

PHYSICOCHEMICAL AND SENSORY PROPERTIES OF RESISTANT STARCH-BASED
CEREAL PRODUCTS AND EFFECTS ON POSTPRANDIAL GLYCEMIC AND
OXIDATIVE STRESS RESPONSES IN HISPANIC WOMEN

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ABSTRACT

The incidence of type 2 diabetes is considered an epidemic in Western countries, and its prevalence is more common in the Hispanic population than in non-Hispanic whites. Postprandial hyperglycemia has been associated with oxidative stress (OS), thus; reducing postprandial glycemia and/or OS through dietary consumption of resistant starch (RS) may be one approach to help modulate glucose and insulin responses. The purpose of this study was twofold: 1) to evaluate the physicochemical and sensory properties of cereal food products supplemented with RS. 2) to compare the effects of a single ingestion of granola bars with high (~18 grams of RS) and low (~0 grams of RS) RS compositions on the postprandial glucose and insulin responses (n=14) and oxidative stress parameters (cellular glutathione peroxidase, F2-isoprostanes, and oxygen radical absorbance capacity) in Hispanic women (n=9). Granola bars and cereals were developed to provide 2 levels (10% and 15%) of RS; isocaloric (0% RS) control samples were prepared with readily digestible (high amylopectin) starch. Samples were stored for up to 4 weeks at 20 °C. Mean composition of the high RS granola bars was 6% protein, 15% moisture, and 18% lipid. RS levels slightly increased from 14 to 16 g/serving after 4 weeks of storage, supporting published research that RS increases with storage due to retrogradation and crystallization of amylose chains. Color became lighter as the level of RS increased (p<0.001). Granola bars containing RS were less brittle (p=0.0043) than control granola bars. Sensory results indicated granola bars/cereals were acceptable. RS-supplemented granola bars were then used for the evaluation of RS ingestion in humans.

There was no difference in postprandial glucose and insulin responses after a single ingestion of a RS-supplemented (18 g) granola bar. No differences were found in the oxidative stress parameters measured. In a subgroup of subjects (n=9), a lower glucose response 30 minutes after RS consumption was found (p=0.0496). Thus, RS consumption may lower fluctuations in blood glucose, which may help manage glucose levels in individuals at risk of type 2 diabetes. Further studies of short term RS consumption are warranted to elucidate its benefits in glucose management.

In memory of my father

Federico Aigster

This dissertation is entirely dedicated to my father. My dad was my number #1 fan, and he was always supportive of my graduate research and education. There is not a single day I do not think about him. Especially today, September 1, my dad's birthday: this is for you!

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CHAPTER 1: INTRODUCTION

Starch, a glucose homopolymer, is comprised of two fractions: a linear fraction, amylose, and a branched fraction, amylopectin. Most naturally-occurring starches contain 30% amylose and 70% amylopectin. The linear backbone consists of $\alpha(1\rightarrow4)$ linked glucose units, with branches arising through $\alpha(1\rightarrow6)$ linkages. High amylose corn starch, also known as resistant starch (RS), is a corn hybrid that contains a high percentage of amylose regions (70% amylose, 30% amylopectin) (Whistler and BeMiller, 1997). Resistant starch was first recognized in the 1980s when it was found to be resistant to enzymatic hydrolysis. It was later shown in human ileostomates that RS bypassed the small intestine and reached the colon where it was fermented to short chain fatty acids (Goldring, 2004). Resistant starch has been defined as 'the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals' (Asp, 1992). Resistant starch has been classified into four major types. (RS1) is the physically entrapped or inaccessible RS found in the cell wall of whole or partially milled grains and seeds as well as legumes. (RS2) is the native resistant ungelatinized granules found in raw potatoes, green bananas, and high amylose maize starches. (RS3) is the retrograded crystalline starch formed during conventional food processing methods such as cooked and cooled potatoes and pasta, bread, ready-to-eat cereals, and retrograded high amylose maize starches. (RS4) is chemically modified starch due to cross-linkage with chemical agents (Brown, 2004; Topping et al., 2003; Patil, 2004; Sajilata et al., 2006).

A physiological effect ascribed to resistant starch (RS) is related to the extent of digestibility of RS in the small intestine and its effects on postprandial glycemia (Kendall et al., 2004). Attenuating the glycemic response by consumption of foods containing RS may help individuals manage their blood glucose levels. Proper blood glucose management is associated with decreased risk of developing diseases and conditions such as diabetes, hyperlipidemia, and cardiovascular disease (Brown et al., 2003).

Resistant starch consumption should result in delayed glucose absorption, suggesting that RS will attenuate the glucose and insulin responses after a meal, thus decreasing the likelihood of developing insulin resistance (Higgins, 2004). According to Kendall and coworkers (2004), RS doses of 20-30 g/day are needed to observe physiological effects. This level of consumption is 3-4 times higher than actual levels of RS consumption in Western countries, which are estimated to be 5-10 g RS/day. The estimated resistant starch intake in the U.S. is between 3-8 g/day

(Murphy et al., 2008). These estimates provide valuable information for food scientists and nutrition professionals to develop food products supplemented with RS and to evaluate the potential health benefits of these products.

Type 2 diabetes (T2D) is a disease that has reached epidemic proportions in developed countries. Type 2 diabetes is characterized by high blood glucose, high blood insulin, and insulin resistance. There are over 23 million people in the U.S. who have diabetes (American Diabetes Association, 2008), and diabetes is more common in the Hispanic/Latino population than Caucasians (Center for Disease Control, 2005). Diabetes is the fifth leading cause of death in the U.S. (American Diabetes Association, 2003). The number of people suffering from diabetes worldwide in the year 2000 was reported to be 171 million, and projections estimate at least 366 million people worldwide will have diabetes by 2030 (World Health Organization, 2008).

The increased incidence of type 2 diabetes may be related to an increase in reactive oxygen species (ROS), leading to oxidative stress. The imbalance between the concentration of ROS and antioxidant defense mechanisms has been associated with health conditions such as obesity, impaired blood glucose, insulin resistance and chronic diseases such as type 2 diabetes (Dotan et al., 2004; Wright et al., 2006).

Postprandial hyperglycemia has been associated with oxidative stress and the development of chronic diseases such as diabetes and heart disease (Kendall et al., 2008). Resistant starch consumption may help modulate glucose levels thus reducing the risk of developing type 2 diabetes. Epidemiological studies have consistently shown an association between whole grain consumption and a decreased risk (20-42%) of developing type 2 diabetes (Fung et al., 2002; McKeown, 2002; Meyer et al., 2000; Montonen et al., 2003). The role of resistant starch in this protective effect has been suggested but has not been directly measured. Specific effects of RS on oxidative stress parameters are not well understood.

Our interest was in testing whether there are beneficial health effects of acute consumption of RS on glycemic control and oxidative stress. The purpose of this study was to evaluate a food product supplemented with 18 g RS/serving and to determine clinical parameters associated with development of type 2 diabetes and oxidative stress.

Research Objectives

Research Goal 1:

To develop cereal food products supplemented with RS and characterize the physicochemical and sensory properties of these products.

Objective 1: Incorporate RS into cereal food products (granola bar, granola cereal) to meet the following requirements:

- deliver up to 15g RS/100g serving;
- physicochemical properties equivalent to the control (no RS) products;
- achieve a sensory hedonic rating of at least 6.0 (“like slightly”) on a 9-point hedonic scale.

Objective 2: Evaluate the stability of these products during storage for 1 month.

Research Goal 2:

To determine the acute effects of ingestion of RS granola bars compared to the control (no RS) on postprandial glucose and insulin responses and oxidative stress parameters in Hispanic women.

Objective 3: Evaluate blood glucose and plasma insulin responses before and after ingestion of RS and control cereal products.

Objective 4: Evaluate oxidative stress parameters: cellular glutathione peroxidase (cGPx), plasma isoprostanes, and oxygen radical absorbance capacity (ORAC) before and after ingestion of RS and control cereal products.

Research Goal 3:

To evaluate the potential of polysaccharides including RS in food packaging applications.

Justification

Research Goal 1: To develop cereal food products supplemented with RS and characterize the physicochemical and sensory properties of these products.

Dietary fibers, including RS, promote beneficial physiological effects including laxation, blood cholesterol attenuation and blood glucose attenuation (Patil, 2004). Feeding uncooked RS (24 g /day) to overweight individuals for 21 days resulted in lower blood cholesterol and glucose levels compared to regular corn starch (Park et al., 2004). Short term supplementation of uncooked RS (60 g/day for 24 hours) resulted in improved insulin sensitivity (Robertson et al.,

2003). Formulation and evaluation of food products (granola bars and cereals) containing high levels of RS and glycemic responses has not been evaluated.

Food product development efforts have been driven by consumers' demands for healthy products. These efforts have been directed towards the emergence of functional foods and functional ingredients. Functional foods, defined as food or food components (including fortified, enriched) that have physiological benefits beyond basic nutrition (Institute of Food Technologists, 2005) is a dynamic and growing sector of the food industry. Globally, the functional foods market is estimated to be between \$7-63 billion. By 2010, the functional foods market is expected to increase to \$167 billion (Anonymous, 2008). Cereals are an excellent vehicle for functional food development including RS since cereals are rich in fiber and non-digestible carbohydrates. Cereals and cereal bars provide nutrition, convenience, value and taste consumers' demand. Cereals and cereal bars are not only consumed for breakfast but also as snacks. The U.S. cereal bar market increased 43% between 2002-2007 accounting for \$1.6 billion in sales (Faron, 2008). Functional ingredients like RS can help increase dietary fiber intake. Resistant starch has been shown to improve textural and organoleptic qualities, and increase the amount of dietary fiber in some food products (Kendall et al., 2004). Resistant starch acts as a prebiotic component by promoting the growth and activity of probiotic bacteria and can interact with other prebiotic dietary fibers such as beta-glucans (Brown, 2004; Goldring, 2004; Topping et al., 2003). Resistant starch levels in foods have been shown to increase during storage as a result of retrogradation and amylose chain crystallization (Kumari et al., 2007; Namratha et al., 2002; Niba, 2002; Niba, 2003; Sajilata et al., 2006).

Research Goal 2: To determine the effects of ingestion of RS cereal products compared to the control (no RS) on postprandial glucose and insulin responses and oxidative stress parameters in Hispanic women.

Resistant starch consumption has been shown to result in favorable changes in postprandial glucose and insulin responses in studies involving overweight/obese as well as healthy individuals (Behall & Hallfrish, 2002; Park et al., 2004; Robertson et al., 2003); however, the effects of RS on glucose and insulin responses in Hispanic-Americans have not been evaluated. Hispanic-Americans are a population at risk for type 2 diabetes development and they are 1.7 times as likely to have diabetes as non-Hispanic whites. In addition, Hispanic-

Americans are the fastest growing minority population in the U.S. (Center for Disease Control, 2005).

The relationship between RS consumption and oxidative stress has not been studied in humans. Shih and coworkers (2007) found decreased levels of malondialdehyde (MDA), increased superoxidase (SOD) activity, and increased total radical-trapping antioxidant levels in diabetic rats consuming RS compared to native starch (Shih et al., 2007).

To our knowledge, only one study has evaluated the effects of RS and lipid oxidation in humans. Higgins and coworkers (2004) showed that ingestion of 5.4% RS as percentage of total carbohydrates increased postprandial lipid oxidation *in vivo*, based on indirect calorimetry and oxidation of labeled [¹⁴C] triolein to ¹⁴CO₂. The authors concluded that incorporation of 5.4% RS in the diet could decrease lipid accumulation in the long term (Higgins et al., 2004).

Hundreds of methods exist to evaluate oxidative stress in biological samples, and various oxidative stress biomarkers are available. In this study, cellular glutathione peroxidase (cGPx), Isoprostanes, F₂-isoprostanes (F₂-IsoPs), and oxygen radical absorbance capacity (ORAC) were used to monitor the effects of RS consumption on oxidative stress parameters in humans. Cellular glutathione peroxidase (cGPx) is an endogenous antioxidant enzyme that reduces H₂O₂ and hydroperoxides (ROOH). Determination of GPx activity is related to lipid oxidation, and reduced GPx levels is an indication of oxidative status (Dotan et al., 2004). Isoprostanes, F₂-isoprostanes (F₂-IsoPs) are produced *in vivo* by a non-enzymatic free radical-induced lipid oxidation of arachidonic acid. F₂-IsoPs are specific products of lipid oxidation; they are stable and their level is not affected by lipid composition of the diet (Roberts & Morrow, 2000). The oxygen radical absorbance capacity (ORAC) is the gold standard method for determining the antioxidant capacity of foods and blood samples (Cao & Prior, 1999). The ORAC assay is one of the most commonly used antioxidant activity assays in biological research.

Research Goal 3:

To evaluate the potential of polysaccharides including RS in food packaging applications.

High amylose corn starch (HACS) also known as resistant starch (RS) alone or more importantly in combination with other polysaccharides such as chitosan can form strong and highly oxygen impermeable films. High amylose corn starch is very useful as a film-forming material because it improves mechanical strength to form stronger, flexible films with good gas

barrier properties compared to regular corn starch, possibly due to amylose crystallization (Han et al., 2006; Myllarinen et al., 2002). Films made with waxy starch containing mainly amylopectin (~99%) result in films that are brittle and have low tension resistance (Phan et al., 2005). Amylose is responsible for the film-forming capability of starch-based films. Addition of a plasticizer, usually glycerol, further promotes film formation. Without a plasticizer, HACS films are brittle due to extensive intermolecular forces; the addition of plasticizers improves flexibility and extensibility of edible films (Bertuzzi et al., 2007). In addition, HACS could be used as films for packaging of fruits and vegetables, snacks, and dry products (Sorrentino et al., 2007).

Preliminary results in our laboratory indicated that chitosan/resistant starch films (60% chitosan and 40% RS) showed oxygen transmission rates of 17.5 cc/m²/day (Aigster et al., 2007), which are comparable to the oxygen transmission rate of Mylar films (~ 60 cc/m²/day), known to be excellent oxygen barriers.

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CHAPTER 2: LITERATURE REVIEW

Starch, Resistant Starch, and Dietary Fiber

Starch

Starch provides 70-80% of the calories consumed worldwide. Starch is a homopolymer of glucose units, and it is the main storage form of glucose in plants. The starch granule consists of two glucose polymers, amylose and amylopectin. Amylose is a long, unbranched chain consisting of glucose units linked by α (1-4) glycosidic linkages. Amylopectin is a branched glucose chain consisting of α (1-4) glycosidic linkages and branches with α (1-6) glycosidic linkages. In humans, starch is degraded by hydrolases, in particular α amylase (Whistler and BeMiller, 1997).

Commercially available starches are obtained from the seeds of corn, waxy corn, high amylose corn, rice, wheat, and from tubers such as potatoes, and tapioca. In food products, starches have been widely used as binding, film-forming, gelling, stabilizing, moisture retention, texturizing, and thickening agents (Whistler and BeMiller, 1997).

Starches are also classified based on *in vitro* enzymatic activity. Rapidly digestible starch (RDS) is the amorphous starch found in starchy foods cooked by moist heat (bread, potatoes). It is the starch that is hydrolyzed to its constituent glucose units after 20 minutes of enzymatic digestion. Slowly digestible starch (SDS) is slowly hydrolyzed, and it is composed of physically inaccessible amorphous starch and raw starch. It is the starch that is converted to glucose after 100 minutes of enzymatic digestion (Sajilata et al., 2006).

Resistant Starches

Resistant starch (RS) is the starch fraction that resists hydrolysis by enzymatic digestion, and it is not hydrolyzed after 120 minutes of incubation (Sajilata et al., 2006).

Resistant starch has several physical, chemical, and physiological properties that are paramount to understanding the amount and types of RS found in foods. First, raw starches are poorly digested; however, when cooked, starch gelatinizes in the presence of water and improves the accessibility to digestive amylases and digestion is enhanced. Second, when the physical structure of a starchy food is disrupted, for instance by milling or mastication, amylase enzymes have greater access to the starch improving digestion. Third, cooking results in gelatinization of starch and increases starch digestibility. With subsequent cooling, some of the starch forms

crystals that are resistant to digestion. This process is known as retrogradation. Retrogradation is an irreversible process that results in increased RS formation by recrystallization and restructuring of the amylose chains (Topping et al., 2003; Sajilata et al., 2006).

Chemically, the starch granule is composed of amylose and amylopectin, and the ratio of the two determines the amount of RS present. Most starches are composed of approximately 70% amylopectin; however, some high amylose starches can contain approximately 70% amylose. The higher the amount of amylose, the more difficult it is for starch to gelatinize and the greater the susceptibility to starch retrogradation resulting in RS formation. The gelatinization temperature range of high amylose corn starch is 66-170 °C, corn starch is 62-80°C, and waxy starch (99% amylopectin) is 63-72°C (Whistler and BeMiller, 1997). The amylose:amylopectin ratio of the starch source is a determining factor in the formation of RS. High amylose maize is more resistant to enzymatic hydrolysis compared to its counterpart amylopectin (Leeman et al., 2006; Lehman & Robin, 2007) and has been used commercially to increase the RS content of processed foods. Physiological factors such as mastication influence the particle size of the starchy food. Large particles travel faster whereas increased chewing results in increased digestibility (Topping et al., 2003). Because physical, chemical, and physiological factors influence the amounts of RS found in foods, it is not surprising that RS may exist in different forms. In addition, because RS escapes digestion in the small intestine, it provides fewer calories than regular starches. It is estimated that RS caloric value based on retrograded commercially available products ranges between 1.6 to 2.8 Kcal/g (Croghan, 2001; Goldring, 2004).

The amount of RS formed depends on the amylose:amylopectin ratio from the starch source. High amylose starch is more resistant to digestion compared to amylopectin, and these differences are explained by the structural and chemical differences of the two starch fractions (Patil, 2004; Tapsell, 2004) (Figure 2.1).

Resistant starch (RS) was first recognized although not identified as such in the 1980s when it was found to be resistant to enzymatic hydrolysis (Englyst et al., 1992). It was later shown in human ileostomates that RS bypassed the small intestine and reached the colon where it was fermented to short chain fatty acids (Goldring, 2004). Resistant starch has been defined as 'the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals' (Asp, 1992). Resistant starch has been classified into four major types.

(RS1) is the physically entrapped or inaccessible RS found in the cell wall of whole or partially milled grains and seeds as well as legumes. (RS2) is the native resistant ungelatinized granules found in raw potatoes, green bananas, and high amylose maize starches. (RS3) is the retrograded crystalline starch formed during conventional food processing methods such as cooked and cooled potatoes and pasta, bread, ready-to-eat cereals, and retrograded high amylose maize starches. (RS4) is chemically modified starch due to cross-linkage with chemical agents (Brown, 2004; Topping et al., 2003; Patil, 2004; Sajilata et al., 2006) (Figure 2.2).

Formation of RS in foods depends on the botanical source, processing methods, and storage conditions (temperature and time) (Namratha et al., 2002). In this study, a commercially available high amylose maize starch (RS2) was used for the RS supplemented cereal based products.

Dietary Fiber

The amount of starch that escapes digestion and absorption has been conventionally quantified as total dietary fiber. Dietary fiber consists of edible plant materials that are resistant to enzyme hydrolysis in the small intestine. These plant materials include cellulose, hemicellulose, lignin, gums, mucilages, oligosaccharides, and pectins (DeVries, 2004). Although much debate exists regarding a consensus on the definition of dietary fiber, the American Association of Cereal Chemists (AACC) has developed a definition that it is quite extensive. Dietary fiber includes: 'The edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation' (American Association of Cereal Chemists, 2001; Patil, 2004; Prosky 2001). This definition encompasses not only the physical attributes of dietary fiber such as nonstarch polysaccharides, cell wall plant materials, and carbohydrates, but also the physiological implications of dietary fiber. Resistant starch has similar physiological responses to dietary fiber; thus, it has been traditionally classified as dietary fiber. Because both, RS and dietary fiber, bypass the small intestine and may be partially or completely fermented in the colon, the possibility that RS may have similar physiological effects to dietary fiber has been debated.

Beta (β) Glucans

Beta glucans are important components of dietary fiber and are classified as soluble fibers. Chemically, β -glucans are unbranched polysaccharides composed of 1 \rightarrow 4 and 1 \rightarrow 3 linked β -D-glucopyranosyl units. Consumption of these compounds has been associated with improvements in heart disease, cholesterol attenuation and reductions in glycemic response (Charalampopoulos et al., 2002). Barley and oats are the major grain sources of β -glucans with ranges of 3-11% and 3-7% on a dry basis, respectively (Charalampopoulos et al., 2002). According to Whistler and BeMiller (1997), ingestion of β -glucans reduces postprandial glucose levels and the insulin response in normal and diabetic individuals possibly due to the increased viscosity in the intestine, in addition to reducing cholesterol levels.

Starch Digestion

Starch is the most important carbohydrate in the human diet, and it is the main source of dietary energy (Rahman et al., 2007; Whistler & BeMiller, 1997). Starch provides most of the dietary energy in developing countries while less dietary contribution is found in Western countries. In developed countries, starches are processed at home or in industrialized settings and are usually cooked before consumption.

Starch changes occur after the starch is heated in the presence of water (i.e. gelatinized). After the starch granules swell, the crystalline and amorphous regions are disrupted allowing hydrolytic enzymes access to glucose units. Enzymatic hydrolysis of starch is initiated in the mouth by salivary α -amylases during chewing and mastication of foods resulting in exposure of starch to amylases. In the small intestine, starch hydrolysis occurs through the action of pancreatic α -amylase and brush border hydrolytic enzymes. α -amylase (salivary and pancreatic) is a random endoamylase that cleaves glucose molecules from the inside of the starch chain. The result is a mixture of dextrans, maltose and maltotriose oligosaccharides. Hydrolysis of oligosaccharides occurs through the action of brush border enzymes in the small intestine epithelium. α -glucosidase (maltase) cleaves one glucose at a time, and isomaltase (debranching enzyme) cleaves the α -1,6 linkages of amylopectin. Most of the enzymatic activity of α -amylase occurs in the duodenum in the small intestine with minor contributions from salivary α -amylase. Once starch reaches the small intestine, its digestion and absorption occurs within minutes resulting in increased blood glucose levels after consumption of starchy foods.

However, not all starches are rapidly digested. Resistant starch bypasses digestion and absorption in the small intestine, and continues to the colon where it is fermented to short chain fatty acids resulting in lower increases in blood glucose levels compared to readily digestible starch (Rahman et al., 2007) (Figure 2.3)

Resistant Starch in Foods

Formation of RS in foods is determined by the starch botanical source, processing methods, and storage conditions (i.e. temperature and time) (Namratha et al., 2002; Niba, 2003a; Niba, 2003b).

During storage, the starch polymers whether intact or gelatinized undergo a series of chemical reactions where a fraction of starch resistant to α -amylase activity re-crystallizes, a process known as retrogradation. Retrogradation is the process of amylose chains re-arranging into crystalline forms (resulting in RS3 formation), and it has been shown by researchers to increase RS content in processed foods (Whistler & BeMiller, 1997; Namratha et al., 2002). Although discrepancies exist among researchers, starch complexes with protein and fat seem to favor RS formation in foods (Namratha et al., 2002; Niba, 2002). It is believed that during storage, starch molecules, in particular amylose, may form molecular complexes with other macronutrients rendering them less susceptible to α -amylase activity.

Factors affecting RS formation include processing conditions (i.e. moist heat vs. dry heat), starch botanical sources (Niba, 2002; Niba 2003a; Niba 2003b), and presence of other ingredients in the food matrix such as protein, fat, dietary fiber, and minerals (Brown, 2004; Kendall et al., 2004; Nugent, 2005; Sharma et al., 2008). Moist heat treatments have been shown to increase RS levels in processed foods, and these levels further increased during storage (Namratha et al., 2002; Niba 2003a). Niba (2003a) evaluated the effects of storage period and temperature on RS content in cornbread. The levels of RS increased from 2.59 g/100g to 4.52 g/100g after 4 days of storage at refrigerated (4 °C) temperature. Namratha and coworkers (2002) found a significant increase ($p < 0.05$) in RS content of various autoclaved ready to eat foods after 4 months of storage.

Processing conditions such as baking resulted in increased RS content in some bread types (Liljeberg, et al., 1996; Waring, 2005). Baking of starchy foods of relatively high moisture content resulted in the formation of retrograded starch which is resistant to enzymatic activity

(Niba 2003a, Namratha et al., 2002). Partial or incomplete gelatinization of starch during baking (Niba, 2003a) may explain the small increased of RS in food products. Some starch may gelatinize and retrograde upon cooling and storage contributing to higher RS content of foods. Prolonged storage for up to 4 months at ambient temperature of ready to eat foods resulted in increased RS levels (Namratha et al., 2002).

Addition of high amylose to foods results in lighter colored products (Baixauli et al., 2008; Brown, 2004; Waring, 2005). This is important in RS supplemented foods where dietary fiber increase is desired. Incorporation of whole grains is associated with dark crust and dark crumb color in baked products. Incorporation of RS results in lighter color compared to traditional dietary fibers (Brown, 2004; Waring 2005). Resistant starch supplementation affects the texture of foods. According to Baixauli and coworkers (2008), muffins supplemented with 20% RS were softer than the control muffins. Sensory evaluations of milk puddings containing 1 to 4% RS indicated that the overall acceptability score of milk puddings containing 4% RS was 4.5 on a 9-point hedonic scale. Incorporation of 1% RS in milk puddings resulted in an overall acceptability score of 6.6 (Ares et al., 2009)

Functional Foods

The emergence of functional foods has been driven by consumers' demands for new products that provide healthy alternatives. Functional foods, defined as food or food components (including fortified, enriched) that have physiological benefits beyond basic nutrition (Institute of Food Technologists, 2005) is a dynamic and fast growing sector of the food industry. The functional foods market worldwide is estimated to be between \$7-63 billion. By 2010, the functional foods market is expected to increase to \$167 billion (Anonymous, 2008). Cereals and whole grains including RS are excellent vehicles for functional food development because cereals are rich in fiber and non-digestible carbohydrates. Cereals and cereal bars provide nutrition, convenience, value and taste consumers demand. Cereals and cereal bars are not only consumed for breakfast but also are consumed as snacks. The U.S. cereal bar market increased 43% between 2002-2007 accounting for \$1.6 billion in sales (Faron, 2008). Functional ingredients like RS can help increase dietary fiber intake.

Resistant Starch and Health Implications

The development and occurrence of chronic diseases have been major causes of concern for health professionals, the government, scientists, and the general public. Some chronic diseases such as heart disease, diabetes, and obesity have been classified as "epidemics" due to their prevalence among populations, raising health concerns and increasing medical costs. In an attempt to lower the incidence of such diseases, researchers have investigated the effect of diet compositions, lifestyle factors, exercise, and heredity factors, among others. Although conflicting results from epidemiological studies and clinical trials exist, there is strong evidence suggesting that dietary modifications may help ameliorate chronic diseases.

In recent years, the term 'metabolic syndrome' has been used to describe a cluster of conditions related to heart disease (i.e. coronary heart disease) and type 2 diabetes. The conditions associated with metabolic syndrome include obesity (in particular abdominal obesity), abnormal glucose metabolism (high blood glucose even though insulin is present or there is insulin resistance), high blood cholesterol and abnormal lipid profiles, and hypertension (Tapsell, 2004).

Starch is a major dietary component of many populations. It is believed that starch consumption can vary from more than 50% of energy intake in some agricultural societies to less than 25% in Western societies (Topping et al., 2003). Although discrepancies exist regarding the definitions of dietary fiber and resistant starch, there is certainly a link between colon health and fermentation of dietary fiber and/or resistant starch by colonic microflora (Nugent, 2005; Sajilata et al., 2006). Dietary fiber and RS, produce short chain fatty acids (SCFA) (i.e. acetate, propionate, and butyrate) as energy fuel for colonocytes. In particular, butyrate seems to be the preferred fuel for the colonic mucosa microflora, and RS fermentation produces large amounts of this particular fatty acid. In vitro animal studies and human studies have shown that RS is fermented in the colon to SCFA; however, the source and structure of RS, processing conditions, and interaction of RS with other dietary components may affect the rate and amount of RS fermentation (Nugent, 2005; Sajilata et al., 2006).

Topping and Clifton (2001) showed that the amount of products derived from the fermentation of dietary fiber were greater than expected based on dietary fiber as the only substrate. This indicated that another compound was being fermented, and this compound was shown to be RS. Resistant starch consumption has been linked to colonic health and possibly

colon cancer prevention. SCFA production results in lower intestinal pH. This acidic environment prevents the conversion of primary bile acids to secondary bile acids which are implicated in colonic cell apoptosis (Kendall et al., 2004). In an in vivo animal study, pigs were fed raw potato starch for 19 days and butyrate formation resulted in decreased colonocyte apoptosis (cell death) (Mentschel and Claus, 2003). The low pH seen in the colonic mucosa may be associated with lower accumulation of protein metabolism byproducts such as phenolics, ammonia, and other nitrogenous compounds which may induce tumor formation (Champ, 2004).

In addition, RS may result in a modest increase in fecal bulking. This was demonstrated by Jenkins and colleagues (1998) when 24 healthy individuals were fed diets containing a high amylose starch (RS2) and retrograded high amylose (RS3). In addition, RS has been proposed as a prebiotic substrate. Prebiotics are nondigestible food components that promote the growth and beneficial activity of health "friendly" bacteria classified as probiotics improving intestinal health. These probiotics include *Bifidobacteria* and *Lactobacillus acidophilus* which are lactic acid bacteria (Topping et al., 2003). Although there is potential for the prebiotic health benefits associated with RS, more research is needed to determine the specific effects of RS on probiotics. Other potential health benefits of RS include stool speed transient time, feeling of satiety, cholesterol lowering effects, and improved blood glucose and insulin levels by ameliorating insulin resistance and preventing development of type 2 diabetes. Current research highlighting the potential benefits of RS on glucose and insulin metabolism and the prevention of type 2 diabetes is discussed in the following section.

