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Adaptation and Validation of Existing Analytical Methods for Monitoring Prebiotics Present in Different Types of Processed Food Matrices

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ADAPTATION AND VALIDATION OF EXISTING ANALYTICAL METHODS FOR MONITORING PREBIOTICS PRESENT IN DIFFERENT TYPES OF PROCESSED FOOD MATRICES

by

Pei Tze Ang

A THESIS

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Interest in resistant starch (RS) as a prebiotic is increasing due to its putative impacts on the gastrointestinal microbiome and thus colon health. Application of RS as a food ingredient is also increasing because of its unique functionality. However, a major obstacle when correlating RS containing food systems to the potential health benefits is the discrepancy in the analytical tests applied and the limitations of those methods used throughout the literature. Therefore, the Megazyme resistant starch assay was adapted and developed to monitor RS present in different types of products commonly consumed in the United States, including an extruded ready-to-eat breakfast cereal, a cookie, a muffin, a sport drink, and a nutritional bar. The linearity of the calibration curve based on glucose standards (0.1-1.0 mg/mL) from the validation of the Megazyme RS assay was

\[ Y = 0.9918x + 0.0057 \]

with a correlation coefficient of \( R^2 = 0.9995 \). The low detection (0.0013%) and quantitation limits (0.0048%) of blank samples were needed to assay the final food products. Each processed food required minimal optimization, with the exception of the sports drink, that included increasing the centrifuge time (cookie), to changing the entire formulation (extruded cereal), to removing the moisture and fat prior to analysis (granola, muffin). As a result, % recoveries of 1% spiked samples ranged from 66-100% and the detection limit was lower than 1% for all the processed foods.
except for muffin and cookie. Application of the validated method to 1% supplemented processed foods showed processing effects, especially for the extruded cereal. RS was also significantly affected when the sports drink was reformulated (sweetener composition and pH) but only non-significant trends resulted when changing the extrusion parameters (barrel temperatures and screw speeds) for the cereal. In summary, application of a more robust and reliable RS assay is important in determining the chemical fate of RS under different processing treatment in processed food supplemented with RS.
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Chapter 1. LITERATURE REVIEW

1.1. Background of Prebiotics

The prebiotic concept was introduced by Gibson and Roberfroid in 1995 and is defined as “non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve health” (Gibson & Roberfroid, 1995). This term was updated by Roberfroid in 2007 as “a selectively fermented ingredient that allows specific changes, both in composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health.”

By definition, a dietary agent must comply with several important features to be considered a prebiotic. First, a prebiotic must be “non-digestible” in that it cannot be degraded or otherwise altered by passage through the stomach, where it is exposed to hydrolytic enzymes and strong acid. Second, a prebiotic must not be digested by enzymes in the small intestine, nor can it be absorbed across the intestinal wall. Instead, a prebiotic must ultimately reach the large intestine, where it can be metabolized by a select group of colonic bacteria (Gibson & Roberfroid, 1995). However, a prebiotic substrate is not available to all bacterial species that inhabit the GI tract but are expected to affect the health benefiting bacteria lactobacilli and bifidobacteria. Moreover, a prebiotic should not stimulate the growth of potentially pathogenic bacteria such as toxin-producing clostridia, proteolitic bacteroides and toxogenic Escherichia coli (Manning & Gibson, 2004). As a result, a “healthier” gut microbiota composition is achieved by the consumption of prebiotics.
The compounds considered to be the first generation prebiotics include oligosaccharides and polysaccharides, which can be further separated into the fructans (Casc & Rastall, 2006), (inulin and various forms of fructooligosaccharides (FOS)) and the non-fructan prebiotics, (galactooligosaccharides (GOS) and soy-derived oligosaccharides (SOS)). Other first generation prebiotics include disaccharides and lactulose. Recently, a second generation group of prebiotics has been identified and includes resistant starch, polydextrose, pectin, xylooligosaccharides, lactitol, and other dietary fibers (Casc & Rastall, 2006).

1.2. Chemistry of Prebiotics

In theory, any nutrient that is selectively metabolized in the gastrointestinal environment by certain organisms of the microflora, but not by others, can be considered a prebiotic. The chemistry of current prebiotics typically includes di-, oligo- or polysaccharides. These compounds are not absorbed in the upper part of the gastrointestinal tract as the β-configuration of glycosidic bonds, prevents hydrolysis by gastric or microbial enzymes. On the contrary, most digestible or fermentable dietary carbohydrates contain α-linked glucosides, which are easily attacked by amylases and other digestive enzymes (Hutkins, unpublished).

