all three carotenoid standards used during this experiment. Measureable carotenoids, by treatment, were in the order as follows for beta carotene: medium > fine > coarse, for all samples. For zeaxanthin, results were in the order as follows: fine > medium > coarse. The highest concentrated carotenoid was lutein. Whole bran samples all contained lutein in the order as follows: fine > coarse > medium

Discussion

After multiple analyses of the three samples, only some constituents were further extracted with reduction in particle size, as some assays are more sensitive to certain compounds (Huang, Ou & Prior, 2005). Additional grinding of wheat bran has increased extractability of water-extractable arabinoxylan (van Craeyveld et al., 2009), produced greater concentrations of short-chain fatty acids when fermented (Stewart & Slavin, 2009), and increased bioavailability niacin, pantothenic acid and thiamin (Shewry et al., 2012), to improve nutritional potential. In some cases, biochemical composition of sterols, folates and alkylresorcinols were not altered with changes in milling conditions; while, compounds such as phytic acid and ferulic constituents had some statistical differences (Hemery et al., 2011).

As some differences were seen in the current study, wheat bran fraction and composition should be considered in product formulations, as previously noted (Stewart & Slavin, 2009). Particle size effects have been observed specifically in grains (Kahlon et al., 1986, Yu & Kies, 1993, Hemery et al., 2007, Engelsen & Hansen, 2009). Researchers have noted that differences in lower particle size compositions compared to non-ground material from the same stock are most likely due the increased accessible surface area as particle size decreases (Stewart & Slavin, 2009). The researchers have determined that multiple dry milling processes reduce wheat bran particle size (Hemery et al., 2007). For the use of wheat bran as a functional food, Hemery e al. (2011) optimized the equipment and conditions by which to produce finely textured wheat bran. Change in particle distribution was made by additional milling, as additional processing has been noted to increase the bioavailability of some phenolic compounds and phytochemicals (Anson et al., 2011, Anson et al., 2012). Heat and/or aggregation enhanced some micronutrients and components of wheat bran, and inversely can destroy others (Yang, Tsou & Lee, 2002, Opara & Rockway, 2006, Hotz & Gibson, 2007).

Comparative results on grain antioxidant properties are available relating samples based on TPC, FRAP, and/or DPPH scavenging capacity (Thaipong et al., 2006, Roy et al., 2010, Herald, Gadgil & Tilley, 2012). Wheat bran has been investigated as a source of dietary fiber before and after micronization, with an increase in chelating activity, reducing power and TPC was reported after size reduction (Zhu et al., 2010). However, DPPH decreased with increased surface area to mass and their material was lower in TPC than that of unaltered wheat bran before and after ultrafine grinding. The difference could arise from sample preparation, i.e. actually particle size achieved by the chosen method (or the method of the assays themselves, which will be further discussed). In Zhu et al., (2010a), the distribution of particle size within the

bran material was reported in ranges for the average composition produced; not in particle size distribution. Small angle X-ray scattering determined the bran material ranged from 10–620 nm (averaging 300–620 nm) and transition electron microscopy determined a range from 30–450 nm (Zhu et al., 2010). However, such ranges could have various distributions within. In this study, DPPH inhibition did not increase with particle size reduction, total (soluble plus bound) and soluble TPC the highest for coarse treatment. This is not in agreement with the work of Zhu et al. (2010). Therefore the methods and difference in particle size composition can provide alternate trends in antioxidant properties measured with similar methods.

Based on the coarse treatment particle size distribution as approximately 91% particles more than 900 µm, for development of the two milled treatments, the majority of the particle sizes (approximately 50%) were chosen to fall in either 500 and 200 µm whole bran distributions, as no previous research of this nature has been conducted at the 100 µm scale. Names were provided for each fraction, as no standard of identity is available for milling whole bran. Coarse and fine bran have been previously defined as unmilled bran and bran milled, on equipment not specified, to 0.35 - 0.59 mm particles (de Silveira & Badiale-Furlong, 2009), additionally, it is reported that only coarse to medium bran particle sizes should be used to increase fecal production or rate (Brodribb & Groves, 1978); although no definition of particle size exists. However, in the current study, all whole bran samples were sieved utilizing several compositional appropriate mesh sizes to more accurately display the particle sizes of the various whole bran samples, as composition and size distribution were found to be important when conducting assays with heterogeneous materials such as with wheat bran (Noort et al., 2010).

The coarse treatment extract TPC (soluble and bound) was similar to previously published results using similar extraction methods (Liyana-Pathirana & Shahidi, 2006). In

cereals, phenolic acids are the most common form of phenolic compound, and it is known that these compounds are present in free and bound form, and the bound fraction represents 80–95% total phenolic acids in the kernel (Moore et al., 2005, Irakli et al., 2012). For this study, an *in vitro* extraction was chosen to best represent the fraction that could be available from digestion, not the total amount present. With the utilized method, extraction yield was approximately 10-15%, and similar to previously reported work on grains (Liyana-Pathirana & Shahidi, 2006). Phenolic compounds may contribute directly to antioxidant action (Awika et al., 2003a); therefore, TPC was measured in conjunction with antioxidant properties. This data was in agreement with previous reports that extracts with the highest TPC showed the greatest antioxidant properties. McCarthy et al., (2012) noted that extracts with high TPC could have the greatest protection against oxidant induced DNA damage.

The TPC method, used measured TPC without distinguishing between phenolic structures (Adom, Sorrells & Liu, 2003), therefore, the current study specifically measured phenolic compounds of importance to the bran layers by determining flavonoid and anthocyanin concentrations. Medium treatment flavonoid contents were determined to be significantly different from that of the coarse treatment, against a standard curve of catechin, a major plant secondary metabolite. The determined flavonoid concentrations for the coarse treatment were similar to that of previous determinations of unmilled wheat bran reported by (Feng & McDonald, 1989), though the current study is the first investigation monitoring multiple whole wheat bran particle size distributions and flavonoid content. In this study, the coarse and medium treatments were not significantly different in flavonoid content, yet their anthocyanin concentrations were significantly different. Differences in these subclasses of phenolic compounds may be due to their extractions (Stalikas, 2007) or the chemistry that differentiates

flavonoids from anthocyanins (Wolfe & Liu, 2008). Anthocyanin concentration in whole red wheat samples (not measured separately to obtain the bran fraction) has been previously reported as low, in comparison to that of whole ground blue wheat (211.9 µg/g) and to blue bran (495.5 µg/g) (Abdel-Aal, Young & Rabalski, 2006).

Antioxidant properties of wheat bran are derived from multiple compounds (Lachman et al., 2012). Chemically distinct antioxidant quantification methods are based on different reaction mechanisms, it is necessary to evaluate whether different methods can provide comparable antioxidant values for the same sample (Ou et al., 2002). Antioxidant quantifications are mechanistically based on either electron or hydrogen atom transfer between an oxidant and a free radical. DPPH assay measures single electron transfer to determine antioxidant reducing capacity. The DPPH method has been widely used to quantitatively test the free radical scavenging ability of various samples, due to ease and method reliability (Hatano et al., 1997, Huang, Ou & Prior, 2005, Kubola & Siriamornpun, 2008). The medium treatment had the lowest IC_{50} value, and therefore, the largest DPPH radical scavenging activity; thus suggesting that the medium treatment provided the best single electron transfer in solution; whereas, the soluble and bound extract DPPH value for fine treatment was less than medium and coarse treatments, with the only difference between these samples being surface area to mass. The current results coincide with the theory that antioxidant compounds on the surface of wheat bran may alter with particle size reduction and exposure of once protected chemical compounds (due to processing); perhaps with oxidization occurring prior to extraction. Mensor et al. (2001) and Mielnik et al. (2003) suggested that the structural conformation of the antioxidants present in solution affects the results of the DPPH assay. The conformation of soluble antioxidants may be more susceptible to oxidation with an increase of surface area to mass.

FRAP measures antioxidant power by reducing power of the electron donating antioxidants present in the extract. During FRAP assay, a single electron is transferred from the antioxidant molecule to the oxidant. However, FRAP is nonspecific and compounds with lower redox potential than Fe^{3+} will initiate Fe^{2+} formation (Benzie & Strain, 1999). Soluble extract power was affected by particle size as determined by FRAP analysis. Both methods (FRAP and TPC) are measured by reducing capacity, and from the two methods, soluble extracts had similar trends. Previous researchers have noted that FRAP activity correlated with TPC of brewers' spent grain extracts (McCarthy et al., 2012). With the correlation between the two assays, both can be used in conjunction to validate each other. Bound extract FRAP values provided a response unseen in the other antioxidant measurements utilized, where the coarse and fine treatment provided approximately the same value, with the medium treatment over three times larger. Further investigations are needed to understand the bound FRAP value, as produced by the medium treatment.

ORAC measures antioxidant properties by hydrogen atom transfer, assessing antioxidant donating capacity (Huang, Ou & Prior, 2005). The soluble and bound TPC and the hydrophilic and lipophilic ORAC values were not similar in trend; ORAC and FRAP are based on different mechanism and were reported to not correlate well (Ou et al., 2002), however FRAP has been reported to correlate with TPC (McCarthy et al., 2012), therefore the results were expected. Extracts had significantly higher '% inhibition' for coarse treatments, with higher ORAC values for the fine treatment. This may be due to the ORAC extractions utilizing hydrophilic and lipophilic properties and the present lipid-soluble compound surface properties. Roy et al. (2010) proposed that extracts exhibiting lower ORAC values compared that of their DPPH value are more powerful pro-oxidants than extracts with higher ORAC than DPPH, as the ORAC assay

uses extract to eliminate peroxyl radicals and protect the fluorescence probe utilized by a biologically sound reaction.

Total antioxidant capacity measures cumulative capacity to scavenge free radicals (Pellegrini et al., 2003). Unlike, ORAC, total antioxidant soluble and bound extract capacity had opposing results. However, combining the soluble and bound total antioxidant extract quantities within a treatment, the treatment values were relatively the same, when compared to total antioxidant capacity of ascorbic acid. In this assay, the overall cumulative capacity to scavenge free radicals of wheat bran seems equal at various particle sizes.

Carotenoids protect the kernel from oxidative damage and when consumed also serve as antioxidants (Adom, Sorrells & Liu, 2003). Carotenoids required a separate extraction from TPC and antioxidant constituent extractions, as carotenoids are lipid soluble and would be underdetermined in an extraction that utilizes hexane. The extraction methods chosen were appropriate for the material and wheat bran lutein and zeaxanthin concentrations were similar to that of previous research (Adom, Sorrells & Liu, 2005). Recent studies have utilized similar extractions on soft wheat flours to obtain the lutein and zeaxanthin concentrations in differ wheat varieties, noting a tight range of carotenoid variation in the cultivar analyze (Lv et al., 2012). However, the previous research did not analyze the bran fraction.

Several carotenoids were examined due to the lipophilic nature and chemical similarities of these compounds to previously investigated tocopherols, however additional carotenoids (α-carotene, β-crytpoxanthin) are present in wheat bran (Britton, 1995, Adom, Sorrells & Liu, 2003, Adom, Sorrells & Liu, 2005), and further investigation is required to understand carotenoid composition and distribution within bran layers. Lipid soluble tocopherols (chemically similar to carotenoids) been noted to differ with particle size (Engelsen & Hansen, 2009), however a

positive trend was not observed with lipid soluble extracts in this study. Previous researchers have noted that not all lipid-soluble vitamins in wheat bran are available at every particle size distribution, as changes in availability of vitamin E were seen with the reduction of particle size (Engelsen & Hansen, 2009). This property may not be a function of vitamin E absorption on the surface of the bran, as in the determination of the availability of vitamins A and E, a difference was found only in vitamin E (Kahlon et al., 1986).

Conclusions

For whole wheat bran at different particle size distributions, anthocyanin extractability, bound total antioxidant capacity, carotenoids and ORAC value increased as particle size distribution decreased (greater for 200 µm than unmilled bran). Therefore, changes in particle size could affect functional food claims with notably quantities bran in the formulation. Further studies are needed to determine specified food products where health claims could be affected by changes in particle size of wheat bran.

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Figure 3.1. Descriptions and photographs of whole bran treatments milled from the same wheat kernels.

Descriptions and photographs of whole bran treatments (coarse, medium and fine) milled from the same wheat kernels. The description is reported as percent volume, determined by sieving. Coarse is described as control and stock material, with particle size varying 90-5000 µm. The medium treatment was milled from coarse bran to less than 3% over the 500 µm sieve. The fine treatment was milled from stock wheat bran material to less than 3% over the 200 µm sieve.

Table 3.1. Particle size distribution of coarse, medium and fine whole wheat bran treatments by sieving.

Coarse		Medium		Fine	
Sieve µm	whole bran %	Sieve µm	whole bran %	Sieve µm	whole bran %
900	91	1041	2	355	2
750	7	500	43	200	48
500	2	355	24	150	17
355	0	240	24	100	12
PAN*	1	PAN*	10	PAN*	24

**The pan is noted as any material that passed through all sieve mesh dimensions utilized.*

Table 3.2. Phenolic compound contents in whole wheat bran extracts as function of particle size distribution.

Whole bran composition	Phenolic compounds*			
	Total phenolic content** (mg FAE/g defatted bran)		Total flavonoid content (µg catechin/g bran)	Total anthocyanin content (µg/g bran)
	Soluble	Bound		
Coarse	0.56±0.05a	0.11±0.03a	185.96±1.31b	36.6±0.10c
Medium	0.38±0.01c	0.11±0.05a	177.05±6.74b	40.5±0.10b
Fine	0.46±0.01b	0.08±0.01a	206.74±4.80a	63.0±0.20a

**Column data with like letters are not significantly different (p>0.05); n=3*

*** The total phenolic contents are expressed as ferulic acid equivalents (FAE)*

Table 3.3. Antioxidant properties of soluble and bound coarse, medium, and fine whole wheat bran treatments. Diphenylpicrylhydrazyl (DPPH) (% inhibition) is reported in terms of an ascorbic acid standard that had 96% DPPH inhibition.

Bran	Antioxidant properties*							
	DPPH (% inhibition)**		FRAP (µmol FeSO4/g of efatted bran) ǂ		ORAC (µM TE of extract) †		Total antioxidant capacity (equivalent to ascorbic acid [mg/g])	
	Soluble	Bound	Soluble	Bound	Hydrophillic	Lipophilic	Soluble	Bound
Coarse	40.71±0.16b	14.58±1.97b	140.70±0.14a	24.62±0.02c	304.48b	121.16b	0.56±0.08a	0.23±0.03a
Medium	43.37±0.16a	21.55±0.27a	99.06±0.10c	85.64±0.09a	1824.70a	1255.60a	0.42±0.02b	0.25±0.04a
Fine	32.06±0.32c	13.39±0.87b	107.41±0.11b	25.61±0.03b	1787.78a	1322.00a	0.42±0.01b	0.30±0.01a

** Column data with like letters are not significantly different (p>0.05); n=3, n=3, n=2, n=3, respectively for each experiment*

***% inhibition was calculated using IC50 value calculate DPPH value. It is defined as the concentration of the sample necessary to cave 50% inhibition, interpolated by linear regression.*

ǂ *FRAP -Ferric reducing/antioxidant power*

† *ORAC – Oxygen scavenging antioxidant capacity, where TE is the Trolox equivalent per gram of extract*

Table 3.4. Carotenoid concentrations in whole wheat bran extracts as function of particle size distribution.

Bran	Carotenoid concentrations (µg/100g)*		
	Beta carotene	Zeaxanthin	Lutein
Coarse	6.11±0.05c	16.68±0.23c	164.67±1.02b
Medium	17.64±0.13a	17.92±0.24b	132.93±0.82c
Fine	14.25±0.12b	35.21±0.47a	174.59±1.08a

** Column data with like letters are not significantly different (p>0.05); n=3*

Chapter 4 - Particle size effect on fermentation of wheat bran in rumen fluid

Abstract

To study the effects of particle size on fermentation of wheat bran in rumen fluid, unmilled bran was fermented and compared to bran milled to volume distributions of 200 and 500 µm from the same commercial stock. To portray the effect from bran only, by excluding residual starch, additional samples included destarched and destarched, cooked wheat bran. Compositional analyses were determined for all samples. The hypothesis was that an increase in substrate surface area to mass would increase gas production from microbes within rumen fluid and decrease remains that would be available to travel to the omasum. Using ANKOM models, *in vitro* rumen fermentations of five wheat bran treatments were executed to observe gas production from the substrate. The composition of the starting material was compared throughout the study to the *in vitro* remains after 24 h rumen fermentation to determine the utilization of cellulose, hemicellulose, lignin, protein, and starch during fermentation. After 24 hour *in vitro* fermentation, unmilled, uncooked wheat bran produced significantly higher gas production (123 mL) and lower remains (0.64 g), by dry weigh, than all processed samples. Starch containing samples produced distinctly different gas production curves than destarched wheat bran and destarched, cooked wheat bran samples. Destarched wheat bran and destarched, cooked wheat bran samples produced gas (mL) at a slower rate from 0-8 h, than starch containing samples. Destarched wheat bran and destarched, cooked wheat bran samples also had significantly higher amounts of digesta remains (approximately 85% and 86% remaining, respectively) after 24 h. Fermentation of wheat bran in rumen fluid using ANKOM models

revealed differences in gas production with change in particle size that were unexpected. With reduction of wheat bran particle size products of fermentation and rate of fermentation was lowered.

Introduction

Wheat contains approximately 71% of the metabolizable energy found in corn, therefore, wheat bran is not a major portion of the finishing cattle diet (National Research Council (US). Subcommittee on Dairy Cattle Nutrition, 2001). Consumption of wheat bran increases ammonia, propionate and acetate in ruminal fluid of supplemented steers, in comparison to that of cracked corn supplemented steers. Wheat bran has been shown to increase energy intake, but does not reverse decreased forage intake that is seen with corn supplementation. However, if economically justified, wheat bran is supplemented to steers (Hess et al., 1996).

Ruminant digestion utilizes microbes to digest polymers and produce volatile fatty acids (VFA), which are absorbed and transported to circulation (Beever, 1993, Stewart, 1997). [**Appendix E** provides a drawing of the ruminant digestive system, while **Appendix F** provides chemical structures of all VFA studied within this dissertation.] The microbial population is housed in the rumen. Within the rumen there are 10-50 billion bacteria, one million protozoa, and variable amounts of fungi and yeasts (Hungate, 1966). There are greater than 10^{10} bacteria per ml of rumen fluid, and with liquid turnover every 12 h, foodstuffs are normally passed in less than 48 h. Products of various strains may change depending on substrate availability, culture conditions and dominances (Russell and Hino, 1985).

The purpose of the rumen is to provide means for substrates to undergo extensive alteration and degradation by the microbial population residing within the organ (McCarthy, 1962). The location of the rumen as the first organ in the digestive tract enables ruminants to

utilize materials with chemical bonds indigestible to vertebrates (Morrison, 1979). Conditions within the rumen vary, and there are limits to digestion in the rumen (Titgemeyer, 1997). Multiple methods are available for *in vitro* rumen digestion, and development of these methods has been described and reviewed (Cheng et al., 1955, Getachew et al., 1998). It has been previously reported (*in vivo*) that processing corn and sorghum by particle size reduction (Theurer, 1986) or altering the protein matrix, increases the extent of starch digestion in the rumen and in the small intestine (Owens et al., 1986), as noted by performance data from growing cattle fed processed corn and sorghum. However, while substrate effect on animals is monitored in the majority of feed studies, the fate of the substrate is not well understood. In this study, an *in vitro* rumen model, utilizing active rumen fluid, was used to observe the digestive pattern of whole wheat bran at various particle sizes and with processing to determine if rumen bacteria have a preference to degrade certain types of particles that enter the alimentary canal.

Materials and methods

Materials

Unmilled, food grade wheat bran was obtained from a flour mill in Commerce City, CO operated by Conagra Mills (Omaha, NE), and all chemicals, reagents, and standards were ACS or HPLC grade. A control sample (unmilled bran) was kept in tinted, air tight containers and stored less than six month prior to compositional analysis.

Milling and preparation of cooked and destarched wheat bran

The commercial wheat bran was used to produce all samples. Whole wheat bran samples of approximately 50% by volume, 200 and 500 µm distributions were milled via three passes through a corrugated (20/22 corr. per square inch; 2.5:1 differential) Ross experimental roller mill (serial # 915, size 9x6; Oklahoma City, OK) in the Experimental Milling Lab (Manhattan,

KS) with an experimental gap, to grind less than 3% over the designated sieve size. Destarched bran was prepared from the stock material by digestion with 0.5% Stargen 001 (Genencor, Palo Alto, CA) for 24 h at 37°C. The destarched bran was washed five times with distilled water, centrifuged at 2,000 g. The sample was recovered, frozen overnight, and lyophilized for 16 h. A portion of destarched bran was used to produce unmilled whole, destarched and cooked bran (referred to throughout as 'destarched, cooked'). After incubating the bran for 24 h at 37°C with 0.5% Stargen 001, washing (five times), recovering the sample, freezing and lyophilizing, the lyophilized bran was subsequently cooked for 1 h in a pressure bottle at 100°C (1/10 bran:water), after settling, cooking water was decant, sample frozen overnight, and lyophilized (16 h).