Oxidative Stress and Type 2 Diabetes

Type 2 diabetes (T2D) is a disease that has reached epidemic proportions in developed countries. In 2002, this disease affected approximately 15 million Americans (Liu, 2002). In 2008, there were over 23 million people in the U.S. who have diabetes and 57 million who have pre-diabetes, a condition where blood glucose level is high (100-125 mg/dl) but not considered diabetic (blood glucose > 126 mg/dl) (American Diabetes Association, 2007). Diabetes is more common in the Hispanic/Latino population (10.4% incidence) than in non-Hispanic whites (6.6% incidence) (American Diabetes Association, 2007; Center for Disease Control, 2005).

Diabetes is the fifth leading cause of death in the U.S.; in 2007, the estimated medical costs associated with this disease were estimated to be \$172 billion dollars (American Diabetes

Association, 2007). The number of people suffering from diabetes worldwide in the year 2000 was reported to be 171 million, and projections estimate at least 366 million people worldwide will have diabetes by 2030 (World Health Organization, 2008). Type 2 diabetes is characterized by high blood glucose and high insulin levels, insulin resistance, and pancreatic beta cell dysfunction (Whitney & Rolfes 2002).

The increased incidence of type 2 diabetes may be related to an increase in reactive oxygen species (ROS), leading to oxidative stress. Oxidative stress is defined as the imbalance between the concentration of reactive oxygen species and antioxidant defense mechanisms (Dotan et al., 2004). This imbalance has been associated with health conditions such as obesity and insulin resistance and chronic diseases such as cardiovascular disease and type 2 diabetes. Oxidative stress resulting from the production of ROS has been suggested as the cause for development of insulin resistance, beta-cell dysfunction, impaired blood glucose tolerance and type 2 diabetes (Wright et al., 2006).

Reducing oxidative stress through dietary consumption of resistant starch may be one approach to reducing the risk of developing type 2 diabetes. Epidemiological studies have consistently shown an association between whole grain consumption and decreased risk of developing type 2 diabetes by 20-42% (Fung et al., 2002; McKeown et al., 2002; Meyer et al., 2000; Montonen et al., 2003). The role of resistant starch in reducing this risk has been suggested but has not been directly measured. The specific effects of RS on reducing oxidative stress are not well understood.

Measures of Oxidative Stress

F₂-Isoprostanes (F₂-IsoPs) are compounds formed by non-enzymatic reactions as a result of free-radical-mediated peroxidation of arachidonic acid. F₂-IsoPs are the most accurate biomarkers of lipid oxidation to assess in vivo oxidative stress status. F₂-IsoPs are stable molecules detected in biological fluids (blood, urine) and tissues in humans (Dotan et al., 2004). Quantification of F₂-IsoPs in urine or plasma is not invasive and it is convenient. The gas chromatography negative ion chemical ionization mass spectrometric (GC/NICI-MS) method utilizing stable isotope dilution is the most preferred method for the quantification of F₂-IsoPs. For quantification purposes, the 15-F_{2t}-IsoP (also known as 8-Iso PGF_{2α}), and other F₂-IsoPs that co-elute with this compound are measured. The GC/NICI-MS method results in high resolution

of GC separation and specificity and sensitivity of MS which allows for quantitative results in pg/ml. Elevations of IsoPs in plasma are associated with lipid oxidation and diseases such as atherosclerosis, diabetes, obesity, and other disorders. Thus, supplementation with dietary antioxidants and antidiabetic treatments has been shown to decrease F₂-IsoPs levels (Liu et al., 2009).

Aerobic reactions lead to the formation of ROS. An increase in ROS such as superoxide radicals, hydroxyl radicals, and hydrogen peroxide in the intracellular environment can lead to oxidative stress. Aerobic organisms have developed endogenous enzymatic systems to neutralize ROS, and these include superoxide dismutase (SOD), catalases (CA), and glutathione peroxidases (GPx) (Margis et al., 2008). Enzymatic assays involve measurements of the enzyme's activity by monitoring the formation of end products and/or the decrease in the levels of substrate. The enzymes commonly measured in relation to the “oxidative status” include superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) (Dotan et al., 2004).

Glutathione peroxidase is the general term for a group of isozymes who function to detoxify peroxides from the cells. Glutathione peroxidases catalyze the reduction of H₂O₂ or hydroperoxides to water or alcohols using reduced glutathione (GSH) as the electron donor ($H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$) and ($ROOH + 2GSH \rightarrow ROH + GSSG + H_2O$). Because hydroperoxides can form highly reactive radicals, glutathione peroxidases play a critical role in protecting the cells against free radical damage in particular, lipid oxidation. In humans, the GPx enzymes are comprised of 4 subunits with each subunit containing a molecule of selenocysteine in the enzyme active site. The selenocysteine is believed to participate in electron donation to the peroxide substrate and becomes oxidized in the process. The enzyme then uses GSH as an electron donor to regenerate the reduced form of selenocysteine. In mammals, there are 4 selenium dependent GPx isozymes: 1) Classical GPx (cGPx) which is found in red blood cells, liver, lung, and kidney. 2) Gastrointestinal GPx (GPx2). 3) Plasma GPx (GPx3) which is present in organs such as kidney, lung, epididymus, placenta, seminal vesicle, heart, and muscle. 4) Phospholipid GPx (PHGPx4 or GPx4) present in various tissues (Dotan et al., 2004; Margis et al., 2008).

Resistant Starch and Type 2 Diabetes

Starch that bypasses digestion and absorption in the small intestine and reaches the colon provides low calories and low glucose and insulin responses compared to more digestible dietary starch. Because RS results in smaller post-prandial glucose and insulin responses, it is reasonable to suggest that RS has a low glycemic index (GI) (Niba, 2002). However, there is no conclusive evidence on the connection between RS and low GI because such studies are scarce, suggesting research in this area is necessary. Resistant starch seems to lower postprandial glucose and insulin responses, lower cholesterol and triglyceride levels, improve insulin sensitivity, and increase satiety (Park et al., 2004; Robertson et al., 2003). These potential health benefits indicate RS may be an appropriate dietary component for the prevention of diseases characterized by abnormal lipid profiles, and insulin resistance which may lead to cardiovascular disease and/or development of type 2 diabetes (Higgins, 2004).

Conflicting results in the literature suggest that total diet composition (i.e. macronutrient composition - fat, protein, carbohydrates), sources of RS, cooking and/or processing conditions, and dietary fiber content need to be considered when evaluating glycemic and insulin responses and RS consumption. It is equally important to note that humans consume RS in a cooked or somehow processed form such as cereals, bread, pasta, and potatoes (Higgins, 2004); therefore, careful attention is needed when evaluating research data based on uncooked RS.

Resistant Starch and Type 2 Diabetes - Animal Studies

In a study conducted to determine the effects of RS on glucose and insulin responses, and blood lipid concentrations, diabetic rats were fed 16% RS (w/w) from either corn or rice for 3 weeks. The RS mixes were prepared in house by cooking/cooling cycles; therefore, it was retrograded starch. The diets were the same among treatments except RS from corn or rice was replaced for part of the corn starch already present in the diet. The authors found that glucose and insulin levels decreased with RS supplementation but the decrease failed to achieve statistical significance; however, both RS resulted in lower serum cholesterol concentrations by 30-36% compared to the corn starch control group (Kim et al., 2003).

In a study conducted by Brown and colleagues (2003), rats were fed RS2 corn at different levels, and the starch was uncooked and cooked. The 8 treatments were 0, 270, 600, or 850 g corn amylose/Kg total starch, and the diets were similar in composition except for the levels of

amylose and amylopectin. The authors speculated that diets high in amylose starches result in decrease post-prandial insulin and glycemic responses compared to high amylopectin starches. Different carbohydrate sources lead to different responses in glucose and insulin concentrations. Moreover, the authors were interested in determining a dose-response relationship between amylose ingestion and glucose-insulin levels as well as the effects of cooking of corn starches. Prior to an overnight fasting, rats were fed the test meal and blood glucose and insulin were measured for 2 hours, so the study measured acute responses. Insulin levels were decreased for all the uncooked treatments whereas glucose levels were unaffected by treatments. The authors concluded that a small proportion (270 g/Kg diet) of uncooked amylose resulted in decreased insulin responses; however, for the cooked amylose, a higher proportion (600-850 g/Kg diet) was needed to achieve a similar insulin effect. Therefore, cooking corn starch attenuates insulin responses when rats are fed an acute test meal (Brown et al., 2003).

Lopez and colleagues (2001) compared raw potato starch (RPS) and high amylose starch (Hi-Maize) by feeding rats 20% RS2 for 21 days. They found that blood cholesterol levels decreased by 27% to 31% respectively, and triglycerides decreased by 22% to 28%, respectively. In addition, enhanced fermentation of RS2 to SCFA was observed as well as increased mineral absorption (Fe, Ca, Mg, etc.). The authors acknowledged the results in rats may not be the same for humans. For instance, the intestinal tract and products of fermentation between rats and humans are different, but there is an indication of the potential health benefits associated with consumption of RS2 (Lopez et al., 2001).

Although the above studies were short-term, with differences in the sources of RS, and experimental designs, there seems to be a link between starch that is resistant to digestion and glucose, insulin, cholesterol, triglycerides, and colonic fermentation. These factors are markers for the development of chronic diseases such as coronary heart disease and type 2 diabetes, and they could help elucidate the potential health benefits of RS as a dietary component in disease prevention.

Resistant Starch and Type 2 Diabetes - Human Studies

In humans, limited evidence is available on the role of RS in chronic diseases such as type 2 diabetes. In addition, the studies are short-term which suggest a need for long-term research to fully elucidate the impact of RS on disease prevention and management. Resistant

starch consumption results in smaller glucose and insulin responses. This is due to the lack of enzymatic attack on RS and its indigestibility in the small intestine. Resistant starch could be a potential candidate for the management of type 2 diabetes.

Supplementation of RS was evaluated in a study of 24 female overweight/obese subjects (Park et al., 2004). The experimental design was a double blind study where a control group received 24 g/day of corn starch (CS), and the experimental group received the same amount of corn RS. Participants received a freeze dried liquid once a day for 21 days in addition to their regular diets. The supplement contained the same amount of protein and fat, and it was made of starch powder, dried whole grains, vegetables, mushrooms, and seaweed. Supplementation of RS resulted in lower total and LDL cholesterol levels and reduced fasting glucose levels compared to the control group. However, there were no differences in serum insulin levels between the RS and control groups. Because RS resulted in lower cholesterol and glucose levels, its palatability was acceptable, and gastrointestinal discomfort was not observed, the authors suggest that RS may be a potential candidate for preventing type 2 diabetes (Park et al., 2004).

Resistant starch supplementation (60 g/day) for 24 hours was given to 10 healthy men and women (Robertson et al., 2003). The RS source was high amylose maize (RS2) manufactured by National Starch. The RS2 was Novelose260 and contains 60% RS and 40% Dietary starch (DS) from corn. The experimental group was given a supplement containing 60g RS and 40 g DS, and the control group received 40 g of waxy maize starch in a cross over design. The supplements were mixed with jelly, and the participants were instructed to consume 4 doses of the jelly throughout the day in addition to the diets provided by the researchers. The diets were designed to meet the energy requirements of the participants based on weight, physical activity, and age. Results from acute, high RS supplementation resulted in lower plasma glucose and insulin levels compared to the control group. The authors concluded this experiment resulted in improved insulin sensitivity, and even though it is a short-term study, there are implications for RS consumption and prevention of type 2 diabetes by mediating insulin resistance (Robertson et al., 2003).

In another short term study, 24 healthy men and women were given cornstarch breads varying in the amount of amylose (Behall & Hallfrish, 2002). Amylose content of the cornstarch breads were: 30%, 40%, 50%, 60%, and 70%. The breads were fed for 3 days in addition to a

diet prepared by the researchers. The study was designed to determine the dose-response of amylose in bread, and its effects on plasma glucose and insulin responses. Results show that plasma glucose levels were lower in the 50%-70% amylose breads compared to the control; however, the greatest glucose lowering effect was seen in the breads containing 60%-70% amylose. Insulin concentrations were also lower in the 60%-70% amylose bread compared to the control bread. The authors concluded that RS starch levels in corn starch bread should exceed 50% amylose which translates to more than 8 g RS to observe a significant decrease in blood glucose and insulin concentrations (Behall & Hallfrish, 2002). These authors reported similar findings to studies in which levels of RS supplementation of 12g resulted in lower glucose and insulin levels. However, difficulties in interpreting data across studies exist due to the different sources of RS used, content of RS in foods, methodology used to quantify RS, and food processing conditions. In addition, the above studies are short-term, and long-term controlled clinical trials are necessary to understand the potential health benefits of RS in the human diet, and its effects on development of chronic diseases.

Resistant Starch Implications

RS occurs naturally in many food products such as whole grains, cereals, bread, pasta, potatoes, and legumes; however, the RS content and types in these food products varies. Consumption of RS is low in many Western countries. In Europe, Australia, and New Zealand, RS intakes have been estimated to be between 3-7 g/day (Brown, 2004). In China and India, RS intakes are higher (10-18 g/day) (Goldring, 2004). Although starch is a major component of the diet, advances in food processing techniques, and the availability of processed foods, has resulted in lower RS consumption in Western societies. Starches have been widely used in the food industry in processed foods as texture enhancers, thickening and bulking agents, and for nutritional qualities. However, the nutritional effects of the starch that escapes digestion (RS) and the food products that contain RS need careful analysis and monitoring.

Traditionally, RS have been analyzed and quantified using the Association of Analytical Chemists (AOAC) methods for total dietary fiber (TDF AOAC 985.29 and others). The AOAC method 2002.02 is the current method used to quantify RS2 (Brown, 2004). In vivo studies are only possible with human ileostomy patients; however, in vivo studies are expensive, time consuming, and impractical for repeated measurements in laboratory settings. In vitro

measurements are practical for repetitive analyses and are good estimates of RS content (Goldring, 2004). The difficulties in RS measurements, lead to conflicting results of RS determination in the literature. In addition, many countries use different methods of RS analysis as approved by regulatory agencies. These various methodologies may result in different RS levels reported in the literature. However, there seems to be a general consensus about the association of RS and chronic disease prevention. Research on the physiological benefits of RS has been conducted during the past few decades. The major focus has been on RS2 and RS3 and high-amylose corn starch (Brown, 2004).

It has also been shown that some RS types enhance the functionality of food products. For instance, some RS improve textural and organoleptic qualities, and increase the amount of dietary fiber in the food product (Brown, 2004).

In addition, RS has been shown to act as a prebiotic component by promoting the growth and activity of healthy bacteria such as *bifidobacteria and lactobacillus* and decreasing levels of harmful bacteria in the colon (Topping et al., 2003; Brown, 2004; Goldring, 2004). Moreover, RS can have synergistic interactions with other dietary components of the starchy containing food product or other prebiotics such as indigestible oligosaccharides (Brown, 2004).

Resistant starch has been proposed as a functional food ingredient (Niba, 2002). Functional foods include foods that not only provide nutritional value but also provide some physiological benefits when consumed in a regular diet (Niba, 2002). In order to maximize the potential of RS as a functional food, foods that are high in RS should be identified and processed without affecting the organoleptic properties of RS so it will be accepted by consumers. Also, the use of food processing techniques that would enable RS stability in foods is another important consideration (Niba, 2002).

Resistant starch has potential as a low-carb food ingredient (Brown, 2004; Patil, 2004). Because of its physicochemical characteristics (i.e. not absorbed), high dietary fiber content, and low caloric content, RS is a good candidate for low-carb food product development.

Resistant starch ingredients have applications in many food products such as breads, muffins, pasta, and breakfast cereals, among others. In addition, RS provides dietary fiber and organoleptic properties that are acceptable and appeal to consumers (Brown, 2004). Resistant starch has also been used in diabetic snack bar formulations. Some nutrition bars such as Choice DM Nutrition Bar and Glucerna Snack Bar include RS on the label. Other bars such as Extend

Bar and Nite Bite Timed Release Glucose have uncooked cornstarch on their labels (Hayes, 2002). This is significant since RS can be identified on the ingredients list as maltodextrin, cornstarch, and dextrin, among other names (Goldring, 2004).

Commercially available physically modified starches such as Hi-Maize (RS2), Novelose (RS3) have diverse functional properties (Brown, 2004; Patil, 2004). Resistant starch is palatable, white in color, of small and uniform particle size, improves texture in baked goods, has low water holding capacity, good mixing properties, extrudes well with good filming properties, low bulk density high fiber, improves crispiness, provides nutritional benefits, and a chance for developing innovative and creative foods (Brown, 2004; Patil, 2004). In Australia, a white bread fortified with high-maize RS increased the dietary fiber content from ~3% to 5.6%. This allowed the marketing of such bread as containing more dietary fiber than multi-grain bread. The product was well accepted by consumers and successful in the market. The product met consumers' demands by providing a healthy product of high quality (Brown, 2004). The white bread fortified with RS resulted in improved physical properties. Addition of RS, improved loaf volume, lighter crust, uniform texture, and increased dietary fiber content (Brown, 2004).

Consumers are well aware of the health benefits of whole foods and dietary fiber; however, modifying the diet sometimes means less palatability and acceptability. Therefore, successful incorporation of RS into food products, development of high RS foods, and processing methods to ensure RS stability are some of the needs food scientists must address based on sound research. In addition, consumer understanding about the physiological consequences of dietary starch and RS is paramount for the success of RS as a functional food ingredient.

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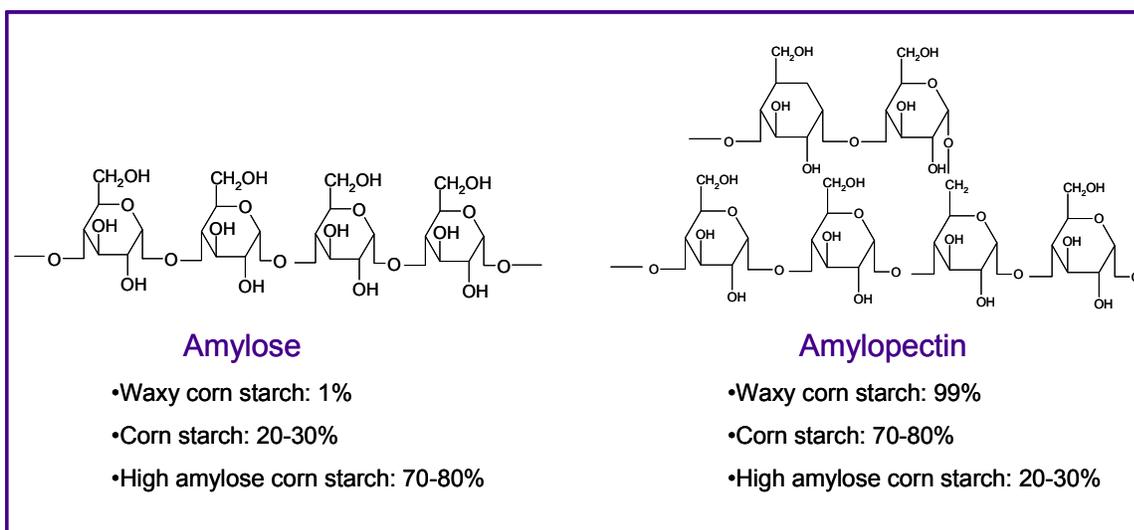


Figure 2.1. Chemical structures of amylose and amylopectin, and their percentages in corn starches.

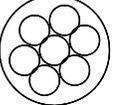
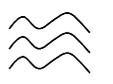
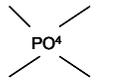
Type of RS	Description	Food sources	Resistance reduced by
RS 1 	Physically inaccessible	Whole/partly milled grains, seeds, legumes	Milling, chewing
RS 2 	Ungelatinized resistant granules	Raw potatoes, green banana, high amylose maize	Food processing, cooking
RS 3 	Retrograded starch	Cooked and cooled potatoes, bread, cornflakes, cook/cool moist heat treatment	Processing conditions
RS 4 	Chemically modified starches –cross-bonded	Fiber-drinks, foods with modified starches	Less susceptible to digestion <i>in vitro</i>

Figure 2.2. Classification of resistant starch types, food sources, and factors affecting digestion resistance.

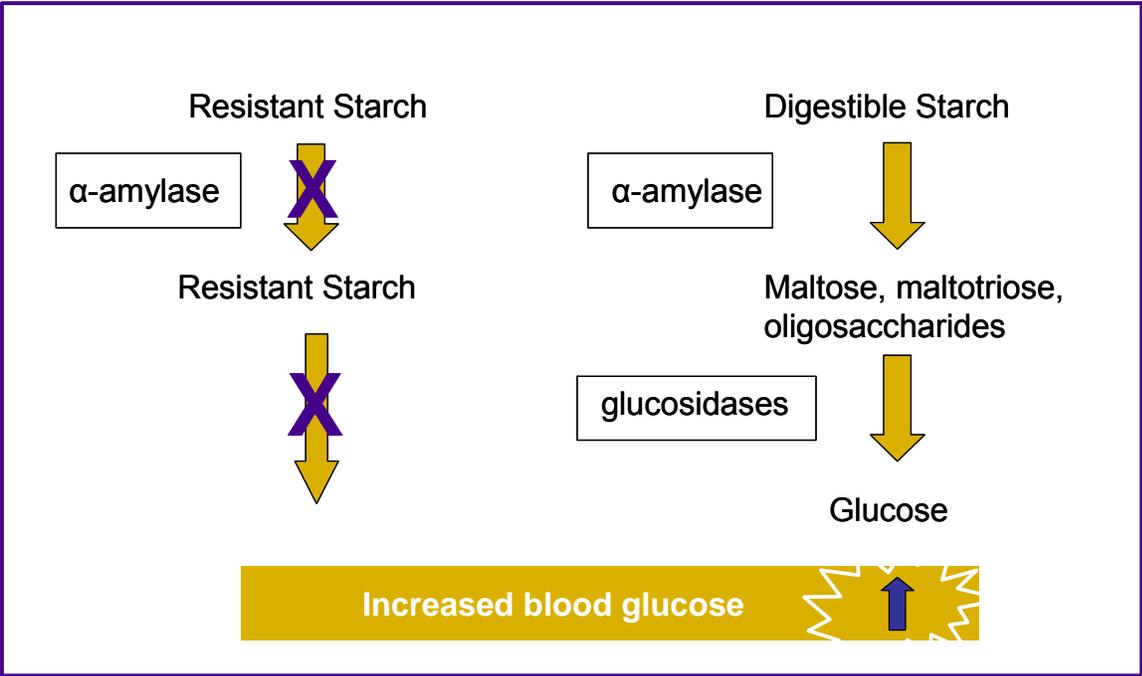


Figure 2.3. Starch metabolism and enzymatic activity.

CHAPTER 3: PHYSICOCHEMICAL AND SENSORY PROPERTIES OF RESISTANT STARCH-CEREAL BASED PRODUCTS DURING STORAGE

Abstract

The purpose of this study was to develop cereal-based food products supplemented with high amylose corn starch (resistant starch, RS) and to evaluate their physicochemical and sensory properties as influenced by storage time. Resistant starch granola bars and cereals were developed to provide 2 levels (10% and 15%) of RS; isocaloric (0% RS) control samples were prepared with readily digestible (high amylopectin) starch. Samples were stored for up to 4 weeks at 20 °C. Proximate composition, RS, and beta-glucan concentrations were measured. Physical characteristics included texture, starch viscosity, and color. Sensory acceptability was evaluated using a 9-point hedonic scale with a targeted mean acceptability of 6.0 ("likes slightly"). Mean composition of the high RS granola bars was 6% protein, 15% moisture, and 18% lipid. RS levels increased from 14 to 16 g/serving after 4 weeks of storage, supporting published research that RS increases with storage due to retrogradation and crystallization of amylose chains. Soluble starch and beta-glucan concentrations were not changed during storage. Color became lighter as the level of RS increased. Sensory results indicated that the granola bars/cereals were acceptable. Incorporation of high levels of RS in food systems is feasible without compromising product acceptability.

Keywords: Resistant starch; Amylose; Granola bars; Cereals; Storage time and temperature; Consumer acceptability.

To be submitted to *Food Chemistry*

Introduction

Starch is a major component of the human diet (Lehman & Robin, 2007), and it is widely used in processed foods (Brown, 2004). Starch is found inside granules in plants, and it is composed of two large glucose polymers: amylose (straight chain) and amylopectin (branched chain) (Whistler & BeMiller, 1997). Processing conditions may change the granular starch structure to nongranular forms (Murphy et al., 2008). The starch structure affects the digestibility of the starch; therefore, not all starches are hydrolyzed equally by digestive enzymes (Patil, 2004; Tapsell, 2004). The starch that is not digested in the small intestine is called resistant starch (RS). The amylose:amylopectin ratio is a determining factor in the formation of resistant starch. High amylose starch is more resistant to enzymatic hydrolysis compared to amylopectin (Leeman et al., 2006; Lehman & Robin, 2007).

Resistant starch was first recognized, although not identified as such, in the 1980s when it was found to be resistant to enzymatic hydrolysis (Englyst et al., 1992). Resistant starch has been defined as 'the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals' (Asp, 1992). There are four major types of RS (Figure 3.1). (RS1) is the physically entrapped or inaccessible RS found in the cell wall of whole or partially milled grains and seeds as well as legumes. (RS2) is the native resistant ungelatinized granules found in raw potatoes, green bananas, and high amylose corn starches. (RS3) is the retrograded crystalline starch formed during conventional food processing methods in products such as cooked and cooled potatoes and pasta, bread, ready-to-eat cereals, and retrograded high amylose corn starches. (RS4) is chemically modified starch cross-linked with chemical agents (Brown, 2004; Topping et al., 2003; Patil, 2004; Sajilata et al., 2006).

Dietary fiber is composed of nondigestible carbohydrates and lignin. Functional fiber is nondigestible carbohydrates that have beneficial physiological effects to humans. In recent years, RS has been classified as a functional fiber. Resistant starch is not digested in the small intestine and, therefore, has many of the physiological health benefits associated with dietary fiber. The recommended dietary fiber intake is 25 g/d and 38 g/d for women and men, respectively; however, most Americans consume 14 g/d of dietary fiber, well below the recommended intakes (Institute of Medicine, 2005).

Dietary fibers, including RS, promote beneficial physiological effects including laxation, blood cholesterol attenuation and blood glucose attenuation (Patil, 2004). The physiological

effects of RS have been studied in animals and humans to elucidate the potential health benefits in gastrointestinal health and glycemic responses. The benefits associated with intestinal health include fermentation of RS to short chain fatty acids (SCFA) including acetate, propionate, and butyrate. Other benefits include laxation and prebiotic effects. The SCFA have been implicated in lowering the colonic pH which results in acidification of the colonic environment. These acidic conditions prevent the growth of carcinogen/tumor promoting compounds, and provide substrate for probiotics in particular *lactobacillus* and *bifidobacterium*. The benefits of RS on glycemic control have been shown in healthy, overweight, and diabetic subjects (Brown, 2004). Feeding a liquid beverage containing uncooked RS3 (retrograded starch) (24 g /day) to overweight individuals for 21 days resulted in lower blood cholesterol and glucose levels compared to feeding regular corn starch (Park et al., 2004). Prior acute consumption of uncooked RS2 (60 g/day for 24 hours) resulted in lowered postprandial plasma glucose and insulin levels in healthy subjects the following day after a meal tolerance test (Robertson et al., 2003).

Increasing the dietary fiber content of foods by supplementation with RS is one approach to increase dietary fiber intake. Food product development efforts have been driven by consumers' demands for healthy products. These efforts have been directed towards the emergence of functional foods and functional ingredients. Granola bars and cereals are excellent vehicles for functional food development since granola bars and cereals are rich in fiber and non-digestible carbohydrates. According to Mintel International Group Ltd, cereal bars sales were \$1.4 billion in 2007, an increase of 43% from 2002 (Teen, 2008). Consumers value convenience (on-the-go foods) and healthy alternatives such as granola bars that are low-calorie, low-fat, and high-fiber. Thus, incorporating RS into cereal bars increases the dietary fiber content and the nutritional value of cereals while providing convenience for the on-the-go consumers.

Resistant starch has been shown to improve textural and organoleptic qualities, along with increasing the amount of dietary fiber in some food products (Kendall et al., 2004). Resistant starch acts as a prebiotic component by promoting the growth and activity of probiotic bacteria and can interact with other prebiotic dietary fibers such as beta-glucans (Brown, 2004; Goldring, 2004; Topping et al., 2003). Resistant starch levels in foods have been shown to increase during storage as a result of retrogradation and amylose chain crystallization (Kumari et al., 2007; Namratha et al., 2002; Niba, 2002; Niba, 2003a; Sajilata et al., 2006).

According to Kendall and coworkers (2004), RS doses of 20-30 g/day are needed to observe physiological effects of RS consumption. This level of consumption is 3-4 times higher than the actual levels of RS consumption in Western countries, which are estimated to be 5-10 g/day RS. The estimated resistant starch intake in the U.S. is between 3-8 g/day. In addition, most foods have RS contents of 3 g or less per serving (Murphy et al., 2008). These estimates provide useful information for food scientists and nutritionist to develop food products supplemented with RS and to evaluate the potential health benefits of these products.

Formation of RS in foods is determined by the starch botanical source, extent of processing, and storage conditions (Namratha et al., 2002; Niba, 2003a; Niba, 2003b). Most physiological studies of the health benefits of RS have been conducted by adding RS as uncooked powders to beverages and jellies at levels of 24-60 g RS/day (Park et al., 2004; Robertson et al., 2005). However, the problem with this approach is that RS would not normally be used in un-heated foods. There is a dearth of information on the effects of RS in processed/cooked foods on parameters related to glucose control in humans. Thus, the purpose of this study was to formulate a cooked cereal food with RS supplemented at levels that may have health significance and determine effects of formulation and storage time on the physicochemical properties, RS conversions, and sensory quality and acceptability of these cereal based products.