1.3. Resistant Starch

Resistant starch (RS) is the portion of starch that is not broken down by enzymes in the stomach and small intestine within 120 min of being consumed, making it available for fermentation in the colon. Resistant starch is different from “digestible starch”, which is rapidly digested to glucose in the small intestine and then absorbed, and is considered by many to be part of dietary fiber (McCleary & Monaghan, 2002). Englyst
et al. (1982) were the first to identify an enzymatic resistant starch fraction based upon their research on non-starch polysaccharides. Studies have shown that RS is a linear molecule of α-1, 4-D-glucan and has low molecular weight of $1.2 \times 10^5$ Da. (Tharanathan, 2002) (Figure 1.1).

There are four proposed reasons for the indigestibility of resistant starch. First, the molecular structure of raw starch granules is compact thereby limiting the accessibility of digestive enzymes, such as different amylases. These raw starches include grains, seeds or tuber starches (Haralampu, 2000). Second, some starch granules structures, such as raw potatoes, unripe bananas and high-amylose maize starch, resist hydrolysis by digestive enzymes (Nugent, 2005). Also, some starches are resistant to enzymes hydrolysis due to the presence of other plant components that limit enzyme accessibility such as in whole grain. Third, starch granules are disrupted by heating in an excess of water by a process commonly known as gelatinization, which allows the starch molecules to be more accessible to digestive enzymes. In preparing starchy food for consumption, rapid digestibility of the starch is rendered during the cooking process where water is present. However, when starch gels are cooled, starch crystals are formed that cannot be broken down by enzyme digestion. This form of retrograded starch is found in small quantities (approximately 5%) in food, such as “corn-flakes” or cooked and cooled potatoes usually used in potato salad (Haralampu, 2000). Lastly, certain starches that have been chemically modified by etherisation, esterisation or cross-bonding, are resistant to digestive enzymes (Lunn & Buttriss, 2007).
Figure 1.1. Amylose and amylopectin.

Source: (Practical Chemistry)
1.4. Types of Resistant Starch

Resistant starch has been classified into four general subtypes, including RS\textsubscript{1}, RS\textsubscript{2}, RS\textsubscript{3}, and RS\textsubscript{4} that are natural while others are synthetically produced. Because natural RS types are frequently destroyed during processing, manufacturing of a RS usually involves partial acid hydrolysis and hydrothermal treatments, heating, retrogradation, extrusion cooking, chemical modification and repolymerisation (Charalampopoulos, Wang, Pandiella, & Webb, 2002).

RS\textsubscript{1} is a physically-protected form of starch that is isolated from coarsely ground or chewed cereals, legumes, and grains (Englyst & Cummings, 1992). RS\textsubscript{1} is also available in some very dense types of processed starchy food (Sajilata, Singhal, & Kulkarni, 2006). The presence of intact cells in certain food such as grains, seeds or tubers leads to the physical inaccessibility of RS\textsubscript{1} to digestion in the gastrointestinal tract (GI). RS\textsubscript{1} is heat stable and able to tolerate most cooking operations. This enables RS\textsubscript{1} to be used as an ingredient in different kinds of conventional food products (Sajilata, Singhal, & Kulkarni, 2006).

RS\textsubscript{2} is an un-gelatinized starch that is slowly degraded by amylases. It is found in uncooked potato, green banana, and high amylose starch. The crystallinity of RS\textsubscript{2} native starch granules makes them poorly susceptible to hydrolysis (Hernandez, Emaldi, & Tovar, 2008). High-amylose maize starch, a type of RS\textsubscript{2} is unique as it retains its structure and resistance even during processing and cooking (Wepner, Berghofer, Miesenberger, & Tiefenbacher, 1999).

RS\textsubscript{3} is essentially a retrograded amylose that is formed during cooling of gelatinized starch and it is considered the most resistant fraction of resistant starch
(Englyst & Cummings, 1992). As RS₃ may be formed in cooked foods that are kept at low or room temperature (Hernandez, Emaldi, & Tovar, 2008), most moist-heated foods contain some RS₃ (Sajilata, Singhal, & Kulkarni, 2006). RS₃ is stable in most cooking operations allowing its use as an ingredient in a wide variety of conventional foods (Haralampu, 2000). Food processing that involves heat and moisture destroys RS₁ and RS₂ in most cases but may form RS₃ due to retrogradation of starch (Faraj, Vasanthan, & Hoover, 2004). RS₃ has higher water holding capacity compared to granular starch (Sanz, Salvador, & Fiszman, 2008a). Some examples of RS₃ containing foods are cooked and cooled potatoes and corn-flakes (Wepner, Berghofer, Miesenberger, & Tiefenbacher, 1999).