General methods

All samples were analyzed for ash (AOAC Method 923.03), moisture (AACC International Method 44-19.01), protein (AOAC Method 984.13), total dietary fiber (TDF) (AOAC Method 991.43), total starch (AOAC Method 996.11), cellulose, hemicellulose (Van Soest et al., 1991), and lignin (AOAC Method 973.18). Cellulose, hemicellulose, and lignin content were analyzed using a fiber evaluation method common in animal nutrition (Horwitz, 2000, Van Soest et al., 1991). Neutral detergent fiber (NDF) was determined; where hemicellulose was the difference between NDF and acid detergent fiber (ADF). Cellulose was the difference between ADF and acid detergent lignin (ADL). In addition, oil (Caprez et al., 1986) and water absorption (AACC Method 56-30.01), modified to determine water holding capacity (WHC) (Caprez et al., 1986, Chen et al., 1984), were determined for each sample prior to fermentation. The method for oil absorption was modified as follows, for the nature of the material (Caprez et al., 1986). All vials were pre-weighed prior to the addition of 1.5 g bran and 10.5 g corn oil (C8267, Sigma-Aldrich, Inc., St. Louis, MO). A magnetic stirring bar was added

and samples were mixed for 30 min at 25°C. Subsequently, the samples were centrifuged at 1,500 × g for 30 min. The excess oil was decanted from the surface and oil absorption expressed as the amount of oil in grams bound by one gram of wheat bran (dry matter basis).

Particle size determination

Laser diffraction sizing (LDS) was used to measure and confirm particle size distributions of wheat bran target sizes after grinding. Particle size distributions were measured using a LS 13 320 SW, Dry Powder System (Beckman/Coulter Particle Characterization, Miami, FL) (Wilson et al., 2006).

In vitro rumen fermentation

Five whole, wheat bran samples were analyzed by *in vitro* rumen fermentation: unmilled, 500 µm, 200 µm, destarched, and destarched, cooked bran. The samples were randomized and bottle set-up time recorded, to reduce bias and equalize conditions, respectively. *In vitro* rumen fermentations were carried out as previously described (Leibovich et al., 2009, Quinn et al., 2010, Quinn et al., 2011). McDougal's buffer (McDougall, 1948, McDougall et al., 1996) was prepared within 2 h of the experiment, and held at 39°C. Ruminal fluid was collected through roughage fed (adult fistulated steer, KSU Dept. of Animal Science, Manhattan, KS) ruminal fistula, transported in pre-heated (39°C), sealed thermoses, and strained through eight layers of cheesecloth. The strained fluid was placed in a sealed, separatory funnel, and held at 39°C for 30 min to obtain a clarified portion. Although the microbial population in the rumen includes both bacteria and protozoa, clarified rumen fluid is typically used and contains only bacteria (Titgemeyer, 1997). Use of this clarification method has been justified in knowledge that protozoa are not digested by the ruminant in the same manner as the bacterial population and are a small portion of the total microbial mass (Weller and Pilgram, 1974). The pH (model 230,

Thermo Orion, Waltham, MA) of the McDougal's buffer and clarified rumen fluid was measured prior to use. Whole bran samples were weighed (1.000 g DM) into 250-mL bottles (ANKOM Technology Corp., Macedon, NY) with 50 ml of clarified ruminal fluid and 100 ml of McDougall's buffer. The bottles were flushed with nitrogen and placed on a rotational shaker at 40 rpm for 24 h at 39°C. Gas production was monitored using ANKOM gas production modules, and calculation of gas production was determined as previously described (López et al., 2007). Total gas production is reported with the blank removed.

VFA

Preparation of VFA samples for gas chromatography (GC) was carried out as previously described (May et al., 2009). Fluid (4 ml) after 24 h was collected from each ANKOM bottle and combined with 1 ml 25% (weight/volume) metaphosphoric acid, vortex mixed and stored at −20°C until analysis. The day of analysis, acidified samples were thawed and centrifuged (accuSpin Micro 17R, Thermo Fisher Scientific, Waltham MA) at 13,300 *g* for 10 min at 20°C. The supernatant was analyzed for VFA by GC (Hewlett-Packard 5890A, Hewlett-Packard, Palo Alto, CA; 2 m × 2 mm column, Supelco B-DA 80/120 4% Carbowax 20-m column packing, Supelco, Bellefonte, PA), with nitrogen as the carrier gas, a flow rate of 24 mL/min, and column temperature of 175°C. The carboxylic acid derivatives measured via GC were: acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate (as drawn in **Appendix 6**).

Digesta analysis

Digesta remaining in the bottles after 24 h were tested for pH and emptied into pre-weighed aluminum pans, to dry in an oven (105°C), overnight, for dry matter determination. Dry matter is reported with the blank removed. After dry matter was recorded, the remains were hydrated and removed from the pans. All collected material was washed and centrifuged for

analysis of protein (AOAC Method 984.13), cellulose, hemicellulose (Van Soest et al., 1991), lignin (AOAC Method 973.18) and total starch (AOAC Method 996.11).

Statistical analysis

All tests were performed in triplicate, unless otherwise noted. Means and standard deviations were calculated for all analyses. Significance of differences between groups were compared using column analysis of one-way ANOVA with Tukey's post hoc test at a significance level of α: 0.05 (GraphPad, GraphPad Software Inc, La Jolla, CA). P values (two-tailed) of less than 0.05 were considered to be a sign of statistical significance. N is listed where n equals the number of replicate assays.

Results

Composition of wheat bran

Prior to fermentation, sample compositional data was recorded. **Table 1** contains analyses of ash, cellulose, hemicellulose, lignin, moisture, oil absorption, protein, TDF, and WHC for all samples. Ash content increased with a reduction of starch in the sample. While, total starch seemed to largely impact gas production. Cooking wheat bran increased the amount of oil absorption and WHC. Cellulose, hemicellulose, and lignin of unmilled, 500 µm, and 200 µm brans were not significantly different. While, lignin was present as the smallest constituent measured, it showed the most alteration from sample to sample and after fermentation (**Table 4.1 & 4.2**). TDF, oil and water absorption were higher for unmilled bran, than milled brans (200 and 500). There was no linear trend observed for TDF to fermentation or gas production. However, oil and water absorption decreased with an increase in surface area to mass.

ANKOM fermentation and gas production

At 0 h, pH of clarified rumen fluid was 6.30 and McDougal's buffer 6.98 (39°C). Quantitative measures of rumen digesta after *in vitro* fermentation are shown in **Table 4.2**. The pH values of the fermentations, within the ANKOM bottles at 24 h, were close, with the exception of destarched bran, which was significantly higher. There was no significant difference in the pH of the wheat brans at various particle size distributions (unmilled, 500 µm, 200 µm). After 24 h, digesta remains from each bottle were dried and weighed. Weighed amounts within **Table 4.2** are reported with the blank removed. Dry remains for processed bran samples (destarched, 0.85 g, and destarched, cooked, 0.86 g) were significantly higher than unprocessed samples. **Table 4.2** displays cellulose, hemicellulose, lignin, and protein content as percent change, from that of the value determined for the material before 24 h fermentation. Blanks from the ANKOM fermentations were also tested for cellulose, hemicellulose, and lignin. For the blanks, a difference of 2.02 was found between NDF and ADF; therefore, that value was assigned as 'hemicellulose'. The concentration of lignin was calculated from the dry material after fermentation with the use of an equation by Hale (1947), to compensate for the material present. The p-values for raw material to digested material for cellulose, hemicellulose, and lignin were calculated at 0.036, 0.001, and 0.027, respectively. Total gas production of this experiment is reported in **Figure 4.1**. The order of gas production (mL) at 24 h for all whole wheat brans was as follows: unmilled > 500 µm > 200 µm > destarched > destarched, cooked. Destarched and destarched, cooked whole bran samples were significantly different from unprocessed samples (unmilled, 500 µm and 200 µm) in total gas production *in vitro*.

VFA

VFA produced from *in vitro* fermentations were measured from all bottles at 24 h. The carboxylic acid derivatives generated from the blank (total of 44.07 mM) were individually subtracted from the bran values represented in the graph (**Figure 4.2**). The order for total VFA produced was as follows: unmilled > 500 µm > 200 µm > destarched, cooked > destarched, for all bran fermentations. Unmilled bran had the highest concentration of VFA produced (102.91 mM), while the particle size reduced whole brans contained significantly less (500 µm: 77.09 mM > 200 µm: 70.06 mM). The unmilled bran is highest in all VFA, except butyrate. Total values of destarched and destarched, cooked bran were close, not significantly different from one another (66.50 mM and 66.81 mM, respectively), yet significantly different from unmilled, 500 µm and 200 µm whole bran compositions. Starch removal significantly lowered VFA production.

Discussion

In vitro fermentations

Previous use of ANKOM models has observed ruminant feed relationships between chemical composition, digesta remains and *in vitro* gas production (Getachew et al., 2004). Similarities between this study and a study on wheat middlings (Getachew et al., 2004) were found in protein and fiber content not increasing with the trend of gas production and digesta remains decreasing with increase of gas production. However, it was noted that only valerate production followed the trend previous researchers observed in *in vitro* gas production at 24 h, not isovalerate and valerate, as previously reported (Getachew et al., 2004). Differences between the studies can be attributed to difference in the rumen fluid utilized and the different substrates, as wheat middlings vary considerably (Cromwell et al., 2000). *In vitro* gas production curves and

total VFA showed that wheat middling produced less gas production than that of corn silage and hominy. This result may be related to the metabolizable energy of wheat compared to that of maize (National Research Council (US). Subcommittee on Dairy Cattle Nutrition, 2001). This study was the first on substrate effects with particle size distribution using ANKOM models; however several *in vitro* wheat bran particle size studies have been reported (Kahlon et al., 1986, Noort et al., 2010, Stewart and Slavin, 2009, Zhang and Moore, 1999).

Previous reports have also noted that observations of only late fermentation imply a constant relationship between substrate disappearance and fermentation products over time (Doane et al., 1997). Researchers noted that gas and VFA production varied in less than 8 h with forage feeds, and remained stable thereafter. However, in this experiment particle size reduction of wheat bran did not produce differences between samples (unmilled, 500 µm, 200 µm) until approximately 8 h. As there are fermentative differences between foodstuffs (Owens et al., 1986), the period of said differences can vary within the digestion process as well. pH within ANKOM models may have inhibited or slowed fermentation rate, since rumen pH ranges from 5.5 to 6.5 (Stewart, 1997). The pH of destarched, cooked bran was higher than the pH used in the testing procedure (McDougall, 1948), which may have slowed the fermentation process for this sample, lowering gas production and degradation activity of bacteria.

Effect of WHC

Coarse bran holds more water than fine bran (Zhang and Moore, 1997), as noted (**Table 4.1**). Due to surface area, and intact structure, unmilled bran can hold more water and oil, therefore, oil absorption and WHC of this material was highest among all samples. It is possible that unmilled bran held more rumen fluid then 200 µm and therefore underwent increased fermentation, as with less processing and under the same conditions, rumen bacteria initiated

more fermentation on large fractions. Although, cooking wheat bran increased the amount of oil absorption and WHC, and did not increase the amount fermented *in vitro.* Therefore, it is also plausible that commensal bacteria could monitor for the hydration levels prior to passing the foodstuffs to the omasum, and into the abomasum for water removal, however, further investigation is needed. Within sheep, researchers observed particles with higher density passing from the rumen into the omasum compared to less dense particles (Kaskea et al., 1992). However, the study also noted that defining particles by length supported the adverse theory that smaller particles digest faster. Overall this study concluded that rumen action was independent of particle density and size, but the probability of a particle exiting the rumen first was higher for particles with added density, as particle size was not assigned as a defining parameter (Kaskea et al., 1992).

Effect of protein content

Protein content differed slightly with increased surface to mass, as availability of macromolecules has been noted to alter with processing (Camire, 1998, Kahlon et al., 2006). While crude protein differed statistically, all wheat bran was in range for the material, as is (Beaugrand et al., 2004) and on dry matter basis (Lamsal et al., 2008), and was assumed to contribute greatly to a biological difference. Available protein that enters the rumen is converted into organic acids and ammonia for use of the bacteria (McCarthy, 1962), and the deamination of valine and leucine by the ruminal bacteria leads to the formation of isobutyric and isovaleric carboxylic acids (Annison and Lewis, 1959). The protein content after fermentation was measured after washing the digesta carefully. The microbial population of rumen fluid is ~40% of the solids content and is composed of amino acids and peptides (Stewart, 1997). Although, some soluble peptides may have been lost during washing, the high concentration of microbes

from in the fluid could have altered substrate compositional analysis by adding protein content or skewing the proteinaceous dry weight. With increased processing (more cooking, further reduction of size) the change in protein content before to after fermentation showed a significant and linear change and the availability of protein may be a contributing factor to reduced gas production in the processed samples.

Effect of fiber components

The TDF value of the control (unmilled) was within range of previous studies (Bourquin et al., 1992, Fahey Jr et al., 1990, Idouraine et al., 1996, Monro, 2004, Vitaglione et al., 2008). Bacteria do not contain cellulose, hemicellulose, or lignin, however, the dried remnants from the *in vitro* rumen fermentation blanks were analyzed to determine if incomplete cleaning of the digesta, prior cellulose, hemicellulose, and lignin, measurement, could affect determination. Rumen fluid microbes may raise NDF slightly, as the blank was measured to have a small amount of NDF in high concentration. Since the digesta were washed, and NDF for the blank was low, it is assumed that the variation from the microbes would be low in analyses with bulk removal of dried rumen fluid. This assumption is made because ADF of the blank at high concentrations was zero, and ADF is the portion which contains some ash and nitrogen compounds (Colburn and Evans, 1967). ADF and NDF of unmilled bran were within range of previous studies (Fahey Jr et al., 1990). Lignin represents a diverse composition of phenolic compounds (Hartley and Morrison III, 1991). Commensal bacteria may have cleaved ester linkages rendering lignin as available (Stalikas, 2007). After fermentation, all values increased due to the decrease in percent of other constituents, except the hemicellulose portion of destarched bran. Increase in constituents (ash, protein, etc.) for destarched and destarched, cooked brans (compared to the other samples) were partly due to the absence of residual starch in

the material, which increased the percent remaining material. Although, minor in some cases, there is lignin, cellulose, and hemicellulose loss in the rumen (Bailey, 1967), there was a linear decrease in the cellulose and lignin from raw material to after fermentation with an increase in surface area to mass.

The utilization of cellulose is noted as a primary factor in ruminant nutrition (Louw et al., 1949). The rate of cellulose digestion in the rumen increases around 6 h (Hale et al., 1947), and cellulose content after milling was only significantly different between starch containing and nonstarch containing fermentations. Disrupting the cellulose matrix can account for an increase in protein digestion (Saunders et al., 1972). Change in particle size rendered changes in protein content after digestion, however, differences were present prior to digestion. *In vitro* digestion of unprocessed wheat bran achieved over 69% degradation, and after reduction of particle size, and disruption of the protein matrix, the digestion increased to 72% with cellulosic enzymes. The percent cellulose digested was in range of a previous *in vitro* study (Cheng et al., 1955).

Effect of total starch content

In vivo studies have noted that during the first 6 h there is a rapid disappearance of soluble nutrients, proteins and carbohydrates in the rumen (Hale et al., 1947). Wheat bran contains residual endosperm from the dry milling process (Sugden, 2001). While, using the same stock material throughout reduces variability due to sample, cooking portrays the effects of processing, and also gelatinizes any starch that could have escaped amylase digestion after 24 h. It has been reported that the type of processing, i.e., finely grinding, steam-flaking, or cracked grain, can alter the level of rumen digestion (Owens et al., 1986). Food processing can also affect other digestive processes (Anson et al., 2012, Kahlon et al., 2006).

Investigations of starch digestion within the rumen have long been discussed (Waldo, 1973). For destarched and destarched, cooked brans, versus samples containing starch, the difference between these gas production curves from 0 to 8 h, and the relative loss of starch after 24h note the digestion of starch fermentation within the rumen. Previous studies have noted increased fermentation of starch from initial to 8 h (Hale et al., 1947). Some starch escapes the rumen (Owens et al., 1986), however digests that contained starch after 24 h fermentation had highly variable assays. This varialbility is known with the use of Glucose Oxidase/Peroxidase Reagent and small sample sizes (McCleary et al., 1997).

Effects of total gas production and contributing VFA

In vitro gas production does not consider multiple *in vivo* effects, and therefore is only an estimate of rumen activity (Blümmel and Ørskov, 1993). The contents within the rumen (food particles, microorganisms) travel out in frequent intervals, to the reticulum and omasum (McCarthy, 1962), and extensive fermentation of larger particles seen in this *in vitro* experiment, may be due to the function of the bacterial population within the rumen and not in the end results from the material within the animal. Digestive speed and differences from the substrate may cover one or the other (Owens et al., 1986). However, total digestion, from all organs, is generally increased by processing, conditions, and particle size reduction (Adeeb et al., 1971). Previous studies have suggested that microbial action and retention in the rumen for digestion diverges with grain type (McNeil et al., 1971) and that digestive responses to treatment of foodstuffs may differ (Owens et al., 1986).

Wheat bran fraction influences short chain fatty acid development as well as bran particle size during *in vitro* fermentation (Stewart and Slavin, 2009). The carboxylic acid derivatives produced by rumen fluid fermentation of whole bran samples followed the same trend as total

gas production, until processing (destarching) of unmilled bran, however, with all samples propionate production followed the trend of VFA production (Beuvink and Spoelstra, 1992). Wheat bran fermentation produces mostly acetate (Bourquin et al., 1992). Unmilled wheat bran producing the highest concentrations of acetate would increase lipogenesis over that of the processed samples (Illius and Jessop, 1996). Previous reports of high production levels of acetate *in vitro* (Durand et al., 1988) were significantly higher than the current reported value, but approximately the same as the values obtained before subtraction of the blank.

Conclusions

The rate of fermentation and the rate of products produced by the ruminal microbes were not equal for wheat bran with different particle size distributions. Unmilled bran produced more total gas, higher carboxylic acids, and fewer remains after 24 h fermentation. Starch containing samples had a faster rate of fermentation from 0 to 7 h compared to the destarched samples. Commensal microbes further fermented larger, more unprocessed particles within a 24 h period, which may be due to adsorption of bacteria or rumen fluid by wheat with different particle sizes or the purpose of the rumen as an organ and the role of these commensal bacteria. These results suggest that minimum processing of wheat bran increases fermentation in the rumen, and possibly rumen health after consumption.

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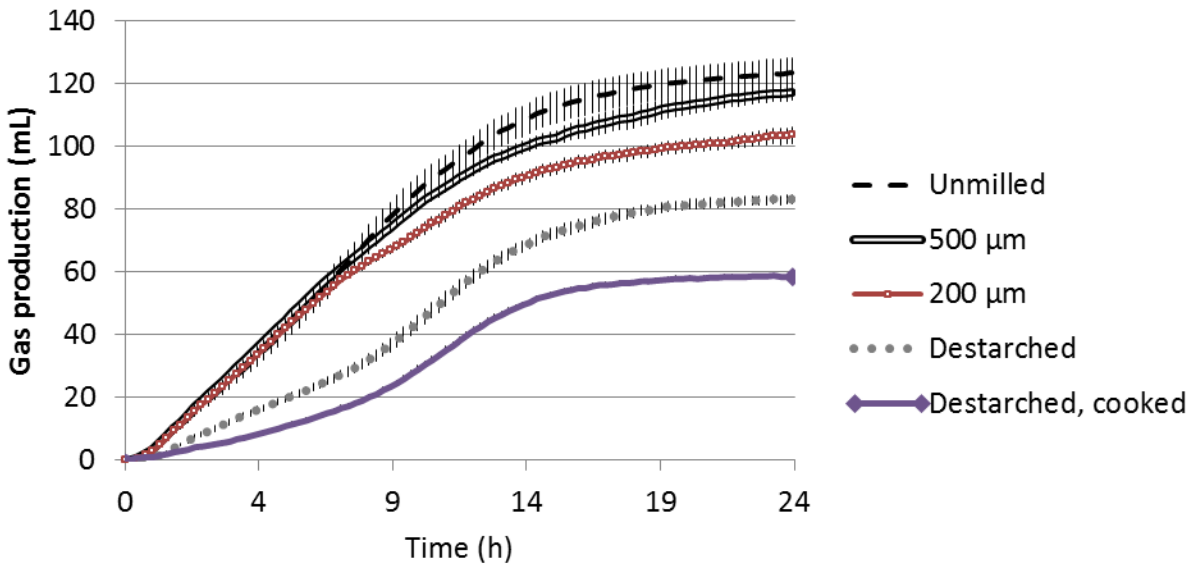

Figure 4.1 Gas production curves (mL per h) of unmilled, 500 µm, 200 µm, destarched, and destarched, cooked whole wheat bran, after *in vitro* fermentation with rumen fluid. Within each curve, standard deviation is reported every 20 min.