Materials and Methods

Product Preparation

Two granola products (granola bars and cereals) were formulated to contain levels of RS that reportedly have dietary and health significance. A commercially available RS2 was used (Hi-Maize[®] 260, donated by Food Innovation, National Starch, Bridgewater, NJ) which contained 60% RS based on total dietary fiber analysis (TDF). Commercially available waxy corn starch was used in the formulation of the isocaloric reference (control) granola bars and granola cereals. The waxy corn starch contained 40% readily digestible starch (Amioca[®], donated by Food Innovation, National Starch, Bridgewater, NJ). The waxy corn starch contained <0.5 g RS/100g.

Granola bars and cereals were formulated to target 0, 10, and 15g RS/100 g serving of product using the formulations described in Table 3.1. Products were prepared by mixing dry

ingredients (oats, RS2 at 10 and 15g RS supplemented bars and cereals) or waxy corn starch (control bars and cereals), coconut, almonds, and baking powder) in a large bowl. For the granola bars, liquid ingredients (honey, canola oil, and egg whites) were whisked until smooth in a separate bowl. Blended liquid ingredients were added to the dry ingredients in three portions. The mixture was stirred until dry ingredients were coated and distributed. Granola cereals contained the same ingredients excluding the egg whites, gum arabic, and baking powder and were prepared similarly. Each formulation mixture was placed in aluminum baking pans. Products were baked in a conventional oven preheated to 163 °C (325 °F) for 35 minutes until golden brown and were then cooled to room temperature. The baking process was controlled by ensuring use of the same pans, oven loading and pan placement within the oven.

The granola bars and cereals for each formulation were prepared twice, on different days, with 8 granola bars and 8 granola cereal servings in each batch (Appendix A). Granola bars and cereals were analyzed weekly at 0, 7, 14, and 28 days for subsequent analyses.

Sample Preparation for Analyses

Fifty grams each of granola cereals or bars were ground using a coffee grinder (Black and Decker Smart Grind Coffee Grinder) to pass through a 1.0 mm sieve, approximately 60 seconds. Ground, sieved samples were transferred to a plastic jar where the contents were mixed thoroughly. Samples were freeze-dried (Virtis Freeze Dryer, SP Industries Inc., Gardiner, NY) at -70 °C for 48 hours. Samples were stored in metalized multilayered flexible pouches (Mylar®MC2, DuPont Teijin Films US, Wilmington, DE) at ambient temperature (20 °C) for up to 4 weeks. This packaging material is an excellent barrier to moisture, oxygen, and light. The packaging material also resembles commercial packaging materials for granola bars and cereals in the marketplace. Products were analyzed weekly at 0, 7, 14, and 28 days of storage.

Physical and Chemical Properties of RS Cereal Based Products

Chemical Analyses

Determination of Resistant Starch (RS) and Soluble Starch (SS)

RS and SS concentrations were determined using the procedure described by McCleary and Monaghan (2002), based on the Association of Official Analytical Chemists (AOAC)

method 2002.02. The assay was performed using a commercially available kit for RS determination (Megazyme International Ireland Ltd., Bray, Co. Wicklow, Ireland).

Freeze-dried ground sample material (100 mg) was added to a screw-cap glass test tube (16 x 100 mm) and 4 ml of sodium maleate buffer (pH =6.0) containing pancreatic α -amylase and amyloglucosidase (AMG) were added. Sample was mixed and incubated in a shaking water bath (linear motion at 100 revolutions per minute) for 16 hr at 37 °C. During this stage, the non-resistant starch was solubilized and hydrolyzed to glucose by the two enzymes. The reaction was stopped by adding an equal volume (4 ml) of 50% ethanol (EtOH). The tube was mixed with a vortex mixer for 60 sec and centrifuged at 1000g for 10 min. The supernatant was decanted. The pellet was re-suspended in 50% EtOH, and the process repeated twice with supernatants from each combined and subsequently used for determination of solubilized starch.

Resistant Starch Determination

A magnetic stirrer bar (5 x 15 mm) was added to the tube containing the pellet and 2 ml of 2M KOH were added. The resistant starch fraction was re-suspended and dissolved by stirring for 20 min in an ice water bath over a magnetic stirrer. Eight milliliters of 1.2M sodium acetate buffer (pH=3.8) and 0.1 ml of AMG were added, mixed and incubated in a water bath for 30 min at 50 °C. For samples containing greater than 10% RS content, tube contents were transferred to a 100 ml volumetric flask, adjusted to volume with water and mixed. A 10 ml aliquot was centrifuged at 1000 g for 10 min. Samples containing less than 10% RS were not diluted and the tube was centrifuged directly at 1000 g for 10 minutes.

Aliquots (0.1 ml) were transferred to two glass test tubes (either the diluted or the undiluted aliquots) and were treated with 3 ml glucose oxidase-peroxidase-aminoantipyrine (GOPOD) reagent. Samples were incubated for 20 min at 50 °C and absorbance read at 510 nm (Beckman Coulter™ DU® 530 Life Sciences UV/VIS Spectrophotometer, Fullerton, CA) against a reagent blank. Glucose solution (1 mg/ml) was used as a standard (Figure 3.2).

Soluble Starch (Non-Resistant Starch) Determination

Collected supernatant from the initial centrifugation was adjusted to volume with 100 mM sodium acetate buffer (pH = 4.5) in a 100 ml volumetric flask and mixed. Duplicate (0.1 ml) aliquots of solution were combined with 10 μ l of AMG solution and incubated for 20 min at

50 °C. Three milliliters of GOPOD reagent were added and incubated for 20 min at 50 °C. Absorbance was measured at 510 nm (Beckman Coulter™ DU® 530 Life Sciences UV/Vis Spectrophotometer, Fullerton, CA) against a reagent blank.

Duplicate samples were analyzed for each treatment. Absorbance data were converted by a reference formula (Megazyme International Ireland Ltd., Bray, Co. Wicklow, Ireland) into g/100 g sample based on dry weight basis (dwb) (Appendix B).

Soluble Fiber - Beta-Glucan Determination

The beta-glucan content was determined using the procedures described by McCleary and Homes (1985), McCleary and Codd (1991), and McCleary and Mugford (1992), based on the AOAC method 995.16. The assay was performed using a commercially available enzyme kit (Megazyme International Ireland Ltd., Bray, Co. Wicklow, Ireland) (Figure 3.3).

Ground, freeze-dried, sample material (200 mg) was placed in a screw cap glass test tube. EtOH (50%, 5 ml) was added to remove free sugars and reduce the level of lipids. Samples were incubated in a boiling water bath for 5 min, mixed on a vortex mixer, and another 5 ml of 50% EtOH were added. Samples were mixed and centrifuged at 1000 g for 10 min. The supernatant was discarded and the pellet re-suspended in 10 ml of 50% EtOH, centrifuged, and supernatant discarded. The pellet was suspended in 5 ml of 20mM sodium phosphate buffer (pH=6.5) and the mixture equilibrated at 50 °C for 5 min. Lichenase enzyme (0.2 ml) was added and mixed (vortex). Tubes were covered with parafilm and incubated for 60 min at 50 °C with occasional vortex mixing (4 times). Two ml of 200 mM sodium acetate buffer (pH=4) were added to each sample, mixed, equilibrated at room temperature for 5 min and centrifuged at 1000 g for 10 min. Aliquots (0.1 ml) were transferred to three clean tubes and two tubes (duplicates per sample) were treated with 0.1 ml β -glucosidase in 50 mM sodium acetate (pH=4). The third aliquot (blank) was treated only with 0.1 ml of 50 mM sodium acetate buffer (pH=4). Tubes were incubated at 50 °C for 10 min. GOPOD reagent (3 ml) was added and the tubes incubated for 20 min at 50 °C. The absorbance was read at 510 nm (Beckman Coulter™ DU® 530 Life Sciences UV/Vis Spectrophotometer, Fullerton, CA) against a reagent blank (Appendix C).

Proximate Analysis, Caloric Content, and Water Activity

Ground samples of control and RS-supplemented granola bars and cereals were analyzed for crude fat, ash, moisture, and crude protein using AOAC official methods of analysis sections 960.39, 923.03 and modified sections 950.46 and 981.10 respectively. Carbohydrate percentages were determined by difference. The caloric content (kcal/100g) of the RS and control granola bars and cereals was calculated using the Food Processor software (Food Processor 8.6.0, ESHA Research, Professional Nutrition Analysis Software and Databases, Salem, OR). Water activity of the samples was analyzed using a water activity instrument (Aqua Lab CX-2, Decagon Devices, Inc., Pullman, Washington).

Physical Properties

Starch Viscosity

The viscosity of the raw starches, RS2 and waxy corn, were measured using the Brabender Micro-Visco-Amylograph (Brabender Micro-Visco-Amylo-Graph, C.W. Brabender Instruments Inc., Hackensack, NJ). Eight grams of raw corn starch (RS2 or waxy corn starch) were mixed with 110 ml of deionized water to make a paste. The starch paste was heated from 30 °C to 92 °C. The heat/cool temperature was 7.5 °C/min. The hold up time was 5 min with the final up hold temperature at 55 °C/min.

The gelatinization temperature range of the waxy starch was determined from the viscoamylograph readings to be (68-74 °C) which is in agreement with other studies (Whistler & BeMiller, 1997); however, the gelatinization temperature of RS2 was not measurable using the visco-amylograph, as the gelatinization temperature range exceeded 100 °C, the maximum heating temperature of this equipment. The reported gelatinization range of RS has been estimated to be 66-168 °C (Whistler & BeMiller, 1997).

Knowing the gelatinization temperature of starches provides valuable information of the potential digestibility behavior of the starch. When the starch granule is disrupted, there is greater access for hydrolytic enzymes to break down and digest starch. The higher the gelatinization temperature range, the more resistance to hydrolases and the more starch that is bypassed by the enzymes in the gastrointestinal tract.

Texture

Fracturability of the granola bars can be measured using a knife attachment that can break or shear the bar (Charley and Weaver 1998) or using the three point bend ring attachment using the bend or snap method (Brennan and Samyue 2004). The fracture properties of resistant starch and control granola bars were analyzed using a texture analyzer (EZ Test, Shimadzu, Nakagyo-Ku, Kyoto, Japan) calibrated with a 25 kg load cell. Fracturability of the granola bar samples (8 cm length X 5 cm height) was determined by the bend method using a three-point bend attachment. Samples were tested in duplicate for the 0, 7, 14, and 21 days and measurements were taken in the central part of the granola bar. Samples were compressed once with a cross head speed of 2 mm/sec and a distance of 10 mm and the force (g) required to break the bars was recorded.

Color

A HunterLab colorimeter system (Minolta Chroma Meter CR-300 Series, Osaka, Japan) was used to determine surface color (L, a*, b* values) of the granola bars and granola cereals. The values were recorded as L* = lightness (where 0 = black, 100 = white), a* (+a* = redness and -a* = greenness) and b* (+b* = yellowness and -b* = blueness). These values were compared to a standard white calibration plate (CR-A44) with a wide-area illumination (measuring area 50 mm) /0 °C viewing angle.

Sensory Evaluation

A consumer acceptability test was conducted to assess the palatability of the experimental (control and 15% RS) granola bars and cereals. A second test was conducted to evaluate the specific attributes (texture, flavor, color, appearance) of the control and 15% RS granola bars and 2 commercial granola bar products.

Granola bar and cereal samples (control-0% RS and 15% RS) were evaluated by a consumer panel (100 participants) according to the procedures described by Meilgaard and coworkers (2007). Granola bars and cereals (0% RS and 15% RS were prepared the day before sensory testing. Products were baked and allowed to cool to ambient temperature. Product (5 g of each granola bar (0% RS, 15% RS) and 5 g of each cereal (0% RS, 15% RS)) was placed into 2-ounce souffle cups with lids (SoloCup Company, Highland Park, IL, U.S.A). The untrained

consumer sample population was selected from students, staff, and faculty in the Human Nutrition, Foods, and Exercise and the Food Science and Technology Departments at Virginia Tech. Consumers (n=100) aged 18-60, approximately two thirds female, one third male, who consumed granola products frequently and reported no allergies to any of the ingredients in the granola bars and cereals were selected to participate in the sensory evaluations.

Sensory evaluations were conducted in the Food Science and Technology sensory laboratory. Panelists were seated in isolated booths under incandescent light, and were asked to complete informed consent forms (Appendix D). Panelists were encouraged to ask any questions to the investigator regarding the study and products during the informed consent process. However, participants were not explicitly told the potential health benefits of RS. Participants were aware the experimental granola bars and cereals may contain RS, but were not informed of the health benefits associated with RS to avoid bias. Participants were first asked to evaluate samples presented to them (2 samples at a time) following a balanced complete block design. Samples were coded with 3 digit numbers to avoid potential identification of the treatments presented. This allowed for randomization of the order of the samples being presented to them. Panelists were oriented to their individual booth and were oriented to the ballot and test procedures. After signing informed consent forms, panelists were asked to complete a demographic survey to assess characteristics (gender, age, education level, income level, employment status, and personal preferences for granola bars and cereal products) (Appendix E). The Sensory Information Management System sensory software (SIMS 2000) (Sensory Computer Systems, Morristown, NJ) was used to record the panelists' acceptability responses and demographic information.

A nine-point verbal hedonic scale (1= “dislike extremely”; 9 = “like extremely”) was used to evaluate acceptability of RS and control granola bars and cereals among consumers (McWilliams 1997; Larmond, 1982; Meilgaard et al., 2007). A value of 6.0 ("like slightly") on the 9-point hedonic was considered the minimum acceptability level for the purposes of this project, indicating that the two product formulations were generally liked and would be palatable for subsequent metabolic studies (Appendix F).

Attributes (appearance, flavor, and texture) of the RS granola bar, control granola bar, and two commercial granola bars (Kashi and Quaker Oats) similar to the ones used in this study were assessed by 50 consumer panelists in a separate sensory test. Samples (5 g) of each of the 4

granola bars (0% RS, 15% RS, Kashi, Quaker) were randomly assigned using 3-digit codes. The 2 commercial bar samples (Kashi, Quaker) were manually crumbled and the raisins present in one of the commercial bars were removed since the experimental bars did not contain raisins. This provided uniformity among the granola bars being compared. The attributes sensory test was conducted under the same conditions as previously described for the acceptability test described above. A “Just About Right” (JAR) test was used. A verbal seven point scale (1= "not enough", 4= "just right", and 7= "too much") was tested for each attribute for each product according to the procedure defined by Meilgaard and coworkers (2007) (Appendix H). This information was used to screen for attributes that might have affected acceptability scores for the experimental and control formulations and to compare formulated products against commercial products.

Approval from the Institutional Review Board (IRB) for research involving human subjects was received (IRB # 06-619) and informed consent was obtained from all participants.

Statistical Analysis

The study was replicated twice, and two measurements of each experimental unit were collected for analytical analysis. Data were expressed as the mean with standard error, and statistical significance was determined at $P < 0.05$. Data were analyzed for main effects of treatment and time, and the interaction between treatment and time by 2-way analysis of variance (ANOVA). Means were compared using Tukey's HSD multiple comparisons procedure.

Sensory results were analyzed by ANOVA for the acceptability test and for the attributes diagnostic sensory test. Means were compared using Tukey's HSD multiple comparisons procedure. The analyses were performed using JMP for Windows version 5.1, 2004, SAS Institute.

Results

Physicochemical Results

The RS level of the high amylose corn starch (RS2) was 43 g/100g dry weight basis (dwb). The levels of RS incorporation for granola bar products were 0.73, 10.75, and 14.33 g RS/100g (dwb) for the targeted 0%, 10%, and 15% RS formulations, respectively. One of the objectives of this study was to monitor RS levels of the granola bars and cereals after 28 days of

storage because there is scientific evidence (Niba, 2003a) that RS levels may increase with time. At day 0, the measured RS levels of the granola bars were 11.5 g, and 14.1 g for the 10% and 15% RS treatments (Table 3.2). At day 28, the RS levels of the granola bars slightly increased to 13.4 g, and 15.8 g, respectively although not statistically significant. The measured RS levels of the granola cereals at day 0 were 7.04 g, and 9.71 g for the 10% and 15% RS treatments. After 1 month of storage, the RS levels slightly increased to 11.7 g, and 11.8 g for the 10% and 15% RS treatments. The resistant starch levels of granola bars and granola cereals were not affected after 28 days storage ($p=0.2541$ and $p=0.1442$, respectively).

The soluble starch content of granola bars and granola cereals did not change with storage time (Table 3.3). Soluble starch levels ranged from 45.1 g to 43.2 g for the granola bars and the levels ranged from 38.3 g to 37.2 g for the granola cereals for the 10% and 15% RS treatments, respectively ($p=0.4793$ and $p=0.5090$, respectively) (Table 3.3). The β -glucan content of granola bars and granola cereals was affected by storage time and slightly increased during storage ($p=0.0283$ and $p=0.0297$, respectively). The β -glucan levels varied from 1.35 g, 1.05 g, to 1.08 g for the granola bars and the levels varied from 1.19 g, 0.97 g, to 1.01 g for the granola cereals for the 0%, 10%, and 15% RS treatments, respectively (Table 3.4).

Proximate composition of granola bars and granola cereals is presented in Table 3.5. Moisture and protein concentration of granola bars were higher than granola cereals and this is attributed to the presence of egg whites. Fat and carbohydrate concentration of granola cereals were higher than those of granola bars. This is attributed to the additional honey and canola oil used for granola cereal formulation. The RS supplemented and control granola bars were formulated to be isocaloric (~380 Kcal/serving) and the RS supplemented and control granola cereals were also isocaloric (~430 Kcal/serving). The difference in the caloric values between the granola bars and granola cereals is attributed to the formulation differences. The granola cereals contained higher amounts of honey and canola oil to compensate for the lack of egg whites compared to the granola bars to help disperse the RS in the products. Water activity of the granola bars significantly increased over time ($p=0.0009$) and ranged from 0.69 to 0.77. Water activity of the granola cereals significantly increased with time ($p<0.0001$) and ranged from 0.22 to 0.45 (Table 3.6). The increase in water activity could be attributed to the retrogradation of the starch molecules. During retrogradation, water is released and may be responsible for the increase in water activity in both, granola bars and cereals.

Results for the physical properties of the RS supplemented and control granola bars and cereals indicated color differences with the addition of RS (Table 3.7). The higher the concentration of RS in the formulation of the granola bars and cereals, the lighter the products. The L-value (whiteness) was significantly different for the 0, 10, and 15% RS granola bars ($p < 0.001$) and cereals ($p < 0.001$). Both RS granola bars were lighter in color than the control. The same observations were true in the cereal products as well. There was an increase in *b* values (yellowness) and a decrease in *a* (redness) values with the addition of RS in the granola bars although these results were not found in the RS cereals. In general, incorporation of RS in food products resulted in lighter color compared to the control granola bars and cereals.

Results for the texture measurements of the granola bars indicated that at RS levels of 10% and 15%, the bars were more resistant to fracture or to disintegration ($p = 0.0043$) than the control granola bars. The average amount of force (g) required to break the bars over 21 days was 423.7g, 1358.8g, and 1440.4g for the control, 10%, and 15% RS products, respectively. There were no significant differences in the force required to break the granola bars over time (Table 3.8).

The Brabender micro visco-amylo-graph results for the waxy corn starch solution (7.5% starch in distilled water) showed an initial gelatinization temperature of 68.9 °C with a maximum viscosity at 78 °C. The gelatinization temperature range for the Hi-Maize® solution was not obtained since the maximum temperature that the Brabender micro visco-amylo-graph can hold is 100°C. The gelatinization temperature range of RS is reported to be between 66-170 °C (Whistler & BeMiller, 1997).

Sensory Evaluations Results

Sensory acceptability tests of the granola bars and cereals were conducted on the control and 15% RS only. The samples were freshly prepared on the day before tasting, and the one day stored samples were evaluated by the consumers' panel. Consumer panel ($n = 100$) acceptability rating of control and RS supplemented granola bars and granola cereals are shown in Figure 3.4. The results from the hedonic acceptability tests showed the control granola bars were rated 6.6 and the control granola cereals were rated 6.1 on the 9 point hedonic scale (1= "dislike extremely", 6= "like slightly, and 9= "like extremely"). The RS granola bars were rated 5.6 and the RS granola cereals were rated 6.4. The control products and the RS cereal products were

considered acceptable by consumers based on our predetermined minimum acceptability level of 6.0 (“like slightly”). Seventy-eight percent (78%) of the panelists rated the RS supplemented granola cereal and control granola bar 6 and above (“like slightly to like extremely”), 68% rated the control cereal 6 and above (“like slightly to like extremely”), and 62% rated the RS supplemented granola bar 6 and above (“like slightly to like extremely”). The RS granola bar was slightly below the predetermined acceptability level; however the RS-supplemented bar scored 5.6 which is higher than the value of 5 (“neither like nor dislike”). There was no significant difference between the control and RS-supplemented cereals ($p=0.217$). There was a significant difference between the control and RS supplemented bars ($p<0.0001$). Consumers' volunteered comments for the acceptability test of the RS granola bar included the bar was "a bit fragile and did not stick together", "good taste but too soft", should be crunchier and a little sweeter", "cake like structure".

Results for attribute diagnostics test for the characteristics of color, sweetness, moistness, crunchiness, stickiness, and chewiness of the RS-supplemented, control, Kashi, and Quaker oats granola bars are shown in Table 3.9. Statistical analysis showed that the RS granola bars differ significantly in color ($p<0.0001$) stickiness ($p<0.0001$), and chewiness ($p=0.0007$) compared to the other 3 bars. The means of each attribute of the RS granola bar were significantly different ($p<0.01$) from the targeted ideal score of 4 (“just right”). In general, the RS granola bar scored lower than four for all of the attributes evaluated (Table 3.9, Figure 3.5).

Discussion

The results of the present study on the physicochemical and sensory properties of RS supplemented and non RS supplemented cereal based products indicate that levels up to 15% RS could be incorporated in granola products with some changes in taste and the physicochemical properties of such products. Resistant starch levels slightly increased after 4 weeks of storage, although this was not statistically significant. Processed foods are usually stored before reaching consumers (Namratha et al., 2002). During storage, the starch polymers whether intact or gelatinized undergo a series of chemical reactions where a fraction of starch resistant to α -amylase activity re-crystallizes, a process known as retrogradation. Retrogradation is the process of amylose chains re-arranging into crystalline forms (resulting in RS3 formation), and some studies demonstrate an increase RS content in processed foods during storage (Whistler &

BeMiller, 1997; Namratha et al., 2002). Although different explanations for this phenomenon exist among studies, starch complexes with protein and fat seem to favor RS formation in foods (Namratha et al., 2002; Niba, 2002). It is believed that during storage, starch molecules, in particular amylose, may form molecular complexes with other macronutrients rendering them less susceptible to α -amylase activity.

Factors affecting RS formation include processing conditions (i.e. moist heat vs. dry heat), starch botanical sources, presence of other ingredients in the food matrix such as protein, fat, dietary fiber, and minerals (Brown, 2004; Kendall et al., 2004; Niba, 2002; Niba 2003a; Niba 2003b; Nugent, 2005; Sharma et al., 2008). In this study, RS levels in the final products varied based on the ingredients used. Granola bars contained egg whites which provided more moisture in the final product compared to the RS cereals. Moist heat treatments have been shown to increase RS levels in processed foods, and these levels further increased during storage (Namratha et al., 2002; Niba 2003a). Niba (2003a) evaluated effects of storage period and temperature on RS content in cornbread. RS concentration increased from 2.59 g/100g to 4.52 g/100g after 4 days of storage at refrigerated (4 °C) temperature. Namratha and coworkers (2002) found a significant increase ($p < 0.05$) in RS content of various Indian autoclaved ready-to-eat foods after 4 months of storage. In this study, the RS levels of granola bars and cereals slightly increased over a 4 week period although this was not statistically significant. It is possible that a longer storage period would result in higher RS content. The shelf-life of granola bars/cereals is usually 10-12 months.

Processing conditions such as baking resulted in increased RS content in some breads (Liljeberg, et al., 1996; Waring, 2005). The baking temperature used in the cereal based products was 163 °C, and the gelatinization temperature range of the Hi-Maize® has been reported to be between 66-170 °C. Baking of starchy foods of relatively high moisture content resulted in the formation of retrograded starch, which is resistant to enzymatic activity (Niba 2003a, Namratha et al., 2002). It is possible that processing methods such as baking of the granola bars promoted the formation of retrograded RS compared to the granola cereals.

Partial or incomplete gelatinization of starch during baking (Niba, 2003a) may explain the small increase of RS in the cereal products. Some starch may have gelatinized and retrograded upon cooling and storage contributing to the higher RS content of these products. Prolonged storage for up to 4 months at ambient temperature of ready-to-eat foods resulted in

increased RS levels (Namratha et al., 2002). In this study, RS levels slightly increased after 4 weeks of storage. The granola bars and cereals were stored in polyester films with an aluminum coating (Mylar®MC2, DuPont Teijin Films US, Wilmington, DE). This type of packaging provides excellent moisture, oxygen, and light barrier properties, and it resembles commercial packaging of granola bars. During storage, the starch retrograded resulting in increased water activity and moisture content within the enclosed environment. A longer storage time could further increase RS in cereal based products.

Color of RS-supplemented cereals was lighter than control cereal products. This is in agreement with other researchers (Baixauli et al., 2008; Brown, 2004; Waring, 2005). Addition of high amylose corn starch to foods results in lighter colored products. This is important in RS-supplemented foods where dietary fiber intake is desired. Incorporation of whole grains is associated with dark crust and dark crumb color in baked products. Addition of RS to foods may result in products that do not look like conventional high-fiber foods. This is important as consumers tend to associate high-fiber foods with dark color and may be unwilling to eat high-fiber foods. Consumers may be interested in eating high-fiber foods with an appealing light color; thus, increasing dietary fiber intake. Incorporation of RS results in lighter color compared to traditional dietary fibers (Brown, 2004; Waring 2005). The RS-supplemented bars were more resistant to fracture compared to the control bars. Texture of the control bars was crumbly especially after 4 weeks of storage. According to Baixauli and coworkers (2008), muffins supplemented with 20% RS were softer than the control muffins. Incorporation of high levels of RS may result in a product that is less brittle than the control counterpart. Brennan and Samyue (2004) showed that biscuits made with up to 10% RS2 resulted in similar textural properties compared to the control biscuits. Sensory evaluation of the RS supplemented cereal products indicated that the RS bars were somewhat dried, needed to be sweeter and were "cake-like" based on consumers' responses. Baixauli and coworkers (2008) showed that muffins containing 7.3 g RS/100g had an overall acceptability score of 6.4; however the levels used in this study were much higher (15g RS/100g granola bar) which are levels thought to provide health benefits. Ares and coworkers (2009) found the overall acceptability score to be 4.5 in milk puddings supplemented with 4% RS. In our study, incorporation of 15 g RS/granola bar serving resulted in overall acceptability of 5.6. Further sensory evaluation based on appearance, texture, and flavor of the RS granola bars revealed differences in those attributes compared to the control

granola bar and 2 commercial brands of granola bars. These results are in agreement with Ares and coworker (2009) who evaluated the effects of RS on milk puddings. The RS levels used in milk puddings were 1% to 4% RS. As the RS level increased sensory changes occurred melting, creaminess, sweetness and floury taste. However, the levels used in their study were much lower (up to 4% RS) compared to our study in which RS levels were more than 3 times their levels.

Conclusion

Incorporation of high levels of up to 15% RS in cereal based products such as granola bars and cereals is feasible. The RS-supplemented products had similar physicochemical and sensory properties compared to the control bars/cereals. However, incorporation of high levels of RS resulted in changes in color, stickiness, and chewiness. The RS supplemented products were acceptable to consumers and contained RS levels that slightly increased after four weeks of storage. Resistant starch incorporation into a cereal matrix may increase dietary fiber intake in the U.S. It is estimated that Americans consume less than half the recommended dietary fiber intake. Incorporation of RS in foods may help against conditions such as obesity and chronic diseases such as type 2 diabetes and cardiovascular disease. RS has a potential as a functional fiber by providing health benefits such as promoting a healthy colon environment, glucose homeostasis, and satiety.

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Table 3.1. Resistant starch and control granola bar formulation¹.

Ingredients	Measures per Batch	Weight (g) per Batch	Weight (g) per Bar	Percent Ingredients (wt %)
Oats	2 cups	180	22.5	21.1
RS2: Hi Maize®	2 cups	268	33.5	31.5
Shredded Coconut	1/2 cup	35	4.4	4.1
Almonds	1/2 cup	45	5.6	5.3
Baking Soda	1 Tsp.	5	0.6	0.6
Honey	1/2 cup	166	20.8	19.5
Canola Oil	1/2 cup	116	14.5	13.6
Egg Whites	2	22	2.8	2.6
Vanilla Extract	1/2 tsp	2.5	0.3	0.3
Vanilla Imitation	1/2 tsp	2.5	0.3	0.3
TIC Gum Arabic	-	10	1.3	1.2
Total		852	106.5	100

Ingredients	Measures per Batch	Weight (g) per Batch	Weight (g) per Bar	Percent Ingredients (wt %)
Oats	2 cups	180	22.5	26.0
Waxy corn starch Amioca®	2 cups	107.2	13.4	15.5
Shredded Coconut	1/2 cup	35	4.4	5.1
Almonds	1/2 cup	45	5.6	6.5
Baking Soda	1 Tsp.	5	0.6	0.7
Honey	1/2 cup	166	20.8	24.0
Canola Oil	1/2 cup	116	14.5	16.8
Egg Whites	2	22	2.8	3.2
Vanilla Extract	1/2 tsp	2.5	0.3	0.4
Vanilla Imitation	1/2 tsp	2.5	0.3	0.4
TIC Gum Arabic	-	10	1.3	1.4
Total		691.2	86.4	100

¹Eight granola bars per batch. Control and RS cereals contain the same ingredients above with the exception of the egg whites, TIG gum arabic, and baking soda. The honey content was increased to 250g (3/4 cup) and the oil content was increased to 142g (3/4 cup) to aid in dispersion of the starch.