Lastly RS₄ is a chemically formed fraction produced via cross-linking reactions (Wang, Brown, Khaled, Mahoney, Evans, & Conway, 2002). RS₄ is a group of starches that have been chemically modified to decrease their digestibility. These starches have been etherised, esterified or cross-bonded with chemicals. In 2005, Nugent proposed that RS₄ may be subdivided into four subcategories according to their solubility in water and experimental methods to analyze RS₄. In producing RS₄, chemical modifications such as conversion, substitution, or cross linking are applied to the starches. Also the chemical modifications of RS₄ prevent digestion of RS₄ by blocking enzyme access to the starches (Kim, et al., 2008); (Sharma, Yadav, & Ritika, 2008).

Resistant starch products derived from corn, wheat and potato are also commercially available. These RS types are marketed based on both their functional and nutritional properties, such as their ability to act as fiber, as a replacement for high glycemic carbohydrates, for their hypocholesterolemic effects and as a prebiotic. RS₂
and RS₃ are the most widely used (Wang, Brown, Khaled, Mahoney, Evans, & Conway, 2002). Examples of commercial resistant starch products derived from high-amylose corn starch include Hi-maize® whole grain corn flour (RS₁ and RS₂), Hi-maize®260 corn starch (RS₂), and Novelose®330 (RS₃) resistant starch.

1.5. Health Promoting Properties of Resistant Starch

Approximately 80-90% of glucose is produced during metabolism of standard starch in the human body during digestion. Some studies have shown that 30-70% of RS is broken down to short chain fatty acids when it is metabolized by colonic bacteria in the colon. The remaining non-degraded RS escapes colonic fermentation and is excreted in the feces (Ranhotra, Gelroth, Astroth, & Eisenbraun, 1991a); (Ranhotra, Gelroth, & Glaser, 1996); (Behall & Howe, 1995); (Cummings, Beatty, Kingman, Bingham, & Englyst, 1996). The overall digestibility of RS depends on the type and source of RS consumed in food. For example, approximately 84% of RS₃ present in corn and 65% of RS₃ present in wheat are degraded by bacterial fermentation in the colon. Similarly, for RS₂ present in foods such as raw potato and green banana, 89% and 96% respectively are degraded by bacterial fermentation in the colon. Digestibility of RS also varies among individuals, which may be attributed to individual differences in enzymatic responses to consumed RS (Sharma, Yadav, & Ritika, 2008). Table 1.1 shows the types of RS, their resistance to digestion in small intestine and food sources.

Physiological changes in the host induced by resistant starch are health promoting (Figure 1.2). Resistant starch exerts a prebiotic effect as it undergoes larger bowel bacterial fermentation that in turn produces short-chain fatty acids (SCFA) (Topping, Bajka, Bird, & et al., 2008). The physiological properties of RS can vary widely
depending on the design of studies conducted such as in-vivo or in-vitro studies, human or animal model studies and differences in the source, type, and dose of resistant starch consumed (Buttriss & Stokes, 2008); (Nugent, 2005).

Resistant starch is a non-digestible carbohydrate that is widely available as dietary sources in foods. As a result, RS could be as important as non-starch polysaccharides (NSP) in promoting large bowel health and preventing inflammatory bowel disease (IBD) and colorectal cancel (CRC) (Topping, Anthony, & Bird, 2003). In today’s society, modern processing and food consumption practices may result in the lower consumption of RS, which as a result could contribute to the rise in Western diseases in developed countries (Topping, Anthony, & Bird, 2003). Due to the unique functionality of resistant starch, there is an increasing interest in using RS to lower the caloric value and digestible carbohydrate content of foods. RS can also be used to enhance the fiber content. Research is on-going on potential of RS to increase satiation and lower the glycemic response. Consumption of RS might be beneficial in prevention of colonic cancer. Similar to dietary fiber, RS is fermented in the large intestine where short chain fatty acids such as acetate, propionate, and butyrate are produced. These results have been supported by in vitro experiments with human fecal inocula where butyrate yield in starch was high (Asp & Bjorck, 1992). Also, research that studied rats fed with RS has showed an increase in fecal bulking and lower fecal pH, as well as greater production of SCFA. These higher SCFA levels have been associated with the decreased incidence of colon cancer, and have been suggested to resemble the effects of soluble dietary fiber (Ferguson, Tasman-Jones, Englyst, & Harris, 2000); (Tharanathan & Mahadevamma, 2003).
Table 1.1. Types of resistant starch, their resistance to digestion in small intestine and food sources.