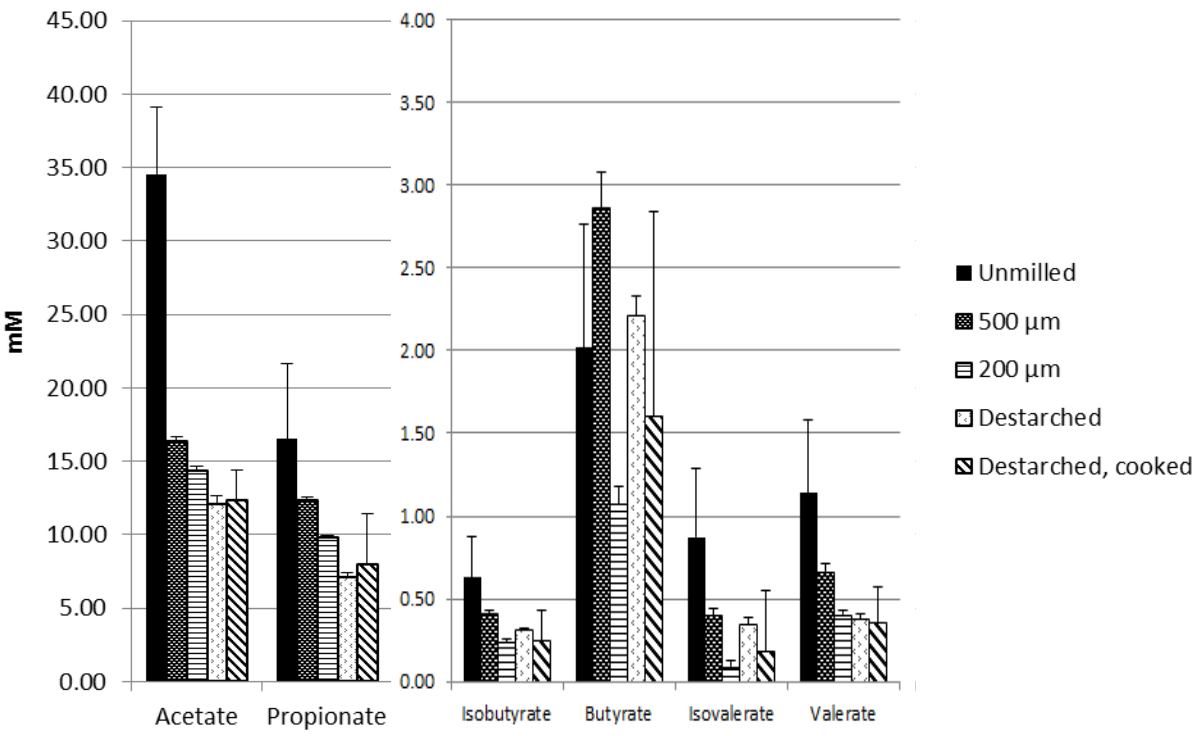

Figure 4.2. Volatile fatty acid derivatives produced from *in vitro* rumen fermentation of unmilled, 500 µm, 200 µm, destarched, and destarched, cooked whole wheat bran.

Table 4.1. Composition and oil and water absorption of wheat bran.

	wheat bran sample*				
	unmilled	500 µm	200 µm	destarched	destarched, cooked
ash %	5.0±0.3c	4.7±0.2c	5.0±0.1c	6.5±0.3a	5.8±0.1b
moisture %	13.0±0.3a	8.3±0.1c	8.2±0.0c	8.0±0.1c	8.9±0.1b
cellulose %	8.5±0.1c	8.3±0.4c	8.7±0.2c	16.9±0.1a	12.5±0.0b
hemicellulose %	29.4±0.9c	27.8±0.5c	28.6±1.2c	49.3±0.6a	45.3±2.1b
lignin %	2.7±0.0c	2.7±0.1c	2.8±0.0c	5.6±0.1a	4.3±0.1b
protein %	16.9±0.0a	16.3±0.0c	16.5±0.0b	16.9±0.1a	16.9±0.1a
total starch %	16.6±0.5a	15.9±0.6a	16.0±1.4a	4.1±0.3b	3.7±0.1b
total dietary fiber %	49.8±0.2c	41.2±2.6d	44.5±0.6d	73.1±1.0a	66.3±0.3b
oil absorption, ml/g	3.2±0.3b	2.0±0.2c	1.6±0.1c	6.6±0.0a	6.2±0.2a
water absorption WHC, ml/g	4.7±0.0c	3.3±0.0d	2.5±0.0e	6.2±0.1b	6.7±0.1a

**Row data with like letters are not significantly different (p>0.05); n=3, with 2-3 subreplicates*

Table 4.2. Quantitative measures of whole wheat bran rumen digesta after *in vitro* fermentation.

				% change after fermentation			
whole wheat bran	**digest pH***	**dry remains at 24 h (g)***	total starch%	cellulose	hemicellulose	lignin	protein
unmilled	6.7a	0.64±0.01b	0.0±0.0a	4.5	3.6	2.7	0.5
500 µm	6.8a	0.64±0.05b	0.1±0.0a	3.6	3.5	2.2	1.5
200 µm	6.8a	0.65±0.04b	0.3±0.5a	2.9	4.2	1.8	4.2
destarched	6.9a	0.85±0.03a	0.2±0.0a	3.1	-3.8	2.1	-7.3
destarched, cooked	6.9a	0.86±0.04a	0.1±0.0a	7.5	0.6	3.3	-8.0

**Column data with like letters are not significantly different (p>0.05); n=3*

Chapter 5 - Mechanism and enzymatic contribution to *in vitro* test method of digestion for maize starches differing in amylose content

Brewer LR, Cai L, Shi YC. Mechanism and Enzymatic Contribution to In Vitro Test Method of Digestion for Maize Starches Differing in Amylose Content. J Agric Food Chem 2012;60:4379-87http://dx.doi.org/10.1021/jf300393m.

Abstract

To determine the rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) content in a starch sample, the addition of amyloglucosidase is often used to convert hydrolyzates from α-amylase digestion to glucose. The objectives of this study were to investigate the exact role of amyloglucosidase in determining the digestibility of starch and to understand the mechanism of enzymatic actions on starch granules. Four maize starches differing in amylose content were examined: waxy maize (0.5% amylose), normal maize (≈27% amylose), and two high-amylose starches (≈57% and ≈71% amylose). Notably, without amyloglucosidase addition, RS content increased from 4.3 to 74.3% for waxy maize starch, 29.7 to 76.5% for normal maize starch, 65.8 to 88.0% for starch with 55% amylose, and 68.2 to 90.4% for the starch with 71% amylose. In the method without α-amylase addition, less RS was produced than without added amyloglucosidase, except in maize at 71% amylose content. Scanning electron microscopy (SEM) revealed the digestive patterns of pinholes with α-amylase and burrowing with amyloglucosidase as well as the degree of digestion between samples. To understand the roles of amyloglucosidase and α-amylase in the *in vitro* test, multiple analytical techniques including gel permeation chromatography, SEM, synchrotron wide-angle X-ray diffraction, and small-angle X-ray scattering were used to determine the molecular and crystalline structure

before and after digestion. Amyloglucosidase has a significant impact on SDS and RS content of granular maize starches.

Introduction

Starch is a major component in cereal grain foods and the most important source of food energy. Understanding starch digestibility is of great interest to the food industry and of importance for diet-related disorders such as obesity, diabetes and cardiovascular disease. Not all starch can be digested in the small intestine, where the portion of starch that is not digested is termed resistant starch (RS) [1].

Granular starch is considered a form of type 2 RS [2-7]. The mechanism of resistance to enzymatic digestion of starch granules is complex and factors such as dense packing and restricted mobility of starch molecules, long amylopectin branches, helix form, crystallinity, lamellar organization as well as structural features of granules are considered [8]. Physiological benefits have been correlated to RS consumption [2, 3, 5, 7] which notably alters fecal bulk and short-chain fatty acid metabolism, thus promoting colonic health [7]. *In vitro* measurement of rapidly digestible starch (RDS), slowly digestible starch (SDS), and RS in granular starches often employs both α-amylase (pancreatin) and amyloglucosidase [9-17]; however, the roles of each enzyme in the *in vitro* tests are not well documented. Amyloglucosidase converts oligomers produced from α-amylase digestion to glucose and is not believed to affect SDS or RS content in normal maize starch [2, 4]. In many cases, the level of glucose produced in the digest is measured and used to calculate the RDS, SDS, and RS content [4, 14, 15, 18]; whether the addition of amyloglucosidase affects the SDS and RS content is not clear. Moreover, improved *in vitro* methods are needed to truly reflect starch digestibility *in vivo* systems [19].

In this study, we selected four native maize starches ranging from 0 to about 70% amylose content and examined the roles of α-amylase and amyloglucosidase on digestibility of the starches. RS and SDS results have been published for normal maize and waxy maize starches [2, 4, 11, 14, 20, 21] and the two high-amylose starches [13, 14, 20, 22]. The high-amylose starches are generally more resistant to enzyme digestion. The maize starches with different amylose contents are known to have different morphology [23-25], molecular structure [4, 13, 26, 27], molecular order and crystallinity [12, 28], and gelatinization properties[26, 29-33]. The goals of this study were to determine if the addition of amyloglucosidase affects the RDS, SDS, and RS content and if amyloglucosidase affects the digestibility of starches with different amylose contents. Multiple analytical techniques including gel permeation chromatography (GPC), scanning electron microscopy (SEM), synchrotron wide-angle X-ray diffraction (WAXD), and small-angle X-ray scattering (SAXS) were used to probe the short and long range orders of the structural changes of the starches after enzyme digestion and understand the mechanism of enzymatic actions on starch granules.

Materials and Methods

Samples

Waxy, normal, and two high-amylose (HYLON V and HYLON VII) maize starches were obtained from National Starch LLC (Bridgewater, NJ) and their amylose content was 0.5, 27.0, 56.8, and 71.0%, respectively, as determined by the potentiometric iodine method [26]. Moisture content for all samples was determined by AACC standard method 44-16.01. Porcine pancreatin (catalogue no. P7545) and amyloglucosidase (catalogue no. A7255) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). Enzyme activity was calculated spectrometrically by measuring substrate decrease over time at 520 nm, using a procedure described previously [34, 35]. α-amylase

(EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase) had an activity of 300 U, with 0.9 mg glucose released from soluble starch in 3 min at 37 °C and pH 5.8. One U is defined as the amount of the enzyme that hydrolyzed the conversion of one micromole of starch per minute to glucose. Amyloglucosidase (EC 3.2.1.3; 1,4- α-D-glucosidase) had an activity of 234 U, with 0.7 mg glucose released from soluble starch in 3 min at 37 °C and pH 5.8. Amyloglucosidase (catalogue no. A7255) has been recently discontinued from Sigma-Aldrich, Inc. The replacement is amyloglucosidase catalogue no. A9228 which is reported as 40000 units/g solid where 1 mg glucose is released from soluble starch in 3 min at pH 4.5 at 55°C [35]. Other chemicals were reagent grade.

Methods

Digestion method and modifications

The control samples were analyzed via the Englyst method [10]. Modified digestion methods were as follows: digestion samples were prepared with only one enzyme, referred to throughout as "digestion with α-amylase," and "digestion with amyloglucosidase". Vials designated as digestion with amyloglucosidase were prepared at the same volume as the control and were run according to the Englyst method of resistant starch determination, only without α-amylase added. At 20- and 120-min intervals, 250 µl of supernatant was added to 10.0 ml 66.6% ethanol solution. [**Appendix G** provides visual representation of this step as supplemental clarification.] All 10.0 ml 66.6% ethanol vials were centrifuged at 1,580 g for 5 min and analyzed by the same colorimetric method as the control digestion method samples. For the digestion with α-amylase samples, the vials were also prepared at the same volume and run under the same method as the control [10], only without amyloglucosidase initially added. At 20 and 120 min, 18 mg of amyloglucosidase was added to all 10.0 ml 66.6% ethanol vials

containing a 250 µl sample from the digestion with α-amylase experiment and held at 37 °C for 30 min. We found that the added amyloglucosidase was able to completely convert oligomers to glucose under the test conditions. All colorimetric determinations of glucose were performed by AACC Method 76-13.01, using a glucose assay kit (Megazyme, International Ireland Ltd. Wicklow, Ireland).

RDS and RS were collected and calculated at 20 and 120 min, respectively, for all samples. The values for SDS and RS were produced from equations. Equations for the two calculated values are SDS = DS-RDS and RS = 100-DS, which are explained in detail in the original publication [10].

High-performance anion-exchange chromatography (HPAEC)

The results of the *in vitro* digestion methods were verified by HPAEC with pulsed amperometric detection (Dionex ICS-3000, Dionex Corp., Sunnyvate, CA). To ensure all digested material was converted to glucose, at 20 and 120 min, 0.5 ml of supernatant from all digestion experiments was introduced to 100 µl of amyloglucosidase (aqueous; 10 µg/10 µl H_2O) and the vials held for 30 min at 70 °C. Amyloglucosidase is active on carbohydrates at 70 °C [36]. The digest was diluted to 1:10000 with deionized water and analyzed by HPAEC-PAD. The eluent was prepared as previously reported [37]. The eluent was 150mM NaOH and the separations were carried out at 25 °C with a flow rate of 1 ml/min. Glucose (catalogue no. P3761, Sigma-Aldrich, Inc., St. Louis, MO) was used as a standard.

SEM

At the conclusion of the *in vitro* digestions, the 120 min vials were collected from the water bath and centrifuged at 1,480 g for 10 min. The supernatant was discarded, and the remaining starch in the vials was washed three times with deionized water and centrifuged at

1,480 g for 10 min. After the starch was cleaned and supernatant was removed, vials were stored at 4 °C for 24 h and freeze-dried for 16 h. The freeze-dried samples were mounted on carbon paper and gold-palladium sputter-coated with a Desk II Sputter/Etch Unit (Denton Vacuum LCC, Moorestown, NJ). Images were collected at 1000× and 5000× resolution by an S-3500N SEM with an absorbed electron detector (S-6542) (Hitachi Science Systems, Chiyoda, Tokyo, Japan) operating at an accelerating voltage of 20 kV.

Molecular size distribution by GPC

All native and freeze-dried digested samples from SEM preparation were used for GPC as previously described [37].

WAXD and SAXS

WAXD and SAXS experiments were carried out at the Advanced Polymers Beamline (X27C) in the National Synchrotron Light Source, Brookhaven National Laboratory, in Upton, NY. Details of the experimental setup of the X27C beamline were previously reported [38-41] The wavelength used was 0.1371 nm, and the sample-to-detector distances were 129.37 mm and 2392.70 mm for WAXD and SAXS, respectively. A 2D MAR- X-ray detector CCD (Rayonix, LLC, formerly Mar USA, Inc., Evanston, IL) was used for data collection. To prepare the samples for WAXD and SAXS, the native maize starches (ca. 11% moisture) and the digested, freeze-dried maize starches (ca. 4% moisture) were mixed with water to form starch pastes (45% moisture) prior to X-ray detection. The relative crystallinity was calculated by the ratio of the peak areas to the total diffractogram area [42].

Statistics

Means and standard deviations were calculated for all collected digestive data. All values are expressed as means ± the standard deviations. The significances of differences between

groups were compared using two-sample t tests (Excel 2003). P values (two-tailed) of less than 0.05 were considered to be a sign of statistical significance.

Results and discussion

RDS, SDS, and RS content of maize starches

Because the molecular structure of maize starch is determined by the amylose and amylopectin fine structure [4], starches with a full range of amylose content percentages were studied. All digestions with α-amylase and amyloglucosidase (**Table 5.1**) were within the acceptable range of previously reported data [2, 4, 10, 11, 20] Results from the modified digestion methods with amyloglucosidase only or with α-amylase only are also presented in **Table 5.1**. The RS content increased, whereas the SDS content decreased, with the increasing percentage of amylose present in the maize starch samples. RS content of the waxy maize and normal maize starches increased from 4.3 to 74.3%, and 29.7 to 76.5%, respectively, when amyloglucosidase was not used. In contrast, when α-amylase was not used, RS content of the waxy maize and normal starches was 41.5 and 66.0%, respectively, suggesting that amyloglucosidase plays a more important role in digestion of starches with low amylose content.

Amyloglucosidase was thought not to affect the digestion results. In the determination of resistant starch content, α-amylase is believed to be the most important enzyme to measure digestion of the starch fractions, whereas amyloglucosidase is employed to combat any potentially inhibitory factors on α-amylase [2, 4]; however, when amyloglucosidase or α-amylase was not used during the digestion, results were dramatically different (**Table 5.1**). When comparing digestion with α-amylase and amyloglucosidase, the digestion without amyloglucosidase yielded an RS content increase in all starches, but the increase in RS content

was not equal among the starches: A greater increase in RS was observed in waxy maize and normal maize starches. Our results are in agreement with the work by Kimura and Robyt (1995), who reported that waxy and normal maize starches were more susceptible to amyloglucosidase hydrolysis than high amylose maize starch [43]. Previously, in studying wheat starch, Shetty et al. showed that pure amyloglucosidase can attack native starch [44].

Studying the effects of amyloglucosidase in conjunction with the effects of α-amylase was essential to determine the synergy of the enzymes as they work together. Amyloglucosidase and α-amylase worked together in digestion of native starch granules. The impact of amyloglucosidase was greater on waxy maize and normal maize starches compared with high-amylose starches. No stepwise, linear correlation was seen in the rapidly digestible or slowly digestible starch content in methods of digestion with α-amylase added.

HPAEC was used as a secondary method to confirm the results of the colorimetric *in vitro* test. Of the HPAEC RS determinations (**Table 5.2**), only normal and waxy maize are reported for comparison. HPAEC data were within range of both our results reported from the colorimetric determination and previously reported data [4, 20]. To samples without amyloglucosidase initially added, amyloglucosidase was later added, remote to any remaining starch granules, to convert the α-amylase digested material in solution to glucose for analysis via the colorimetric method and HPAEC. Without full conversion to glucose, digested material would be present, but would remain unrecognized by the colorimetric or identification of glucose by HPAEC.

Significant differences were found between enzyme addition methods. Notably, amyloglucosidase could change the outcome 30-60% of the expected slowly digestible starch fraction. No official AOAC method exists for SDS measurement. Of the available methods, the

Englyst method of digestion [2] is often used to measure SDS because it was designed to calculate this nutritional concept. Only from *in vivo* studies has SDS been found to alter gastrointestinal response [41].

Starch granular morphology before and after enzyme digestion

SEM photomicrographs provide an overall picture of digestion for multiple granules and are grouped together for comparison (**Figure 5.1-2**). Maize starches containing amylose are naturally irregularly shaped [45]. Classically, SEM starch digestion analysis focuses on comparing the uniformity of sample, pore, and digestive residue geometry [25, 41, 45]. The digestion of native starches by amylases seems to be inversely related to the amylose content [15, 45]. For high-amylose starches, granules with pinholes were observed after 2 h *in vitro* digestion, but the majority of digestive residues still resembled the native starch granules (**Figures 5.1D and 5.2D**), suggesting that digestion on high-amylose maize starches were heterogeneous. Using transmission electron microscopy (TEM), Evans and Thompson [20] reported that after digestion, most of the residual granules from high-amylose maize starches showed little evidence of digestion [7, 15], and partially digested granules had a radial digestion pattern [15, 45, 46] in the interior.

Figures 5.1 and 5.2, A1-D1 show the native starches and provide objective views of the basic morphology in undigested starches. In **Figure 5.2**, magnification is at 5000× to examine the details of digestion within the same sample and across the sample set. Degradation and digestive progression decreased as amylose content increased. In consistent with the level of RS content (**Table 5.1**), waxy maize starch granules were extensively digested by α-amylase and amyloglucosidase (**Figure 5.1A2 and 5.2A2**). Amyloglucosidase alone generated more and large pinholes on waxy and normal maize starches (**Figure 5.1A4 and 5.5A4**) compared with the granules digested by α-amylase only (**Figure 5.1A3 and 5.2A3**). It has been suggested that

during digestion, enzymes migrate inside the granule [4, 45, 47]. The digestion of the starch material has been theorized to occur from the inside, and enzymes return to the surface after all material is consumed [48]. A side-by-side mechanism has been proposed to explain the digestion of amylose and amylopectin as well as crystalline and amorphous regions in normal maize starch granules [4]. The digestion via pores route [4] is applicable only in A-type starches [45]. Amyloglucosidase degrades maize starch surfaces in a surface pattern resembling the native starch pore distribution [4, 46, 47].

Molecular size distribution

The GPC chromatograms visually portray the molecular size distribution for each starch and enzyme combination before and after digestion (**Figure 5.3A-D**). Dextrans with different molecular weights (MW) were used to calibrate the columns in this study. An equivalent molecular size to dextran standards was used. However, the absolute MW was not obtained [49]. For native starches, the peak around the retention time (RT) of 18 min represented amylopectin, whereas the peak around the RT of 23 min (about $1.6x10^5$ g/mole) was amylose. Although SEM microphotographs provide a visual of the degradation, GPC analysis displays the relative MW distribution of products digested by the enzymes [4, 14, 37]. The molecular size distribution of waxy maize with amyloglucosidase appeared to follow the same trend as the native starch, reflecting the localized attack of the granules by amyloglucosidase. The waxy maize digested by α-amylase had more low MW fraction and was detected around the same retention time (RT) as the samples digested with amyloglucosidase and α-amylase (**Figure 5.3A**). Chromatograms for normal maize with amyloglucosidase and with α-amylase had a large peak at 23 min RT (about $1.6x10^5$ g/mole), whereas the normal maize starch digested by both α-amylase and amyloglucosidase had more low MW molecules appearing at 31 min RT (about $1.5x10^3$ g/mole)

(**Figure 5.3B**), suggesting a synergistic attack by the two enzymes. For the moderately high-amylose (57%) maize starch, the area under the peak starting at 18 min RT for the combined digestion with amyloglucosidase and α-amylase was notably smaller than that of the native and two modified digestions (**Figure 5.3C**). For the starch with 71% amylose, a shoulder was noted at 29 min RT for the residues digested by α-amylase and amyloglucosidase and a smaller shoulder was observed for the residues digested by α-amylase only (**Figure 5.3D**). The relative small changes in molecular size distribution between native high amylose starches and digestive residues suggest that digestion on high-amylose starches was heterogeneous and limited.

Starch structure by SAXS and WAXD

Figure 5.4 shows the synchrotron SAXS curves of four native starches and their digestion residues. For native waxy maize and normal maize starches, the 9 nm lamellar peak was clearly observed at q 0.65 1/nm (**Figure 5.4A, B**). This peak was thought to be attributable to the alternative repeating stacks of amorphous and crystalline lamella in starch granules [50-52]. Due to lower amylopectin content, fewer crystalline lamella were formed for high-amylose starch granules, resulting in a broader and less clear lamellar peak (**Figure 5.4C, D**).