Table 3.2. Concentration of resistant starch in control and resistant starch-supplemented (10%, 15%) granola bars and granola cereals over 28 days of storage (20 °C).

Storage period	Storage treatments		
	Control (0% RS) g/100g	10% RS g/100g	15%RS g/100g
Granola Bars	$(\bar{x} \pm \text{SEM})^1$	$(\bar{x} \pm \text{SEM})^1$	$(\bar{x} \pm \text{SEM})^1$
0 days	0.16 ± 0.03^a	11.5 ± 0.00^b	14.1 ± 0.98^c
7 days	0.15 ± 0.01^a	12.7 ± 0.56^b	15.3 ± 0.78^c
14 days	0.22 ± 0.01^a	12.6 ± 0.33^b	13.8 ± 0.33^c
21 days	0.24 ± 0.01^a	11.7 ± 1.13^b	15.7 ± 0.88^c
28 days	0.22 ± 0.04^a	13.4 ± 1.48^b	15.8 ± 0.66^c
Granola Cereals			
0 days	0.21 ± 0.13^a	7.04 ± 2.60^b	9.71 ± 1.54^c
7 days	0.14 ± 0.17^a	7.52 ± 0.03^b	13.1 ± 2.49^c
14 days	0.49 ± 0.09^a	9.17 ± 0.88^b	9.74 ± 0.43^c
21 days	0.44 ± 0.28^a	9.34 ± 0.57^b	11.9 ± 0.28^c
28 days	0.13 ± 0.06^a	11.7 ± 3.70^b	11.8 ± 0.84^c

Data expressed on a dry weight basis (dwb).

¹Mean of two samples. Data expressed as mean \pm standard error of the mean ($\bar{x} \pm \text{SEM}$).

Means in the same row without a common letter differ ($p < 0.05$) according to Tukey's HSD multiple comparisons procedure test.

Table 3.3. Concentration of soluble starch in control and resistant starch-supplemented (10%, 15%) granola bars and cereals over 28 days of storage (20 °C).

Storage period	Storage treatments		
	Control (0% RS) g/100g	10% RS g/100g	15% RS g/100g
Granola Bars	($\bar{x} \pm \text{SEM}$) ¹	($\bar{x} \pm \text{SEM}$) ¹	($\bar{x} \pm \text{SEM}$) ¹
0 days	46.9 ± 2.18 ^a	45.1 ± 0.64 ^b	43.2 ± 2.34 ^{ab}
7 days	46.8 ± 1.18 ^a	44.9 ± 1.21 ^b	43.7 ± 2.17 ^{ab}
14 days	56.8 ± 9.55 ^a	42.9 ± 0.56 ^b	45.5 ± 0.89 ^{ab}
21 days	49.1 ± 0.20 ^a	44.3 ± 1.48 ^b	46.7 ± 0.83 ^{ab}
28 days	47.0 ± 0.78 ^a	42.6 ± 0.97 ^b	45.5 ± 0.16 ^{ab}
Granola Cereals			
0 days	41.5 ± 0.34 ^a	38.3 ± 0.34 ^b	37.2 ± 1.19 ^b
7 days	43.9 ± 0.39 ^a	39.5 ± 1.96 ^b	38.0 ± 3.95 ^b
14 days	42.8 ± 0.67 ^a	38.6 ± 0.95 ^b	39.0 ± 0.90 ^b
21 days	45.3 ± 0.19 ^a	36.2 ± 1.38 ^b	38.1 ± 0.66 ^b
28 days	36.4 ± 5.79 ^a	38.3 ± 4.85 ^b	38.9 ± 0.19 ^b

Data expressed on a dry weight basis (dwb).

¹Mean of two samples. Data expressed as mean ± standard error of the mean ($\bar{x} \pm \text{SEM}$).

Means in the same row without a common letter differ ($p < 0.05$) according to Tukey's HSD multiple comparisons procedure test.

Table 3.4. Concentration of β -glucans in control and resistant starch-supplemented (10%, 15%) granola bars and cereals over 28 days of storage (20 °C).

Storage period	Storage treatments		
	Control (0% RS) g/100g	10% RS g/100g	15% RS g/100g
Granola Bars	$(\bar{x} \pm \text{SEM})^1$	$(\bar{x} \pm \text{SEM})^1$	$(\bar{x} \pm \text{SEM})^1$
0 days	1.35 ± 0.22^a	1.05 ± 0.04^b	1.08 ± 0.02^b
7 days	1.52 ± 0.04^a	1.19 ± 0.00^b	0.92 ± 0.05^b
14 days	1.38 ± 0.15^a	1.05 ± 0.16^b	1.01 ± 0.01^b
21 days	1.58 ± 0.11^a	1.35 ± 0.13^b	1.33 ± 0.05^b
28 days	1.41 ± 0.07^a	1.09 ± 0.11^b	1.05 ± 0.10^b
Granola Cereals			
0 days	1.19 ± 0.18^a	0.97 ± 0.25^b	1.01 ± 0.01^b
7 days	0.97 ± 0.06^a	0.77 ± 0.12^b	0.90 ± 0.06^b
14 days	1.12 ± 0.02^a	0.84 ± 0.09^b	0.85 ± 0.05^b
21 days	1.43 ± 0.10^a	0.97 ± 0.24^b	0.96 ± 0.06^b
28 days	1.66 ± 0.06^a	1.11 ± 0.25^b	0.96 ± 0.05^b

Data expressed on a dry weight basis (dwb).

¹Mean of two samples. Data expressed as mean \pm standard error of the mean ($\bar{x} \pm \text{SEM}$).

Means in the same row without a common letter differ ($p < 0.05$) according to Tukey's HSD multiple comparisons procedure test.

Table 3.5. Proximate composition of control and resistant starch-supplemented (10%, 15%) granola bars and cereals at day 0 at 20 °C (% wt).

GRANOLA BARS					
Sample	Moisture ($\bar{x} \pm \text{SEM}$) ¹	Protein ($\bar{x} \pm \text{SEM}$) ¹	Lipid ($\bar{x} \pm \text{SEM}$) ¹	Ash ($\bar{x} \pm \text{SEM}$) ¹	Carbohydrate ^a ($\bar{x} \pm \text{SEM}$) ¹
Control	16.6 ± 0.40	6.42 ± 0.10	18.6 ± 0.19	2.40 ± 0.10	55.9 ± 0.02
10% RS	15.3 ± 0.56	6.38 ± 0.02	18.6 ± 0.03	2.35 ± 0.15	57.3 ± 0.68
15% RS	15.1 ± 0.81	5.87 ± 0.07	17.8 ± 0.36	2.30 ± 0.10	58.9 ± 0.48
GRANOLA CEREALS					
Sample	Moisture ($\bar{x} \pm \text{SEM}$) ¹	Protein ($\bar{x} \pm \text{SEM}$) ¹	Lipid ($\bar{x} \pm \text{SEM}$) ¹	Ash ($\bar{x} \pm \text{SEM}$) ¹	Carbohydrate ^a ($\bar{x} \pm \text{SEM}$) ¹
Control	7.14 ± 0.55	6.06 ± 0.09	22.4 ± 0.96	2.25 ± 0.05	62.2 ± 1.46
10% RS	6.02 ± 0.48	4.89 ± 0.09	21.7 ± 0.35	2.00 ± 0.00	65.4 ± 0.05
15% RS	5.74 ± 0.49	4.47 ± 0.08	21.5 ± 0.61	1.95 ± 0.35	66.4 ± 0.83

Data expressed as % wt.

¹Mean of two samples. Data expressed as the mean ± standard error of the mean ($\bar{x} \pm \text{SEM}$).

^aCalculated by difference

Table 3.6. Water activity (A_w) content of control and resistant starch-supplemented (10%, 15%) granola bars and cereals over 28 days of storage (20 °C).

Granola Bars

	Day 0 ($\bar{x} \pm \text{SEM}$) ¹	Day 7 ($\bar{x} \pm \text{SEM}$) ¹	Day 14 ($\bar{x} \pm \text{SEM}$) ¹	Day 21 ($\bar{x} \pm \text{SEM}$) ¹	Day 28 ($\bar{x} \pm \text{SEM}$) ¹
Sample					
Control ^a	0.69 ± 0.03 ^a	0.74 ± 0.00 ^b	0.75 ± 0.01 ^b	0.76 ± 0.00 ^b	0.77 ± 0.01 ^b
10% RS ^a	0.68 ± 0.07 ^a	0.74 ± 0.00 ^b	0.74 ± 0.02 ^b	0.74 ± 0.03 ^b	0.77 ± 0.02 ^b
15% RS ^a	0.69 ± 0.02 ^a	0.73 ± 0.00 ^b	0.75 ± 0.02 ^b	0.76 ± 0.01 ^b	0.76 ± 0.03 ^b

Granola Cereals

	Day 0 ($\bar{x} \pm \text{SEM}$) ¹	Day 7 ($\bar{x} \pm \text{SEM}$) ¹	Day 14 ($\bar{x} \pm \text{SEM}$) ¹	Day 21 ($\bar{x} \pm \text{SEM}$) ¹	Day 28 ($\bar{x} \pm \text{SEM}$) ¹
Sample					
Control ^a	0.41 ± 0.02 ^a	0.45 ± 0.09 ^a	0.29 ± 0.06 ^b	0.39 ± 0.03 ^a	0.34 ± 0.00 ^b
10% RS ^a	0.42 ± 0.02 ^a	0.40 ± 0.03 ^a	0.30 ± 0.01 ^b	0.37 ± 0.02 ^a	0.31 ± 0.04 ^b
15% RS ^b	0.26 ± 0.00 ^a	0.33 ± 0.01 ^a	0.23 ± 0.01 ^b	0.30 ± 0.00 ^a	0.22 ± 0.01 ^b

¹Mean of two samples. Data expressed as the mean ± standard error of the mean ($\bar{x} \pm \text{SEM}$). Means in the same row without a common letter differ ($p < 0.05$) according to Tukey's HSD multiple comparisons procedure test.

Table 3.7. Color (L, a, b values) of control and resistant starch-supplemented (10%, 15%) granola bars and cereals after 28 days of storage (20 °C).

Granola Bars

Sample	L-value ($\bar{x} \pm \text{SEM}$) ¹	a-value ($\bar{x} \pm \text{SEM}$) ¹	b-value ($\bar{x} \pm \text{SEM}$) ¹
Control	48.9 ± 0.73 ^a	11.1 ± 0.40 ^a	30.3 ± 0.76 ^a
10% RS	55.2 ± 1.19 ^b	8.48 ± 0.87 ^b	32.8 ± 1.47 ^b
15% RS	59.7 ± 0.66 ^c	6.99 ± 0.70 ^c	32.5 ± 1.42 ^b

Granola Cereals

Sample	L-value	a-value	b-value
Control	50.3 ± 0.51 ^a	6.58 ± 0.12 ^a	24.8 ± 0.36 ^a
10% RS	54.6 ± 0.65 ^b	7.32 ± 0.26 ^a	28.8 ± 0.16 ^b
15% RS	59.4 ± 0.56 ^c	6.35 ± 0.19 ^a	25.9 ± 0.42 ^a

¹Mean of two samples. Data expressed as mean ± standard error of the mean ($\bar{x} \pm \text{SEM}$).

Means in the same column without a common letter differ ($p < 0.05$) according to Tukey's HSD multiple comparisons procedure test.

Color parameters: L = lightness (0 = black, 100 = white), a (+a = redness and -a = greenness) and b (+b = yellowness and -b = blueness).

Table 3.8. Texture analysis of control and resistant starch-supplemented (10%, 15%) granola bars and over 21 days of storage (20 °C). Results expressed as grams (g) of force required to break the bars.

Granola Bars

Sample	Day 0 ($\bar{x} \pm \text{SEM}$) ¹	Day 7 ($\bar{x} \pm \text{SEM}$) ¹	Day 14 ($\bar{x} \pm \text{SEM}$) ¹	Day 21 ($\bar{x} \pm \text{SEM}$) ¹
Control	333 ± 47.4 ^a	219 ± 110 ^a	545 ± 74.4 ^a	487 ± 111 ^a
10% RS	908 ± 190 ^b	1204 ± 254 ^b	1734 ± 629 ^b	1629 ± 883 ^b
15% RS	1492 ± 20.9 ^b	1583 ± 643 ^b	1288 ± 145 ^b	1471 ± 87.2 ^b

¹Mean of two samples. Data expressed as the mean ± standard error of the mean ($\bar{x} \pm \text{SEM}$). Means in the same column without a common letter differ ($p < 0.05$) according to Tukey's HSD multiple comparisons procedure test.

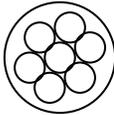
Table 3.9. Sensory description ($\bar{x} \pm SD$) of granola bars with 0%, 15% resistant starch using the just-about-right method.

Attribute	15 % RS	Control	Commercial 1	Commercial 2	P-value
Color	2.98 ± 1.12 ^c	3.72 ± 1.23 ^b	3.72 ± 0.67 ^b	4.62 ± 0.99 ^a	<0.0001
Sweetness	2.72 ± 1.05 ^b	4.20 ± 1.25 ^a	3.04 ± 1.21 ^b	4.46 ± 1.15 ^a	<0.0001
Moistness	2.78 ± 1.68 ^c	5.62 ± 1.10 ^a	2.84 ± 1.22 ^c	4.70 ± 1.28 ^b	<0.0001
Crunchiness	1.76 ± 1.00 ^c	1.80 ± 1.11 ^c	4.06 ± 1.20 ^a	2.42 ± 1.09 ^b	<0.0001
Stickiness	2.56 ± 1.55 ^b	3.76 ± 1.44 ^a	4.14 ± 1.46 ^a	3.98 ± 1.27 ^a	<0.0001
Chewiness	3.20 ± 1.65 ^b	3.64 ± 1.44 ^{a,b}	4.06 ± 1.27 ^a	4.30 ± 1.22 ^a	0.0007

Consumers' perception (n=50) for the intensity of six sensory attributes for RS-supplemented (15%) and control (0% RS) granola bars, and two commercial granola bars on day 1 of storage (20°C). A score of 1 = "not enough", 4 = "just right", and 7 = "too much".

^{a,b,c}Indicates significant differences in a given row at $\alpha = 0.05$ using Tukey's HSD mean separation on JMP

¹Commercial bars: Kashi and Quaker.

Type of RS	Description	Food sources	Resistance reduced
RS 1 	Physically inaccessible	Whole/partly milled grains, seeds, legumes	Milling, chewing
RS 2 	Ungelatinized resistant granules	Raw potatoes, green banana, high amylose maize	Food processing, cooking
RS 3 	Retrograded starch	Cooked and cooled potatoes, bread, cornflakes, cook/cool moist heat treatment	Processing conditions
RS 4 	Chemically modified starches –cross-bonded	Fiber-drinks, foods with modified starches	Less susceptible to digestion <i>in vitro</i>

(Adapted from Brown, 2004; Patil, 2004; Topping et al., 2003; Sajilata et al., 2006)

Figure 3.1. Classification of resistant starch types, food sources, and factors affecting digestion resistance.

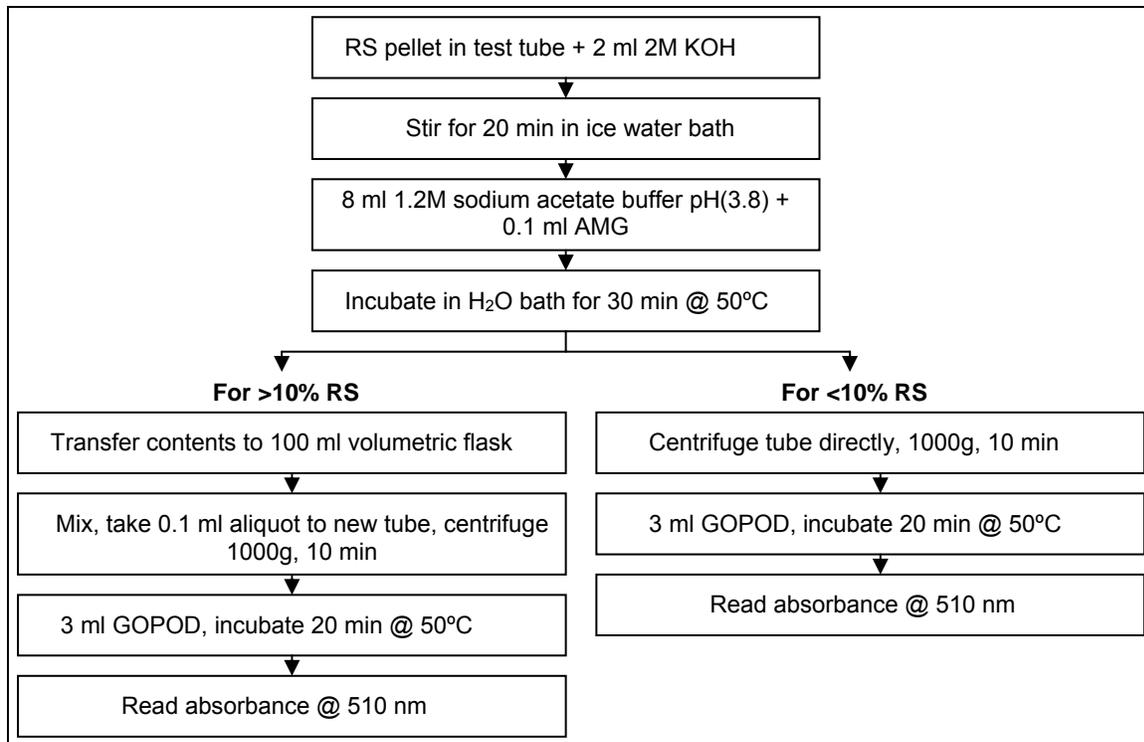


Figure 3.2. Flow diagram for extraction and measurement of resistant starch from granola bars and cereals.

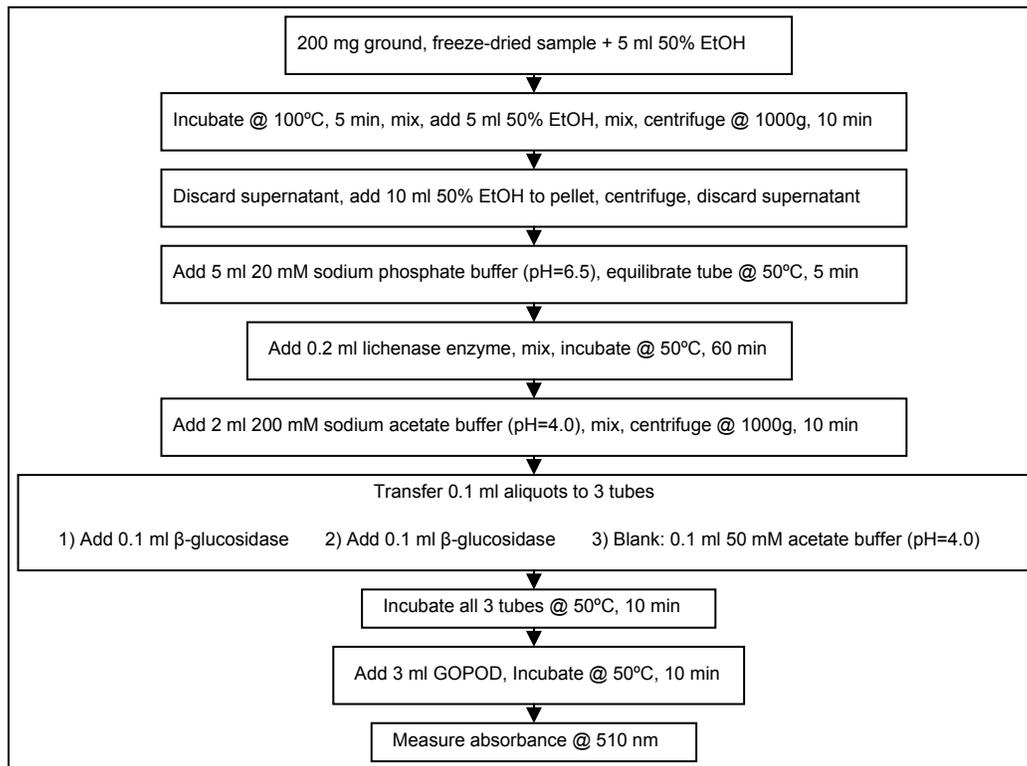
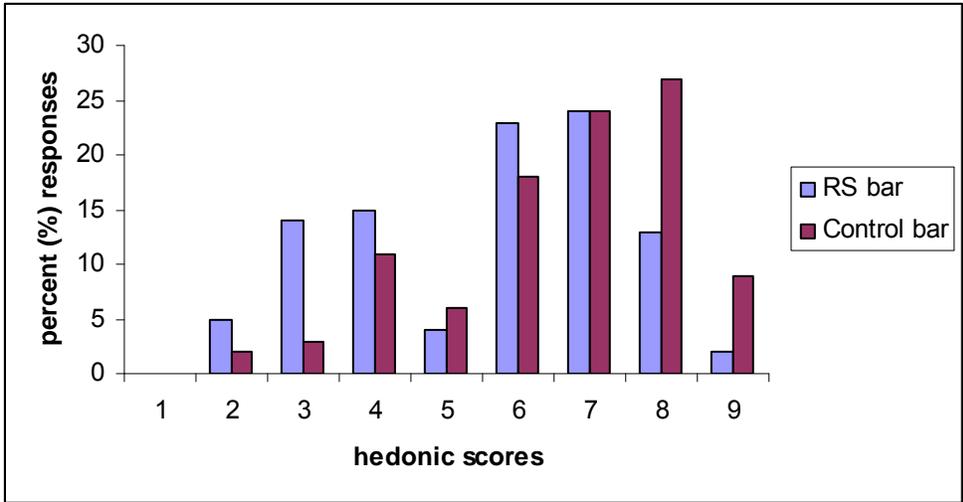
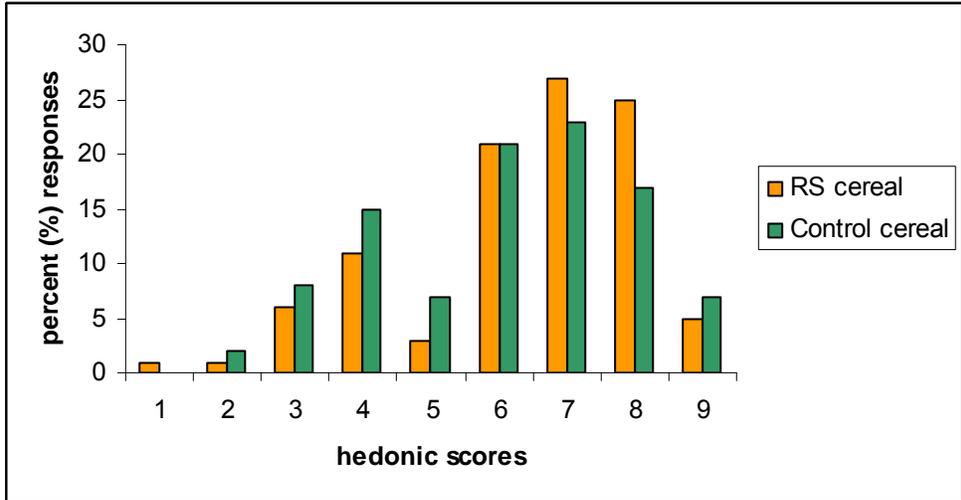


Figure 3.3. Flow diagram for extraction and measurement of Beta-glucans from granola bars and cereals.



(a)



(b)

Figure 3.4. Overall consumer acceptability (n=100) of RS-supplemented and control granola bars (a) and RS-supplemented and control granola cereal (b) after 1 day of storage at 20 °C. Nine point intensity scale: 1 = "dislike extremely", 6 = "like slightly", 9 = "like extremely". (RS-bar: mean hedonic score and standard deviation = 5.6 ± 1.9 ; hedonic score distribution 1-5 (38%) 6-9 (62%)). (Control bar: mean hedonic score and standard deviation = 6.6 ± 1.7 ; hedonic score distribution 1-5 (22%) 6-9 (78%)). (RS-cereal: mean hedonic score and standard deviation = 6.4 ± 1.7 ; hedonic score distribution 1-5 (22%) 6-9 (78%)). (Control cereal: mean hedonic score and standard deviation = 6.1 ± 1.8 ; hedonic score distribution 1-5 (32%) 6-9 (68%)).

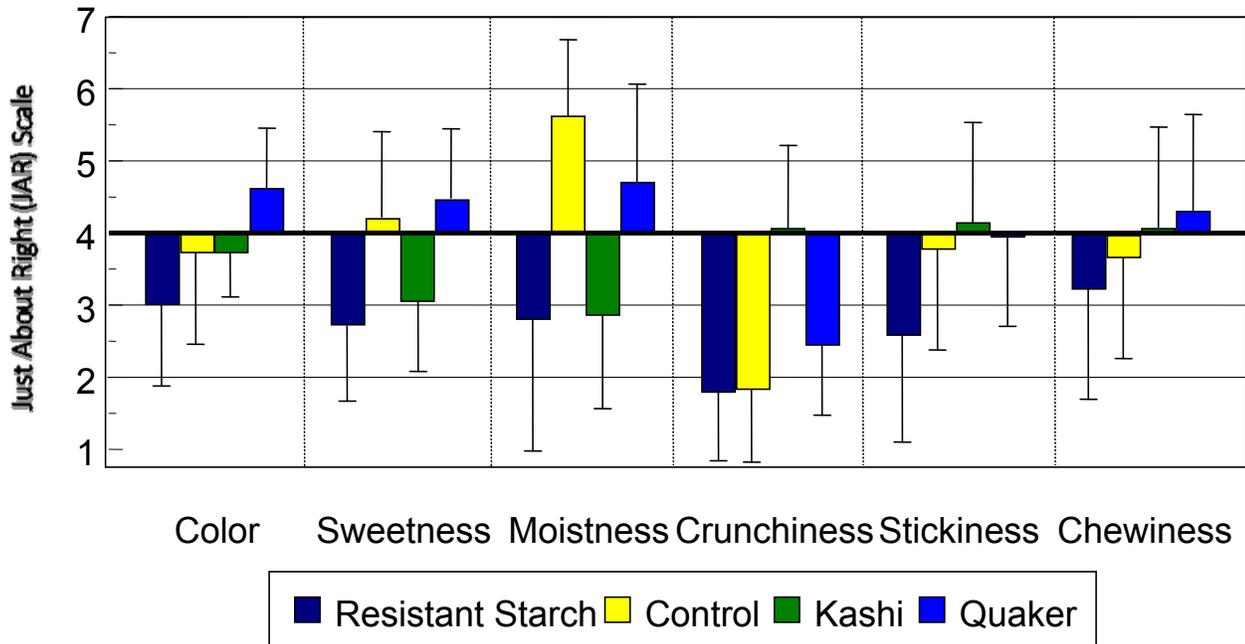


Figure 3.5. Consumers' perception (n=50) for the intensity of six sensory attributes for RS-supplemented (15%) and control (0% RS) granola bars, and two commercial granola bars (Kashi and Quaker) on day 1 of storage (20°C). Sensory description of granola bars using the just about right scale. A score of 1 = "not enough", 4 = "just right", and 7 = "too much".

CHAPTER 4: EFFECTS OF ACUTE RESISTANT STARCH CONSUMPTION ON POSTPRANDIAL GLUCOSE AND INSULIN RESPONSES AND OXIDATIVE STRESS PARAMETERS IN HISPANIC WOMEN

Abstract

The incidence of type 2 diabetes is considered an epidemic in Western countries, and its prevalence is more common in the Hispanic/Latino population than in non-Hispanic whites. Oxidative stress has been associated with obesity and chronic diseases such as heart disease and diabetes. Reducing oxidative stress (OS) through dietary consumption of resistant starch (RS) may be one approach to help modulate glucose and insulin responses. The purpose of this study was to compare the effects of a single ingestion of granola bars with high (~18 grams of RS) and low (~0 grams of RS) resistant starch compositions on the postprandial glucose and insulin responses (n=14) and oxidative stress parameters (cellular glutathione peroxidase, F2-isoprostanes, and oxygen radical absorbance capacity) in Hispanic women (n=9). Subjects received both treatments (RS, control) in a cross-over design, two weeks apart. Mean composition of the RS granola bar was 6% protein, 15% moisture, and 18% lipid. There was no difference in glucose and insulin responses after a single ingestion of a RS-supplemented granola bar over the entire 120 minute period. No differences were found in the oxidative stress parameters measured. When subjects with abnormal blood glucose levels (glucose levels decreased after 30 minutes of consumption) were excluded from analysis (n=9), a lower glucose response 30 minutes after RS consumption was found (p=0.0496). Thus, RS consumption may lower fluctuations in blood glucose, which may help manage glucose levels in individuals at risk of type 2 diabetes. Further studies of short term RS consumption are warranted to elucidate its benefits in glucose management.

Keywords: glucose response, high amylose corn starch, oxidative stress, postprandial, resistant starch, reactive oxygen species.

To be submitted to *Nutrition Research*

Introduction

A physiological effect ascribed to resistant starch (RS) is related to the extent of digestibility of RS in the small intestine, and its effects on postprandial glycemia (Kendall et al., 2004). Attenuating the glycemic response by consumption of foods containing RS may help individuals manage their blood glucose levels. Proper blood glucose management is associated with decreased risk of developing diseases and conditions such as diabetes, hyperlipidemia, and cardiovascular disease (Brown et al., 2003).