<table>
<thead>
<tr>
<th>Type of starch</th>
<th>Description</th>
<th>Digestion in small intestine</th>
<th>Resistance reduced by</th>
<th>Food sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS₁</td>
<td>Physically inaccessible to digestion by entrapment in a non-digestible matrix</td>
<td>Slow rate; partial degree. Totally digested if properly milled</td>
<td>Milling, chewing</td>
<td>Whole or partly milled grains, seeds, legumes, and pasta</td>
</tr>
<tr>
<td>RS₂</td>
<td>Ungelatinized resistant granules with type B crystallinity, slowly hydrolyzed by α-amylase</td>
<td>Very slow rate; little degree. Totally digested when freshly cooked</td>
<td>Food processing and cooking</td>
<td>Raw potatoes, green bananas, some legumes, high-amylose starches</td>
</tr>
<tr>
<td>RS₃</td>
<td>Retrograded starch formed when starch-containing foods are cooked and cooled</td>
<td>Slow rate; partial degree. Reversible digestion: digestibility improved by reheating</td>
<td>Processing conditions</td>
<td>Cooked and cooled potatoes, bread, corn flakes, food products with prolonged and/or repeated moist heat treatment</td>
</tr>
<tr>
<td>RS₄</td>
<td>Selected chemically-modified resistant starches and industrially processed food ingredients</td>
<td>As a result of chemical modification, can resist hydrolysis</td>
<td>Less susceptible to digestibility in vitro</td>
<td>Some fiber: drinks, foods in which modified starches have been used (certain breads and cakes)</td>
</tr>
</tbody>
</table>

Sources: (Fuentes-Zaragoza, Riquelme-Navarrete, Sanchez-Zapata, & Perez-Alvarez, 2010); (Sharma, Yadav, & Ritika, 2008); (Lunn & Buttriss, 2007); (Sajilata, Singhal, & Kulkarni, 2006); (Nugent, 2005).
Figure 1.2. Proposed mechanism by selective fermentation of prebiotics and subsequent production of short chain fatty acids (SCFA) resulting in improved bowel habit, increased dietary mineral absorption, and might reduce the risk of colon cancer (adopted from Dr. Walter gut micro notes).
Resistant starch has been reported to have a specific role in stimulation of colonic bacteria able to produce butyric acid (Champ, Langkilde, & Brovns, 2003). As butyrate is one of the main energy substrates for large intestinal epithelial cells and inhibits the malignant transformation of such cells in vitro, these results support that easily fermentable RS fraction by colonic bacteria may be able to prevent colonic cancer (Asp & Bjorck, 1992). In addition, several studies have also shown that butyrate can have an inhibitory effect on the growth and proliferation of tumor cells in vitro by arresting one of the phases of cell cycle (G1) (Sharma, Yadav, & Ritika, 2008); (Mentschel & Claus, 2003). Butyrate can also affect gene expression by inducing apoptosis of colonocytes where unwanted dead cells are removed (Mentschel & Claus, 2003).

Research has demonstrated that the production of SCFA in the colon improves the metabolic absorption of various ions, including Ca, Mg and Fe (Cummings, MacFarlane, & Englyst, 2001). Morais et al. (1996) compared the apparent intestinal absorption of calcium, phosphorus, iron, and zinc in the presence of either resistant or digestible starch in a study. This study showed that the consumption of meal containing 16.4% RS resulted in greater apparent absorption of calcium and iron compared with the consumption of a meal that contained completely digestible starch (Morais, Feste, Miller, & Lifichitz, 1996).

Resistant starch is a popular ingredient for many food manufacturers of breads, cakes, muffins, or similar products as a dietary means to lower to lower the overall glycemic index (GI) value of the food, particularly if it is replacing existing readily absorbed forms of carbohydrate (Nugent, 2005). The slow digestion of RS has implications for its use in controlled glucose release applications (Sajilata, Singhal, &
Kulkarni, 2006). Therefore, a lowered insulin response and greater access to the use of stored fat could be expected (Nugent, 2005). Studies have shown that the metabolism of RS occurred 5-7 h after consumption, which reduced postprandial glycemia and insulinemia, and as a result has the potential for increasing the period of satiety (Raben, Tagliabue, Christensen, Madsn, Holst, & Astrup, 1994); (Reader, Johnson, Hollander, & Franz, 1997). The study conducted by Truwell (1992) has shown that food containing RS decreased postprandial blood glucose and as a result may play a role for type II diabetes patients by improving their metabolic control.