For waxy maize starch (**Figure 5.4A**), the 9 nm lamellar peak remained intact when digested by amyloglucosidase only, decreased by α-amylase only, and disappeared with the combination of α-amylase and amyloglucosidase. These results indicated that α-amylase had more profound effects on the lamellae structure of waxy maize starch than amyloglucosidase. For normal maize starch (**Figure 5.4B**), the 9 nm lamellar peak remained the same as that of native starch when treated by amyloglucosidase only, α-amylase only, and a combination of α-amylase and amyloglucosidase, signifying that these two enzymes had similar digestion effects on normal maize starch. After digestion by amyloglucosidase only, α-amylase only, and

combined α-amylase and amyloglucosidase, the 9 nm lamellar peak of the high-amylose maize starches was still evident but decreased (**Figure 5.4C,D**), suggesting that bulk amorphous starch within the amorphous growth rings was hydrolyzed [53]. However, this hydrolysis was relatively small because little change in molecular size was observed (**Figure 5.3**) and the degree of crystallinity of the starch was not increased (**Figure 5.5D**).

Synchrotron WAXD curves of four native starches and their digestion residues are shown in **Figure 5.5**. Native waxy maize starch and normal maize starch showed a typical A-type X-ray diffraction pattern (**Figure 5A,B**), whereas two native high-amylose maize starches displayed a B-type starch structure (**Figure 5C,D**). Compared with native starch, the relative crystallinity of waxy maize starch decreased from 37.8% to 29.2% and 26.9%, respectively, after digestion by both α-amylase and amyloglucosidase and α-amylase only, but increased to 56% when digested by amyloglucosidase only (**Table 5.3; Figure 5A**). The degree of crystallinity of normal maize starch undigested, digested by both α-amylase and amyloglucosidase, α-amylase only, and amyloglucosidase only was 34.7%, 26.4%, 23.5%, and 32.6%, respectively (**Table 5.3; Figure 5.5B**). Similar trends were observed for high-amylose maize starches (**Table 5.3; Figure 5.5C, D**). Our results suggested that α-amylase was able to hydrolyze both crystalline and amorphous regions for all maize starches and were consistent with the side-by-side mechanism [4]. Previous studies also show that α-amylase hydrolyzes both amorphous and crystalline domains of wheat starch and maize mutant starches [54]. In addition, a new α-amylase from *Anoxybacillus flavothermus* [55] and a fungal α-amylase from *Rhizomucor sp.* [56] were reported to be very efficient in hydrolyzing the crystalline fraction of maize starch.

Our experiments noted significant differences between enzyme addition methods. Amyloglucosidase had a significant impact on SDS and RS content of granular maize starches.

Amyloglucosidase digestion was greater for waxy maize and normal maize starches than for high-amylose maize starches. The extent of enzyme digestion is largely controlled by the granule architecture and diffusion of the enzymes within densely-packed starch granules. Future project aspirations involve applying knowledge of enzyme digestion and synergy to starch morphological impacts on digestion to diagram the influence of resistant starches in the human digestive system as functional foods.

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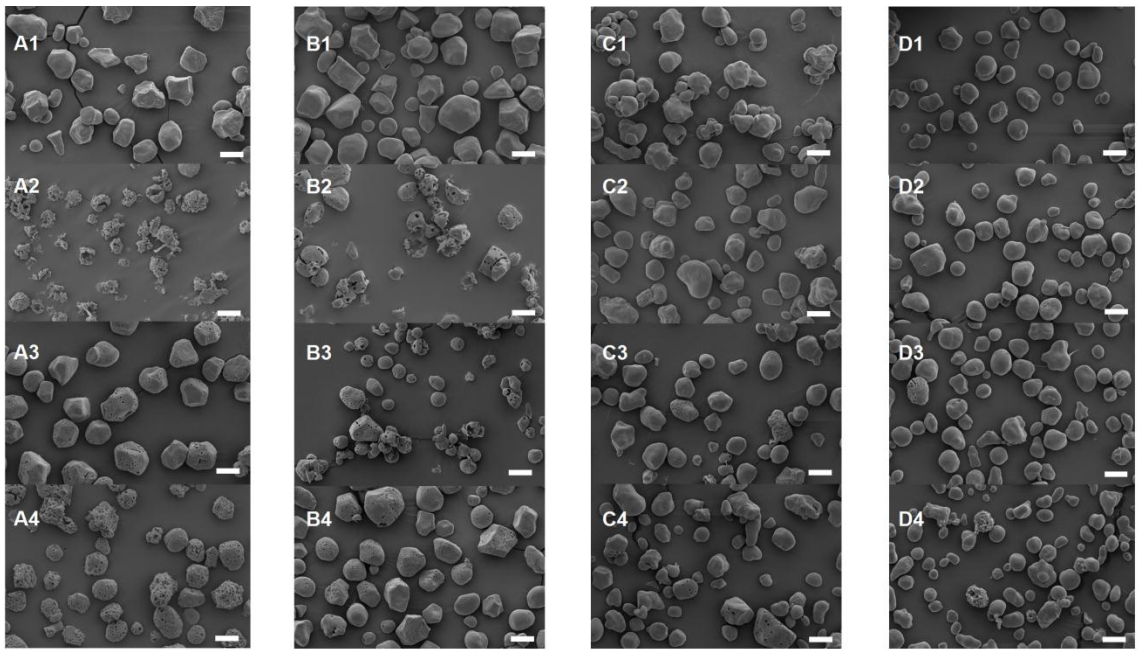

Figure 5.1. Scanning electron micrographs of maize starch at 1000X magnification with 10 µm scale bars: A) waxy, B) normal, C) HYLON V, and D) HYLON VII maize starch are all represented with 1) native maize starch, 2) maize starch after digestion with α-amylase and amyloglucosidase (control method), 3) maize starch digestion with α-amylase only, and 4) maize starch after digestion using only amyloglucosidase in the method.

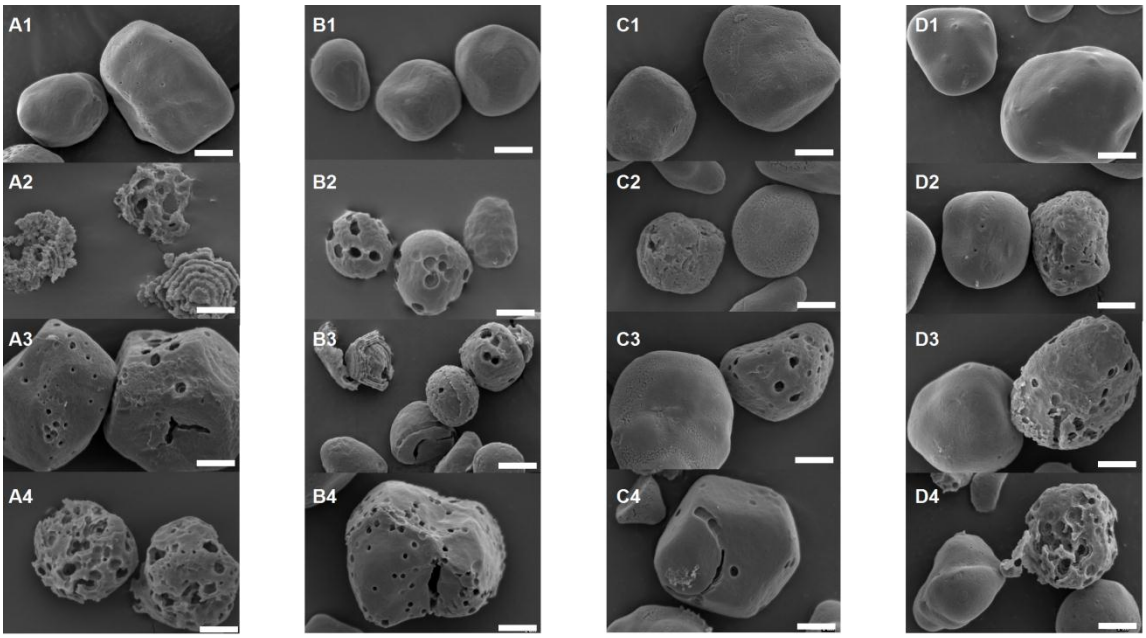

Figure 5.2. Scanning electron micrographs of maize starch at 5000X magnification with 3 µm scale bars: A) waxy, B) normal, C) HYLON V, and D) HYLON VII maize starch are all represented with 1) native maize starch, 2) maize starch after digestion with α-amylase and amyloglucosidase (control method), 3) maize starch digestion with α-amylase only, 4) maize starch after digestion using only amyloglucosidase in the method.

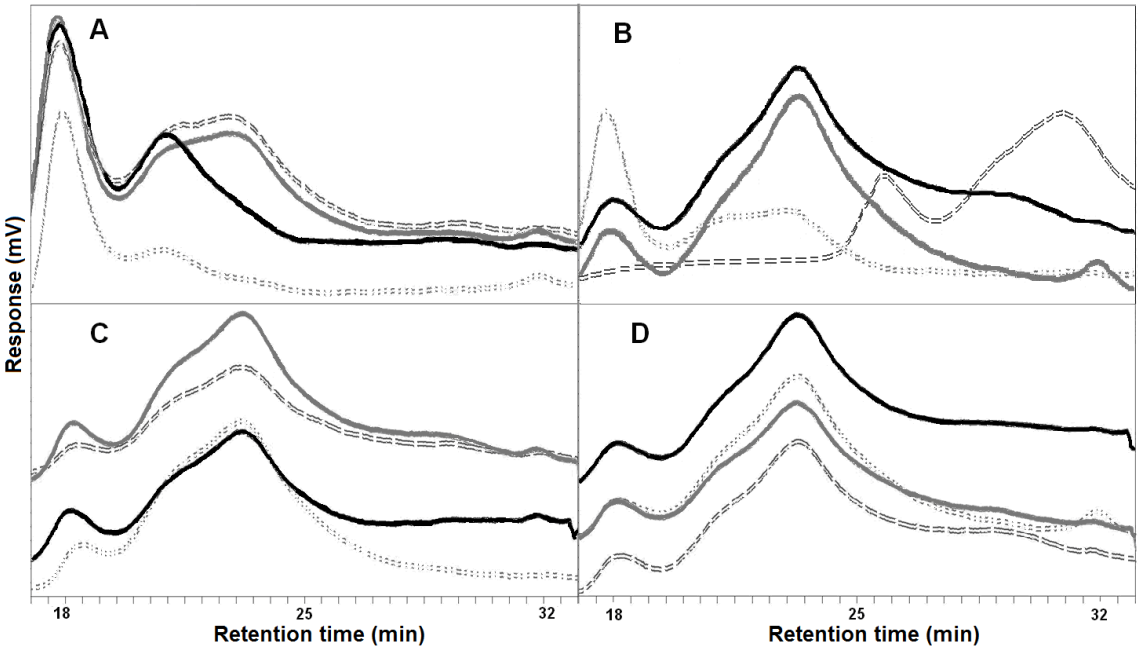

Figure 5.3. Gel permeation chromatography plots of retention time versus response: A) waxy, B) normal, C) HYLON V, and D) HYLON VII maize starch. For all chromatographs, the patterns are as follows: native (dot), maize starch after digestion with alpha amylase and amyloglucosidase (dashed), maize starch digestion with only α-amylase (gray) and maize starch after digestion using only amyloglucosidase (black).

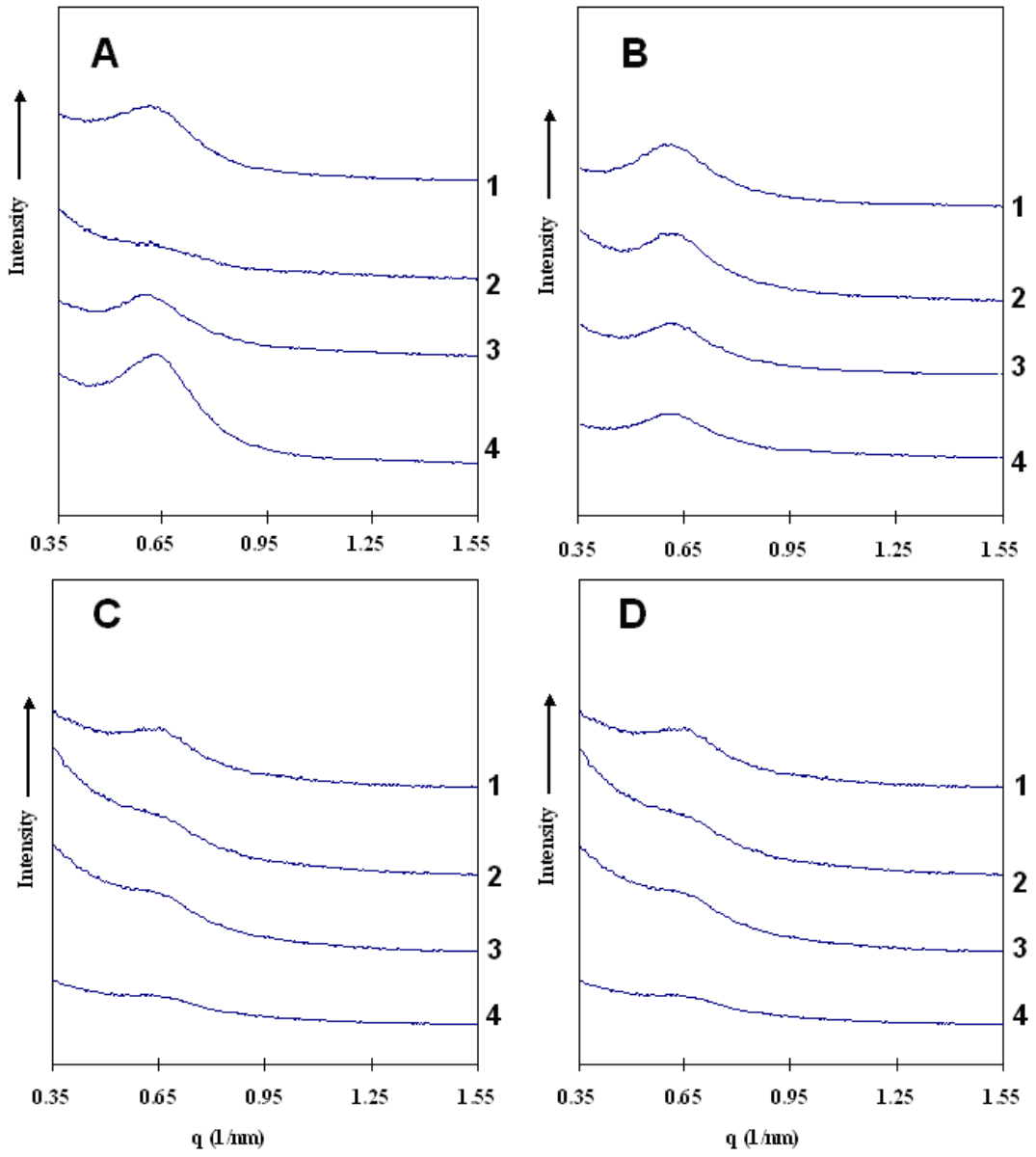

Figure 5.4. Synchrotron small -angle X-ray scattering curves of native starch (1) and native starch digested by α-amylase and amyloglucosidase (2), by α-amylase only (3), and by amyloglucosidase only (4) in descending order for A) waxy, B) normal, C) HYLON V, and D) HYLON VII maize starch.

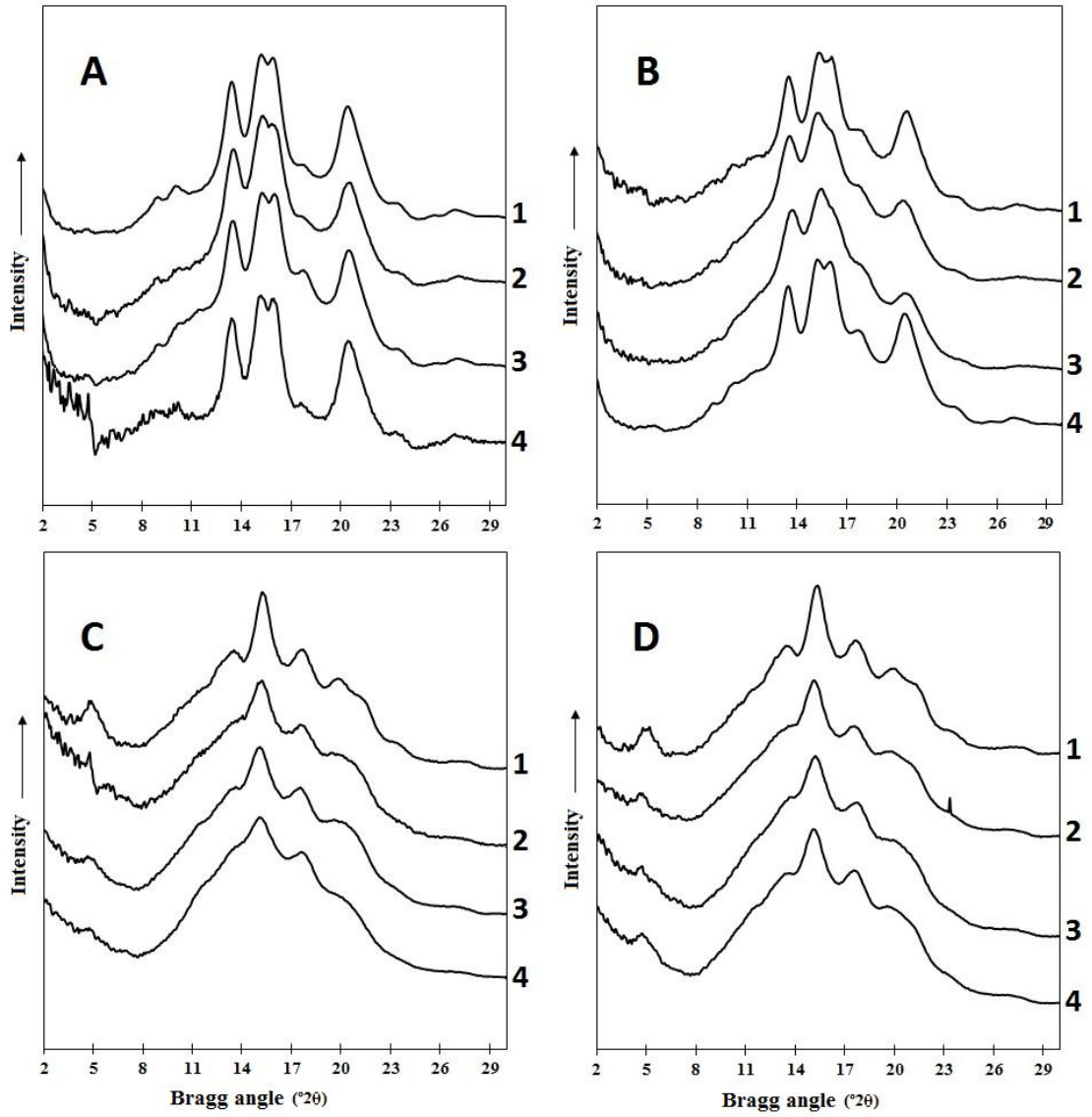

Figure 5.5. Synchrotron wide-angle X-ray diffraction curves of native starch (1), native starch digested by α-amylase and amyloglucosidase (2), by α-amylase only (3), and by amyloglucosidase only (4) in descending order for A) waxy, B) normal, C) HYLON V, and D) HYLON VII maize starch.

Table 5.1. Rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) *in vitro*. Rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) *in vitro* results as determined by α-amylase and amyloglucosidase digestion and modified digestive methods.

maize starch	α-amylase and amyloglucosidase digestion (control method)			digestion with α-amylase			digestion with amyloglucosidase		
	RDS%	SDS%	RS%	RDS%	SDS%	RS%	RDS%	SDS%	RS%
waxy	29.0±0.4aA	67.2±0.4	4.3±0.8	10.2±1.8	15.5±2.2	74.3±1.0F	31.8±2.2aA	26.6±4.1	41.5±3.8
normal	15.1±1.3B	50.2±2.8	29.7±2.0c	5.4±0.7bC	18.1±2.8D	76.5±0.4F	5.2±0.7bC	28.8±1.5c	66.0±2.4
HYLON V	5.9±0.3C	28.3±0.8	65.8±0.7	2.2±0.2d	9.8±0.8DE	88.0±0.7F	1.6±0.7d	19.3±0.8D	79.1±0.3F
HYLON VII	7.7±0.1C	24.0±1.8	68.2±0.7	4.3±1.0eC	5.3±1.5eE	90.4±2.1G	0.8±0.5	4.7±1.2eE	94.4±1.8G

Data with lowercase letters indicate no significant difference at (P>0.05) for the sample. Capital letters denote no significant difference among category groups (P>0.05); n=4

* Values shown are mean ± standard deviation

** Starch portions are reported in percentage of sample

Table 5.2. Resistant starch (RS) as determined by high-performance anion-exchange chromatography (HPAEC). Resistant starch (RS) as determined by the α-amylase and amyloglucosidase digestion method and modified methods *in vitro* results analyzed via high-performance anion-exchange chromatography (HPAEC).

maize starch	enzymes used in starch digestion	RS %
waxy	Amyloglucosidase and alpha amylase	2.7±1.1
	Alpha amylase	71.2±4.8ab
	Amyloglucosidase	56.7±4.3a
normal	Amyloglucosidase and alpha amylase	32.1±4.7
	Alpha amylase	78.4±0.5b
	Amyloglucosidase	69.5±2.5b

Data with like letters are not significantly different ($p>0.05$); n=3

* Values shown are mean ± standard deviation

** Starch portions are reported in percentage of sample

Table 5.3 Crystallinity of native starch and native starch digested starches. Crystallinity of native starch and native starch digested by α-amylase and amyloglucosidase, by α-amylase only, and by amyloglucosidase only in descending order for A) waxy, B) normal, C) HYLON V, and D) HYLON VII maize starch.

maize starch	crystallinity (%)			
	undigested	α-amylase and amyloglucosidase digestion	digestion with α-amylase	digestion with amyloglucosidase
waxy	37.8±0.3	29.2±0.4	26.9±0.1	56.0±0.6
normal	34.7±0.3	26.4±0.1	23.5±0.4	32.6±0.1
HYLON V	28.3±0.4	21.3±0.3	17.7±0.4	17.4±0.1
HYLON VII	23.7±0.3	17.5±0.1	17.6±0.3	17.2±0.4

Chapter 6 - Glycemic response and breath hydrogen testing by human consumption of crystalline short-chain amylose

Abstract

Background

Starch is a major component in cereal foods and an important source of food energy. Understanding starch digestibility is of great interest to the food industry and of importance for diet-related disorders such as obesity, diabetes and cardiovascular disease. Glycemic response is a nutritional parameter used to classify foods high in carbohydrate. The glycemic index (GI) of a food is used to classify foods based on their potential to raise postprandial blood glucose levels in comparison to an equal available carbohydrate amount of glucose. Not all starch and non-digestible oligosaccharides (NDO) can be digested in the small intestine, and the undigested portions are termed resistant starch (RS). A high portion of slowly digestible starch and/or RS + NDO indicates a low GI. However, RS and NDO have modified dietary fiber assays, and so available carbohydrate may not be reflected *in vivo* results. Recent methods used to calculate available carbohydrate include RS and NDO in total dietary fiber, which affect GI value.