Resistant starch consumption should result in delayed carbohydrate absorption bypassing the small intestine, suggesting RS will attenuate the glucose and insulin responses after a meal, thus decreasing the likelihood of developing insulin resistance (Higgins, 2004). According to Kendall and coworkers (2004), RS doses of 20-30 g/day are needed to observe physiological effects. This level of consumption is 3-4 times higher than actual levels of RS consumption in Western countries, which are estimated to be 5-10 g RS/day (Kendall et al., 2004). The estimated resistant starch intake in the U.S. is between 3-8 g/day (Murphy et al., 2008). Type 2 diabetes (T2D) is a disease that has reached epidemic proportions in developed countries. In 2002, this disease affected approximately 15 million Americans (Liu, 2002). Type 2 diabetes is characterized by high blood glucose, high blood insulin, and insulin resistance. In 2008, there were over 23 million people in the U.S. who had diabetes and 57 million who had pre-diabetes, a condition where blood glucose level is high (100-125 mg/dl) but not considered diabetic (blood glucose > 126 mg/dl) (American Diabetes Association, 2007). Diabetes is more common in the Hispanic/Latino population (10.4% incidence) than in non-Hispanic whites (6.6% incidence) (American Diabetes Association, 2007; Center for Disease Control, 2004).

Diabetes is the fifth leading cause of death in the U.S.; in 2007, the estimated medical costs associated with this disease were estimated to be \$172 billion dollars (American Diabetes Association, 2007). The number of people suffering from diabetes worldwide in the year 2000 was reported to be 171 million, and projections estimate at least 366 million people worldwide will have diabetes by 2030 (World Health Organization, 2008).

Oxidative stress resulting from overproduction of reactive oxygen species (ROS) has been associated with conditions related such as insulin resistance, beta-cell dysfunction, impaired blood glucose tolerance, and type 2 diabetes. The imbalance between the concentration of ROS

and antioxidant defense mechanisms has been associated with health conditions such as obesity and chronic diseases such as type 2 diabetes (Dotan et al., 2004; Wright et al., 2006).

Dietary consumption of resistant starch may be one approach to reducing the risk of developing type 2 diabetes by reducing ROS. Epidemiological studies have consistently shown an association between whole grain consumption and decreased risk of developing type 2 diabetes by 20-42% (Fung et al., 2002; McKeown et al., 2002; Meyer et al., 2000; Montonen et al., 2003). The role of resistant starch in this protective effect has been suggested, but has not been directly assessed. Specific effects of RS on oxidative stress parameters are not well understood.

Resistant starch consumption has been shown to result in favorable changes in postprandial glucose and insulin responses in studies involving overweight/obese, as well as healthy individuals (Behall & Hallfrish, 2002; Park et al., 2004; Robertson et al., 2003); however, the effects of RS on glucose and insulin responses in Hispanic-Americans have not been evaluated. Hispanic-Americans are a population at risk for type 2 diabetes development and they are 1.7 times as likely to have diabetes as non-Hispanic whites. In addition, Hispanic-Americans are the fastest growing minority population in the U.S. (Center for Disease Control, 2005).

The relationship between RS consumption and oxidative stress has not been studied in humans. Shih and coworkers (2007) found decreased levels of malondialdehyde (MDA), increased superoxidase (SOD) activity, and increased total radical-trapping antioxidant levels in diabetic rats consuming RS compared to native starch (Shih et al., 2007).

To our knowledge, only one study has evaluated the effects of RS on lipid oxidation in humans. Higgins and coworkers (2004) showed that ingestion of 5.4% RS as percentage of total carbohydrates increased postprandial lipid oxidation *in vivo*, based on indirect calorimetry and oxidation of labeled [¹⁴C] triolein to ¹⁴CO₂. The authors concluded that incorporation of 5.4% RS in the diet could decrease long term lipid accumulation (Higgins et al., 2004).

Our interest was in testing whether there are beneficial health effects of acute consumption of RS on glycemic control and oxidative stress. The purpose of this study was to evaluate potential effects of a food product supplemented with 18 g RS/serving on clinical parameters associated with development of type 2 diabetes and oxidative stress.

Experimental Design

Granola Bars Preparation

Granola bars were formulated to contain 18 g RS per bar that might have dietary and health significance and a control bar to contain ~0 g RS per bar. A commercially available RS2 was used (Hi-Maize[®] 260, donated by Food Innovation, National Starch, Bridgewater, NJ) which contained 60% RS based on total dietary fiber (TDF). Commercially available waxy corn starch was used in the formulation of the isocaloric reference (control) granola bars. The waxy corn starch contained 40% readily digestible starch (Amioca[®], donated by Food Innovation, National Starch, Bridgewater, NJ). The waxy corn starch contained <0.5 g RS/100g.

Products were prepared by mixing dry ingredients (oats, RS2 (supplemented bars) or waxy corn starch (control bars), coconut, almonds, and baking powder) in a large bowl. Liquid ingredients (honey, canola oil, and egg whites) were whisked until smooth in a separate bowl. Blended liquid ingredients were added to the dry ingredients in three portions. The mixture was stirred until dry ingredients were coated and distributed. The two formulation mixtures were placed in aluminum baking (13x9x2 in) pans. Products were baked in a conventional oven at 163 °C (325 °F) for 35 minutes until golden brown and were cooled to room temperature. The oven and baking pans were always the same, and the baking pans were placed at the same level in the oven and the amount of granola bars and cereals baked was always the same.

The granola bars were prepared the day before each test day according to the study design (described below).

Subject Selection and Recruitment

Fourteen (n=14) volunteer free living Hispanic women, 20 years of age and older, were recruited for this study. Since the Hispanic population in South West Virginia is limited (~2% men and women, ~1% women) (<http://www.census.gov>), Hispanic women who responded to the study advertisement at Virginia Tech and local businesses within three months of the initial advertisement were invited to participate. The inclusion criteria for the study were as follows: Hispanic women 20 years or older, were healthy, non-pregnant nor breastfeeding, and were not diagnosed as diabetic were recruited for the study. The exclusion criteria of the study were as follows: subjects who were not complaint to the study protocol were removed from the study. Once recruited, participants completed a diabetes risk test developed by the American Diabetes

Association (<http://www.diabetes.org>) (Appendices I, J, K). The risk test was composed of seven simple questions about weight, age, lifestyle and family history, which are potential risk factors for diabetes. The scores of the test were used as a guide to determine if individuals were at risk of type 2 diabetes. The study was approved by the Institutional Review Board (IRB) at Virginia Polytechnic Institute and State University, prior to subject recruitment (IRB # 08-199) (Appendix L). All participants signed informed consent forms prior to enrolling in the study protocol (Appendix M).

Study Design

Subjects received both treatments (RS, control) in a cross-over design, with testing dates two weeks apart. Subjects were randomly assigned to order of treatment. As subjects were free living, they were instructed to follow their normal dietary pattern prior to and during the study. On test day, participants arrived at the laboratory at 8:00 am after a 10-12 hour fast. Blood samples were drawn at time 0 (during fasting) then treatment or control granola samples (described below) were provided and consumed within 15 minutes. Blood samples were drawn at 30 minutes, 1, and 2 hours after consumption of the test samples. Additional food was not allowed during the 2 hour test period, but water was allowed *at libitum*.

Measurements of height and weight were obtained, and body mass index (BMI - Kg/m²) was calculated. Three-day food records were kept by the participants to evaluate their regular eating patterns and to determine their caloric, macronutrient, and fiber intakes (Appendix N).

Seven subjects received the control treatment first. The control treatment contained < 0.5 gram of RS by providing granola bars formulated with a waxy corn starch (Amioca®, Food Innovation, National Starch, Bridgewater, NJ). The other seven subjects received the RS treatment first. The RS treatment contained ~18 grams of RS in the granola bars formulated with Hi-Maize 260® (Food Innovation, National Starch, Bridgewater, NJ). Subjects in the control group were subsequently assigned to the RS treatment group, and subjects in the RS group were assigned to the control group two weeks apart; thus each subject served as her own control. The RS granola bars and control bars were isocaloric. Each bar provided ~450 Kcal/serving (The serving size of the RS granola bar was 120 g, and the serving size of the control bar was 100g). The difference in the serving size between the two bars is due to the amount of RS added.

Analyses of Granola Bars

Ground samples of control and RS-supplemented granola bars were analyzed for crude fat, ash, moisture, and crude protein using AOAC official methods of analysis sections 960.39, 923.03 and modified sections 950.46 and 981.10 respectively. The Kjeldahl nitrogen method ($N \times 6.25$) was used to estimate protein. Total lipids were measured using the Soxhlet method. Ash was analyzed by burning a sample in a furnace at 530 °C for 12 hours. Carbohydrate percentages were determined by difference. The caloric content (kcal/100g) and dietary fiber content of the RS and control granola bars and cereals was calculated using the Food Processor software (Food Processor 8.6.0, ESHA Research, Professional Nutrition Analysis Software and Databases, Salem, OR).

Resistant starch content was determined by the procedure of McCleary and Monaghan (2002) using a commercially available enzymatic kit (Megazyme International Ireland Ltd.)

Alpha-Amylase Activity Assay

The α -amylase activity of various starches was examined *in vitro* using a colorimetric assay. Briefly, 200 μ l of the enzyme, porcine pancreatic α -amylase (4U/ml), was added to 400 μ l of substrate (0.5% potato starch, 0.5% high amylose starch (RS2), or 0.5% waxy starch in PBS) and incubated at room temperature for 3 min. Four hundred μ l of 3,5-Dinitrosalicylic acid (DNS) reagent was added and incubated for 15 min at 85-90 °C to develop color and stop enzyme activity. The samples were cooled for 10 minutes. One hundred forty μ l of deionized water and 60 μ l of each reaction mixtures were loaded into the wells of clear 96 well plates. The 540 nm absorbance was measured using a Victor³ multilabel plate reader (Perkin-Elmer®, Turku, Finland).

Blood Sample Collection

Blood samples were collected in Vacutainers containing heparin as the anticoagulant at 0, 30 minutes, 1, and 2 hours postprandial and immediately placed on ice until processing for blood glucose and plasma separation. Blood samples were taken by a certified phlebotomist in the Human Nutrition, Foods, and Exercise Department at Virginia Tech. Blood glucose was measured in whole blood samples immediately after the blood was drawn and placed on ice. Blood samples were centrifuged at 3500 rpm at 4 °C for 15 minutes (Eppendorf 5810 Laboratory

Centrifuge, Hamburg, Germany) and plasma separated from other blood components for insulin and antioxidant status parameters (isoprostanes (F₂-Isops), oxygen radical absorbance capacity (ORAC) assays). Red blood cells (RBCs) were separated from plasma for cellular glutathione peroxidase (cGPx) activity. Plasma and RBCs were aliquoted into individual cryovac tubes, and snapped frozen at -60 °C in a dry ice/ethanol bath. Plasma samples were stored at -80 °C until further analyses.

Laboratory Methods

Whole blood glucose

Whole blood glucose concentrations (mg/dl) were measured using a glucose monitoring system (Accu-Chek® AVIVA Diabetes Meter, Roche Diagnostics Corporation, Kalamazoo, MI) after a 12-hour fast, and at 30 min, 60 min, and 120 min after consumption of the RS or control granola bars.

Plasma insulin

Plasma insulin was measured using a sandwich immunoassay ELISA kit (insulin EIA 80-INSHU-E01, Alpco Diagnostics™, Salem, NH) according to the manufacturers' instructions after a 12-hour fast, and at 30 min, 60 min, and 120 min after consumption of the RS or control granola bars. Briefly, 25 µl of each standard, control, or sample were added into the respective wells on the 96-well plate. A 100 µl of working enzyme conjugate was added to each well. The plate was incubated for 1 hour, shaking at 700-900 rpm on an orbital microplate shaker at room temperature (18-25 °C). The plate was then washed 6 times with working strength wash buffer using a wash bottle. Substrate (100 µl) was added to each well. The plate was incubated for 15 min at room temperature. Stop solution (100 µl) was added to each well. The intensity of the yellow color is directly proportional to the concentration of insulin in each well. The plate was read using a µQuant™ microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) and the absorbance at 450 nm with a reference wavelength of 620-650 nm was measured. Results were expressed as (µIU insulin/ml).

Glutathione peroxidase (cGPx) activity of red blood cells (RBCs)

The cellular GPx enzymatic activity of the RBCs was measured using a colorimetric enzymatic kit according to the manufacturer's specifications (BIOXYTECH® GPx-340™ 21017, OXIS international, Inc. Portland, OR) after a 12-hour fast, and at 120 min after consumption of the RS or control granola bars. Briefly, RBCs were diluted (1:100) with assay buffer and mixed for 30 seconds. The following reagents were added to wells of a 96-well microplate (75 µl assay buffer, 75 µl NADPH). Diluted RBCs samples (15 µl) were added and the reaction initiated with 75 µl of 0.007% tert-butyl hydroperoxide. The activity of the cGPx enzyme was measured spectrophotometrically with a microplate reader (Victor³ multilabel plate reader, Perkin-Elmer® precisely, Turku, Finland) at an absorbance of 340 nm. The rate of decrease in absorbance is directly proportional to the cGPx activity in the sample.

The bicinchoninic acid (BCA) was used for the quantification of protein (µg/ml) for RBCs samples according to manufacturer's recommendations (Pierce Biotechnology, Thermo Scientific, Rockford, IL). Results for the cGPx activity for the RBCs samples were expressed as nmol NADPH/min/mg protein = mU cGPx activity/mg protein (Appendix O).

Oxygen Radical Absorbance Capacity (ORAC)

The ORAC_{FL} assay measures the peroxy radical scavenging activity of plasma samples compared to Trolox, a water-soluble analogue of vitamin E (Cao and Prior, 1999). Briefly, fluorescein (FL) stock solution (100 µM) in phosphate buffer (75 µM, pH 7.4) was prepared and kept at 4 °C protected from light. A fresh working FL solution (100 nM) was prepared daily by diluting the FL stock solution in phosphate buffer. Two hundred µL of the working FL solution were added to the 40 µL of each plasma sample (1:50 dilution) or Trolox standard in phosphate buffer (5, 10, 20, 40, 80 µM) in a black 96-well microplate and incubated for 20 min at 37 °C. The radical scavenging reaction was initiated by adding 35 µL of the peroxy radical generator 2,2'-azobis (2-aminopropane) dihydrochloride (AAPH) (0.36 M), and the fluorescence was measured ($\lambda_{\text{ex}} = 485 \text{ nm}$ and $\lambda_{\text{em}} = 535 \text{ nm}$) each minute using a Victor³ multilabel plate reader (Perkin-Elmer®, Turku, Finland). The temperature was maintained at 37 °C until the fluorescence had declined to less than 5% of the initial reading. Plasma samples and Trolox standards were run in duplicate. Results for the ORAC_{FL} were calculated using a regression equation relating Trolox concentrations and the area under the kinetic fluorescein decay curve.

The ORAC_{FL} value of each plasma sample was expressed as micromoles of Trolox equivalents per liter of plasma ($\mu\text{mol Trolox/L}$). The ORAC_{FL} values were calculated for the RS (treatment) and control groups at time 0 (fasting levels) and 2 hours postprandial.

Plasma 8-epi-prostaglandin-F₂-alpha (F₂-isoprostanes)

Plasma F₂-isoprostanes were quantified at the Virginia-Maryland Regional College of Veterinary Medicine, Toxicology laboratory (Blacksburg, VA). F₂-isoprostanes in plasma were measured after purification and derivatization by selected ion monitoring gas chromatography/negative ion chemical ionization-mass spectrometry (GC/NCI-MS) employing [²H₄] 8-iso-prostaglandin F_{2 α} as an internal standard (Morrow and Roberts, 1999; Roberts and Morrow, 2000) (Appendix P).

Statistics

Data were analyzed for the main effects of treatment and time, and the interaction between treatment and time using repeated measures ANOVA and Tukey's HSD multiple comparisons procedure (JMP 7.0, 2007, SAS Institute, New Jersey, USA). The area under the curve (AUC) for glucose was calculated using the linear trapezoid method. Data were expressed as the Mean \pm SEM. Statistical significance was set a priori $p < 0.05$.

Results

Granola bar composition

To estimate the amount of RS-supplemented granola bars that individuals in the study could consume in 20 minutes, two volunteer subjects were given a 100 g granola bar and 100 g granola cereal. Subjects were asked to consume as much granola as they could within 20 minutes. The average amount of granola products consumed was 130 g. Based on these results, the subjects involved in this study were given 120 g of a granola bar containing 18 g of RS. The RS supplemented and control granola bars were formulated to be isocaloric (~450 Kcal/serving). The weight of the RS granola bar was 120 g whereas the weight of the control granola bar was 100 g. In the final formulations, the weight of the control bars was less than the RS bars due to the incorporation of digestible starch (40% digestible starch) and the need to be isocaloric. The

RS granola bar contained both 40% digestible starch and 60% RS as it is found in Hi-Maize®. The composition of the granola bars are shown in Tables 4.1 and 4.2.

In-vitro α -amylase activity assay

The α -amylase activity of the raw starting starch materials was measured. The results showed potato starch was completely digested (100% digested), waxy starch was 51% digested, and resistant starch was 13% digested under the conditions of this assay. Kelp, a plant material suggested to inhibit alpha amylase activity was used as a reference. The *in-vitro* α -amylase inhibition results indicated that RS was resistant to the enzyme in this experiment (Figure 4.1).

Human subject characteristics

The mean age of the subjects was 32.4 ± 1.56 and the mean BMI (Kg/m^2) was 26.6 ± 1.36 . Subjects were healthy and not taking any medications for diabetes. Out of the 14 subjects recruited, 7 subjects scored 5 or less on the American Diabetes Association diabetes risk test indicating a lower risk for type 2 diabetes while the other 7 subjects scored 10 or above in the diabetes risk test indicating a higher risk for type 2 diabetes. The dietary intake of the subjects based on a 3-day food record is shown in Table 4.3.

Effects of RS consumption on whole blood glucose and plasma insulin

We determined the effects of a single ingestion of a granola bar containing RS (18 g) (treatment food) on the postprandial increase in blood glucose and insulin in Hispanic women. Granola bars containing waxy corn starch (<0.5 g RS) were used as the control food challenge. Whole blood glucose and plasma insulin responses and whole blood glucose area under the curve (AUC) are shown in Figure 4.2 and Table 4.4 and 4.5. Mean whole blood glucose and plasma insulin levels for both treatment conditions (treatment and control) rose to their highest levels at 30 min after consumption of the granola bars, followed by declines after 30 minutes to the lowest level 2 hours after consumption. Whole blood glucose, plasma insulin, and whole blood glucose AUC did not differ significantly between the two groups up to 2 hours after consumption.

Based on the results from blood glucose levels after consumption of the food treatments, glucose responses from all subjects (n=14) were analyzed individually. Because Hispanic women enrolled in this study were healthy with normal baseline glucose levels (~92 mg/dl), subjects

with abnormal blood glucose levels were excluded from subsequent analysis. Subjects whose blood glucose levels declined at 30 minutes after consumption of the food treatments (n=4) and whose baseline blood glucose was ≥ 100 mg/dl (n=1) (an indication of pre-diabetes) were excluded from analysis. Subjects were free living (not confined to a metabolic ward) and were expected to follow the study protocol; however, a decrease in blood glucose at 30 minutes after consumption of the food treatments is indicative of lack of compliance. Based on these assumptions, a subgroup of subjects (n=9) were included for the analyses of blood glucose, insulin responses and parameters of oxidative stress.

Effects of RS consumption on whole blood glucose and plasma insulin (n=9)

Whole blood glucose, plasma insulin, and whole blood glucose AUC are shown in Figure 4.3 and Tables 4.4 and 4.5. Whole blood glucose level for the treatment condition was significantly lower than for the control condition 30 minutes after consumption (p=0.0496). There seems to be attenuation in the glucose response after consumption of RS that may have biological significance in managing postprandial glucose spikes. The blood glucose in the control group reached a peak at 30 minutes after consumption. The blood glucose levels for both the treatment and control groups declined after 2 hours. Plasma insulin levels did not differ significantly between the groups up to 2 hours after consumption.

Effects of RS consumption on oxidative stress parameters (n=9)

Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay measures the free radical scavenging ability of blood plasma samples against the peroxy radicals in an aqueous environment. The fasting and two hour postprandial ORAC values of the RS and control groups expressed as Trolox equivalents (TE) per liter of plasma are presented in Figure 4.4. The ORAC values for the RS group were 1813 and 1464 μmol of TE/L for the fasting and 2 hours postprandial, respectively. The ORAC values for the control group were 1810 and 1284 μmol of TE/L for the fasting and 2 hour postprandial, respectively. There was not a significant difference for time-treatment interaction (p=0.9470); however there was a significant difference with time. The ORAC values decreased at 2 hours for both the RS treatment and control (p=0.0269).

Glutathione peroxidase (cGPx) activity of red blood cells (RBCs)

We determined the enzymatic activity of cellular glutathione peroxidase (cGPx) before and after consumption of a granola bar containing RS compared to a control (no RS) granola bar in Hispanic women. The RBCs cGPx did not differ significantly between the two groups. The baseline cGPx activity values were 87.6 and 84.5 mU/mg protein for the RS and control groups, respectively. The 2-hour postprandial cGPx values were 87.1 and 83.1 mU/mg protein for the RS and control group, respectively.

Plasma 8-epi-prostaglandin-F₂-alpha (F₂-isoprostanes)

We measured F₂-isoprostanes to determine the extent of lipid oxidation before and after consumption of a granola bar containing RS compared to a control (no RS) granola bar in Hispanic women. Plasma F₂-isoprostanes did not differ significantly between the two groups. The baseline F₂-isoprostanes were 79.7 and 85.7 pg/ml for the RS and control groups, respectively. After 2 hours of consumption, the F₂-isoprostanes decreased to 71.7 and 80.7 pg/ml for the RS and control groups, respectively. The plasma F₂-isoprostanes decreased significantly with time (p=0.0250) for both groups although no significant differences were observed between the treatment and control groups (Table 4.6).

Discussion

For more than 20 years dietary starch was believed to be completely digested and absorbed from the small intestine. It was not until later that a fraction of starch was shown to be resistant to digestion and absorption from the intestinal tract. This starch fraction was later defined as resistant starch (Asp, 1982). Much attention has been given to the potential health benefits of RS on glucose and insulin management. However, the inhibitory effects of RS on postprandial increases in blood glucose and insulin have shown equivocal results.

The influence of various starches (resistant starch and waxy starch) on oxidative stress is not well known. In particular, the role of dietary RS in positive health outcomes has not been elucidated. To our knowledge, this is the first study of the influence of RS on oxidative stress responses in Hispanic women after a single consumption of a granola bar containing RS.

The purpose of this study was to examine the effects of a single ingestion of a granola bar containing RS in Hispanic women on glucose control and oxidative stress. In our study, a single

ingestion of 18 g RS (n=14) did not result in lower glucose levels compared to a granola bar containing waxy corn starch (p=0.9892). When subjects who had abnormal glucose levels (i.e. one subject had glucose levels > 100 mg/dl at baseline and four subjects had glucose levels that decreased at 30 minutes after consumption of the control bar) were excluded from the analysis (n=9), an attenuation of the glucose response after 30 minutes of RS consumption (p=0.0496) was found (Table 4.4). This is particularly significant since modulation of postprandial plasma glucose levels has been shown to play a key role in glycemic control in type 2 diabetes and people at risk for type 2 diabetes.

Evidence suggests that cooking RS attenuates glucose and insulin responses in humans and animals. Supplementing 24 g of uncooked RS/day in a liquid beverage for 21 days decreased fasting serum glucose levels (p<0.05) in overweight men and women (Park et al., 2004). Supplementing 50 g of raw potato starch (54% RS) compared to 50 g of pregelatinized potato starch (100% digestible) mixed in a liquid syrup resulted in a postprandial plasma glucose increase nine times more after the 100% digestible starch compared to the raw potato starch in a group of 10 healthy men (Raben et al., 1994). Similar results were found in insulin levels (Raben et al; 1994). Results from Raben and coworkers (1994) showed small increases in blood glucose after consumption of raw potato starch; however, the authors indicated that heating the starch would affect the physical properties of the raw starch rendering it more digestible.

Najjar and coworkers (2004) investigated the effect of temperature of cooked potato meals containing 50 g of carbohydrates in healthy subjects (n=9). Results from their study indicated that temperature (83°C for hot potato and 26°C for cold potato) has an effect on glucose and insulin responses. Glucose levels were significantly lowered after consumption of cold potato; however, the same effect was not achieved with hot potato (Najjar et al., 2004). In healthy rats, a relative small proportion of uncooked amylose from corn starch (270 g/kg starch) was enough to achieve an attenuating effect on postprandial insulin levels compared to 0g amylose/kg starch (Brown et al., 2003). After cooking, a higher amount of amylose (600 g/kg) was needed to achieve a similar effect. There were no effects of feeding uncooked or cooked amylose on glucose response (Brown et al., 2003). Thus, cooking attenuated the ability of high amylose corn starch to reduce postprandial insulin concentrations without affecting glucose responses in healthy rats.

Although we observed a lower 30-minute glucose peak in the RS-supplemented group (n=9), it is possible that molecular changes in the starch during baking of the granola bars increased the starch susceptibility to digestion. During heating, starch gelatinizes and becomes more susceptible to enzymatic hydrolysis, whereas upon cooling, the amylose chains retrograde and become more resistant to enzymatic hydrolysis. The RS formed after cooling is a retrograded starch (RS3) which is a crystalline type of RS (Englyst and Cummings, 1987; Muir and O'Dea, 1992). In our study, a granular type of RS (RS2) was used which may have been partially hydrolyzed during cooking and may have become more digestible. It is possible that feeding raw/uncooked high amylose corn starch (RS2) could have resulted in marked decreases in postprandial glucose and insulin responses compared to the cooked RS-supplemented granola bars in Hispanic women.

Shih and coworkers (2007) investigated the effects of retrograded RS (RS3) from rice and corn in rats with streptozotocin (STZ)-induced diabetes. Lowered glucose and insulin responses with rice starch compared to corn starch after 4 weeks were found. In a similar study, Kim and coworkers (2003) fed RS from corn or rice to STZ diabetic rats. Their results showed no difference in glucose and insulin responses with either type of starch. However, they found RS from rice and corn significantly lowered plasma total lipid and cholesterol concentrations (Kim et al., 2003).

In our study, the reduced glucose response (n=9) 30 minutes after ingestion of the RS-supplemented granola bar also might have been due to the high amylose corn starch (RS2) being digested and absorbed at slower rates and to a lesser extent in the small intestine. It has been suggested that RS will pass undigested into the colon where it is fermented to short chain fatty acids limiting the amount of glucose that can be absorbed from the small intestine (Shih et al., 2007).

In our study, plasma insulin levels were not affected by RS supplementation in all subjects (n=14) or in the group who seemed to respond to the RS treatment by having a lower glycemic response (n=9). This finding is in agreement with Park and coworkers (2004) who found no significant influence on serum insulin concentrations after subjects consumed 24 g RS for 21 days. Studies have shown improvements in insulin levels along with glucose levels due to feeding RS to normal subjects (Raben et al., 1994), normal subjects after a second meal effect (Lilleberg et al., 1999), and subjects with type 2 diabetes (Reader et al., 1997). Changes in

glucose and insulin levels were not found in our study and the reasons for this are unclear. Hispanics are a population at risk for type 2 diabetes; in our study, these women were healthy with normal glucose and insulin levels which may explain the insulin response to the RS-supplemented granola bar.

Resistant starch may be fermented in the colon to short chain fatty acids, acetate, propionate, and butyrate (Cummings et al., 1996; Kendall et al., 2004), which results in lower plasma total lipid and cholesterol concentrations in rats (Kim et al., 2003). Therefore, reduced plasma lipid concentrations may lead to decreased lipid peroxidation and increased levels of antioxidant defense systems. Shih and coworkers (2007) found serum glutathione peroxidase activity was lower in the rice-fed group compared to the corn starch diet in STZ diabetic rats. Plasma glutathione peroxidase activity decreased postprandially after 2 and 4 hours of ingestion of a single dose of olive oil with a concomitant increase in lipid peroxides. A decrease in antioxidant defenses accompanied with an increase in lipid oxidation is indicative of increased oxidative status (Frito, et al., 2002). Plasma glutathione peroxidase activity significantly decreased after 2 hours of ingestion a high-fat (50 g fat) meal indicating consumption of a high-fat meal is associated with augmented oxidative stress as evidenced by the depletion of antioxidant enzymes (Tsai et al., 2004). In our study, feeding a RS-supplemented granola bar containing 18 g/RS resulted in similar levels of glutathione peroxidase in the RS and control groups. The RBCs cGPx was essentially unchanged from baseline (87.6 nmol/min/mg protein) and 2 hours after ingestion of RS (87.1 nmol/min/mg protein). The cGPx activity of the control granola bars followed a similar trend (84.5 nmol/min/mg protein at baseline and 83.2 after 2 hours) although this result was not statistically significant. However, the plasma total radical trapping antioxidant parameter (TRAP) was 37% higher in the rice fed rats than rats receiving corn starch (Shih et al., 2007). No changes in total antioxidant capacity were observed after acute consumption of a low-flavonoid cocoa drink vs. a high-flavonoid rich cocoa drink (Wiswedel et al., 2004). Similarly, Mathur and coworkers (2002) found no changes in plasma ORAC after daily consumption of high-flavonoid vs. low-flavonoid drinks for 6-weeks. Results from Wiswedel and coworkers (2004) and Mathur and coworkers (2002) indicate that consumption of antioxidant containing drinks do not have a significant effect on plasma antioxidant capacity. In our study, the ORAC values were slightly higher 2 hours after consumption of RS (1447.66 $\mu\text{mol TE/L}$) compared to the control granola bar (1310.76 μmol

TE/L) although not statistically significant. Measures of lipid oxidation indicated that the serum malondialdehyde (MDA) in serum and liver were lower in diabetic rats fed rice compared to those fed a corn starch diet (Shih et al., 2007). Wiswedel and coworkers (2004) showed a slight increase in plasma F2-Isprostanes 2 and 4 hours after consumption of a low-flavonoid cocoa drink in healthy subjects, albeit not significantly different from ingestion of a high-flavonoid cocoa drink. However, when subjects were subjected to exercise, the difference in F2-isoprostanes was statistically significant. Thus the authors concluded that dietary flavanoids present in cocoa drinks can lower plasma F2-isoprostanes after 2 or 4 hours of ingestion (Wiswedel et al., 2004). Similarly, a significant increase in plasma F2-Isoprostanes 2 hours after ingestion of a single fast-food meal (McDonald's Big Mac meal) was found by Gopaul and coworkers (2000). In our study, plasma F2-Isoprostane levels were slightly lower after 2 hours of ingestion of RS (71.67 pg/ml) compared to the control granola bar (80.67 pg/ml) although not statistically significant (Table 4.6).