Resistant starch exerts hypocholesterolemic effects where by affecting lipid metabolism, as shown by studies with rats (Nugent, 2005). A number of measures of lipid metabolism were reduced in these in rats such as total lipids, total cholesterol, low density lipoproteins (LDL), high density lipoprotein (HDL), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), triglycerides and triglyceride-rich lipoproteins. Hypocholesterolemic effects in response to RS consumption have been widely demonstrated in other studies involving rats. For examples , it has been reported that there was a raise in rats’ cecal size and cecal pool of SCFA, as well as SCFA absorption in rats fed RS diet (25 % raw potato) (Sajilata, Singhal, & Kulkarni, 2006). The plasma cholesterol and triglyceride levels in rats were lowered as well. Also, lower cholesterol levels in all lipoprotein fractions, especially the HDL1, and lower triglycerides in triglyceride rich lipoprotein fraction were reported in the RS fed mice (Sajilata, Singhal, & Kulkarni, 2006). Moreover, it was shown that RS in bean starches, such as Adzuki and Tebou, lowered the levels of serum total cholesterol, VLDL, IDL, and LDL cholesterol, increased the cecal concentration of SCFA, especially butyric acid
concentration, and increased fecal neutral sterol excretion (Martinez-Flores, Chang, Martinez-Bustos, & Sgarbierid, 2004). However, contradictory results have been reported on hypoglycemic effects in humans. Early studies in humans have shown a beneficial effect of RS on fasting plasma triglyceride and cholesterol levels. Other studies indicated that RS consumption did not affect total lipids, triglycerides, HDL or LDL or VLDL levels in humans (Nugent, 2005). Therefore it is evident that more research is needed to better understand the role that RS plays in human health (Nugent, 2005).

Additional studies have shown the potential of RS to modify fat oxidation and act as a satiety agent to aid in weight management but the results are still not conclusive (Nugent, 2005); (Sharma, Yadav, & Ritika, 2008); (Mikušová, Šturdík, Mošovská, Brindzová, & Mikulajová, 2009). Current studies in humans have also indicated that diets rich in RS do not affect total energy expenditure, carbohydrate oxidation or fat oxidation (Ranganathan, et al., 1994); (Tagliabue, Raben, Heijnen, Deurenberg, Pasquali, & Astrup, 1995); (Howe, Rumpler, & Behall, 1996); (Raben, Andersen, & Karberg, 1997). Tapsell et al. (2004) proposed that eating a diet rich in RS may increase the mobilization and use of fat stores as a direct result of reducing insulin secretion. Another study that examined the potential of RS as a satiety agent on human volunteers has shown that breads rich in RS imparted greater satiety than white breads between 70 and 120 min after eating (Skrabanja, Kovac, Golob, Liljeberg Elmståhl, Björck, & Kreft, 2001). However, another study has indicated that high RS meals caused less satiety than low RS meals 1 h post ingestion (Anderson, Catherine, & Woodend, 2002). Yet, another study that examined the relationship between RS content of a meal and postprandial fat oxidation determined that replacing 5.4 % of total dietary carbohydrates with RS could
significantly increase postprandial lipid oxidation and probably reduce fat accumulation long term (Higgins, Higbee, Donahoo, Brown, Bell, & Bessesen, 2004).

1.6. Structure of Resistant Starch

Different types of RS have different structures. For example, RS₁ is a physically protected form of starch found in whole grains, seeds, and legumes (Sajilata, Singhal, & Kulkarni, 2006). Figure 1.3 shows the microscopic view of the physically inaccessible RS₁ in cell or tissue structures of partly milled grains, seeds, and vegetables. In raw RS₂ starch granules, starch is tightly packed in a radial pattern and is relatively dehydrated. This compact structure accounts for the resistant nature and limits the accessibility of digestive enzyme to hydrolyze this starch fraction (Sajilata, Singhal, & Kulkarni, 2006) (Figure 1.4).
Figure 1.3. Structure of resistant starch type I (RS$_1$).
Source: (Sajilata, Singhal, & Kulkarni, 2006).

Figure 1.4. Structure of resistant starch type II (RS$_2$).
Source: (Sajilata, Singhal, & Kulkarni, 2006).
Alternatively, RS₃ is a retrograded starch forming a completely hydrated granule. The amylose in the starch granules leaches into the solution as a random coil polymer. Upon cooling the polymer chains begin to re-associate as double helices that are stabilized by hydrogen bonds (Wu & Sarko, 1978) with 6 glucose units per turn in a 20.8 Å repeat of an individual strand double helix. The double helices are structured parallel to each other and are left-handed. To obtain A-type crystalline structure of RS₃, (Eerlingen, Deceuninck, & Delcour, 1993b) gelatinized the starch and stored it at high temperature about 100 °C for several hours. The crystalline structure has a dense structure and only a few water molecules present in the monoclinic unit cell. Upon further retrogradation, the double helices pack in a hexagonal unit cell that forms the B crystalline structure. In B crystalline structure, there are about 36 to 42 water molecules per unit cell where they are located in fixed positions within a central channel formed by 6 double helices.