Methods

Crystalline short-chain amylose (CSCA) was produced by a one-pot procedure wherein waxy maize starch was enzymatically debranched with continued stirring at 50°C for 24 h to produce CSCA. CSCA gave 100% carbohydrate when assayed by AOAC Method 996.11, and 0% dietary fiber by AOAC Method 991.43, which translate erroneously to 100% “available carbohydrate”. RS content was determined by the Englyst assay (1992). CSCA was used as a substrate to produce glycemic curves within ten young adults. Using standard methods of the GI, this

controlled trial compares blood glucose response of 50 g available carbohydrate: waxy maize starch (TDF: 0%, RS: 0.6%; the pre-modified starch material), to CSCA, (total dietary fiber 0% and 75% RS). CSCA was consumed either alone or with glucose, to observe its effect in a mixed formulation. Breath hydrogen tests were also provided every 30 min, over a period of 6 h. Breath samples were analyzed on a Quintron BreathTracker SC, using carbon dioxide as an internal reference to normalize hydrogen (H_2) and methane (CH_4) production (ppm).

Results

All volunteers had lower postprandial glucose curves with consumption of CSCA. The rise in glucose (mmol/L) for 25 g CSCA with 25 g glucose was similar to the rise observed with 50 g glucose solution, however, 25 g CSCA with 25 g glucose had a slightly (yet, significant $p<0.05$) reduced peak height and extension. After 50 g available carbohydrate consumption, the following percentage of volunteers passed below baseline at 2 h: waxy maize starch (80%), glucose (50 %), CSCA (20%), and 25 g CSCA to 25 g glucose (0%). CSCA was correlated with subjects' return to basal blood glucose levels at 2 h. After breath hydrogen testing, the minimum production levels for the population with 50 g CSCA samples were 2.50 ppm H_2 and 2.00 ppm CH_4; peak values were 22.38 ppm H_2 and 7.00 ppm CH_4. One volunteer provided elevated H_2 production after 150 min with all samples. Ninety percent of the population produced CH_4, and 70% produced CH_4 with every sample. It was noted that after subtraction of the baseline, the majority of volunteers had lower postprandial fermentation curves with consumption of CSCA over that of waxy maize starch.

Conclusions

Our study concluded that 50 g available carbohydrate of CSCA, determined by the Total Starch AOAC Method 996.11, was not entirely available *in vivo*. In mixed formulation, 25 g CSCA

with 25 g glucose gave a significant effect on extension of plasma glucose elevation within curves, as well as on the initial raise. Four volunteers elevated H_2 production with CSCA and four elevated H_2 production with waxy maize starch. For the population, CSCA did not produce elevated H_2 production more than other samples within the trial. This study shows that the RS content of a food affects glycemic response and GI values, as well as addresses the functions of RS in the colon with noninvasive measures for carbohydrate metabolism.

Introduction

Type 3 resistant starch (RS) is defined as any starch or portion that is resistant to digestion by retrogradation or recrystallization, thereby escaping digestion within the small intestine (1, 2). Retrogradation occurs when heated carbohydrates are cooled (3), usually in the presence of moisture. RS notably alters fecal bulk and short-chain fatty acid metabolism, promoting colonic health (4), and impacts putative markers of colonic health in humans (5-7). However, *in vivo* research on RS digestive mechanism and fermentation are sparse. The role and mechanism of RS in diet-linked diseases such as diabetes, cardiovascular diseases, obesity, or gastrointestinal cancers, is still not well understood. Since RS is not degraded to glucose for passage from the intestinal lumen, across the epithelium to blood, there is a change in glycemia and insulinemia when compared the same quantity of digestible starch. As RS functions in shifting energy value, RS could be beneficial in diabetes management as well as weight management, based on recently developed concepts of energy regulation and macronutrient intake (8).

Due to interest in starch digestibility in relation to carbohydrate nutrition, many researchers have applied debranching techniques to prepare RS (9-13). Detailed structural changes after debranching have been explored recently (14, 15), however, more unknowns

remain. The study of non-digestible oligosaccharides (NDO) has accelerated since their recent association within health and nutrition (16). NDO are a diverse group of monosaccharides configured in multiple arrangements, by multiple methods, with many only slightly differing in chain length (17), that are classified by molecular size or degree of polymerization (18). A detailed review is available on the role of NDO in promoting health by fiber-like and prebiotic actions (19). Several commercial type 3 RS such as NOVELOSE 330 (National Starch, LLC, Bridgewater, NJ), CrystaLean (SunOpta Ingredients Group, Chelmsford, MA), C*Actistar (Cargill, Minnetonka, MI), and Neo-amylose (Aventis Research & Technologies GmbH, Frankfurt, Germany) have interesting digestive properties due to their reduced chain length by modification. The *in vitro* ability to provide protective effects to prevent carcinogenesis have been investigated with retrograded tapioca starch, produced by the methods of U.S. Patent 6 043 229 (obtained from Cerestar-Cargill (Vilvoorde, Belgium) (20). While, *in vivo*, researchers noted the most important colonic effect observed was the high fermentation rate of type 3 RS to high proportions of butyrate (12, 21). When C*Actistar-RS3® was fermented *in vitro*, there was significantly faster substrate disappearance, higher total short-chain fatty acids (SCFA) and butyrate production after 8 h of incubation (22).

The Glycemic Index (GI) ranks foods on how rapidly and extensively blood glucose level is elevated within a set of established parameters (23, 24), but controversies with the GI exist and have been reviewed (25). The nutrition community is divided over the usefulness and reliability of the GI in diabetic management and in the prevention of dietary related chronic diseases, such as obesity, insulin resistance, cardiovascular diseases or certain cancers (25-27). The use of the GI is controversial for many reasons (25, 28), such as: variability in blood sampling (23, 24), carbohydrate determination, sample size and preparation (25), variability between wheat cultivar

(if using a white bread standard) (29), calculation of response (27, 30-33), impressionable human variation, and variation between laboratories (34). Controversy and variability between foods may be due to factors that affect carbohydrate structure, preparation (hydration/temperature), starch retrogradation, level of starch resistance, or starch modifications. For example, modified starches with phosphate esters are less digestible than native of the same type (35), and all structural modifications should be considered as separate ingredients. A recent review describes the controversies with the GI in detail (25).

The GI uses a 50 g available carbohydrate sample, not including total dietary fiber (TDF), as the standard for determination (36). TDF is an *in vitro* measure of soluble and insoluble fiber content (37). The FAO/WHO has defined the method to calculate available carbohydrate for the GI, as the "total carbohydrate minus dietary fibre, as determined by the AOAC method". The organization also notes, the AOAC method does not include RS when they are present (38). Chemical, chromatographic, colorimetric, electrophoretic, enzymatic, gravimetric, and titration methods can be used to measure carbohydrates (37). While the majority of early GI publications rarely report the method for measuring available carbohydrates, the use of total glycemic carbohydrates [total starch plus free glucose (2)] (39), AOAC Method 996.11 (40, 41), or values from food tables/ manufacturers' information (42, 43) have been cited as the determining method for calculating available carbohydrate. Some researchers recognize the measurement of available carbohydrate content leads to overestimations in the presence of RS fractions that are not recorded as dietary fiber (27, 44). Only a few researchers using the GI explain carbohydrate in adequate detail (45), or provide the method of carbohydrate determination (46, 47). The latest AOAC International definition of dietary fiber was approved by the Codex Alimentarius Commission's Committee on Nutrition and Foods for Special Dietary Uses to incorporate the

development of AOAC Method 2009.01 and 2011.25 and advances in fiber research, and describes dietary fiber as carbohydrate polymers of 10 or more monomeric units, which are not hydrolyzed by endogenous enzymes within the small intestine of humans. The use of TDF in the GI calculation of available carbohydrate is to account for material that is present, but indigestible. However, since the development of the GI method, additional dietary fiber determinations have been established (48). While the available carbohydrate test sample and reference food should not include RS, practitioners believe RS is difficult to appropriately measure in clinical settings (28). Determination of available carbohydrate for the GI method with the use of commonly utilized AOAC methods to determine dietary fiber (985.29 or 991.43), in addition to a specified method (resistant starch – 2002.02, resistant maltodextrins – 2001.03, polydextrose – 2000.11, transgalactoligosaccharides – 2001.02; to name a few) can doubly account for portions of constituents. A new integrated total dietary fiber method (AOAC Method 2011.25) attempts to incorporate RS, TDF and resistant maltodextrins in one method (48). This procedure also includes measure of available carbohydrate. Currently, the GI methods do not specify the use a particular dietary fiber method. Developers of the GI method are aware that certain measurements of dietary fiber do not measure carbohydrates appropriately (24) and underestimation of 'available carbohydrate' are present in some published reports (28).

Within healthy humans, carbohydrate digestion begins with mastication and salivary α-amylase, with a majority of fermentation being initiated in the large intestine. During fermentation of polymers comprised of glucose molecules, anaerobic bacteria produce acids, water and gases (49). SCFA production varies with diet and demographic (50), yet the major SCFA derivatives produced from starch digestion are: acetate, butyrate and propionate (51, 52). Acetate is highly produced from starches and is used as an energy source (51), while butyrate is a

preferred energy source of healthy colonic epithelial cells (53) and increases with RS consumption (51, 54-56). Propionate is utilized for energy and gluconeogenesis. Production levels of SCFA have been associated with healthy colonic function (51) by lowering colonic pH, inhibiting growth of pathogenic bacteria, reducing secondary bile acids and increasing mineral absorption (57-60). The major gases produced by anaerobic bacteria in the human colon are: carbon dioxide (CO_2), hydrogen (H_2), methane (CH_4). During metabolism, CO_2 is produced by all cells, but only bacteria can produce H_2 and CH_4 as metabolic by-products (61). A portion of H_2 produced is absorbed into the lungs and excreted with expelled air. Analysis of air expelled through the mouth provides 'semi-quantitative' and qualitative estimations of H_2 production rate by colonic bacteria (61), depending on calculation used and substrate (62). Contamination of the sample with room air and dead space gases is associated with reduced H_2 concentrations (63). An optimum sample is collected at the end of the breath, after clearing the dead space air from the digestive system.

Breath hydrogen testing is usually executed as a functional analysis of the intestine to investigate carbohydrate malabsorption in humans (64, 65). Malabsorption of carbohydrate results in increased H_2 production from fermentation, which can be measured, postpradinal, at as low as 2 g carbohydrate (66). The amount of H_2 produced is proportional to the amount of malabsorbed carbohydrate (67). However, further experimentation is needed to determine the strength of manipulation for various attributes (time, hydration, environment, substrate, etc).

Methane may be monitored during the breath hydrogen test. Studies have shown there is difficulty in interpreting CH_4 production, as variability in overall CH_4 response differs between volunteers (68, 69). For CH_4 production, adults are broken into two categories: producing and nonproducing. Two-thirds of the population is nonproducing, having zero readings or producing

less than 1 ppm above atmospheric CH_4 (atmospheric CH_4 is approximately 0.8 ppm). Methane producers (approximately $^1/_3$ of the population) have mean CH_4 concentrations of about 23 ppm. Breath hydrogen testing is based on the assumption that colonic flora are constant and/or consistent (67), and measurement methods have been reviewed and validity discussed (63, 65, 70). Although human subject to subject variation is present and quantification of carbohydrate is equivocal, the test is highly sensitive, and has promise for studying malabsorbed carbohydrate (63).

Studies utilizing breath hydrogen testing with RS consumption have been reported (7, 71, 72). When breath hydrogen testing was used as a measurement for RS, acute consumption of RS showed increased fermentation through elevated breath hydrogen excretion (7, 72); however, less than satisfactory results have been discussed (62).

The crystalline short-chain amylose (CSCA) used in this study gave 100% carbohydrate when assayed by AOAC Method 996.11, and 0% dietary fiber by AOAC Method 991.43. CSCA is considered available, based on the current GI concept. It is hypothesized that RS is "probably not the cause for differences" in GI, although RS "content may still be of importance in relation to glucose metabolism" (73), however, confirmation is needed. This study uses starch type, preparation, and processing (27) to affect GI value and fermentation. *In vivo* investigations of CSCA have not been previously reported, and the glycemic response and fermentation of CSCA in human subjects is investigated in this study.

Materials and Methods

Production of CSCA

Waxy maize starch (Amioca starch, Batch #: MD-8230) and HYLON VII were obtained from National Starch LLC (Bridgewater, NJ). Pullulanase (EC 232-983-9) was obtained from

Genencor International, Inc. (Rochester, NY). Type 3 RS was produced by debranching waxy maize starch, as previously reported (15), with modification to the enzyme source. Waxy maize starch was mixed with distilled water to produce a 25% solids (by wt.) slurry. The slurry was adjusted to pH 4.0 by adding 0.5N hydrochloric acid and monitored with a calibrated pH meter. The slurry was cooked at 115–120 °C in a Parr reactor (Parr Instrument, Moline, IL) for 10 min, and cooled to 50 °C. Pullulanase (dwb; 1.4×10^6 IAU, 1.0% based on dry starch) was added, and kept 24 h, at 50 °C, with constant stirring. After 24 h, the precipitates of crystallized solids were filtered, washed with deionized water, and dried in an oven at 40 °C for 12 h. Yield of CSCA, by this method, was reported as 60.7% (14). [**Appendix H** provides visual representation of CSCA as a finished product and in preparation as supplemental clarification on the material.]

General methods

For all samples, moisture content was analyzed by a standard method (AOAC Method 925.10). Total carbohydrate was determined for waxy maize starch and CSCA by AOAC Method 996.11, not including TDF, as determined by AOAC Method 991.43. Starch samples were analyzed for %RS, as previously reported (2, 35). [**Appendix I** provides visual representation of RS and TDF determination for sample comparison with these *in vitro* analyses.]

Measurement of D-Glucose was performed on the 50 g commercial glucose solution (Sigma Aldrich, Inc., St. Louis, MO) provided to all volunteers alone and in mixed formulation. D-Glucose was measured using a D-Glucose Kit from Megazyme glucose assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland).

Ethics

All procedures and methods described herein involving the use of human subjects were approved by the Institutional Review Board for Protection of Human Subjects Committee (IRB)

at Kansas State University (KSU). Informed consent for sampling and the use of this data was obtained from each subject. All investigators completed IRB human test subject training. In addition, all investigators collecting human samples have conducted an occupational health and safety program, and blood borne pathogen training/certification with the KSU Research Compliance office, Manhattan, KS, and the American Red Cross, Kansas City, KS, respectively. Hazardous material methods, of handling and disposal, were followed.

Subjects

Volunteers were openly solicited and completed an interest screener prior to inclusion. The random pool contained ten free-living subjects (4 women; 6 men) between the ages of 20-30, whose fasting glucose levels were measured prior to sampling. The use of ten volunteers is implemented when assigning a GI value for a food product (24). Subjects with history of chronic disease, who were pregnant/lactating, or who had gluten/glucose intolerance, were excluded from this study. Proceeding each study day, participants were required to consume a standard evening meal, fast overnight, and avoid strenuous exercise, alcohol, or change in normal diet. During the study, each volunteer visited the laboratory after overnight fasting for 10 h on four separate occasions over a three-week period; up to two visits/week, with at least one washout day between visits. Gas excretion is affected by diet and fasting (74), so all volunteers were asked to fast overnight, to begin at basal levels. Additionally, 60-70 g carbohydrate per day has been estimated to be required for maintenance of the bacterial population within the colon (75, 76), therefore volunteers were only allowed to visit twice per week to maintain a homeostatic state. Non-fermentable snacks (≤ 20 g available carbohydrate) were provided to the volunteers, upon request, after completion of the blood glucose measurement (2 h). Non-fermentable snacks

included eggs, meats, crackers and candies containing no fermentable ingredients; as determined from the listed ingredients on the manufacturer label.

Glycemia

The trial was designed as a controlled, cross-over trial. The investigators, research staff and volunteers had access to sample identity during the clinical portion of the study and the order of the samples was randomized (Excel 2003). Methods were designed from a previously reported study (77), with modification to the use of the 75 g glucose tolerance beverage. Prior to testing the trial samples, volunteers completed a glucose tolerance test, as described previously (77), using standard 50 g oral commercial glucose solution (298 mL). On study days, after the fasting blood glucose sample was collected, the designated prandial sample was consumed (within 10 min), time recorded, and blood samples collected, periodically for 2 h, at 0, 30, 45, 60, 90, and 120 min intervals, by finger stick, and immediately analyzed on a 2300 Stat Plus (YSI Life Sciences, Yellow Springs, OH), in duplicate, for glucose (77). [**Appendix J** provides a photograph of the YSI instrument, with explanation of blood sample analysis.] The samples of 50 g available carbohydrate included: CSCA, 25 g CSCA with 25 g glucose, and raw. Raw waxy maize starch was used throughout, as gelatinized waxy maize starch would provide different digestive results (78). All samples were provided in a disposable plastic cup, with disposable plasticware for mixing, and brought up to 298 ml with purified water to match the fluid consumed during the glucose tolerance test.

Glucose incremental areas under the curve (iAUC) were determined using the trapezoid method (GraphPad Prism v 5.02, La Jolla, CA), as published (77). The highest value within 120 min was designated as the peak value, and the baseline was the fasting value.

Fermentation

A noninvasive measure of fermentation was utilized to observe products of CSCA exiting the small intestine. The ability to observe RS exiting the small intestine and of RS to produce products of fermentation was previously confirmed (72). There is no standard of identity for breath hydrogen examination of absorbed carbohydrate; therefore, the experiments were designed as follows.

Initial gas production was recorded (0 h) from each volunteer, using a Quintron BreathTracker SC (Quintron USA, Milwaukee, WI) to measure CO_2, CH_4 and H_2. Subsequently, the randomized trial sample was consumed within 10 min. Sample collection occurred in 30 min increments, over a period of 6 h (occasionally a supplemental reading was recorded for 6.5-7 h). With the exception of glucose, which was monitored for 4 h, as responses from glucose are acute and have been reported to occur within 120 min (63). Previous studies have shown that 6 h should be a minimum for samples of unknown nature and 14 h provides the return of basal value (79). Volunteers collecting their own breath sample worked best in this study and maintained CO_2 at 4.4%±0.4% ppm. For analysis, CO_2 was the internal standard (80, 81) to normalize the samples between readings and from contamination of room air. The presumed alveolar CO_2 concentration was 5.5%, and the degree of dilution corrected for (80). [**Appendix K** provides a photograph of the Quintron instrument, with explanation of breath sample analysis.]

Quality of breath sample can cause variation of the reading (64). To avoid dilution of breath samples with dead space gases in humans, the volunteers were trained to exhale into a quantified volume to obtain end-expiratory samples for analysis (65) and to use Alveosampler kits (QT0087-P, Quintron USA) to collect 20-30 cc/mL in Air-Tite disposable syringes (Products Co., Inc. Virginia Beach, VA). After collection, all samples were analyzed within 2 h. All volunteers refrained from exercise, extended rest, alcohol and tobacco productions during the

testing period. Gas production curves were produced using GraphPad Prism v 5.02 (La Jolla, CA). The highest value within 6 h was designated as the peak value, and the baseline was established at fasting values. Response curves were drawn, however, iAUC was not calculated as sometimes suggested (7, 65, 79), due to duration of monitoring and response incurred. iAUC values for breath hydrogen are better calculated with quickly fermenting samples or samples analyzed between 0 and 840 min (79).

Statistical analysis

Data was analyzed using AVOVA, Tukey's and column statistics within GraphPad Prism 5. Differences between iAUC values and significance among treatments was declared at $P \leq 0.05$.

Results and discussion

CSCA

Waxy maize starch is high in amylopectin, with branched molecules that are almost fully digested by *in vitro* enzymatic action within 2 h (82). Debranching of the amylopectin chains to produce CSCA, increased the starch resistance to digestion (14, 15). It was discovered in long periods of debranching, aggregation and crystallization, the crystallized portion increased, becoming dense and thick in texture. As enzymes have limited access to double helices in CSCA, type 3 RS was formed (15). The RS content of the CSCA produced in this work was 75.0% (**Table 1**). This trial was designed to test the *in vivo* response to CSCA, as the molecular and crystal structures of this type 3 RS have been investigated and published (15).