Our results for oxidative stress parameters as measured by RBC cGPx, F2-Isoprostanes, and ORAC indicated that after 2 hours of consumption of RS cGPx levels and F2-Isoprostanes were slightly lower and ORAC values slightly higher compared to the control group. Although these results were not statistically significant, it is possible these results may have biological significance if RS was fed for a longer time. In our study, the oxidative stress parameters were measured after a single ingestion of 18 g/RS. It is possible that ingestion of RS for a longer period of time may result in greater attenuation of lipid oxidation and more pronounced antioxidant responses. Long-term studies supplementing RS in a cooked food matrix (i.e. RS-supplemented granola bar) are warranted to elucidate the potential metabolic effects of RS on glucose, insulin, and oxidative stress parameters.

The sample size in our study was small, and this might have contributed to the non significant differences found in the responses measured between the RS and control treatments. The power of the test ($P = 1 - \beta$) was calculated to be 0.74 and 0.55 for $n=14$ and $n=9$, respectively. This indicates that the probability of rejecting the null hypothesis (no differences between treatments) when the null hypothesis is false is 0.74 and 0.55 for $n=14$ and $n=9$. The β (Type II error) is failing to reject the null hypothesis when it is false. As the sample size increase, the power of the test increases. A limitation of this study was the small population of Hispanics

living in this area, and therefore, conducting this type of study where more Hispanics reside will help elucidate the benefits of RS in this population.

Conclusions

This study demonstrates that RS may have potential health benefits by attenuating postprandial glucose response. Results from a subgroup of Hispanic women (n=9) indicated that a single ingestion of RS resulted in a lower glucose peak after 30 minutes compared to the control waxy starch. In addition, RS may have potential health benefits pertaining to lipid oxidation and antioxidant effects although our results were not statistically significant. Further studies incorporating high levels of RS in a food matrix and for a longer period of time are needed to elucidate the health benefits of RS. In addition, the effects of RS on oxidative stress parameters in type 2 diabetic subjects need to be explored. Consumption of a RS-supplemented granola bar may help individuals at risk of type 2 diabetes manage their blood glucose levels.

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Table 4.1. Proximate composition of granola bars containing 0% resistant starch (RS) (control granola bars) and 15% RS (test food)

	Resistant starch (RS)	Waxy corn starch (control)
Moisture	15.1 ± 0.81	16.6 ± 0.40
Protein	5.87 ± 0.07	6.42 ± 0.10
Lipid	17.8 ± 0.36	18.6 ± 0.19
Ash	2.40 ± 0.10	2.30 ± 0.10
Carbohydrate ^a	58.9 ± 0.48	55.9 ± 0.02

Data expressed as the mean ± SEM of duplicate samples (g/100g). ^aCarbohydrate calculated by difference.

Table 4.2. Percent of resistant starch (RS) and available starch (AS), and caloric (Kcal/100g), and dietary fiber content of granola bars containing 0% resistant starch (RS) (control granola bars) and 15% RS (test food)

	Resistant starch (RS)	Waxy corn starch (control)
Resistant starch (g/100g)	14.9 ± 0.38	0.20 ± 0.01
Available starch (g/100g)	44.9 ± 0.67	49.3 ± 1.95
Kcal/100g ^a	380	380
Dietary fiber ^a (g/100g)	23	3

Data expressed as the mean ± SEM of duplicate samples. ^aThe control and RS granola bars provide 380 Kcal/100g. The resistant starch granola bars for the human study provided 450 Kcal/120g serving and 18g RS/120g serving. The control granola bars provided 450 Kcal/100g serving and 0g RS/100g serving.

Table 4.3. Mean dietary intakes of Hispanic women (n=14) based on self-reported 3-day food records (2 weekdays, 1 weekend day).

Variable	Mean \pm SEM
Energy (Kcal)	1745 \pm 107
Protein (g)	71 \pm 6
Carbohydrate (g)	233 \pm 13
Dietary fiber (g)	16 \pm 2
Sugar-total (g)	95 \pm 10
Other carbohydrates (g)	105 \pm 6
Fat (g)	56 \pm 6
Saturated fat (g)	19 \pm 2
Monounsaturated fat (g)	13 \pm 1
Polyunsaturated fat (g)	6 \pm 1

Data analyzed using Food Processor (8.6.0, ESHA Research, Professional Nutrition Analysis Software and Databases).

Table 4.4. Changes in whole blood glucose levels (mg/dl) of Hispanic women before and after ingestion (0-120 minutes) of RS-supplemented (18g RS/120g serving) and control granola bars (0g RS/100g serving).

Subjects	Group	Before ingestion		After ingestion (min)		AUC
		0 min	30 min	60 min	120 min	
All subjects (n=14)	RS	93.3 ± 1.68	97.6 ± 2.82	83.2 ± 1.36	88.9 ± 1.75	10740 ± 283
	Control	92.2 ± 1.14	96.3 ± 1.88	80.9 ± 1.65	87.6 ± 1.43	10544 ± 258
(n=9)	RS	90.7 ± 1.49	93.2 ± 3.58*	85.2 ± 3.65	89.6 ± 2.51	10689 ± 309
	Control	92.8 ± 1.49	102.4 ± 2.04*	84.4 ± 3.14	88.7 ± 2.43	10914 ± 223

Each value for blood glucose is shown as the mean ± SEM (mg/dl). Whole blood samples were collected before ingestion and at 30, 60, 120 minutes after ingestion of the test food or control. Area under the curve (AUC) is the area under the blood glucose concentration-time curve from 0 to 2 hours after ingestion. * indicates significant difference (p=0.0496).

Table 4.5. Changes in plasma insulin levels glucose levels (mIU/ml) of Hispanic women before and after ingestion (0-120 minutes) of RS-supplemented (18g RS/120g serving) and control granola bars (0g RS/100g serving).

Subjects	Group	After ingestion (min)			
		Before ingestion 0 min	30 min	60 min	120 min
All subjects (n=14)	RS	10.4 ± 2.60	54.0 ± 7.44	23.7 ± 3.87	15.1 ± 2.58
	Control	7.10 ± 0.56	44.2 ± 4.94	18.6 ± 1.87	11.2 ± 1.08
(n=9)	RS	9.10 ± 1.34	54.6 ± 7.58	22.4 ± 1.98	14.1 ± 1.72
	Control	7.50 ± 0.78	51.6 ± 5.52	19.6 ± 1.75	13.1 ± 1.09

Each value for plasma insulin is shown as the mean ± SEM (mIU/ml). Plasma samples were collected before ingestion and at 30, 60, 120 minutes after ingestion of the test food or control.

Table 4.6. Plasma oxygen radical absorbance capacity (ORAC), red blood cell (RBC) cellular glutathione peroxidase activity (cGPx), and F₂-Isoprostanes evaluated in Hispanic women (n=9) after 2 hours of consumption of granola bars containing resistant starch (RS) (18g RS/120g serving) and waxy corn starch control granola bars (0g RS/100g serving).

	Resistant starch (RS)	Waxy corn starch (control)
Plasma ORAC TE (μmol/L)	1447.66 ± 151.33	1310.76 ± 76.30
RBC cGPx (nmol/min/mg protein)	87.14 ± 5.10	83.15 ± 5.94
F ₂ -isoprostanes (pg/ml)	71.67 ± 4.64	80.67 ± 11.10

Values are expressed as the mean ± SEM, n=9; different subscript letters in a row indicate significantly different means, P<0.05.

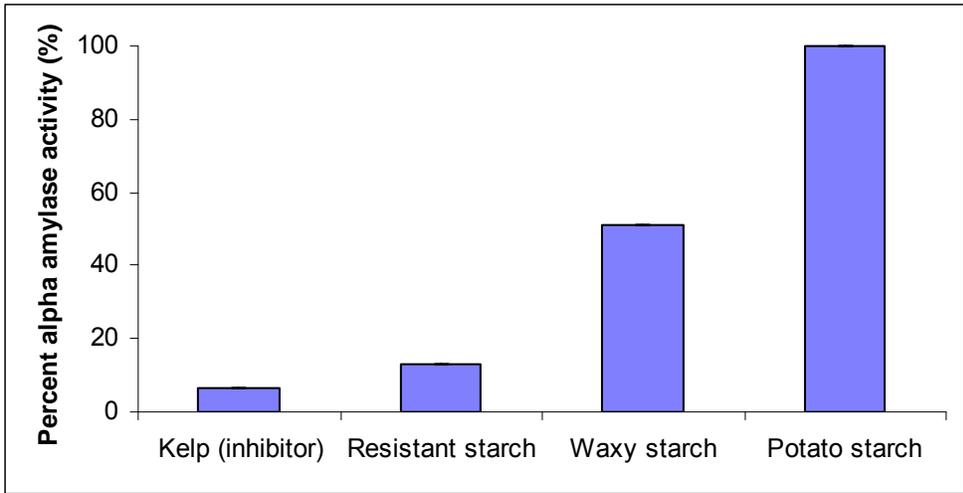
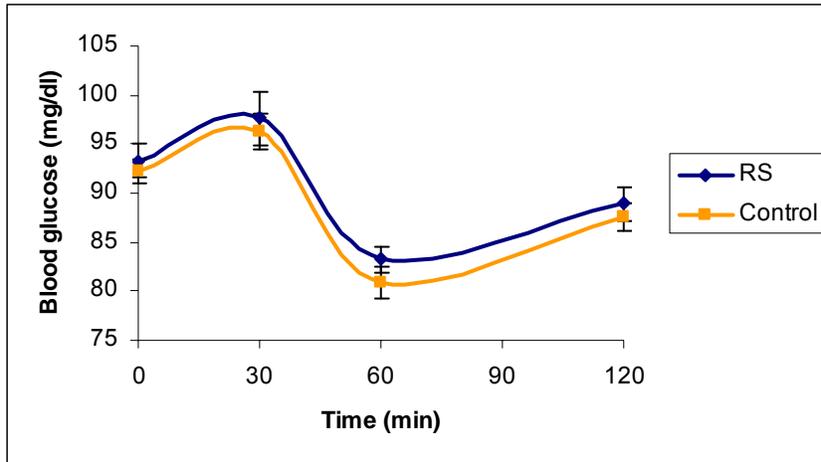


Figure 4.1. In-vitro alpha amylase activity of resistant starch, waxy starch, and potato starch. Kelp extract was used as an example of a compound that inhibits α -amylase activity.

(a)



(b)

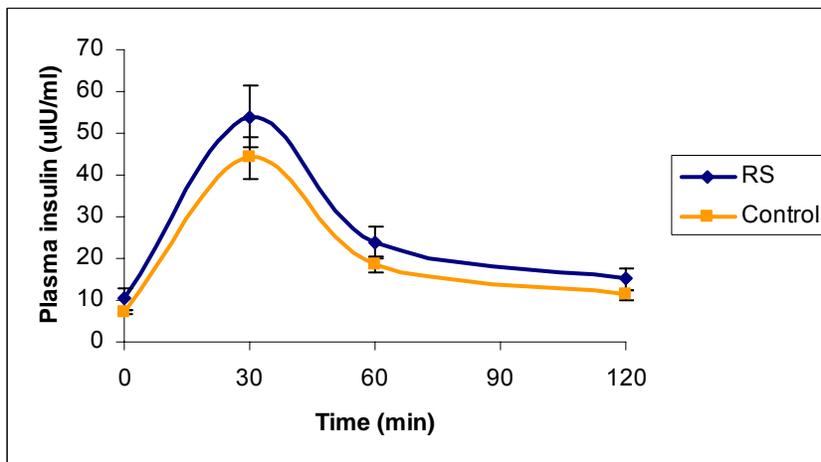
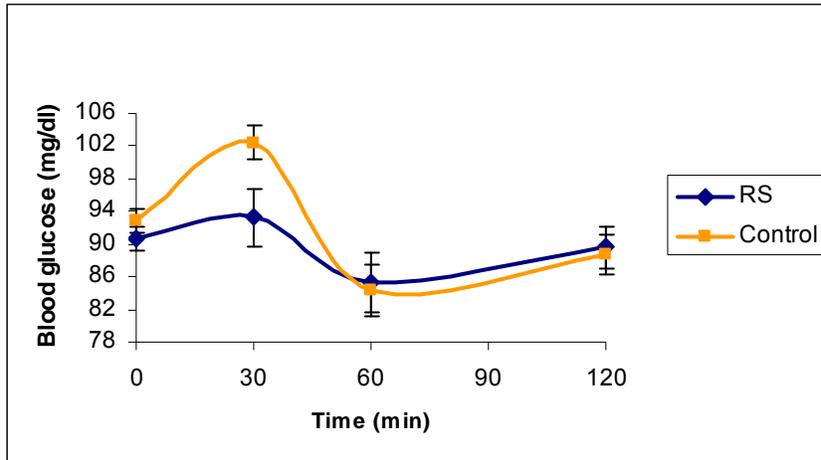


Figure 4.2. Baseline and postprandial whole blood glucose (a) and baseline and postprandial plasma insulin (b) in healthy Hispanic women (n=14) fed a granola bar containing 18 g RS/120g serving and a control granola bar containing 0 g RS/100g serving. Blood samples were taken during fasting (time 0), and after 30, 60, and 120 minutes after consumption.

(a)



(b)

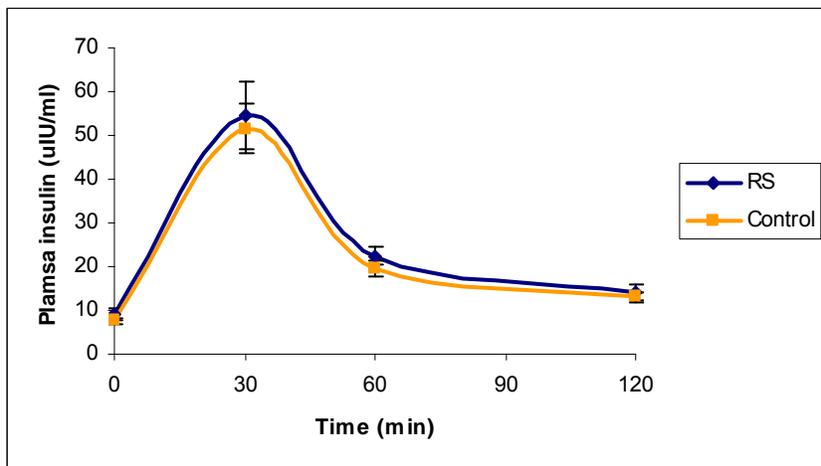


Figure 4.3. Baseline and postprandial whole blood glucose (a) and baseline and postprandial plasma insulin (b) in healthy Hispanic women (n=9) fed a granola bar containing 18 g RS/120g serving and a control granola bar containing 0 g RS/100g serving. Blood samples were taken during fasting (time 0), and after 30, 60, and 120 minutes after consumption.

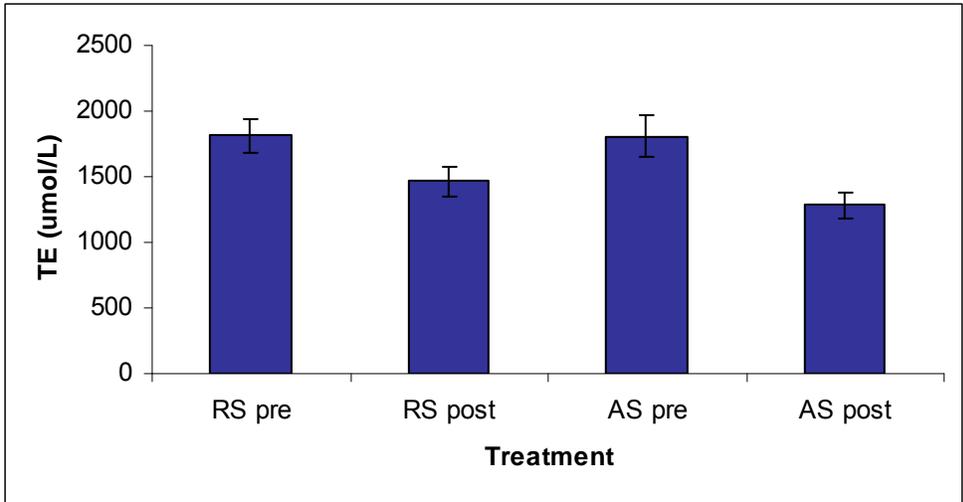


Figure 4.4. Oxygen radical absorbance capacity (ORAC) of blood plasma of Hispanic women (n=9) fed a granola bar containing 18g RS/120g serving and a control granola bar containing 0 g RS/100g serving. Blood samples were taken during fasting (time 0) and 120 minutes after consumption. AS (available starch). Results are expressed as micromoles of Trolox equivalents per liter of plasma (mean \pm SEM).

CHAPTER 5: EVALUATION OF POLYSACCHARIDES INCLUDING RESISTANT STARCH (RS) AND CHITOSAN (CS) AND THEIR POTENTIAL USE IN FOOD PACKAGING APPLICATIONS

Abstract

Polysaccharides, such as resistant starch (RS) with high amylose content and chitosan (CS), can form edible biodegradable films. The functional properties of amylose films are slightly better compared to amylopectin films in terms of film strength and oxygen barrier properties. Chitosan further enhances the properties of RS films. The purpose of this paper is to review the use of polysaccharides as edible films and coatings in foods and to report preliminary findings of the potential use of CS and RS in food packaging. In this work CS:RS films ranging from 100% CS to 100% RS were prepared. Films were mechanically stable with oxygen barrier properties comparable to commercial materials. Vitamin E was successfully incorporated into 100% CS and 60:40% CS:RS films. CS:RS films with incorporated antioxidants could have potential as active packaging materials.

Keywords: biodegradable films, chitosan, resistant starch, glycerol, mechanical properties

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To be submitted to *Packaging World*

Introduction

Traditionally, food packaging materials have been composed of nonrenewable petroleum-based plastic materials. At the beginning of the twenty-first century, attention has been given to the replacement of nonrenewable sources with renewable, mainly plant-derived, products (Krochta and Mulder-Johnston, 1997; Bertuzzi et al., 2007). Although the commercial use of biopolymers such as starch and chitosan as food packaging materials is still in its early years, their use and application is likely to increase in the near future (Robertson, 2006). Consumers are constantly demanding new, healthier, renewable and biodegradable food packaging materials to minimize solid waste, litter, and pollution associated with non biodegradable packaging (Alves et al., 2006; Krochta and Mulder-Johnston, 1997). There is a growing interest in production of biodegradable films from natural polymers such as polysaccharides.

Chitosan (CS) and resistant starch (RS) are two readily available polysaccharides with good film forming properties (Cervera et al., 2004; Garcia et al., 2006; Krogars et al., 2003). Chitosan is the deacetylated form of chitin, a biopolymer of $\beta(1\rightarrow4)$ linked *N*-acetylglucosamine. Starch, a glucose homopolymer, is comprised of two fractions; a linear fraction, amylose, and a branched fraction, amylopectin. Most naturally occurring starches contain 30% amylose and 70% amylopectin. The linear backbone consists of $\alpha(1\rightarrow4)$ linked glucose units, with branches arising through $\alpha(1\rightarrow6)$ linkages. High amylose corn starch (HACS), also known as resistant starch (RS), is a corn hybrid that contains a high percentage of amylose regions (70% amylose, 30% amylopectin) (Figure 1) and a readily available polysaccharide with good film forming properties (Whistler and BeMiller, 1997). The functional properties of amylose films are slightly better compared to amylopectin films in terms of film strength and oxygen barrier properties (Rindlaw et al., 1998). Both CS and RS are nontoxic, biocompatible, renewable, biodegradable, and abundant, economically produced polymers and have been used in the food and pharmaceutical industries extensively (Ban et al., 2006; Harish Prashanth and Tharanathan, 2007; Krogars et al., 2003; Lafargue et al., 2007; Lourdin et al., 1995).

The purpose of this paper is to review the current use of polysaccharides as edible films and coatings and to report preliminary findings of the potential use of CS and RS in food packaging. Films and coatings prepared from CS and RS could have potential as active food packaging materials.

Edible Films and Coatings

Edible films are defined as thin layers of edible materials applied to a food as a surface coating or positioned between food components or layers. Both terms, films and coatings, are used interchangeably but generally coatings are applied directly on the surface of the food and edible films are preformed separately as thin layers and applied on the food (Robertson, 2006). To date, edible films have been used in various commercial applications including casings for sausages, chocolate coatings for nuts and fruits, and wax coatings for fruits and vegetables (Donhowe and Fennema, 1994; Bertuzzi et al., 2007).

Many of the functions of edible films and coatings are similar to conventional food packaging materials. These include providing moisture or gas barriers, preventing migration of lipids, improving mechanical handling of the food product, protecting the food from environmental conditions, retaining volatile flavor compounds, and carrying and delivering antioxidants and antimicrobial agents. Although edible films and coatings will never replace current packaging materials for prolonged food storage, edible films and coatings in close contact with the food product are primarily used to improve overall food quality, extend the shelf-life of foods, promote the use of natural resources, and reduce environmental pollution (Robertson, 2006).

Polysaccharide-based Edible Films and Coatings

Incorporating biodegradable synthetic materials (polylactide, polyesters and polycaprolactone) into films is expensive and their use is limited. An alternative way to develop renewable, biodegradable, and biocompatible films is to use abundant and natural materials such as polysaccharides, proteins, and lipid-based materials (Ban et al., 2006). Polysaccharides such as starch, cellulose, alginates, chitin, and plant gums are readily available and are being studied as edible films and coatings in food packaging and food preservation (Phan et al., 2005). Starch, in particular, is a naturally occurring biomaterial that is abundant, renewable, biodegradable, and produced at relatively low cost for food packaging applications (Lafargue et al., 2007; Lourdin et al., 1995).

In general, polysaccharide films possess poor water vapor barrier properties but good oxygen barrier properties. The hydrogen bonding capability of polysaccharides allows the production of films with good mechanical properties (Wang et al., 2007). To improve film

strength and control water uptake, copolymerization of starch with synthetic polymers have been used to improve the mechanical and barrier properties of starch films; however, synthetic polymers such as polyethylene (PE) are non biodegradable. Other approaches include a combination of cellulose fibers added to modified starches such as hydroxypropylated starch to enhance the mechanical properties (strength, elasticity, and barrier properties) of the films (Ban et al., 2006). A composite flexible film made by blending chitosan and amylose results in strong antibacterial properties. Suzuki and coworkers (2007) explained the improved antibacterial properties of the chitosan-amylose film based on the morphology of the polysaccharide polymers. Chitosan contains amino groups that may be hidden in the film whereas in the blend of amylose and chitosan, the amino groups may be exposed on the surface of the film (Suzuki et al., 2007). The tensile strength of chitosan-corn starch composite films is comparable to those of low-density and high-density polyethylene but lower than that of cellophane (Garcia et al., 2006). Polysaccharide-based coatings (starch, carrageenan and chitosan) extended the shelf-life of strawberries; however, the oxygen permeability of carrageenan was significantly lower than the starch films (Ribeiro et al., 2007).

In the food industry, starch-based films can potentially replace synthetic packaging materials such as polyethylene (PE); however, most naturally-occurring starches contain 70% amylopectin and 30% amylose (Whistler and BeMiller, 1997); and the low amylose-to-amylopectin ratio contributes to starch films of low elasticity and high hydrophilicity (Ban et al., 2006). The oxygen permeability of starch films made of amylose and amylopectin was affected at various relative humidities. Above 80% relative humidity, amylopectin films become more oxygen permeable compared to amylose films (Forsell, et al., 2002).

A composite flexible film made by blending chitosan (degree of N-deacetylation, DDA, 44%) and amylose results in strong antibacterial properties. Suzuki and coworkers (2007) explained the improved antibacterial properties of a chitosan-amylose film compared to chitosan based on the morphology of the polysaccharide polymers. Zeta potential measurements indicated the surface of chitosan (DDA: 44%) in the chitosan-only films had a weak positive potential whereas amylose-chitosan blended films showed a larger positive potential. The authors suggested that the free amino groups of glucosamine residues are less exposed to the surface in the chitosan-only films resulting in loss of antibacterial properties of chitosan. More amino groups are exposed on the surface of chitosan-amylose films with strong antimicrobial

properties. Amylose-only films showed a negative zeta potential, which is expected in neutral polymer films (Suzuki et al., 2007). The tensile strength of chitosan-corn starch composite films (28.7 MPa) is comparable to those of low-density polyethylene (LDPE, 16.2 MPa) and high-density polyethylene (HDPE, 27.8 MPa) but lower than that of cellophane (85.8 MPa). Water vapor permeability of chitosan-corn starch films plasticized with glycerol was lower than those of the single component films and cellophane but higher than LDPE and HDPE. Thus, composite films of chitosan-corn starch were resistant and their flexibility enhanced with the addition of glycerol (Garcia et al., 2006).

Polysaccharide-based coatings (starch, carrageenan and chitosan) have extended the shelf-life of fresh strawberries. Strawberries were sprayed with the three solutions and stored at 5°C for six days. The oxygen permeability of carrageenan-based films was significantly lower than that of starch films. Chitosan coatings with the addition of calcium resulted in antimicrobial protection (Ribeiro et al., 2007).

Starch limitations for commercial film applications include poor film strength, brittleness, and water sensitivity (Shogren, 2007). One advantage of using starch for food protection and preservation is the ability of starch films to act as excellent oxygen barriers (Forssell et al., 2002). Plasticizers such as glycerol and sorbitol are required for polysaccharide-based films to promote flexibility and molecular mobility by reducing internal hydrogen bonding between the polymer chains while enhancing intermolecular space (Krogars et al., 2003; Larotonda et al., 2004; Rojas-Grau et al., 2008). Addition of cellulose fibers and/or chitosan to starch solutions enhances film strength of starch-based films by undergoing hydrogen bonding (Ban et al., 2006). Incorporation of cellulose (22%) or chitosan (33%) of the starch films improved the physical and chemical properties of starch-based films. A 5 to 10-fold increase in tensile strength was achieved by adding these biopolymers to the starch films (Ban et al., 2006).

Cassava starch films containing natural antioxidants from grape pomace were evaluated as active packaging materials, and results indicate grape pomace extracts (1-8%) might be added to biodegradable films as a source of antioxidant components (Hayashi et al., 2006). Chemically or physically modified starches enhance film properties by altering the structure of the starch polymer. High amylose corn starch (HACS), a physically modified starch, can enhance the functionality of starch-based edible films and coatings.

Case Study: Preliminary Work on the Preparation and Characterization of a Chitosan/Resistant Starch (CS/RS) Film

We investigated the use of two natural, biodegradable polysaccharides: chitosan (CS) and resistant starch (RS) for food packaging applications in our laboratory. The chemical structures of CS and RS are shown in Figure 5.1. The goal of this preliminary work was to evaluate the film forming properties and oxygen transmission rate of a film made with two low-cost and abundant polysaccharides. We also investigated the addition of an antioxidant on the film properties. Free films of CS and RS were prepared using solvent casting techniques. Chitosan (CS) at 1% (w/w) was dissolved in aqueous acetic acid at room temperature. Resistant starch (RS) at 1% (w/w) was dissolved in distilled water and prepared in a high-pressure reactor (100 rpm, 150 °C, and 4 bar of pressure) and held for 30 minutes. Film forming solutions were prepared by combining CS and RS solutions with glycerol, as a plasticizer (20% based on polymer weight). The composition of the CS:RS (w/w) solutions studied were as follow: 100:0%, 80:20%, 60:40%, 50:50%, 40:60%, 20:80%, and 0:100%. For antioxidant loaded films, the antioxidant (α -tocopherol acetate) 20% (w/w) based on the weight of the polymer and TWEEN 20 as an emulsifier 1% (w/w) based on polymer weight were added to the film forming solutions. The film forming solutions were poured in polytetra-fluoroethylene (Teflon) molds and dried at room temperature for 48 hours. Films were equilibrated at 60% relative humidity (RH) until analyzed (Aigster et al., 2007).

We measured thickness of the films, degree of stiffness of the films using dynamic mechanical analysis (DMA), tensile strength using an Instron universal testing instrument, oxygen transmission rate of the films. Incorporation of the antioxidant in the films was monitored using Fourier Transform Infrared Spectroscopy (FTIR), and lipid oxidation of the films in an oil model system was monitored using peroxide values (PV). Films with higher levels of RS exhibited an opaque appearance, while films with higher levels of CS displayed a yellow tint (Figure 5.2). The average film thickness was 0.026 ± 0.003 mm. The mechanical and physical properties of the films varied with composition. Dynamic mechanical analysis (DMA) indicated that the modulus varied from 2200 MPa for the 100% CS film to 1250 MPa for the 100% RS film (Figure 5.3). Increasing the concentration of RS resulted in a decrease in the modulus of the films indicating stiffness of the films. Based on the DMA results, the 100% CS

and 60:40% CS:RS films were used for tensile strength analysis, oxygen transmission rate, and peroxide values.

Results from the tensile strength test indicated the films were not elastic, but the 100% chitosan films showed a longer elongation at break compared to the 60:40 CS/RS films. These results are expected due to the ability for tighter chain packing and increased crystallinity enabled by the high amylose content of the RS. Films prepared from entirely RS were too brittle to endure analysis. The oxygen transmission rate of the 60:40% CS:RS film was 17.5 cc/m²/day (Figure 5.4) which are comparable to the oxygen transmission rate of Mylar films (~60 cc/m²/day), known to be excellent oxygen barriers.

Successful incorporation of α -tocopherol acetate was demonstrated using Fourier transform infrared spectroscopy (FTIR) equipped with an attenuated total reflectance (ATR) solid state probe. The appearance of the carbonyl stretch at 1750 cm⁻¹ suggests that the α -tocopherol acetate was successfully incorporated into the film (Figure 5.5).