In addition to the crystalline structure, the degree of polymerization (DP) of amylose affects the overall yield. The yield of RS₃ rises to a maximum DP of 100 and thereafter remains constant (Eerlingen & Decour, 1995). A minimum DP of 10 and a maximum of 100 are necessary in the formation of double helix (Gidley, Cooke, Drake, Hoffman, Russell, & Greenwell, 1995). A schematic of RS₃ formed in aqueous amylose solution depicted as either a micelle or lamella model, is shown in Figure 1.5 and Figure 1.6, respectively. Lastly, the structure of RS₄ includes modified starches obtained by chemical treatment conversion, substitution, or cross linking (Sajilata, Singhal, & Kulkarni, 2006). Figure 1.7 shows preparation of RS₄ by the addition of distarch phosphate ester to the starch.
Figure 1.5. Schematic presentation of enzyme-resistant starch type III (RS₃) formed in aqueous amylase solution. Micelle model.

Double helices are ordered into a crystalline structure (C) over a particular region of the chain, interspersed with amorphous, enzyme degradable regions. Source: (Sajilata, Singhal, & Kulkami, 2006).
Lamellar structures are formed by folding of the polymer chains. The fold zones are amorphous (A), while the center of the lamella is crystalline (C). Source: (Sajilata, Singhal, & Kulkami, 2006).

Figure 1.7. Preparation of cross-bonded starch.
Source: (Sajilata, Singhal, & Kulkami, 2006).
1.7. Resistant Starch in Foods

An extensive interest in RS food applications has occurred by product developers and nutritionists due to the potential physiological benefits and the unique functionality of RS. Addition of RS in foods yields higher quality products that are not attainable with traditional insoluble fibers (Yue & Waring, 1998); (Baixauli, Salvador, Martinez-Cervera, & Fiszman, 2008). Fiber-containing foods have been known to be coarser, denser and sometimes less palatable than refined, processed foods. Resistant starch does not change the taste or significantly change the texture of the food product. Therefore, sensory properties of RS containing food products may improve compared with many other traditionally used fibers, such as brans and gums (Sajilata, Singhal, & Kulkarni, 2006). Because RS has small particle size, a white appearance, and bland flavor, less interference with food texture expected. RS has desirable physiochemical properties such as swelling, viscosity increase, gel formation, and water-binding capacity making it useful in a variety of foods. In addition, RS has low water-holding capacity resulting in improved handling and texture in the final product (Yue & Waring, 1998). For example, RS increased coating crispness of products and the bowl life of breakfast cereals. It is also possible to use most RS to replace flour on a 1-to-1 basis without significantly affecting dough handling and rheology. These properties of RS may be promising for products that undergo different processing conditions such as extrusion and baking. Moreover, RS allows the formation of low-bulk high fiber products with better organoleptic qualities such as improved texture, appearance, and mouth feel compared to traditional fibers, (whole grains, bran or fruit fibers) (Sajilata, Singhal, & Kulkarni, 2006). Also, RS positively affects the sensory characteristics of the final product. This
increases the likelihood that consumers will accept RS enriched food products more and hence increases their dietary fiber intake (Buttriss & Stokes, 2008). Resistant starch may be used as a functional food ingredient for lowering the caloric value of foods and is useful in products for coeliacs, such as bulk laxatives, and in products for oral rehydration therapy. Lastly, potential uses of RS in fermented foods include dry-cured sausages (Nugent, 2005).

RS has been incorporated in different bakery products such as bread, cakes, muffins, and breakfast cereals. The amount of RS used to replace flour depends on the particular starch being used, the application, the desired fiber level, and, in some cases, the desired structure function claims. In general, application tests showed that RS acts as a texture modifier, imparting a favorable tenderness to the crumb. A panel rated cakes fortified with 40% total dietary fiber as RS as having the best flavor, grittiness, moisture perception, and tenderness 24 hr after baking compared to the control cakes and cakes fortified with oat fiber (Sajilata, Singhal, & Kulkarni, 2006).