The use of waxy maize starch to produce CSCA is just one of many modifications to produce resistant carbohydrates. Unlike raw waxy maize starch, raw banana (12), raw high amylose maize (71, 82), or raw potato (9, 71) starches naturally exhibit resistance to digestion (83). Also resistant to activity of amylases are starch pastes containing sago starch and

monoglycerides (84), as well as commercially available C*Actistar, produced from partial enzymatic hydrolysis and retrogradation of tapioca starch (85). While increased resistance by modification has been noted with most starches (12, 14, 85), the hydrolysis of native starches is greatly attributed to botanical origin (86), with smaller granules being more susceptible to enzymatic action regardless origin (87).

RS and TDF content

RS and TDF contents of CSCA, waxy maize starch and HYLON VII are reported in **Table 1**. Waxy maize starch had the lowest RS content (0.6%), while CSCA contained the highest RS content (75%). The TDF content for CSCA and waxy maize starch were statistically the same (0.0%). Waxy maize starch and CSCA had no TDF, but remarkably different RS contents. This *in vivo* trial used waxy maize starch (~100% amylopectin) as a sample for consumption, as waxy maize starch was used to produce CSCA. Starch digestibility and physiological response are greatly attributed to the amylose:amylopectin ratio (45). In most cases, starches with high ratios of amylose are sources of RS, while gelatinized waxy starches are rapidly digestible (78). In addition, starches with high portions of RS differ from starches with large amounts of amylopectin in plasma glucose (postprandial) and insulin response (46, 47). CSCA had higher RS content than type 3 RS products CrystaLean (19.2%–41%) and C*Actistar (53%), but less than Neo-amylose (87 or 95%) (73). Preliminary studies strongly indicate that RS of specified digestive patterns and degrees of resistance are producible with increased sample preparation time (15). A current review offers details of starch structure and digestibility (86), and additional methods are available to determine RS content (88-90).

All samples were tested for RS and TDF contents to confirm the quantification of these *in vitro* analyses prior to human consumption. And the RS and TDF contents for waxy maize starch

and HYLON VII starch were in accordance with previously publications (82, 91). HYLON VII has 71% amylose (92) and was used as a control to confirm the results of the *in vitro* tests. HYLON VII starch was not fed as an *in vivo* response sample, but may have positive fermentation attributes (6, 71, 93). *In vivo* fermentation and glycemic response of HYLON VII has been previously reported (93, 94) Vonk et al (2000) showed fermentation cites of HYLON VII within the colon, in comparison to Novelose 330 (Unilever Research Laboratory, Vlaardingen, Netherlands), with the use of C^{13}. The use of C^{13} confirmed partial digestion/fermentation of HYLON VII *in vivo*.

D-glucose analysis

By spectrometric analysis, the glucose concentration of the commercial 50 g glucose solution was 50±0.14 g of glucose per bottle. The concentration of the control glucose tolerance solution was confirmed by spectrophotometric D-glucose analysis, to confirm the nutritional claim, as well as to confirm accurate available carbohydrate calculations for these experiments.

Glycemia

Glucose response

Figure 1 contains population averages for the glucose responses and iAUC for the trial samples. Glucose responses were reported as population averages for an overall observation of a population, as variability from within volunteers did occur (34). In **Fig. 61.A**, responses of 50 g available carbohydrate treatments: glucose, CSCA, 25g CSCA with 25 g glucose, and waxy maize starch, are depicted as glucose (mmol/L) over time. Diminished peak heights of CSCA and waxy maize starch curve averages show that glucose absorption was lower than that of samples containing the glucose solution (50 g glucose and 25 g CSCA with 25 g glucose). Foods with RS give low glycemic response due to RS transport through the small intestine, undigested

(95). It has been previously reported that RS is likely to be negatively correlated with glycemic response (96), yet there was a similar rise in curves produced from 50 g glucose, compared to that of 25 g CSCA with 25 g glucose. Total glucose concentration of the '25 g glucose to 25 g CSCA' sample may have saturated glucose absorption in some volunteers. The use of high-carbohydrate concentrations, when utilizing the GI methodology, is recommended to optimize responses for comparison (24); some researchers have used <50g available carbohydrate (12, 97) due to ornate food volumes without this modification. It is noted that type 3 RS starches can be dissolved in 0.5-1.0 M potassium hydroxide (98) and is entirely resistant to digestion in near neutral pH by pancreatic amylases. Presumably, the blood glucose response from 25 g CSCA with 25 g glucose is entirely from the glucose present in the sample, as the portion of CSCA that is nonresistant.

Previous studies have noted that uncooked high amylose starches can demonstrate a reduced glucose response (99). Raw waxy maize starch is slowly digestible starch *in vivo* as compared to maltodextrin (100), with similar reports of uncooked normal maize starch compared to glucose (101). The digestibility and therefore, blood glucose level, of a starch will differ depending on whether the starch is consumed raw or cooked. Raw maize starches (normal and waxy) are slowly digesting starches, while high-amylose maize starch is usually characterized as resistant, all partly due to their lamellar semicrystalline and amorphous layers that must be broken down for digestion, systematically resulting in gradual glucose release by enzymatic action (102).

CSCA had low glucose response within the population, as did raw waxy maize starch (**Figure 6.1**). Low blood glucose response may be due to the concentration of the sample in solution, transit time in the digestive system and the need for brush border enzymes to cleave

glucose for absorption (44). A review of slowly digestible starch reported personal communications on the effect of maltodextrin, normal maize and waxy maize starch intake on blood glucose levels of 22 healthy volunteers (12). Researchers noted that with 35 g available carbohydrate, native maize starch exhibited a slower increase of postprandial blood glucose levels, and sustained blood glucose levels for a longer period of time, compared to maltodextrin, which dipped partially under baseline, due in part to fast decline of blood glucose concentration. Their waxy maize starch curve exhibited a peak similar to that of native maize starch, with reduced peak height and area, similar to the population curve for waxy maize starch produced in the current trial. A native maize starch curve with reduced peak height and area was also reported in a patent by Axelsen and Smith (2000) and with the use of PolyGlycopleX® (InovoBiologic Inc, Calgary, AB, Canada) in mixed formation (103). However, PolyGlycopleX® curves have not been graphed within published manuscripts on humans (103, 104) or rodents (105-107) for comparison to current data. In humans, after consumption of PolyGlycopleX®, subjects experienced mild to moderate adverse effects. The volunteers from the current trial reported no adverse effects from CSCA.

CSCA aided in the return to normal plasma glucose and/or glucose stabilization. After consumption of CSCA, only 20% of the volunteers passed below baseline at 2 h (**Fig. 6.1.A**). The remaining volunteers were approximately at baseline value. Another short-chain polymer, maltodextrin has been noted to cause a dip in blood glucose baseline (12). For glucose – 50%, and for waxy maize starch – 80% of all volunteers were below baseline at 120 min; these samples affected the volunteers' glucose absorption differently than CSCA. The return to stable glucose levels is important for diet related disorders and CSCA should be further investigated for additional metabolic responses and at different concentrations in mixed formulations.

The calculated iAUC of CSCA (**Fig. 6.1.B**) significantly differed from the other samples and CSCA curves were lower than the respective glucose curve in all cases (**Figure 6.2**). iAUC is used to calculate and compare GI values between studies (23, 34, 108). High variation between volunteers of iAUC for waxy maize starch caused the sample to not be significantly different from 25 g CSCA with 25 g glucose. The iAUC of waxy maize starch is high due to the extension of the peaks in comparison the other samples. It is possible that a portion of the population was unable to digest raw waxy maize starch; however, further investigation would be required to confirm this.

Table 6.2 lists the curve characteristics for all 50 g available carbohydrate samples. The relative change from baseline to peak value is shown with the peak range for the population and median values for each treatment (**Table 6.2**). The change from baseline to peak (range) was as follows: glucose > 25 g CSCA with 25 g glucose > CSCA > waxy maize starch (raw). The peak range for 50 g CSCA was 4.25–5.57 glucose mmol/L, and calculated as only 3–40% of the iAUC of the glucose peak. The 25 g CSCA with 25 g glucose sample had a peak range of 5.81–9.14 glucose mmol/L and waxy maize starch from 4.82–6.03 glucose mmol/L. The ranges overlapped in most cases, and expressed the high variability between individuals (34). Peak ranges (glucose mmol/L) differed, even though all samples contained the same amount of 'available carbohydrate', by definition of the GI. If the GI for glucose is 100 (109), then GI of CSCA ranged from 3 to 40, with an assigned value of 21. The GI value and RS content of foods are inverse correlated (110). The GI of CSCA was influenced by the sample's modifications, processing and chemical structure. The amount of carbohydrate content in a food is not the only factor determining GI, as multiple factors affect digestion (27, 111). In this study, 25 g CSCA with 25 g glucose had a GI value of 71, and waxy maize starch had a GI value of 50. The high GI

value of 25 g CSCA with 25 g glucose is attributed to the high concentration of available D-glucose from the commercial solution. A recent study to determine the influence of starch substrates to energy expenditure or appetite, waxy maize starch lead to a lowered and extended plasma glucose curve when compared to white bread and maltodextrin (100). Their study did not provide a glucose standard and utilized white bread as the control to calculate total glucose (28), however the calculated reduction of the curve was similar to the current findings.

CSCA had the lowest impact on glycemia and did not appear to initially alter or inhibit the glucose absorption, as noted from the range and median. The median is reported for better depiction of the range values. The median values for the change from baseline to peak were as follows, and were noted to not follow the order of the range values: waxy maize starch > 25 g CSCA with 25 g glucose > glucose > CSCA. The medians (**Table 6.2**) and glucose curves (**Fig. 6.1.A; Figure 6.2**) of CSCA compared to those of 25 g CSCA with 25 g glucose, imply that CSCA also aids to shift either the ability or the use of insulin to control plasma glucose levels (78) and relative reduction of the peak. Adding 25 g CSCA to 25 g glucose invoked curves that were significantly different from the control (glucose solution) GI curve at $p=0.0404$. Treatments peaked blood glucose concentration at various times across the population. In **Figure 6.2**, 60% of the population peaked glucose concentration at the 30 min testing for the standard solution. All volunteers peaked glucose concentration for CSCA at or within 45 min. The response of 25 g CSCA with 25 g glucose was the most variable treatment, and 50% of the population peaked glucose concentration at or before 30 min. For waxy maize starch, 40% of the population peaked glucose concentration at the 45 min glucose testing.

While CSCA had no measured dietary fiber value by AOAC Method 991.43, its classification as type 3 RS proposes CSCA may be a functional fiber (112), and it may contain

dietary fiber with AOAC Method 2009.06 or 2011.25. RS usually analyzes with some TDF value (73), which is a required value by the Nutrition Labeling and Education Act to support any fiber content claims (73). The problem of including RS as a fiber is derived from RS not containing the properties of soluble and insoluble fiber, and has been discussed (48, 73), although there is a tendency for type 3 RS to behave physiologically as a fiber (113). By GI of type 3 RS without TDF value (CSCA), available carbohydrate found to be digestible by *in vitro* analysis was not necessarily available *in vivo*. With present methods, *in vivo* studies seem necessary to define carbohydrates, as chemical structure does not take into account their physiological response on satiety, emptying, or glycemic levels. The use of CSCA in food applications requires further study, as type 3 RS typically has no improved digestibility with reheating (2) and CSCA exhibits thermal stability (14).

Gas production

Gas production

H_2 and CH_4 production was recorded for all treatments with all volunteers; however production of H_2 and CH_4 was variable within the same volunteer and across the population (**Figure 6.2**) in consistence with a previous study (74). One volunteer experienced values for H_2 and CH_4 that were consistently higher than the population, one volunteer fermented all samples, except 50 g glucose, and another volunteer did not produce CH_4 throughout the trial.

The H_2 curves for all samples are graphed in **Figure 6.2**, with the corresponding glucose curve**.** The comparison of individual glucose curves to the corresponding H_2 production curve shows high absorption of glucose with a relatively low H_2 production curve or low absorption of glucose with a relatively high H_2 production curve, in most cases. Within the fermentation curves, a significant peak, denoting production (if present), is identified with a star; while a

significant rise (if present) is identified with an arrow. Significance was calculated statistically and measured against previously published values of significant H_2 increase (71, 114). Multiple H_2 production peaks within a curve are assumed to be attributed to normal fluctuation. Normal H_2 levels regularly fluctuate over a period of hours within a volunteer (74), as observe in the individual graphs. In 40% of waxy maize starch H_2 production curves, volunteers experienced an increase in gas production around 5 h. For these volunteers, a supplemental reading was recorded for 6.5-7 h, until H_2 readings began to decrease. In the current experiment, four volunteers produced increased H_2 values after consumption of CSCA and four with waxy maize starch; with 2 of 4 volunteers producing in both treatments (**Figure 6.2**). Increased starch resistance with octenyl succinic anhydride modification, noted increased H_2 production by breath hydrogen testing (115). However, it was observed that CSCA did not always increase H_2 production more than samples containing lower RS content and further studies with a large number of subjects are needed to determine if level of resistance (%RS) is directly proportional to H_2 production.

Minimum and peak H_2 values for the population are listed in **Table 6.3**. Normal breath H_2 excretion is very low (74), and few H_2 basal levels were higher than 16 ppm, which is generally considered elevated from normal production H_2 (114). In this study, $^1/_{10}{}^{th}$ of the population tested high for H_2 production after 50 g glucose consumption, $^1/_{10}{}^{th}$ from 50 g CSCA, $^3/_{10}{}^{th}$ from 25 g CSCA with 25 g glucose, and $^1/_{10}{}^{th}$ from 50 g waxy maize starch. Rumessen (1990) suggests analyzing and reporting baseline rise to peak for gas production measurements of malabsorbed carbohydrates (65, 116), however, many of the values in **Table 6.3** are not significantly different due to the high variation in the population, and some volunteers did not produce a significantly relevant change in H_2 and CH_4 production following consumption of any of the samples.

Increased H_2 production may also contribute to development of abdominal bloating (63), and one volunteer did report a feeling of increased flatus with consumption of raw waxy maize starch (50 g) with increased H_2 production. However, CSCA concentrations were tolerated by the volunteers. Twenty-eight grams of supplemented RS per day has been reported as tolerable by volunteers, while lower concentrations used in their study showed no change in gas production (117). Increased H_2 excretion was also seen with 10.33 g RS supplementation, but not with lower quantities (118). The sample containing 25 g CSCA only increased H_2 production in one volunteer. Therefore, for the population, 25 g CSCA in mixed formulation (with 25 g glucose) did not produce increased gas production. RS in various mixed formations has provided different digestive results (71).

Figure 6.3 showed mean population curves for all treatments. Wave-like patterns CH_4 production curves influenced us to not display individual CH_4 curves, as with H_2 production curves in **Figure 6.2**. The population graph of 50 g glucose H_2 production (**Fig. 6.3A**) showed a descending power trend that leveled around 150 min, with no trend in CH_4 production. In the population graph of 50 g CSCA H_2 production (**Fig. 6.3B**), there was an ascending power trend that elevated around 210 min, with a CH_4 population graph that steadily increased at 210 min, just under significance ($p \approx 0.05$). The H_2 production population graph for 25 g CSCA with 25 g glucose showed a descending power trend that leveled around 150 min, which then entered into an ascending power trend at 210 min (**Fig. 6.3C**); the CH_4 population graph oscillated from 0-360 min, one spike at 60 min. In the population graph of 50 g waxy maize starch (**Fig. 6.3D**), H_2 and CH_4 production mimicked that of CSCA, with the exception of a CH_4 production spike occurring at 330 min. Digestive transit periods vary between individuals (63), however, after 1 h, mean H_2 values (ppm) for all treatments, except 50 g glucose, were above the mean production

of CH_4 at the same period. There was commonality in the samples containing starch to produce some H_2.

Waxy maize starch contained low portions of RS by the in vitro test (**Table 6.1**) and was expected to be digested in the small intestine. In some cases, waxy maize starch increased H_2 to produce 44-106 ppm H_2 within the colon (**Figure 6.2**). Disconnection of *in vitro* analyses and *in vivo* breath hydrogen results have been previously observed (119). It is hypothesized that the maximum consumption of glucose at this period, for those volunteers, was reached. Therefore, easily fermentable, waxy maize starch may have overloaded glucose absorption and passed into the colon in some cases. Upon entering the colon, it would be quickly fermented, to produce high H_2 values. Methane readings remained constant in these events, with wave-like responses. H_2 can exhibit regular patterns, while CH_4 does not simultaneously change with H_2 (74). In some cases, RS was found to increase measurable end-expiratory CH_4 in volunteers compared to that of lactulose load (71), however no pattern was observed in the measured CH_4 values.

Minimum and peak CH_4 production values are listed in **Table 6.3**, mean basal values for CH_4 production in all volunteers was 5.62±6.27 ppm, and 70% of volunteers produced methane. For CH_4 categories of producing and nonproducing, 40% of the population was categorized as nonproducing, while only 30% averaged approximately 23 ppm CH_4. The probability for whether CH_4 production occurred in an individual or not, is consistent (68, 69), as noted by CH_4 production and nonproduction was categorized for the current population. As CH_4 is only produced in the colon (120), and 30% of the population was over 1 ppm, yet under 23 ppm (Lauren Brewer, personal communication), it is possible that the products provided to the volunteers may have lowered CH_4 production within CH_4 producing individuals, by the only common factor: consumption of samples with no TDF content.

Significant differences for the population graphs were analyzed after AVOVA by Tukey's multiple comparison tests, and percent significance is reported in reference to the population in **Table 6.4**. By 1-way AVOVA, all gas production curves differed significantly within an individual (ranging from $p<0.0001$-0.042), except in cases where no production was found. However, significant differences for all treatments were not present for the population. CSCA treatments were significantly different from other treatments in 26% of measurements. No significant response from over 40% of the population with any sample was found. Previous studies have shown high-RS and low-RS diets (n=10) (72), HYLON VII, cooked, and raw potato starch curves (n=7), where all breath H_2 treatments were significantly different. Retrograded high-amylose maize starch and a lipid complex high-amylose maize starch have been measured (unpublished) by breath hydrogen testing on healthy subjects (62). Within their experiment on intubation (121), the researchers noted that with slower fermentation of RS, the procedure is qualitative under these conditions, and some H_2 may be utilized by the flora over the extended period. Therefore, they do not recommend breath hydrogen testing for only RS quantification (62). In some cases, H_2 production in the current trial may have been lessen by bacteria during the extension of the curve from slow fermentation.

Conclusions

This study confirms that RS content in a product affects GI value as a variable independent of dietary fiber. CSCA alone and in mixed formation reduced all curves, compared to the response from glucose. Development of the GI method to include RS concentration by utilizing current dietary fiber methods (such as AOAC Method 2009.06 or 2011.25) to determine the true value of the test material would provide better representation of the carbohydrate in clinical testing.

Large variations in individual breath hydrogen results were noted and as a result, group observations for the current population were difficult to conclude. Of the ten volunteers tested in this trial, four increased H_2 production when consuming CSCA, and four increased H_2 production when consuming waxy maize starch; with two of those experiencing the change in both treatments. CSCA was found to not significantly differ from the waxy maize starch breath H_2 responses, in the majority of cases (60%).

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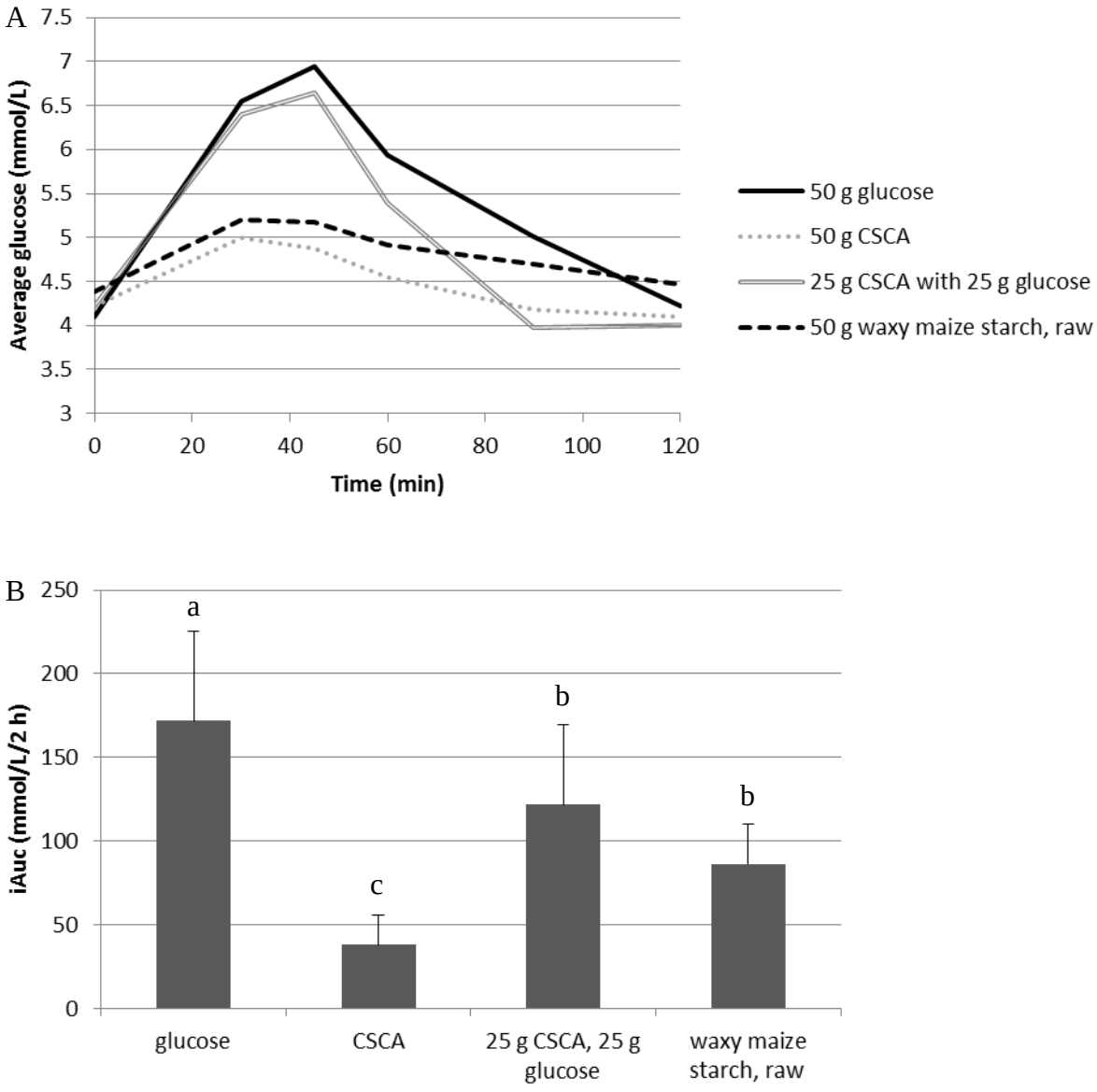


Figure 6.1. Population glucose responses to 50 g available carbohydrate samples of glucose, crystalline short-chain amylose (CSCA), 25 g CSCA with 25 g glucose, and waxy maize starch, raw. A. plasma glucose (mmol/L) over time and includes the control 50 g glucose solution; while B. incremental area under the glucose curve. Data presented are mean ± standard deviation; with significance as $P < 0.05$; and, different letters indicate difference between treatments.