Antioxidant incorporation caused no observable decline in film properties. The PV of the films with α -tocopherol acetate is shown in Figure 5.6

Mechanically stable films were prepared from chitosan and resistant starch. The oxygen barrier properties of the films were comparable to commercial packaging films. The antioxidant, vitamin E, was successfully incorporated into the film. Such films may have potential as food packaging materials or for health applications.

Uses of High Amylose Corn Starch (HACS) in Edible Films

Although the literature on HACS as a film former is scarce, limited research indicate this type of starch alone or more importantly in combination with other polysaccharides can form strong and highly oxygen impermeable films. High amylose corn starch is very useful as a film-forming material because it improves mechanical strength to form stronger, flexible films with good gas barrier properties compared to regular corn starch, possibly due to amylose crystallization (Han et al., 2006; Myllarinen et al., 2002). Films made with waxy starch containing mainly amylopectin (~99%) result in films that are brittle and have low tension resistance (Phan et al., 2005). Amylose is responsible for the film-forming capability of starch-based films. Addition of a plasticizer, usually glycerol or sorbitol, further promotes film formation. Without a plasticizer, HACS films are brittle due to extensive intermolecular forces;

the addition of plasticizers improves flexibility and extensibility of edible films (Bertuzzi et al., 2007). In addition, HACS could be used as films for packaging of fruits and vegetables, snacks, and dry products (Sorrentino et al., 2007).

Potential problems with HACS films are the migration of plasticizer on to the surface and increased crystallinity with aging resulting in embrittlement of the films. Krogars and coworkers (2003) showed that after 9-months of storage, the HACS films plasticized with glycerol and sorbitol (1:1) (100% based on the weight of the polymer) resulted in flexible films similar to freshly prepared films indicating minimal aging. The combination of two plasticizers resulted in improved stability of the HACS films compared to using each plasticizer alone by inducing tight interactions and keeping the plasticizers tightly bonded preventing them from migrating to the surface of the films. X-ray diffraction patterns showed the 9-months old HACS films were similar to the diffraction pattern of the freshly prepared films indicating the degree of crystallinity of the HACS films did not increase during storage. The similar crystallinity of the films is attributed to water incorporation during storage (Krogars et al., 2003). Similarly, Myllarinen and coworkers (2002) found no changes in crystallinity in amylose films prepared from potato starch after 2 months of storage.

Garcia and coworkers (1998) evaluated the effects of native starch and HACS on strawberry quality. The authors concluded HACS extended the shelf-life of refrigerated strawberries by reducing weight loss, maintaining firmness, and reducing microbial decay better than the native corn starch coating. Ryu and colleagues (2002) demonstrated that composite edible films of HACS and corn zein (a corn protein) could be used as an inner packaging material for sliced cheese due to overall strength, flexibility, and barrier properties of this edible packaging material. Thus, HACS films may find applications in fresh fruit and vegetables, and the dairy industry.

Composite films of chitosan and high amylose starch could be used as multilayered, controlled-release systems for oral drug delivery (Cervera et al., 2004). In addition, the films can be used to enhance oxygen and gas barrier properties as high amylose starch has been shown to form films with great oxygen permeability (Cervera et al., 2004). This could be important in protecting foods from lipid oxidation.

Although HACS films can be formed with good film strength and oxygen barrier properties, these films are sensitive to water permeability. Combining HACS with other polysaccharides

(chitosan, alginates, cellulose fiber), proteins (milk protein, corn zein), or lipids (waxes) will improve the mechanical and water barrier properties of HACS films.

Preliminary results in our laboratory indicated that chitosan/resistant starch films (60% chitosan and 40% RS) showed oxygen transmission rates of 17.5 cc/m²/day (Aigster et al., 2007), which are comparable to the oxygen transmission rate of Mylar films (~ 60 cc/m²/day), known to be excellent oxygen barriers. Multilayer or composite films with HACS and a lipid layer dispersed in the polysaccharide will result in films with high oxygen barrier provided by the HACS and moisture barrier provide by the lipid material. However, caution should be taken when comparing composite edible films. Different film compositions are affected by temperature, relative humidity, and type and amount of plasticizer used. Thus, water and oxygen permeabilities are highly dependent on testing conditions. In general, polysaccharides are good oxygen barriers whereas lipid materials provide good moisture barrier properties. Applying multilayer techniques to improve flexibility and durability of edible films is an area of active research. Some techniques involve casting a lipid material onto the dried polysaccharide film forming a bilayered film. Another technique involves adding a lipid material in to a polysaccharide-based film forming solution before casting resulting in an emulsified film material (Robertson, 2006).

Polysaccharides are poor water barriers (corn starch, water vapor permeability, WVP, 17.7 x 10¹¹ g s⁻¹ m⁻¹ Pa⁻¹) compared to synthetic plastic materials such as low-density polyethylene (LDPE, WVP, 0.0914 x 10¹¹ g s⁻¹ m⁻¹ Pa⁻¹) (Garcia et al., 2006), but when combined with lipid materials, better water permeabilities are obtained (Han et al., 2006). However, polysaccharides are better oxygen barriers than lipid materials; thus, combining polysaccharides and lipids will improve the overall film properties (Ryu et al., 2002).

Final Considerations

Increase in consumer demand for healthier, greener, and recyclable packaging alternatives has prompted research in academia and industry to develop new packaging that prolong shelf life of products while the packaging itself is biodegradable. Edible films from animal and plant proteins, lipid materials, and polysaccharides are being investigated with the goal to find the "perfect" ecologically friendly edible packaging. Edible coatings function as gas and moisture barriers, and also as carriers of antioxidants and antimicrobials. High amylose corn

starch has been shown to form strong films with good oxygen barrier properties. In addition, HACS coatings exhibit controlled-release characteristics for delivery of a host of additives such as antioxidants, antimicrobials, and drugs (Dimantov et al., 2004; Palviainen et al., 2001).

The use of HACS as edible films has numerous applications in the food industry. Films and coatings made with HACS can be used to protect nuts from lipid oxidation by providing an oxygen barrier. In addition, HACS coatings could be applied to extend the shelf-life of fresh fruits and vegetables, and in meat packaging to help absorb meat exudates. A future application will be to investigate the effectiveness of HACS films in controlled release packaging. The role of HACS in packaging is still in its infancy. Investigating the release of active compounds (antioxidants and antimicrobials) at different rates to enhance the quality and safety of food products during storage is the next step for the future of HACS films and coatings in food packaging.

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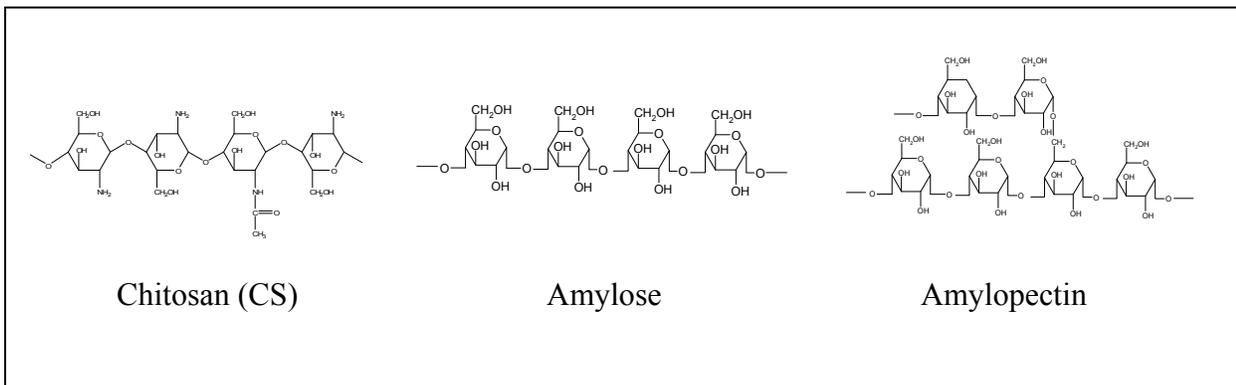


Figure 5.1. Chemical structures of chitosan (CS) and resistant starch (RS).



Figure 5.2. Example of a chitosan/resistant starch film casted on a polytetra-fluoroethylene (Teflon) mold. The film is dried for 48 hours at 20 °C and removed from the mold.

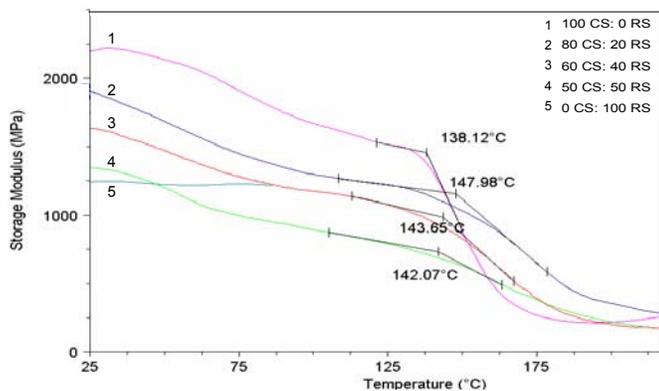


Figure 5.3. Dynamic mechanical analysis (DMA) results for various concentrations of chitosan (CS) and resistant starch (RS) films. The films concentrations are: 100:0% 80:20%, 60:40%, 50:50%, and 0:100% CS:RS.

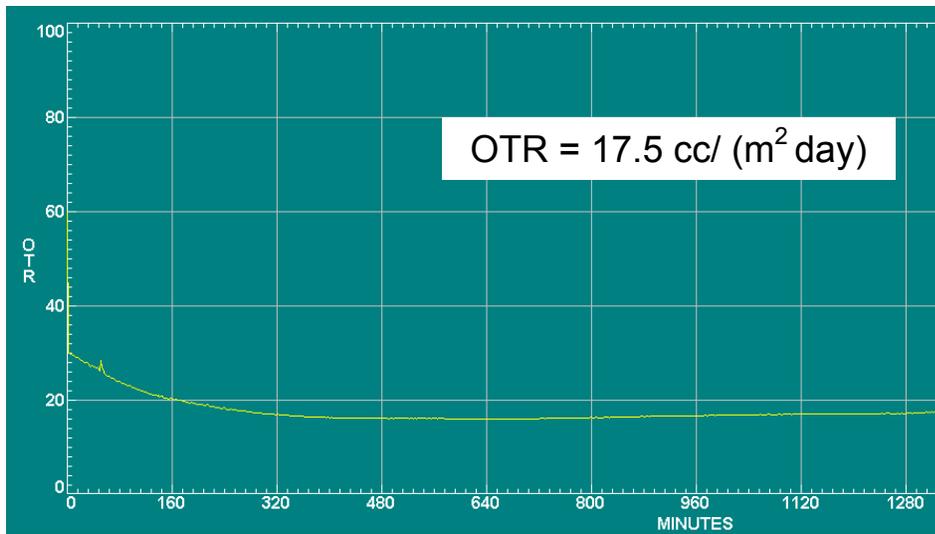


Figure 5.4. Oxygen transmission rate (OTR) measurement of a chitosan (CS) and resistant starch (RS) film (60:40% CS:RS) after the film has been exposed to oxygen for 24 hours.

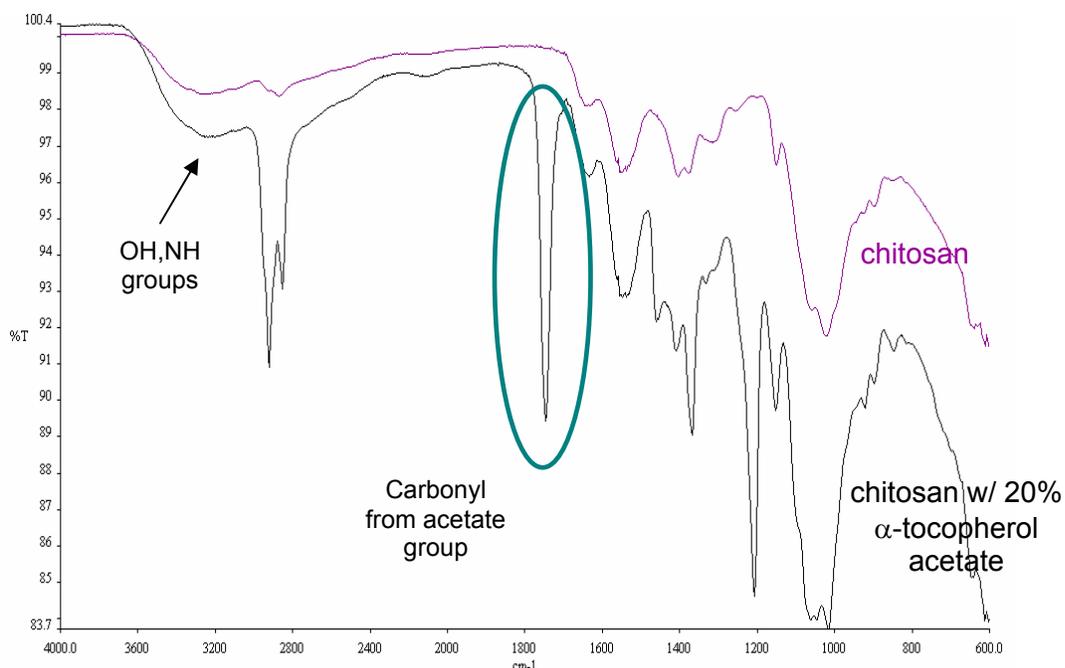


Figure 5.5. Fourier transform infrared spectroscopy (FTIR) spectra of a 100% chitosan film (upper scan) and a 100% chitosan film containing α -tocopherol acetate (lower scan).

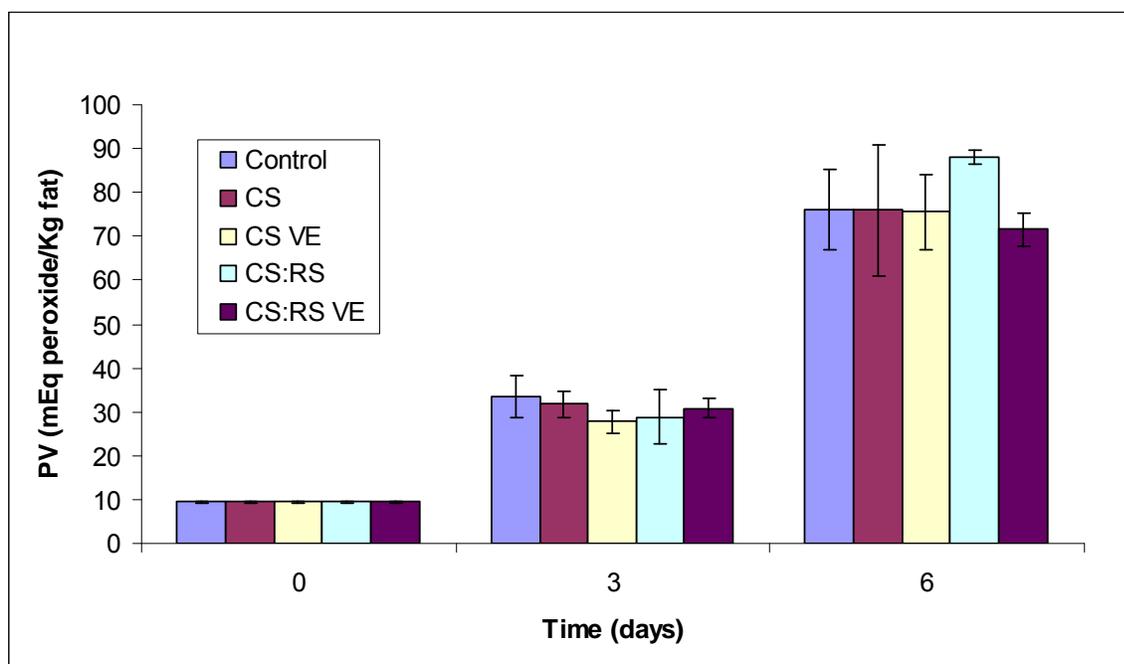


Figure 5.6. Oxidative stability of chitosan (100% CS) and chitosan and resistant starch (60:40% CS:RS) films without vitamin E and with vitamin E (20% vitamin E - VE) over a 6 day period as measured by peroxide values (PV). Films were cut into portions and immersed in stripped corn oil. The oil was stored for 6 days at 60 °C.

CHAPTER 6: CONCLUSIONS

The results on the physicochemical and sensory properties of RS-supplemented cereal based products indicate that levels of up to 15% RS can be incorporated in these food matrices without compromising taste and physical and chemical properties of these products. These levels (up to 15%) of RS in cereal products is higher than the levels used in other food products (muffins, milk puddings) used in other studies (1% to 10%). Levels of RS supplementation in our study are in agreement with literature values of RS amounts to elicit physiological effects in humans. Although these high levels of RS resulted in some changes in texture, color, and flavor of the cereal based products, the potential health benefits of RS may account for consumers' willingness to forgo these attributes in favor of dietary fiber/functional food benefits and healthy behaviors. Incorporating 15% RS in a cereal matrix resulted in physicochemical and sensory changes intuitively (i.e. the higher the amount of an ingredient (RS), the more differences that would be noticeable). Based on the results from this part of the study, RS granola bars were used for the effects of acute consumption of RS on glycemic control and oxidative stress parameters in Hispanic women.

Results for the acute consumption of 18g RS/120 g granola bar per serving in Hispanic women (n=9), indicated the glucose response was attenuated after 30 minutes of RS granola bars consumption. Although Hispanics are a population at risk for type 2 diabetes, the effects of RS on insulin and oxidative stress biomarkers did not show significance in the parameters measured. On the basis of these results, the acute effects of RS in healthy Hispanic women do not indicate amelioration of insulin or oxidative stress parameters measured. The results from the study indicate glucose can be lowered in this group of women and this could be useful for future research on the acute effects of RS on pre-diabetes/diabetes prevention. More importantly, the effects of RS on glycemic control could be explored in long term studies on this population as well as other at risk populations including African Americans, Pacific Islanders, and Native Americans. Also of importance is the fact that dietary fiber intake is well below the recommended levels. The 2005 dietary guidelines for Americans suggest increasing dietary fiber intake. The recommended level of fiber intake is 14 g/1000 Kcal which translates to 25 g fiber/day for women and 38 g fiber/day for men. In addition, whole grain intake recommendations include at least three servings per day replacing refined grains with whole grains. Thus, RS consumption could help Americans increase their dietary fiber and whole grain

intake. In addition to applications for health benefits, RS (HACS) may have future food packaging applications because of its low cost relative to other materials and its film-forming properties.

Future Study Recommendations

Based on the physicochemical and sensory properties of this work, research on the effects of processing conditions should be evaluated. In particular:

- 1) Investigate the effects of raw (uncooked RS) vs. processed (cooked RS) on the behavior of RS (gelatinization temperature range, and melting behavior). Investigate RS recovered by the AOAC RS assay in model systems with uncooked and cooked RS.
- 2) Study levels of RS that could be incorporated in food products for commercial applications based on sensory and statistical analysis (response surface methodology).

Based on the acute effects of RS consumption in Hispanic women, implications for future studies are made:

- 1) Investigate levels of RS that are feasible in food matrices that are physiologically effective. Greater RS amounts higher than this study could be challenging! Use raw (uncooked RS) may not be feasible as humans do not eat raw starch normally.
- 2) Evaluate the effects of RS on short and long term studies with pre-diabetics/diabetics (from an accessible population living in the SWVA area).
- 3) Evaluate sustained intake (various portions throughout a day or long term) of RS in human subjects at risk for type 2 diabetes and evaluate their glycemic and oxidative stress responses.
- 4) Study combinations of dietary fibers and RS at levels that are tolerable and find synergistic effects of various soluble/insoluble fibers.

Based on the review and preliminary results of the use of RS in food packaging applications, suggestions for future studies include:

- 1) Explore the oxygen barrier properties of RS alone or in combination with other polysaccharides such as chitosan as it appears to provide a good oxygen barrier comparable to synthetic materials due to its degree of crystallinity and tight packing.
- 2) Investigate the properties of RS in combination with other biomaterials in food packaging applications to promote the use of natural resources as food packaging materials.

Limitations of this Study

The Hispanic population in South West Virginia (SWVA) is quite limited. According to the U.S. Census Bureau in 2007, 2.1% Montgomery Co, 1.3% in Pulaski Co, and 1.9% in Roanoke Co. of the population are of Hispanic/Latino origin (<http://www.census.gov> accessed June 2009). Additionally, this study evaluated Hispanic women which would account for about half of the reported percentage of the total Hispanic population. Thus, subject selection in the SWVA area was challenging and resulted in recruiting healthy Hispanic women for the study. This study could be continued in an area where a larger Hispanic/Latino people reside to explore the potential health benefits of RS on glycemic control and oxidative stress parameters.

APPENDIX A

Granola Bars and Granola Cereals Containing 0% RS and 15% RS



APPENDIX B

Reference Formulae for Resistant Starch (RS) and Soluble Starch (SS) Calculations

- 1) Resistant starch content (g/100g sample) for samples containing < 10% RS
(Control granola bars and control granola cereals)

$$\Delta A \times F \times 10.3/0.1 \times 1/1000 \times 100/W \times 0.9$$

Where:

ΔA	= absorbance @ 510 nm against the reagent bank
F	= conversion from absorbance to μg (100 μg glucose/absorbance of glucose standard)
10.3/0.1	= dilution factor (0.1 ml taken from 10.3 ml)
1/1000	= conversion factor from μg to mg
W	= dry weight of sample ["as is" weight x (100-moisture content)/100]
0.9	= conversion factor from glucose to anhydro-glucose in starch

- 2) Resistant starch content (g/100g sample) for samples containing > 10% RS
(RS-supplemented granola bars and RS-supplemented granola cereals)

$$\Delta A \times F \times 100/0.1 \times 1/1000 \times 100/W \times 0.9$$

Where:

ΔA	= absorbance @ 510 nm against the reagent bank
F	= conversion from absorbance to μg (100 μg glucose/absorbance of glucose standard)
100/0.1	= dilution factor (0.1 ml taken from 100 ml)
1/1000	= conversion factor from μg to mg
W	= dry weight of sample ["as is" weight x (100-moisture content)/100]
0.9	= conversion factor from glucose to anhydro-glucose in starch

- 3) Soluble starch content (g/100g sample) for all samples
(Control granola bars and cereals and RS-supplemented granola bars and cereals)

$$\Delta A \times F \times 100/0.1 \times 1/1000 \times 100/W \times 0.9$$

Where:

ΔA	= absorbance @ 510 nm against the reagent bank
F	= conversion from absorbance to μg (100 μg glucose/absorbance of glucose standard)
100/0.1	= dilution factor (0.1 ml taken from 100 ml)
1/1000	= conversion factor from μg to mg
W	= dry weight of sample ["as is" weight x (100-moisture content)/100]
0.9	= conversion factor from glucose to anhydro-glucose in starch

APPENDIX C

Reference Formulae for Beta-glucan Calculations

Beta-glucan content of control and RS-supplemented granola bars and granola cereals

$$(\% \text{ w/w}) = \Delta A \times F \times 64 \times 1/1000 \times 100/W \times 0.9$$

Where:

- ΔA = absorbance after β -glucosidase treatment minus the blank absorbance reading
- F = conversion from absorbance to μg (100 μg glucose/absorbance of glucose standard)
- 64 = correction factor (0.1 ml out of 6.4 ml for cooked, toasted, and extruded cereal products)
- 1/1000 = conversion from μg to mg
- W = dry weight of sample
- 0.9 = conversion factor from glucose to anhydro-glucose as it occurs in beta-glucan

APPENDIX D

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

Informed Consent for Participants in Research Projects Involving Human Subjects (Sensory Evaluation)

Title of Project: Sensory Acceptability Test of Resistant Starch Supplemented Cereal Based Products.

Investigators: Annelisse (Annie) Aigster, MS, Susan E. Duncan, PhD, RD, William E. Barbeau, PhD.

I. Purpose of this Research/Project

You are invited to participate in a sensory test to determine the acceptability level of a novel cereal based product supplemented with resistant starch. Resistant starch (RS) behaves like dietary fiber. Inclusion of RS in these cereal based products may have potential health benefits. The purpose of this study is to determine how acceptable the RS supplemented cereal products are to consumers. About 50 individuals who are 18 years old or older will be recruited for sensory testing.

II. Procedures

There will be one sensory testing session lasting approximately 15 minutes. You will first complete a demographic survey. After that, you will be presented with one cereal sample and will be asked to rate how much you like/dislike the sample. A second sample may also be presented for you to taste and evaluate in the same way as the first cereal sample. If you find the RS cereal based product to be unacceptable or offensive, you may spit it out in the cup provided.

Some individuals are sensitive to certain foods such as nuts, honey and eggs. If you are aware of any food or drug allergies you may have please let the investigator know and indicate those allergies in the survey.

III. Risks

There are no more than minimal risks for participating in this study. If you are aware of any allergy reactions to nuts, honeys, and/or eggs, please inform the investigator.

IV. Benefits

Your participation on this study will provide valuable information about the acceptability level of RS supplemented cereal products. Results from this sensory evaluation will be used to determine how likeable these cereal products are to consumers. If you would like a summary of the research results, please contact the researcher at a later time.

V. Extent of Anonymity and Confidentiality

The results of your performance as a panelist will be kept strictly confidential. Individual panelists will be referred to by a code number for data analyses and for any publication of the results.

VI. Compensation

You will not be compensated for participating in this study. You will receive a candy treat for participating.

VII. Freedom to Withdraw

If you agree to participate in this study, you are free to withdraw from the study at any time without penalty. There may be reasons under which the investigator may determine you should not participate in this study. If you have allergies to nuts, honeys, and eggs, you will be asked to refrain from participating.

VIII. Subject's Responsibilities

I voluntarily agree to participate in this study. I have the following responsibilities:

- 1) Complete a demographic survey
- 2) Taste and rate a RS supplemented cereal based product

IX. Subject's Permission

I have read the Consent Form and conditions of this project. I have had all my questions answered. I hereby acknowledge the above and give my voluntary consent:

_____ Date _____
Subject signature

Should I have any pertinent questions about this research or its conduct, and research subjects' rights, and whom to contact in the event of a research-related injury to the subject, I may contact:

Annie Aigster, Graduate Research Assistant, Investigator (540) 231-7708;
aigster@vt.edu

Susan E. Duncan, Faculty/Investigator (540) 231-8675;
duncans@vt.edu

William E. Barbeau, Faculty/Investigator (540) 231-6785;
barbeau@vt.edu

David M. Moore

moored@vt.edu

Chair, Virginia Tech Institutional Review
Board for the Protection of Human Subjects
Office of Research Compliance
1880 Pratt Drive, Suite 2006 (0497)
Blacksburg, VA 24061

(540) 231-4991;

APPENDIX E

No. _____

Demographic Survey

Please circle/complete the following questions:

1) Are you Male or Female?

Male Female

2) What is your age?

18-25 26-35 36-45 46-55 56 or older

3) What is your current marital status?

Single Married Separated Divorced Widowed

4) What is your total household income, including all earners in your household?

>\$20,000 \$21,000-\$40,000 \$41,000-\$60,000 \$61,000-\$80,000
<\$80,000

5) What is the highest education level you have completed?

High School College Graduate School Degree

6) What is your current employment status?

Part-time Full-time Unemployed Student Retired Other

7) Do you eat cereals on a regular basis?

Yes No

8) If you answered yes to question #7, how often do you eat cereals?

1 a day 2 a day > 2 a day 1 a week 2 a week > 2 a week

9) How much cereal do you eat at one time?

1/2 cup 1 cup >1 cup

10) What kind(s) of cereals do you eat? Please list the products:

11) Do you eat granola bars on a regular basis?

Yes No

12) If you answered yes to question #11, how often do you eat granola bars?

1 a day 2 a day > 2 a day 1 a week 2 a week > 2 a week

13) What kind(s) of granola bars do you eat? Please list products:

14) Do you have any known allergies to any of these? If not, just leave blank

Nuts Honey Eggs

APPENDIX F

Example of Rating Acceptability Test Form

Hedonic Scale

Date _____

Product _____

Please taste the sample provided and check in the appropriate box how much you like or dislike it.

Sample _____

dislike
extremely

dislike
very much

dislike
moderately

dislike
slightly

neither
like or dislike

like
slightly

like
moderately

like
very much

like
extremely

Comments:

APPENDIX G

Attribute Diagnostics Example Form- Granola Bars
Please indicate your opinion about the following characteristics

Appearance

1. Color

Much too light Much too dark

Flavor

2. Sweetness

Not at all sweet Much too sweet

Texture

3. Moistness

Not at all moist Much too moist

4. Crunchiness

Not at all crunchy Much too crunchy

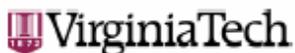
5. Stickiness

Not at all sticky Much too sticky

6. Chewiness

Not al all chewy Much too chewy

APPENDIX H - IRB Approval for sensory evaluation of the granola bar/cereal products



Office of Research Compliance
Institutional Review Board
1880 Pratt Drive (0497)
Blacksburg, Virginia 24061
540/231-4991 Fax: 540/231-0959
E-mail: moored@vt.edu
www.irb.vt.edu
FNA000005721 expires 7/20/07
IRB # is IRB00000587

DATE: November 1, 2006

MEMORANDUM

TO: Susan E. Duncan
William Barbeau
Annelisse Aigster

FROM: David M. Moore 

SUBJECT: **IRB Exempt Approval:** "Evaluation of the Physical, Chemical, and Sensory Properties of Resistant Starch Supplemented Cereal Based Products", IRB # 06-619

I have reviewed your request to the IRB for exemption for the above referenced project. I concur that the research falls within the exempt status. Approval is granted effective as of October 31, 2006.

As an investigator of human subjects, your responsibilities include the following:

1. Report promptly proposed changes in previously approved human subject research activities to the IRB, including changes to your study forms, procedures and investigators, regardless of how minor. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.
2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

cc: File
Department Reviewer:Kathy Hosig

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VIRGINIA POLYTECHNIC INSTITUTE UNIVERSITY AND STATE UNIVERSITY
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Virginia Tech

Effects of resistant starch cereal-based products on glycemic and insulin responses in humans

Volunteers wanted for nutrition research study

- Are you a Hispanic-American woman 20 years old or older?
- Do you eat granola bars/cereals?