Additionally, RS is currently being used in bread-making for dietary fiber fortification due to the negative characteristics associated with high-fiber breads, such as dark color, reduced loaf volume, poor mouth feel, and masking of flavor. The American Institute of Baking conducted a study to evaluate the effect of RS on bread characteristics and to compare their performance to traditional fibers. Bread containing 40% total dietary fiber RS had greater loaf volume and better cell structure compared with traditional fibers tested (Baghurst, Baghurst, & Record, 1996). Another study determined the texture characteristics of RS enriched muffins showed that RS produced a softer texture, more elastic, cohesive and tenderer structure compared to the control muffin. These effects
were more evident at higher concentrations of RS (Baixauli, Salvador, Martinez-Cervera, & Fiszman, 2008).

Along with textural enhancement, RS can improve expansion in extruded cereals and snacks as shown by a study that incorporated oat fiber and RS in extruded cereals (Sajilata, Singhal, & Kulkarni, 2006). Various cereals were formulated to contain 40% total dietary RS (Novelose 240 starch) alone and in combination with oat fiber in ratios of 50/50 and 25/75 based on weight. The RS cereal with no oat fiber had greater volumetric expansion than the control. In blends with oat fiber, cereal with 75% RS had better expansion compared to the sample containing only 50% (Sajilata, Singhal, & Kulkarni, 2006).

It has also been reported that dried pasta products containing up to 15% RS can be prepared with little or no effect on dough rheology during extrusion (Sajilata, Singhal, & Kulkarni, 2006). The pasta containing 15% RS had a lighter color compared to the control. Also a firm “aldente” texture was obtained in the same cooking time as the control pasta. RS may also be used in thickened, opaque health beverages in which insoluble fiber is desired. Insoluble fibers generally require suspension and add opacity to beverages. Compared with insoluble fibers, RS imparts a less gritty mouth feel and masks flavors less (Sajilata, Singhal, & Kulkarni, 2006).

1.8. Interaction of Starch with other Food Components

The formation of RS is influenced by the interaction of starch with different components such as protein, dietary fiber, ions, sugars, lipids, and emulsifiers present in the food system. Escarpa et al. (1997) proposed that starch-protein interaction reduced the RS contents in potato starch when albumin was added then autoclaved and cooled at -20
˚C. Insoluble dietary fiber constituents such as cellulose and lignin were shown to minimally affect RS yields compared with other constituents. Minerals such as calcium and potassium decreased the RS yields in potato starch gels that were autoclaved and cooled. These effects may be caused to the lack of hydrogen bond between amylose and amylopectin chains caused by adsorption of calcium and potassium (Escarpa, Gonzalez, Morales, & Saura-Calixto, 1997). The addition of soluble sugars to food products such as glucose, maltose, sucrose, and ribose has been shown to reduce the level of crystallization and subsequently reduce RS yield (Buch & Walker, 1988); (I’ Anson, Miles, Morris, Besford, Jarvis, & Marsh, 1990); (Kohyama & Nishinari, 1991). Based on these RS studies, the researchers proposed that as soluble sugar molecules interact with the starch molecular chains, the matrix of gelatinized starch changes, and thus inhibits the retrogradation of starch. The sugar acts as an anti-plasticizer and increases the glass transition temperature of the starch.

Lipids and emulsifiers present in food products could also form amylose-lipid complexes during food processing such as autoclaving and cooling. As a result, amylose content may influence the amount of RS present in a food product. For example, retrogradation of amylose was identified as the main mechanism for the formation of RS that can be generated in larger amounts by repeated autoclaving (Berry, 1986); (Bjorck, Eliasson, Drews, Gudmundsson, & Karlson, 1990). Formation of amylose-lipid complexes competed with amylose chain association in generation of RS. Amylose-lipid complexes were enzyme degradable, thus an increased complexed amylose reduced RS yields. However, different mechanisms have been proposed by different scientists working in the RS area. Some have suggested that amylose-lipid complex often reduces
the formation of RS while others have reported the amylose-lipid complex itself to be a form of RS (Sajilata, Singhal, & Kulkarni, 2006).