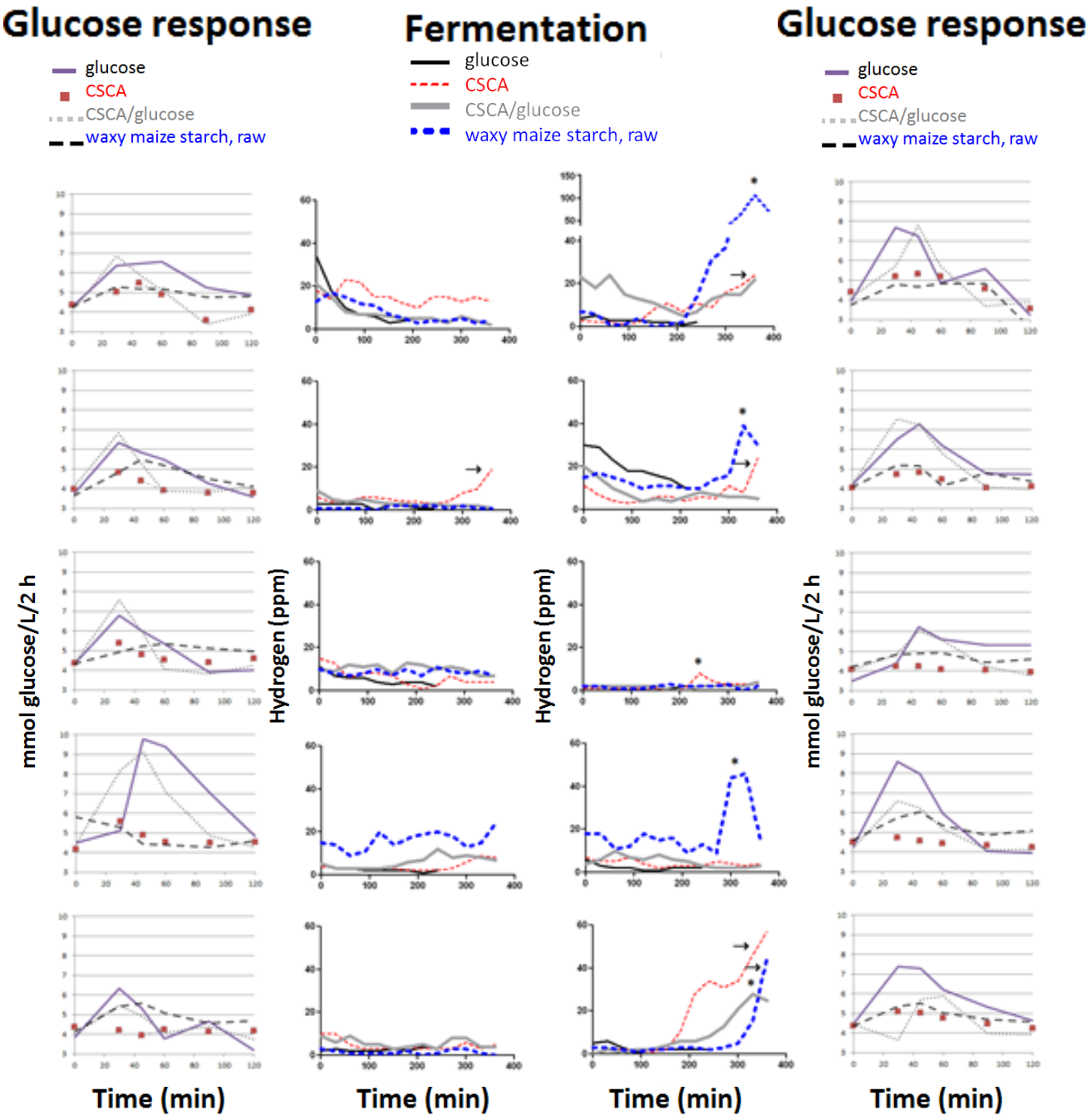


Figure 6.2. Individual breath hydrogen curves (ppm H_2), up to 390 min with corresponding 120 min blood glucose curve. Individual breath hydrogen curves (ppm H_2), up to 390 min, with corresponding 120 min blood glucose curve, after consumption of 50 g available carbohydrate samples of glucose, crystalline short-chain amylose (CSCA), 25 g CSCA with 25 g glucose, and waxy maize starch, raw. A star denotes a significant, peak for that graph, while an arrow denotes a significant raise, $p<0.05$.

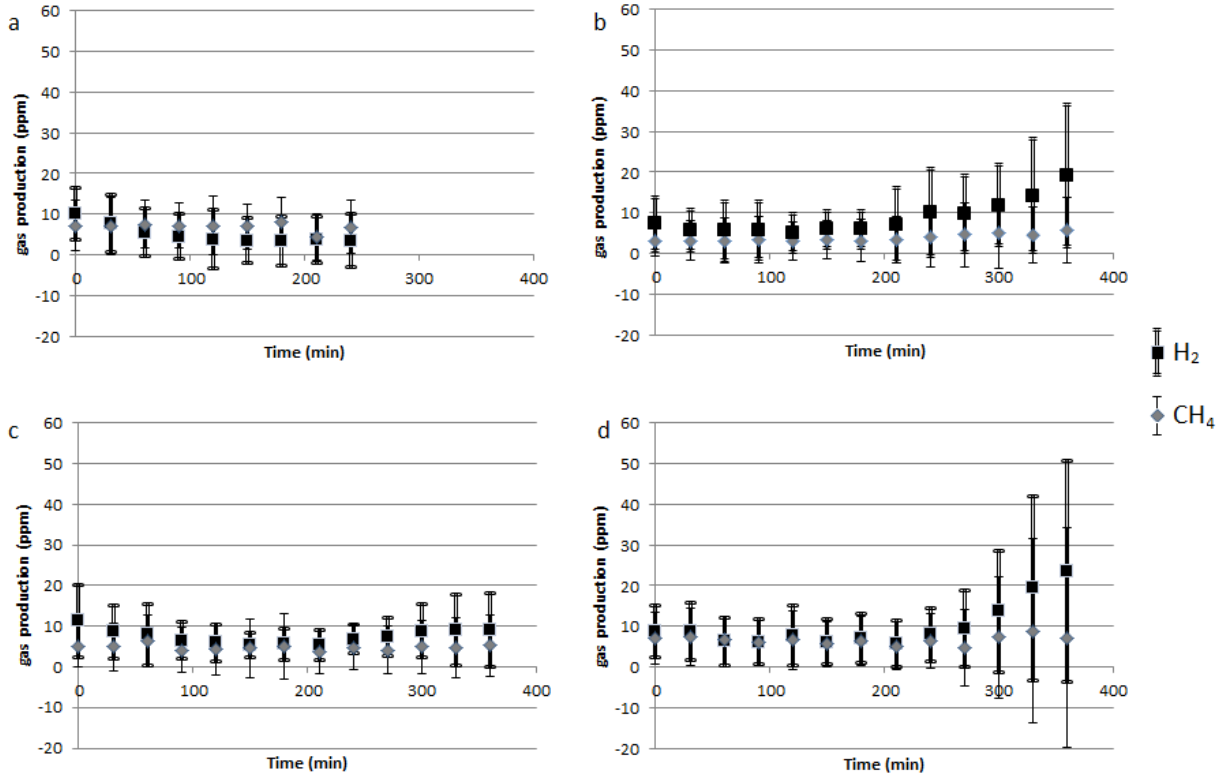


Figure 6.3. Mean and standard deviation of increased gas production (ppm) after consumption of 50 g available carbohydrate samples over time. Squares represent hydrogen and diamonds represent methane (both as ppm) for a) glucose, b) crystalline short-chain amylose (CSCA), c) 25 g CSCA with 25 g glucose, and d) waxy maize starch, raw.

Table 6.1. Resistant starch (RS) and total dietary fiber (TDF) contents of crystalline short-chain amylose (CSCA), waxy maize starch and HYLON VII.

Sample	RS* (%)	TDF† (%)
CSCA	75.0 ± 1.4a	0.0 ± 0.01b
Waxy maize starch	0.6 ± 1.9c	0.0 ± 0.00b
HYLON VII	68.2 ± 0.7b	16.5 ± 0.01a

Column data with like letters are not significantly different (p>0.05); n=3

* RS content was determined by a previously reported method (Englyst et al., 1992), where RS = 100%−% digestible starch at 120 min.

†TDF values were determined by AOAC Method 991.43.

Table 6.2. Variation in blood glucose response among volunteers after consumption of 50 g of available carbohydrate from four treatments: glucose, crystalline short-chain amylose (CSCA), 25 g CSCA with 25 g glucose and waxy maize starch, raw.

Sample (50 g available carbohydrate*)	Peak range (glucose mmol/L)	Relative change from baseline to peak value†	
		R	$\tilde{x}$
Glucose	6.24–9.77	2.30–5.28	2.85
CSCA	4.25–5.57	0.18–1.42	0.88
25 g CSCA with 25g glucose	5.81–9.14	1.43–4.71	2.99
Waxy maize starch, raw	4.82–6.03	0.00–1.45	1.065

* available carbohydrate AOAC Method 996.11

†$\tilde{x}$ = median; R = range; produced from n =10

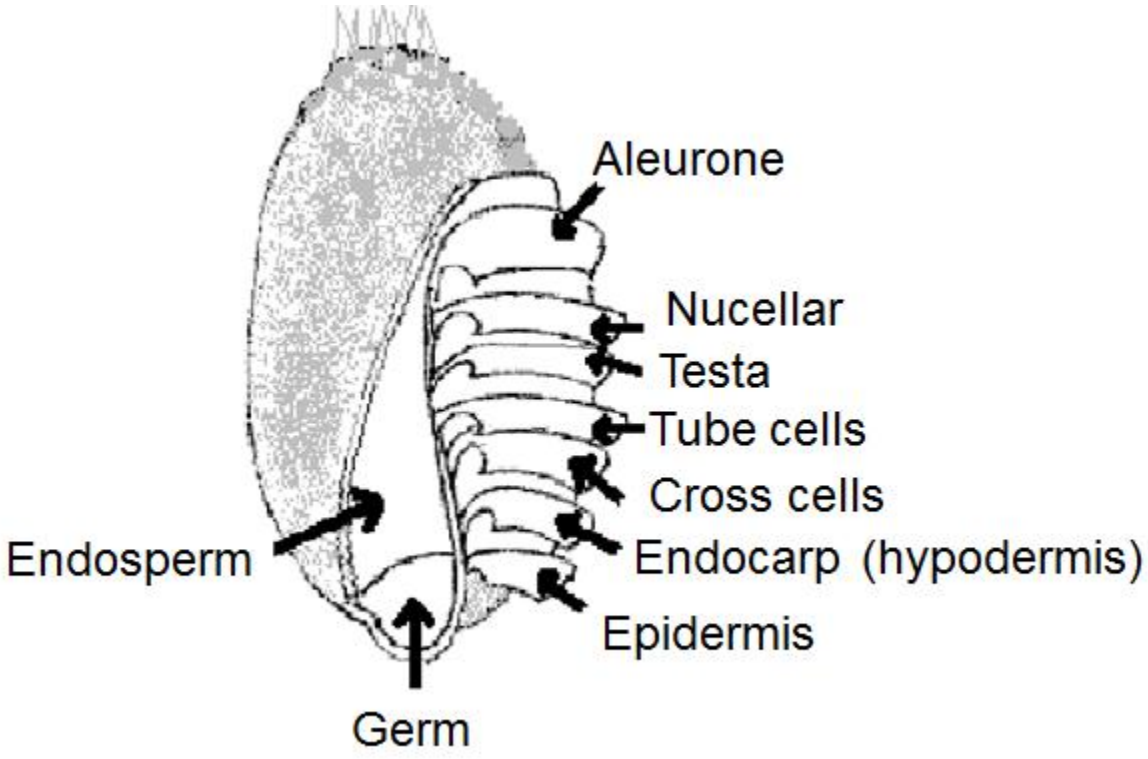
Table 6.3. Mean gas production of minimum and peak values from breath hydrogen testing. Mean gas production of minimum and peak values for 50 g available carbohydrate of glucose, crystalline short-chain amylose (CSCA), 25 g CSCA with 25 g glucose, and waxy maize starch treatments.

	Population treatment response*	
	Hydrogen (ppm)	**Methane (ppm)**
	Minimum value	
50 g glucose	3.40 ± 3.13bc	6.09 ± 8.73a
50 g CSCA	2.50 ± 3.25b	2.00 ± 4.04a
25 g CSCA, 25 g glucose	2.75 ± 2.40b	3.00 ± 5.21a
50 g waxy maize starch, raw	3.90 ± 4.33bc	3.30 ± 4.69a
	Peak value	
50 g glucose	10.60 ± 11.56ab	8.73 ± 11.05a
50 g CSCA	22.38 ± 15.38ac	7.00 ± 8.49a
25 g CSCA, 25 g glucose	16.38 ± 8.16ab	8.50 ± 7.76a
50 g waxy maize starch, raw	29.60 ± 31.88a	11.70 ± 12.42a

* Column data with like letters are not significantly different ($p>0.05$); n=10

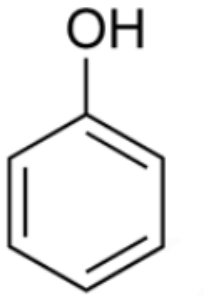
Chapter 7 - Appendix list

Appendix A. Wheat kernel with detailed wheat bran layers

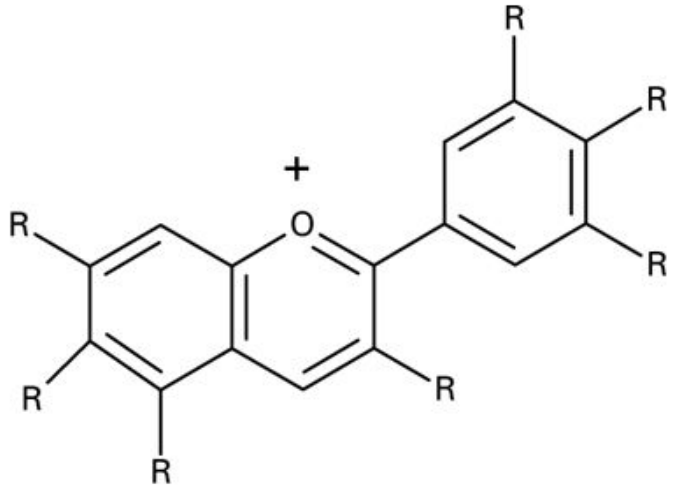


Appendix B. Antioxidant chemical structures

Basic phenolic compound

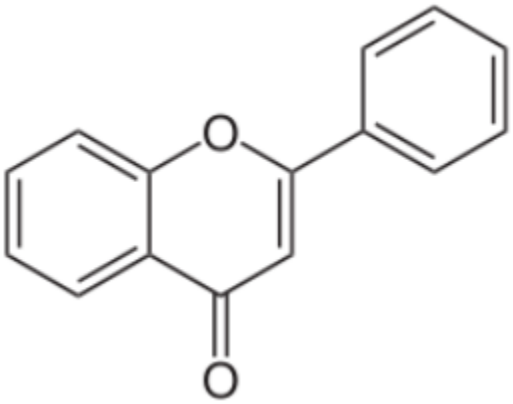


Basic anthocyanin

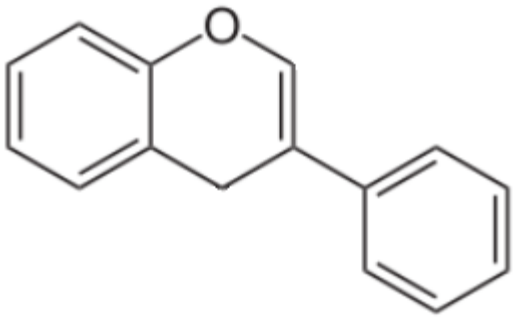


Basic flavonoids

Flavone

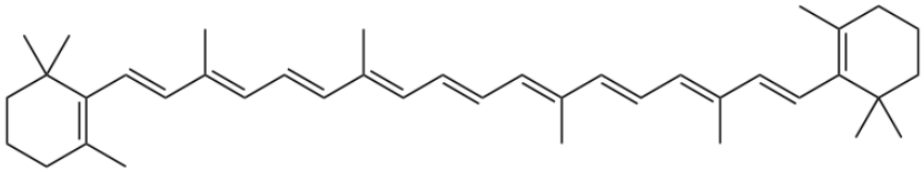


Isoflavone

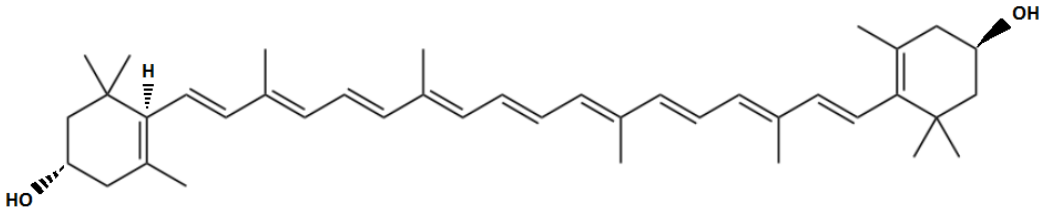


<u>Carotenoids</u>

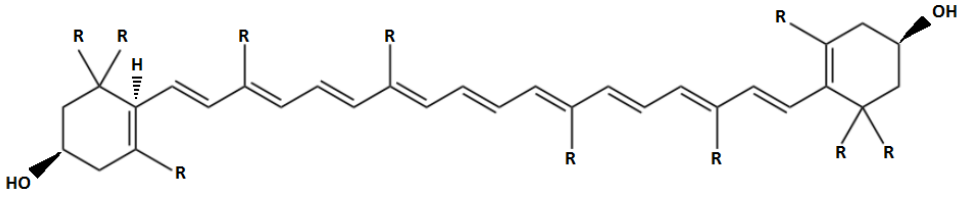
Beta-carotene



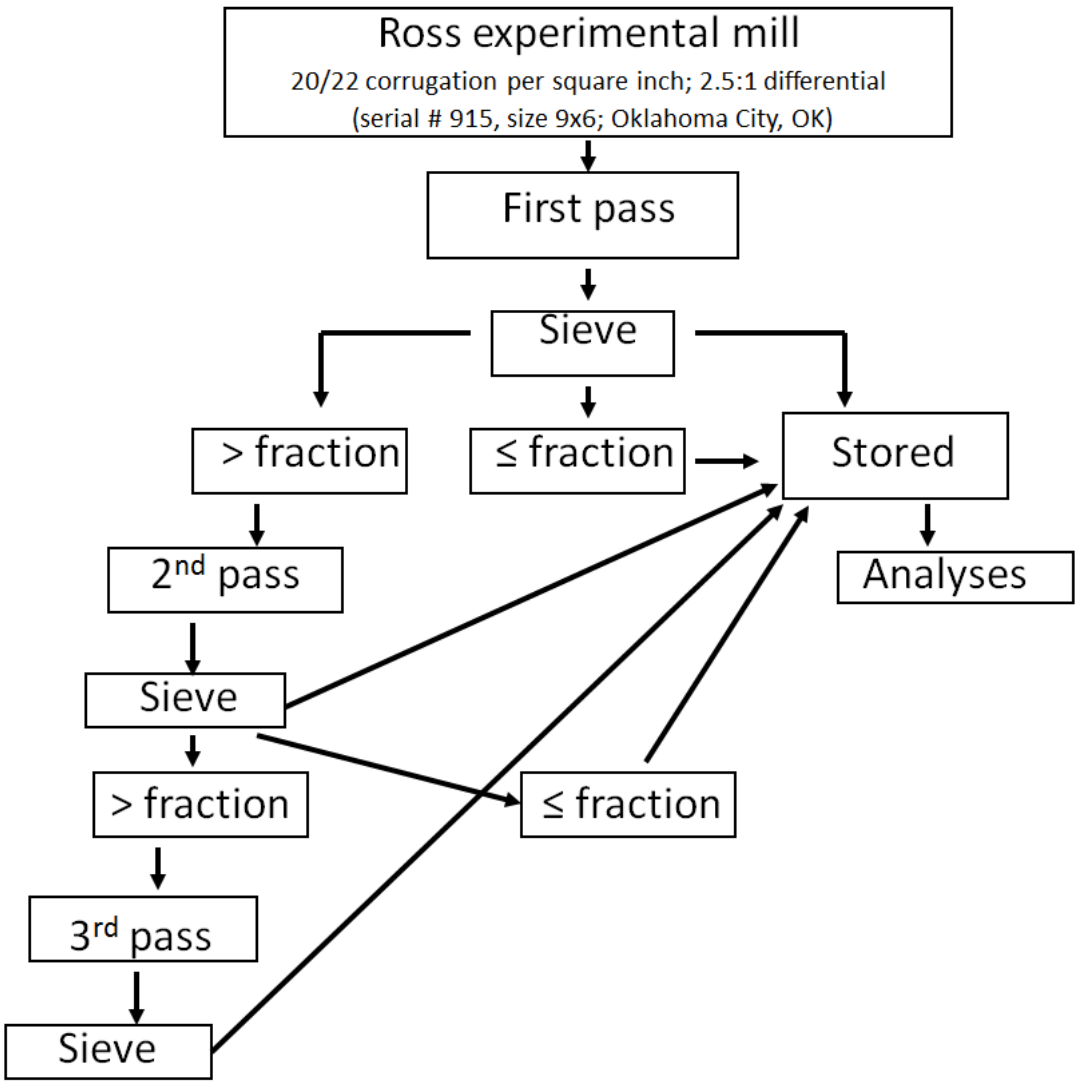
Lutein



Zeaxanthin



Appendix C. Wheat bran particle size reduction scheme



Key

Analyses – the multiple analytical techniques used to quanitify attributes for the each particle size distribution