*If you answered **yes** to these questions you may be eligible to participate in a nutrition research study*

The purpose of this project is to study the health effects of granola bars and cereals that have resistant starch added to them. Resistant starch (RS) acts like dietary fiber. Adding resistant starch to these cereal foods may improve health by lowering blood sugar and insulin. We will compare how people respond when they eat cereal foods that have resistant starch added to them and when they eat cereal foods that do not have resistant starch added to them. We will measure levels of sugar, insulin, and antioxidants in the blood.

Your participation on this study will provide valuable information about the potential health benefits of resistant starch and glucose and insulin responses, as well as oxidative stress amelioration.

You will be compensated for attending two sessions each lasting 3 hours.

For more information please call Annie Aigster, PhD candidate, Human Nutrition, Foods, and Exercise department, Virginia Tech. Phone #: 540-818-5273 or email aaigster@vt.edu

(Se habla español)

APPENDIX J

Participant's Information and Medical History

General Information

Name _____

Address _____

Phone number: (H or Cell): _____ (W): _____

Emergency contact person: _____ Relationship: _____

Emergency contact person phone number: _____

Date of birth (month, day, year): _____

Place of birth: _____

Citizenship: Native Naturalized Alien

If not a US citizen, please indicate country of citizenship: _____

Medical history

Please check (X) the appropriate response:

In your opinion, are you in good general health? Yes No

Are you now taking any drugs or medications? Yes No

If yes, which ones? _____

Are you allergic to any medications or foods? Yes No

If yes, which ones? _____

Do you take dietary supplements (multivitamins, individual vitamins, herbal supplements, etc) Yes No

If yes, which ones, please list brand names if known _____

Do you take anti-contraceptive pills? Yes No

If yes, which one (please include the brand name) _____

When was your last period? (Please approximate the date if unknown) _____

Have you had:

- | | | |
|---|------------------------------|-----------------------------|
| High blood pressure | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| Liver, gall bladder, problems (such as jaundice, hepatitis) | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| Diabetes | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| Heart disease | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| Kidney problems | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| Stomach problems, indigestion, bloating, diarrhea, ulcers | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| Bleeding susceptibility, bruising | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| Paralyzed or numbed | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| Depression or excessive worry | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| Dizziness or fainting | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| Broken bones | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| Back trouble | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| Asthma or other respiratory problems | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| Nervous condition | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| Thyroid problems | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| History of blood clots | <input type="checkbox"/> Yes | <input type="checkbox"/> No |
| Other | <input type="checkbox"/> Yes | <input type="checkbox"/> No |

Please explain: _____

Family illness

Please check (X) if there is any history in your family of:

- | | | | |
|--|--|--|----------------------------------|
| <input type="checkbox"/> Diabetes | <input type="checkbox"/> Easy bleeding | <input type="checkbox"/> Obesity | <input type="checkbox"/> Allergy |
| <input type="checkbox"/> High blood pressure | <input type="checkbox"/> Jaundice | <input type="checkbox"/> High blood fats | <input type="checkbox"/> Gout |
| <input type="checkbox"/> Stroke | <input type="checkbox"/> Alcoholism | <input type="checkbox"/> Cancer of _____ | <input type="checkbox"/> Asthma |
| <input type="checkbox"/> Heart trouble | <input type="checkbox"/> Tuberculosis | <input type="checkbox"/> Psychiatric illness | <input type="checkbox"/> Other |

Please explain: _____

Social history

Do you smoke? Yes No
If yes, how much? _____

Do you drink alcohol? Yes No
If yes, how much? _____

Do you exercise regularly? Yes No
If yes, how often? (ex. once a week for 20 minutes) _____

Demographics (Please circle one)

What is your age range?
18-25 26-35 36-45 46-55 56 or older

What is your current marital status?
Single Married Separated Divorced Widowed

What is your total household income, including all earners in your household?
<\$20,000 \$21,000-\$40,000 \$41,000-\$60,000 \$61,000-\$80,000 >\$80,000

What is the highest education level you have completed?
High School College Graduate School Degree

What is your current employment status?
Part-time Full-time Unemployed Student Retired Other

Do you eat granola bars on a regular basis?
Yes No

If yes, how often do you eat granola bars?
<1 a week 1-2 a week 1-2 a day >2 a day

What kind(s) of granola bars do you eat? **Please list products:**

APPENDIX K

Diabetes Risk Test - from the American Diabetes Association

There are 23.6 million children and adults in the US with diabetes, and nearly a quarter of them (or 5.7 million people) do not know it. Diabetes is more common in African Americans, Latinos, Native Americans, Asian Americans and Pacific Islanders. If you are a member of one of these ethnic groups, you need to pay special attention to this test.

To find out if you are at risk, write in the points next to each statement that is true for you. If a statement is not true, write a zero. Then add all the points to get your total score.

	Yes	No
1. My weight is equal to or above that listed in the chart below?	5 pts	0 pts

Height in feet and inches without shoes	Weight in pounds
4'10"	129
4'11"	133
5'0"	138
5'1"	143
5'2"	147
5'3"	152
5'4"	157
5'5"	162
5'6"	167
5'7"	172
5'8"	177
5'9"	182
5'10"	188
5'11"	193
6'0"	199
6'1"	204
6'2"	210
6'3"	216
6'4"	221

2. I am under 65 years of age and I get little or no exercise during a usual day?	Yes	No
	5 pts	0 pts

3. I am between 45 and 65 years of age?	5 pts	0 pts
---	-------	-------

4. I am 65 years old or older?	9 pts	0 pts
--------------------------------	-------	-------

5. I am a women who has had a baby weighing more than nine pounds at birth?	1 pts	0 pts
---	-------	-------

6. I have a sister or brother with diabetes?	1 pts	0 pts
--	-------	-------

7. I have a parent with diabetes?

1 pts

0 pts

Total points: _____

Scoring 3-9 points

You are probably at low risk for having diabetes now. But don't just forget about it, especially if you are Hispanic/Latino, African American, African Indian, Asian American, or Pacific Islander. You may be at higher risk in the future.

Scoring 10 or more points

You are at greater risk for having diabetes. Only your health care provider can determine if you have diabetes. At your next office visit, find out for sure.

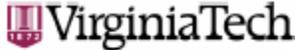
“Copyright © 2009 American Diabetes Association

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APPENDIX L

IRB Expedited Approval Form - Feeding Study



Office of Research Compliance
Institutional Review Board
2000 Kraft Drive, Suite 2000 (0497)
Blacksburg, Virginia 24061
540/231-4991 Fax 540/231-0959
e-mail moored@vt.edu
www.irb.vt.edu

PVA00000572 expires 1/20/2010
IRB # is IRB00000667

DATE: April 1, 2008

MEMORANDUM

TO: William Barbeau
Annelisse Aigster
Susan E. Duncan

Approval date: 4/1/2008
Continuing Review Due Date: 3/17/2009
Expiration Date: 3/31/2009

FROM: David M. Moore 

SUBJECT: IRB Expedited Approval: "Effects of Resistant Starch Cereal-Based Products on Glycemic and Insulin Responses in Humans", IRB # 08-199

This memo is regarding the above-mentioned protocol. The proposed research is eligible for expedited review according to the specifications authorized by 45 CFR 46.110 and 21 CFR 56.110. As Chair of the Virginia Tech Institutional Review Board, I have granted approval to the study for a period of 12 months, effective April 1, 2008.

As an investigator of human subjects, your responsibilities include the following:

1. Report promptly proposed changes in previously approved human subject research activities to the IRB, including changes to your study forms, procedures and investigators, regardless of how minor. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.
2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.
3. Report promptly to the IRB of the study's closing (i.e., data collecting and data analysis complete at Virginia Tech). If the study is to continue past the expiration date (listed above), investigators must submit a request for continuing review prior to the continuing review due date (listed above). It is the researcher's responsibility to obtain re-approval from the IRB before the study's expiration date.
4. If re-approval is not obtained (unless the study has been reported to the IRB as closed) prior to the expiration date, all activities involving human subjects and data analysis must cease immediately, except where necessary to eliminate apparent immediate hazards to the subjects.

Important:

If you are conducting **federally funded non-exempt research**, please send the applicable OSP/grant proposal to the IRB office, once available. OSP funds may not be released until the IRB has compared and found consistent the proposal and related IRB application.

cc: File
Department Reviewer: Kathy Hosig

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APPENDIX M

IRB Approved Informed Consent Form for Feeding Study

08-199

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

Informed Consent for Participants in Research Projects Involving Human Subjects

Title of Project: Effects Of Resistant Starch Cereal-Based Products On Glycemic And Insulin Responses In Humans

Investigators: Annelisse (Annie) Aigster, MS, William E. Barbeau, PhD, Susan E. Duncan, PhD, RD,.

I. Purpose of this Research/Project

You are invited to participate in a research project to study the health effects of granola bars and cereals that have resistant starch added to them. Resistant starch (RS) acts like dietary fiber. Adding resistant starch to these cereal foods may improve health by lowering blood sugar and insulin.

The purpose of this study is to make cereal foods that have about 30 grams (1 ounce) of resistant starch in each serving and then see if they might improve health in people who are at risk for type 2 diabetes. We will compare how people respond when they eat cereal foods that have resistant starch added to them and when they eat cereal foods that do not have resistant starch added to them. We will measure levels of sugar, insulin, and antioxidants in the blood. We will also measure markers of oxidation in the urine.

II. Procedures

There will be 2 sessions that you will need to come to the laboratory to have your blood drawn. A certified phlebotomist will draw the blood samples. Each session will last approximately 3 hours. You will be randomly assigned to receive one of two treatments, at least two weeks apart. One time, you will receive the control treatment granola bar/cereal containing waxy corn starch (donated by Food Innovation, National Starch, Bridgewater, NJ). The other time, you will receive the treatment granola bar/cereal containing 30 grams of resistant starch (donated by Food Innovation, National Starch, Bridgewater, NJ). You will follow your usual diet before you come to the laboratory. On the test day, you will come to the laboratory at 8:00 am in a fasting state (without eating or drinking anything but water for at least 12 hours). We will take a blood sample when you first come to the laboratory, and then you will eat a test meal within 15 minutes. More blood samples will be taken at 30 minutes, 1 hour, and 2 hours after you eat the test meal. You will not be able to eat or drink anything during those 2 hours except water. A total of no more than 200 ml of blood will be taken. You will collect 2 urine samples on the same day, one in the morning when you wake up that day and one around dinner time that evening.

Your height and weight will be measured. You will keep your clothes on but will be asked to remove any outer clothing such as hat, coat, sweater and shoes. You will keep a record of what you eat for three days before you come for the first session. We will send you the instructions for this with the forms in the mail or give them to you in person. You will also be asked to answer a few questions about yourself, such as age, gender and medical history.

III. Risks

There are no more than minimal risks for participating in this study. In some individuals, a bruise may form during blood collection; however, certified phlebotomists will draw your blood to prevent bruise formation. Some individuals may faint during blood drawings. If you faint, you may rest for as long as you want in a comfortable position.

Resistant starch can cause intestinal discomfort in some people. If you experience stomach discomfort, bloating, or any other discomfort, please inform the investigator. Some individuals are sensitive to certain foods such as nuts, honey, and eggs. If you are aware of any food or drug allergies you may please let the investigator know and indicate those allergies in the survey. The following ingredients may be found in the granola bars: starch, oats, almonds, coconut, honey, canola oil, and egg whites

IV. Benefits

Your participation on this study will provide valuable information about the potential health benefits of resistant starch and glucose and insulin responses, as well as oxidative stress amelioration. Your participation will help elucidate the potential health benefits associated with consumption of resistant starch. Your glucose, insulin, and antioxidant results will be available to you by checking the appropriate boxes on this consent form. The benefits to general public include sharing the findings to understand the link between chronic diseases such as type 2 diabetes and dietary practices/modification.

V. Extent of Anonymity and Confidentiality

Your information will be kept strictly confidential. You will be assigned a code number, and you will not be identified by your name. Individual subjects will be referred to by a code number for data analyses and for any publication of the results. Your results and data sheets will be kept confidentially in a locked cabinet. Your code number will be kept separately from your results in a separate locked file cabinet.

VI. Compensation

You will be compensated for participating in this study. You will receive \$50 after completing the first blood drawing session and filled in the required forms. You will receive another \$50 after completing the second blood drawing session. You will receive \$100 after completing all of your forms including the 3-day food records, and conduct an informal exit interview.

VII. Freedom to Withdraw

If you agree to participate in this study, you are free to withdraw from the study at any time without penalty. There may be reasons under which the investigator may determine you should not participate in this study. If you have allergies to nuts, honey, and eggs, you are asked to refrain from participating.

VIII. Subject's Responsibilities

I voluntarily agree to participate in this study. I have the following responsibilities, **for 2 separate sessions**:

- 1) Arrive at the laboratory on the scheduled times after an overnight 12 hour fast
- 2) Consume all the resistant starch or control granola cereal samples as instructed by the investigators
- 3) Allow for measurements to be taken (weight and height)
- 4) Allow for blood drawing at times 0, 30 minutes, 1 hour, and 2 hours
- 5) Collect 2 urine samples
- 6) Keep a 3-day food record

IX. Subject's Permission

I have read the Consent Form and conditions of this project. I have had all my questions answered. I hereby acknowledge the above and give my voluntary consent:

_____ Date _____
Subject signature

Subject information:

Printed name: _____

Phone number: _____ E-mail address: _____

- I would like a copy of my glucose and insulin results.
- I would like a copy of my antioxidant status results.
- I would like a copy of my body measurements.
- I would like a copy of my nutrient analysis results.

Should I have any pertinent questions about this research or its conduct, and research subjects' rights, and whom to contact in the event of a research-related injury to the subject, I may contact:

Annie Aigster, Graduate Research Assistant, Investigator (540) 231-7708; aaigster@vt.edu

Susan E. Duncan, Faculty/Investigator (540) 231-8675; duncans@vt.edu

William E. Barbeau, Faculty/Investigator (540) 231-6785; barbeau@vt.edu

Kathy W. Hosig, HNFE Departmental Reviewer (540) 231-4900; hosig@vt.edu

David M. Moore (540) 231-4991; moored@vt.edu

Chair, Virginia Tech Institutional Review
Board for the Protection of Human Subjects
Office of Research Compliance
1880 Pratt Drive, Suite 2006 (0497)
Blacksburg, VA 24061

APPENDIX N

3-Day Food Record

Instructions for keeping your 3-day food record

1. Write down **everything you eat and drink for 3 days** including 2 weekdays and 1 weekend day.
2. Write down **each** meal/snack or drink immediately after you consumed it.
3. Include **all beverages** such as tea, coffee, soda, water, juice.
4. Write down specific amounts (weight, volume, etc). **For example: 1 slice of bread, ½ cup of cooked rice, 1 banana, 1 cup (8 oz) of orange juice, 1 tablespoon of sugar, 1 can of coke (12 oz), etc.**
5. If you know **the brand name** of the food, write it down.
6. If you eat out please write down the food, the amount (approximately), and the name of the **restaurant/fast food place**.
7. Write down the **method of preparation** of the food: baked, boiled, fried, steamed, etc.
8. **List all the ingredients:** for example: if you ate a chicken sandwich, describe the ingredients of the sandwich. 2 slices of white bread, 6 oz of fried chicken, 1 teaspoon of mayonnaise, a leaf of lettuce, and a slice of tomato.
9. **List condiments:** such as salt, pepper, sugar, etc.
10. List **any dietary supplements** you are taking such as multivitamins. Include brand name and amount taken.

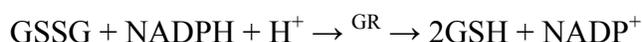
APPENDIX O

Detailed Red Blood Cell Glutathione Peroxidase (cGPx) Activity

Glutathione Peroxidase Assay

Cellular glutathione peroxidase (cGPx) Assay protocol principle

The colorimetric assay for cGPx indirectly measures the activity of the enzyme. Oxidized glutathione (GSSG) produced from the reduction of peroxides by cGPx is recycled to its reduced form (GSH) by the enzyme glutathione reductase (GR):



The oxidation of NADPH to NADP⁺ results in a decrease of absorbance at 340 nm providing an indirect measure of the cGPx activity. To assay cGPx, the red blood cell (RBC) homogenate is added to a solution containing GSH, GR, and NADPH. The enzyme reaction is initiated by adding the substrate, tert-butyl hydroperoxide and the absorbance at 340 nm recorded. The rate of decrease in absorbance is directly proportional to the cGPx activity in the sample.

Cellular glutathione peroxidase (cGPx) Assay protocol steps (96 well microplate)

RBC samples diluted (1:100) with assay buffer

75 µl of assay buffer are added to each well

75 µl of NADPH reagent are added to each well

15 µl of RBC sample are added to each well

75 µl of tert-butyl hydroperoxide are added and mixed

Measure the absorbance @ 340 nm at room temperature (23-25 °C) at 30 seconds intervals for 3 minutes.

Calculations:

Determine the rate of decrease in absorbance at 340 nm per minute

Calculate the net rate for the sample by subtracting the rate observed for a water blank

Convert the net absorbance at 340 nm/min for the sample to NADPH consumed (nmol/min/ml) using the following relationship:

$$1 \text{ mU/ml} = 1 \text{ nmol NADPH/min/ml} = (\text{Absorbance at 340 nm/min})/0.00622$$

Correct for the dilution of the sample: 16 fold dilution in the assay (15 µl/240 µl)

Express units of activity in the sample in relation to the protein content of RBC (mU/mg protein or nmol NADPH/min/mg protein)

Protein Assay based on bicinchoninic acid (BCA)

The BCA assay is a colorimetric assay for the detection and quantification of protein. The method is based on the reduction of Cu⁺² to Cu⁺¹ by protein in an alkaline medium with the

colorimetric detection of the cuprous cation. The purple-colored reaction product of the assay is measured by the absorbance at 562 nm.

Results of cGPx activity are expressed as nmol NADPH or cGPx activity/min/mg protein.

APPENDIX P

Detailed Plasma Isoprostanes GC-MS Protocol and Sample Chromatograms

F₂-Isoprostanes Assay in Plasma

Principle: A derivatization protocol has been developed to make samples suitable for GC-MS analysis. After addition of the deuterated internal standard, solid phase extraction in both C18 and silica Sep-Pak is performed to remove contaminants. Pentafluorobenzyl (PFB) is added to enhance the sensitivity of detection using electron capture chemical ionization technique. The last step is to cap the hydroxyl groups by trimethylsilyl (TMS) derivatization. Analysis of the derivatives of F₂-IsoPs and the internal standard is carried out using selective ion monitoring (SIM) techniques. The ions monitored are m/z 569 for the F₂-IsoPs and m/z 573 for the internal standard (Liu et al., 2009)

Details for the F₂-IsoPs assay in plasma are outlined in the following section, and this method measures only free F₂-IsoPs as quantifying esterified F₂-IsoPs yields no additional advantage (Liu et al., 2009).

1) Plasma Preparation

To 4 ml of HPLC water in a Falcon plastic tube, a 100 µl of the 8-Iso PGF_{2α}-d₄ standard (10 pg/µl) in acetonitrile are added. The standard is dissolved into the water and mixed in a vortex. 2-3 ml of plasma is added and mixed in a vortex.

The pH is adjusted to pH=3.0 with 1 M HCL.

2) Purification

- a) C18 Sep-Pak (Waters Sep-Pak® Plus C18 cartridges)
 - Connect a 10 cc syringe to the C18 Sep-Pak
 - Condition the cartridge with 3 ml MeOH and 7 ml of pH 3.0 water
 - Load the sample at a low flow rate (1-2 ml/min)
 - Wash the sample sequentially with 10 ml of pH 3.0 water and 10 ml of heptanes
 - Elute the sample with 10 ml of ethyl acetate-heptane, 1:1 into a falcon tube
- b) Silica Sep-Pak (Waters Sep-Pak® Plus Silica cartridges)
 - Remove the C18 Sep-Pak from syringe and attach the Silica Sep-Pak
 - Condition the cartridge with 5 ml of ethyl acetate
 - Add a small scoop of anhydrous sodium sulfate to remove any remaining water
 - Load the sample. Push the sample slowly
 - Wash the cartridge with 5 ml of ethyl acetate
 - Elute isoprostanes from the Silica Sep-Pak with 5 ml of ethyl acetate/MeOH (1:1) into a 5 ml Reacti-vial
 - Dry the sample under nitrogen at 37 °C

3) Esterification with Pentafluorobenzyl Bromide (PFBBR)

To the dry (sample) residue,

Add 40 µl of the PFBBR solution in acetonitrile

Add 20 µl of the diisopropylethylamine (DIPEA) 10% solution in acetonitrile

Cap, mix on a vortex, and place at 37 °C for 20 min
Dry under nitrogen at 37 °C
Reconstitute the sample in 50 µl of MeOH-CHCl₃ (3:2)

4) TLC Purification

a) TLC Preparation

- Pre-wash the TLC plate in MeOH, dry in oven for 10-15 min at 100 °C
- Place TLC plates in a dessicator to cool down
- Make fresh solvent mixture of 100 ml CHCl₃-EtOH (93:7)
- Draw a line across the TLC plate, 13 cm from the origin

b) Sample Migration

- Apply 50 µl of sample to the TLC spotting area
- Add 5 µl of the methyl ester standard to one lane on a separate plate
- Place the plates in the TLC tank
- Allow the solvent to move to the 13 cm mark and remove the plate from the tank

c) Recovering the Isoprostanes from the TLC Plate

- Lightly spray ONLY the plate containing the methyl ester standard with phosphomolybdic acid spray reagent
- Place on hot plate and heat until dark bands appear
- Measure the distance of the middle of the methyl ester band from the origin. Scrape each sample lane 1 cm above and below this mark and tap silica onto a piece of weighing paper
- Pour silica into a 2 ml bullet tube and add 1 ml ethyl acetate. Mix
- Centrifuge for 2 min at 13,000 rpm
- Transfer the ethyl acetate into a 1 ml bullet tube
- Dry under nitrogen at 37 °C

5) Derivatization with Bis(trimethylsilyl) trifluoroacetamide (BSTFA)

Silylate for GC-MS analysis by adding 8 µl of dry N,N-Dimethylformamide (DMF) and 20 µl of BSTFA

Mix on a vortex and place at 37 °C for 5 min

Dry under nitrogen and reconstitute with 20 µl of dry undecane.

Transfer sample to an autosampler vial for GC-MS analysis

6) GC-MS Analysis

For quantification of F₂-IsoPs by GC-MS, we used an Agilent Technology 6890N gas chromatograph (GC), and an Agilent 5973 mass spectrometer (MS) with a computer interface. GC: The F₂-IsoPs are separated on a 15 meter DB1701 (J&W Durabond) fused silica capillary column that gives good separation of F₂-IsoPs. The column temperature is programmed from 190 °C to 280 °C at a rate of 20 °C/min. Holding time 3 min and total run time is 8 min/sample. Helium is used as the carrier gas at a rate of 2 ml/min. Injection volume of each sample is 2µl.

MS: Methane is used as the reagent gas at a 2 ml/min rate and helium is used as the carrier gas for negative ion chemical ionization (NICI). The ion source temperature is set 275 °C. The ion monitored for F₂-IsoPs is the carboxylate anion m/z 569. The corresponding carboxylate anion for the deuterated internal standard is m/z 573.

Calculations:

[Isoprostanes] pg/ml = height (d0)/height (d4) * quantity of d4 added in pg/ml of sample

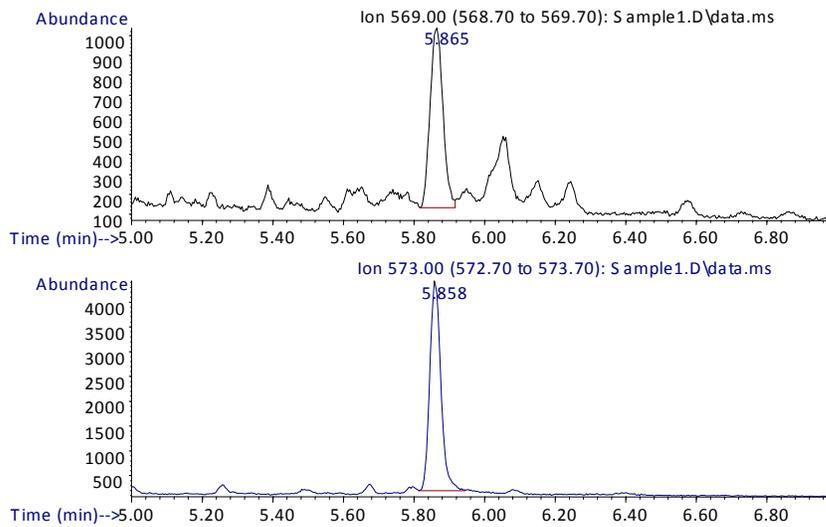
[Isoprostanes] pg/ml = height (d0)/height (d4) * 994 pg/ml of sample

where:

d0 = height of F₂-IsoPs peak; d4 = height of deuterated internal standard peak.

GC-MS chromatograms of plasma at time 0 min and 120 min after consumption of RS or control granola bars

F₂-IsoPs Time 0 RS group

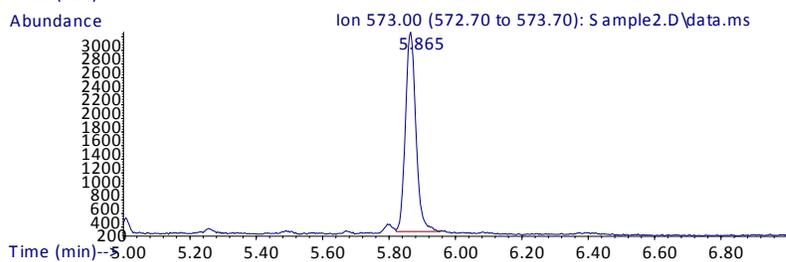
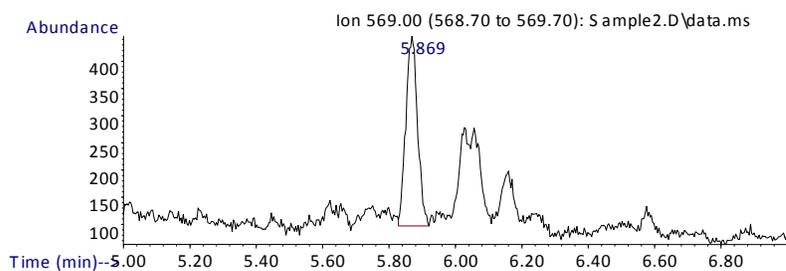


Sample calculation:

$$[\text{Isoprostanes}] = 974/4,363 * 994/2.49$$

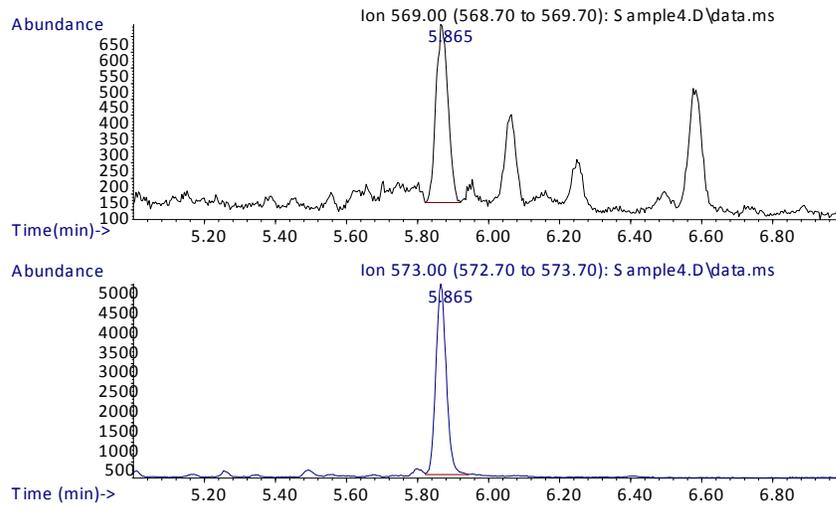
$$[\text{Isoprostanes}] = 89 \text{ pg/ml}$$

F₂-IsoPs Time 120 min RS group



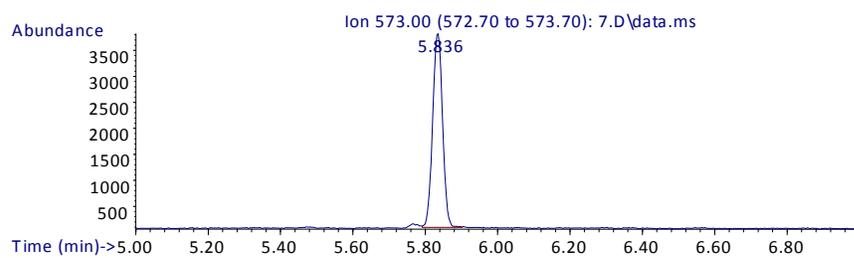
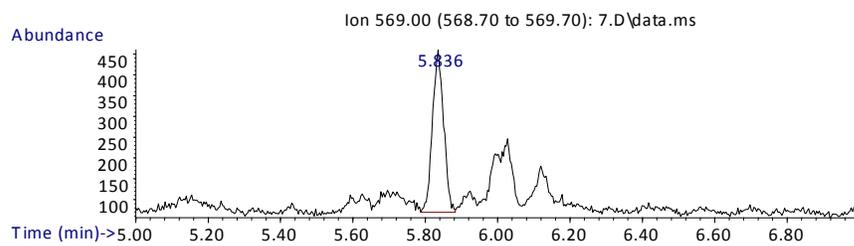
[Isoprostanes] = 74 mg/ml

F₂-IsoPs Time 0 Control group



[Isoprostanes] = 76 mg/ml

F₂-IsoPs Time 120 min Control group



[Isoprostanes] = 90 mg/ml