Fraction – the desired particle size distribution (200 or 500 µm)

Pass – sending the wheat bran through the roller with the experimental gap at the desired setting

Sieve – sieving the material with the predetermined sieves for the desired particle size distribution (as noted in Table 3.1**).**

Appendix D. Antioxidant analyses

Extraction of soluble phenolic compounds

1. 1 g extracted for 2 h with 10 ml of 80% methanol at room temperature on an orbital shaker set at 150 rpm, 37 C
2. Extracts pooled and evaporated at 45 C in a vacuum oven
3. Freeze dry and record weights
4. Redissolve in 5 ml methanol

Extraction of bound phenolic compounds

Adom (2005) procedure is used on the pellet from the previous extraction

1) 1 g sample digested with 2M sodium hydroxide at room temperature for 1 h
    a. Under continuous nitrogen gas
2) Neutralize with HCl and extract with hexane to remove lipids
3) Extract solution 5 times with ethyl acetate
4) Pool ethyl acetate extracts together and evaporate to dryness
    a. Under continuous nitrogen gas
5) Dissolve phenolics compounds in 10 ml methanol and hold in the freeze at -20 C

Extraction of total phenolic compounds (TPC)

1. To one tube add:
    a. 200 µL of extract solution (that was shaken for 1 minute)
    b. 1 ml of diluted (1:10 with water) folin-ciocalteu reagent
2. Shake mixture
3. Add 800 µL of 10% Na_2CO_3
4. Take volume up to 5 ml with distilled water
5. After 2 hour reaction, read the absorbance at 760 nm

Molybdenum(VI) + e → Mo(V)

Theorized that molybdenum is easier to be reduced: the e- transfer occurs between reductants & MoVI

Determination of total antioxidant capacity

1. To one tube add
    a. 0.3 ml sample
    b. 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate)
2. Incubate at 95C for 90 minutes
3. After the mixture cools to RT read absorbance of the solution at 695 nm, against a blank

molybdate + e → molybdate

Theorized that molybdate is easier to be reduced: the e- transfer occurs between reductants & molybdate

---

2,2-diphenyl-1-picryl hydrazl (DPPH) assay

--DPPH radical scavenging assay (von Gadow et al., 1997; Zielinski and Kozlowska, 2000) --

Buffers: Prepare DPPH solution 0.004% in methanol

1. To one tube add
    a. 1.9 ml DPPH solution
    b. 100 µL sample
2. Keep in a dark room to react for 30 minutes
3. Test absorbance at 517 nm

Purple color goes to intense yellow color at that wavelength when DPPH and antioxidants combine

The test relies on the ability to act as donors of hydrogen atoms in the transformation of the DPPH radical to its reduced form (DPPH•-H).

Watch decrease in absorbance at 515 nm over time until stable – calculate % inhibition

(von Gadow et al., 1997)

DPPH• + AH → DPPH•(-H) + A•

Ferric reducing/anti-oxidant power (FRAP) assay

Chemicals

Acetate buffer

- 300 mM pH 3.6
- 3.1 g sodium acetate 0.3 H_2O
- 16 ml glacial acetic acid
- Distilled water to 1 liter
- Check pH, store at 4C

Dilute HCl: 40 mM

- 1.46 ml conc HCl (11M)
- Distilled water to 1 liter
- Store at room temperature

TPTZ (2,4,6-tri[2-pyridyl]-s-triazine): 10 mM

- 0.031 g TPTZ in 10 ml of 40 mM HCl, dissolve at 50C in water bath, MAKE FRESH ON THE DAY OF ASSAY

Ferric chloride: 20mM

- 0.054 g $FeCl_2$ $6H_2O$
- Dissolve in 10 ml distilled water
- MAKE FRESH ON THE DAY OF ASSAY

Standards of ferrous sulfate were used

Prepare 1 mM solution: 0.278 g FeSO4 in 1 liter distilled water.

Dilute for a series of standards

Standard concentration (mM)	$FeSO_4$ $7H_2O$ solution (ml)	Distilled water (ml)
0.1	1	9
0.2	2	8
0.4	4	6
0.6	6	4
0.8	8	2
1.0	10	0

FRAP reagent (10:1:1)

--Keep in water bath at 37C--

100 ml acetate buffer

10 ml TPTZ solution

10 ml $FeCl_3$ solution

(all above are the 10:1:1)

12 ml distilled water

For the FRAP assay:

1. To one tube add:
    a. 1.8 ml FRAP reagent

    b. 300 µl sample
    c. 180 µl distilled water
2. Incubate at 37C for 4 minute
3. Test absorbance at 593 nm

*Blank is the FRAP reagent

Will have an intense blue color at 593 nm

Fe^{3+}-TPTZ to Fe^{2+}-TPTZ

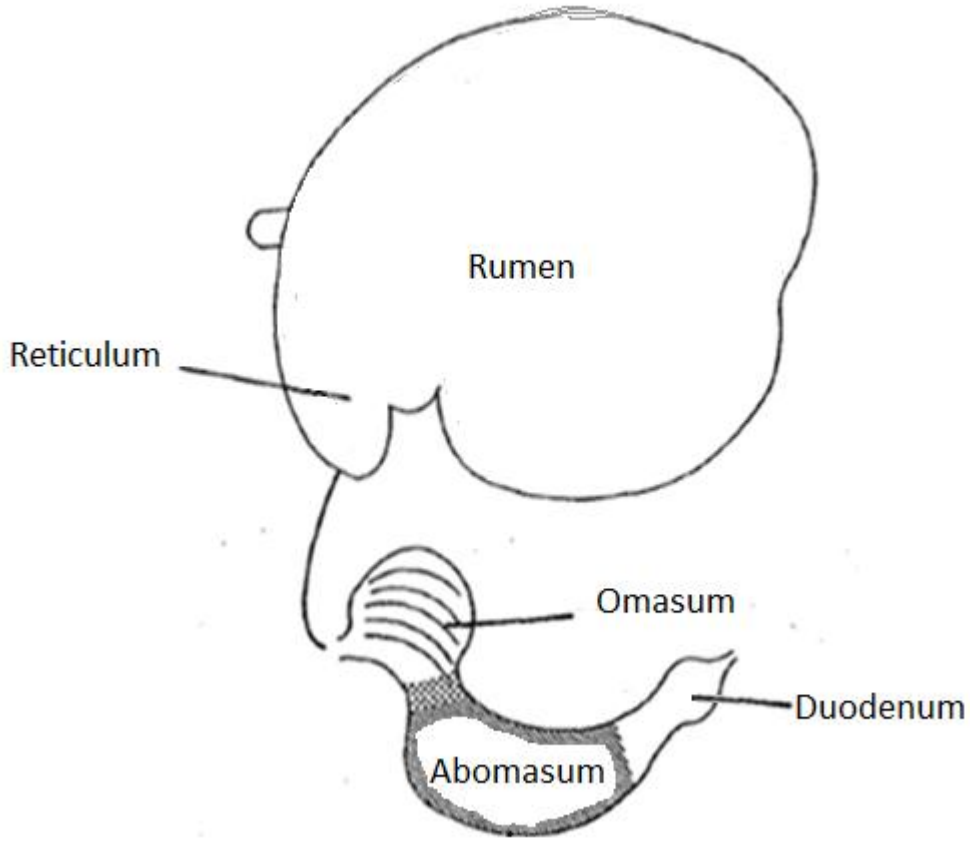
Oxygen radical absorbance capacity (ORAC)

Extraction prep for ORAC analysis at USDA (Ou et al., 2002; Awika et al., 2003b)

1) 0.5 g sample added to 20 ml hexane:dichloromethane (1:1) (HD)
    a. Extract lipophilic antioxidant constituents
2) Mixed to turbulence under nitrogen at 25 °C - 1 h
    a. Modified extraction time (↑ 1 h)
3) Evaporate HD extracts at room temperature (23 °C) under nitrogen to dryness
    a. With 1 ml methanol prior to analysis
4) Previously extracted sample mixed to turbulence at 25 °C for 1 h with acetone:water (70:30) [nitrogen]
    a. Extract hydrophilic antioxidant constituents.
5) Mixed to turbulence under nitrogen at 25 °C - 1 h
6) Centrifuge at 12,100 g for 15 min, and stored at -20°C
7) Supernatants ready for analysis after appropriate dilution with 75 mM potassium phosphate buffer solution (pH 7.4)
    a. Diluted to 1/50 for analysis

The assay measures the oxidative degradation of fluorescein (the loss of fluorescence) over 2 hours using a Synergy 2 microplate reader equipped with Gen5TM data analysis software (Biotek Instruments Inc., Winooski, VT, USA).

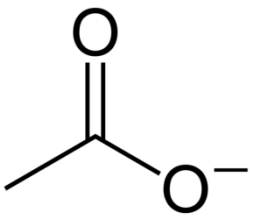
Appendix E. Rumen digestive system

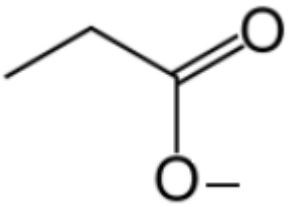


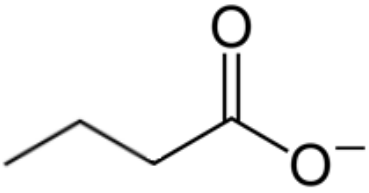
Modified from (Lechner-Doll et al., 1995)

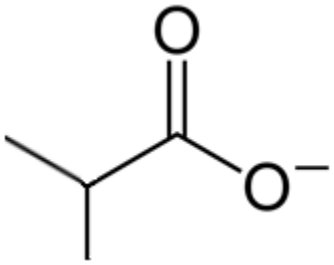
Appendix F. Volatile fatty acids (VFA)*

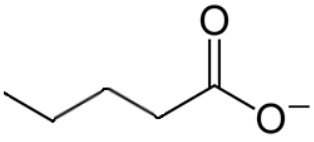
*While other compounds exist, the following were analyzed in this dissertation.

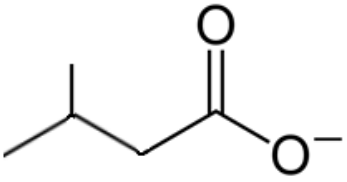
Acetate 

Propionate 

Butyrate 

Isobutyrate 

Valerate 

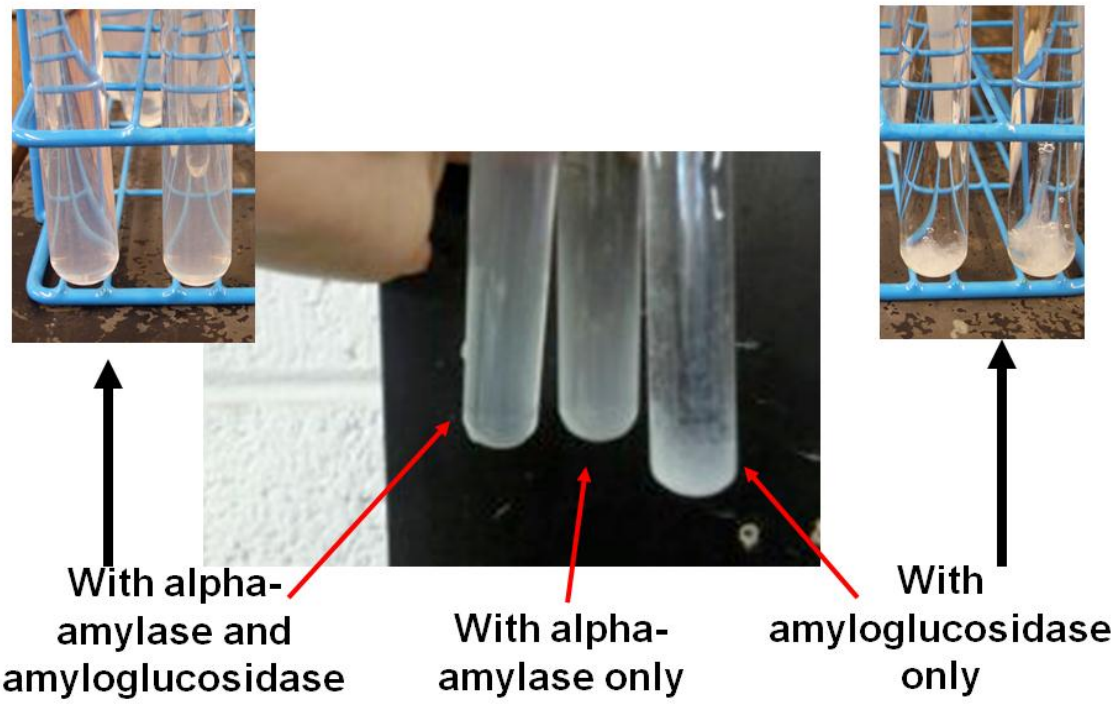
Isovalerate 

Appendix G. Supplemental figures from Chapter 5

Chapter 5, "Mechanism and enzymatic contribution to in vitro test method of digestion for maize starches differing in amylose content" was previously published:

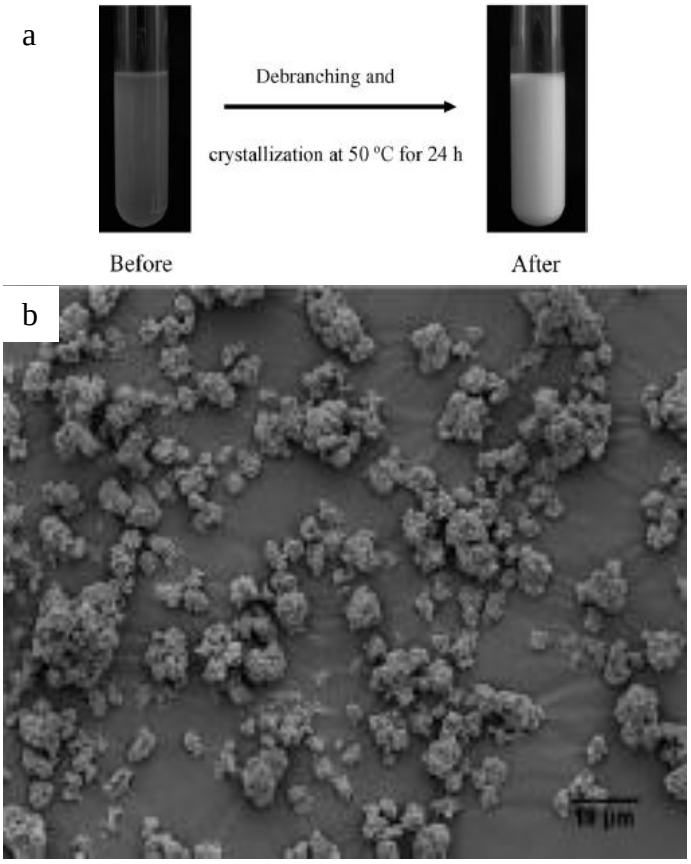
Brewer LR, Cai L, Shi YC. Mechanism and Enzymatic Contribution to In Vitro Test Method of Digestion for Maize Starches Differing in Amylose Content. J Agric Food Chem 2012;60:4379-87http://dx.doi.org/10.1021/jf300393m.

The following figures and descriptions are provided for further clarification of the methods.



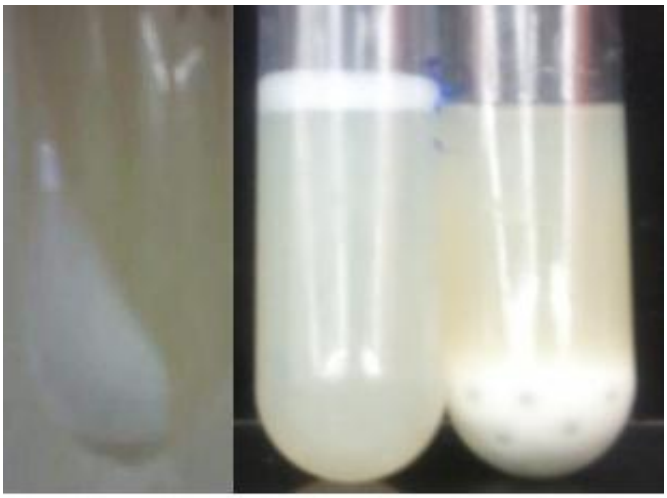
The above figure depicts the addition of 250 µl of in vitro digestion, added to 10 ml of 66% ethanol. Note the failed dispersion of the material within the tubes with amyloglucosidase only due to the lack of alpha-amylase digestion.

Appendix H. CSCA supplemental figures

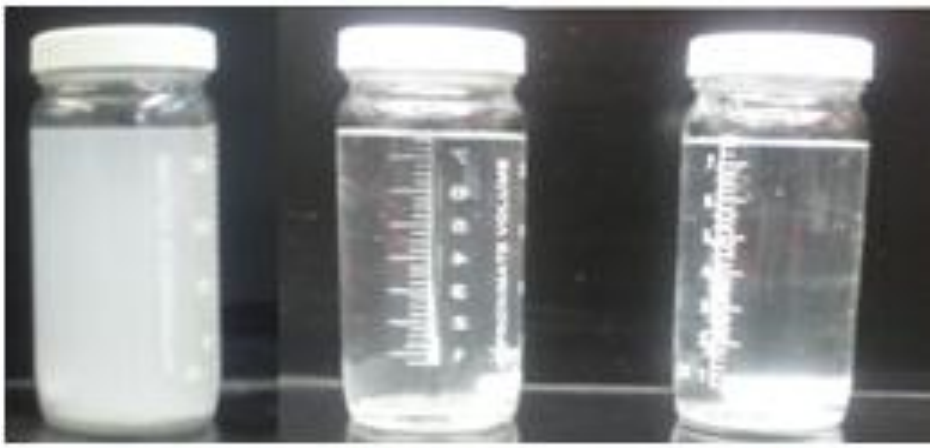


The above figure is modified with permission from author (Cai et al., 2010). A) starch polymer solution at the beginning of debranching (enzymes added) and cloudy slurry after 24 h of crystallization (after debranching) at 50°C; B) SEM image of debranched waxy maize starch, after crystallization at 50°C for 24 h. The sample was recovered by filtration and oven dried (40°C).

Appendix I. RS and TDF analyses



HYLON VII Waxy maize starch CSCA
RS: 68% 0.6% 75%



HYLON VII Waxy maize starch CSCA
TDF: 16% 0% 0%

In the top figure, (from left to right) HYLON VII, waxy maize starch and CSCA are shown at 120 min digestion. %RS is listed below each figure. Note the high concentration of residual starch for HYLON VII and CSCA. In the bottom figure, (from left to right) HYLON VII, waxy maize starch and CSCA are shown after precipitation (during AOAC method 991.43) with 78% (heated) ethanol. %TDF is listed below each figure. Note the cloudiness of HYLON VII, due to the presence of fibrous material.

Appendix J. YSI Stat Plus Glucose (and Lactose) analyzer

(YSI Incorporated, Life Sciences, Yellow Springs, Ohio)

Upon sampling of < 70 µl of blood, glucose oxidase is immobilized between polycarbonate and cellulose acetate membranes within the system. Glucose within the blood sample is oxidized when it enters the glucose oxidase layer. From the reaction hydrogen peroxide is produced. The hydrogen peroxide passes through cellulose acetate to a platinum electrode and is oxidized. The measured current is proportional to glucose concentration in the sample.

The following equation accounts for the enzymatic reaction:

$$\text{D-glucose} + O_2 \xrightarrow{\text{(glucose oxidase)}} \text{D-glucono-}\delta\text{-lactone} + H_2O_2$$

The following equation accounts for the anode reaction:

$$H_2O_2 \xrightarrow{\text{(platinum anode)}} 2H^+ + O_2 + 2e^-$$

For further information on this equipment, please refer to:
http://www.ysiuk.com/uploads/Documents/YSI%202300%20STAT%20Plus%20Leaflet.pdf

Appendix K. Quintron BreathTracker SC

(Quintron USA, Milwaukee, WI)

This instrument separates components by gas chromatography. Room air acts as the carrier gas for the sample. Hydrogen and methane are measured when separated from all other expelled gases (and from each other), and analyzed with a solid-state sensor.

For further information on this equipment, please refer to:
http://www.quintron-usa.com/images/pdfs/Support_Files/QT02444RD1-Catalog-InfoBooklet.pdf

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