1.9. **Food Processing Effects on Resistant Starch**

The resistant starch content in food may be influenced by different processing techniques such as baking, extrusion, heating, cooling, milling, drying, and pasteurization. Gelatinization and retrogradation of starch in these processes predominantly influenced the formation of RS (Sajilata, Singhal, & Kulkarni, 2006). Natural sources of RS in legumes, potatoes, and bananas are affected by processing and storage conditions; however, commercially manufactured RS are not affected by these conditions. For instance, the amounts of RS2 in green bananas decreased with increased ripeness, while a commercial form of RS2, Hi-maize, does not present these difficulties (Nugent, 2005). Foods that are highly processed such as cereal flours, or food made from those flours such as pasta, contained lower amounts of RS. This is because the crystalline structure in cereal grains (type A) are less stable and processing of cereal grains caused a large decrease in RS content. The crystalline structure of starch in legumes (type C) is more stable than starch in cereal grains and legumes were an excellent source of RS. Cooking under conditions of high moisture and temperature can significantly lower RS content, especially RS2 by disrupting crystalline structure of the starch. Extrusion processing followed by cooling increased the level of RS due to crystallization (Haralampu, 2000). In a study to evaluate the effect of baking on RS formation in white bread, samples were baked and divided into 3 fractions that included crumb, inner crust, and outer crust (Westerlund, Theander, Andersson, & Aman, 1989). It was reported that baking increased RS content with highest RS levels present in dough and in crumb after
baking for 35 min (Westerlund, Theander, Andersson, & Aman, 1989). Faraj et al. (2004) conducted a study to determine the effect of extrusion cooking, at different temperatures (90, 100, 120, 140, or 160 °C), moisture contents (20%, 25%, 30%, 35%, or 40%), and screw speeds (60, 80, or 100 rpm) on the formation of RS type III in hull-less barley flours from CDC-Candle (waxy) and Phoenix (regular). In general, the RS3 content of native flour was decreased by extrusion cooking, but not significantly. Storage of extruded flour samples at 4 °C for 24 h before oven drying slightly increased RS3 content (Faraj, Vasanthan, & Hoover, 2004).

Generally, RS increases on storage, especially low-temperature storage (Sajilata, Singhal, & Kulkarni, 2006). The amount of RS in gelatinized food such as corn, ragi, rice, sago and potato flours has increased on low-temperature storage and decreased on reheating the samples. Cooked food samples of rice, unleavened bread, potato, Bengal gram, and green gram also showed increased RS formation on storage (Sajilata, Singhal, & Kulkarni, 2006). It was also shown that the longer the duration of storage of gelatinized wheat flour, the greater was the formation of RS (Kavita, Varghese, Chitra, & Jamuna, 1998). Mitsuda (1993) has reported that rice stored at -20 °C retrograded more than rice stored in the refrigerator.

Despite the growing interest in developing RS as functional food ingredients, relatively little is known about effects on its prebiotic property in response to different types of food processes, such as baking, extrusion, pasteurization, drying, etc. The amount of RS is strongly dependant on the type of food products and the type of RS in the food products. RS that contains reducing ends may participate in Maillard reactions and prebiotic activity could be lost. Addition of prebiotics, especially RS to acid foods
where hydrolysis might occur, or in baked goods, under conditions that promote browning reactions, could result in significant loss of biological and functional activity. Clearly, more information is needed to assess the stability of RS following typical food processing conditions. However, few oligosaccharide standards are available and most food labs are not equipped to perform the established RS analysis (Swennen, Courtin, & Delcour, 2006). Furthermore, validation of methods for analyzing RS present in different food matrices is necessary to effectively account for processing effects.

1.10. RS Determination in Foods

Determination of RS in food ingredients and processed foods is important in establishing nutritional information to consumers, regulatory agents and manufacturers. In order to effectively use the results generated from RS supplemented food product, the different analytical procedures for determining levels must be considered. In comparing the different RS analytical procedures currently available, significant differences exist with respect to sample preparation, the enzyme used, and the establishment of experimental conditions that mimic gastrointestinal digestion of starch. Although ongoing improvements in analytical procedures are essential, these modifications in protocols reduce the availability of comparable data to access process effects and the nutritional quality of foods. Besides that, the food products analyzed with different procedures are different in terms of their genetic origin, composition, processing, and storage condition making it difficult to compare the amount of RS in food products (Perera, Meda, & Tyler, 2010). We have thus chosen to use the current modified method from Megazyme (AOAC Method, 2002.02) for the measurement of RS in different processed food products, which may be a more robust and reliable method compared to other methods.
Chapter 2. OBJECTIVES AND SPECIFIC AIMS

The objective of this project was to adapt and validate existing analytical methods for monitoring RS present in different types of processed food matrices, including extruded cereal, bread, cookie, muffin, sport drink and a nutritional bar. The objective for this project was satisfied by completing the following specific aims.

Specific Aim 1: To develop and adapt extraction and analytical procedures for measuring resistant starch in the different cited matrices.

Specific Aim 2: To validate the methods established from Specific Aim 1 for each food matrix.

Specific Aim 3: To determine RS content in formulated prototype food products containing 1% resistant starch.

Specific Aim 4: To assess the chemical fate of RS in prototype foods during various processing treatments as applied to extruded cereal and a sports drink.
Chapter 3. MATERIALS AND METHODS

3.1. Specific Aim 1: Experimental Design and Procedures

3.1.1. Megazyme Kit

Resistant starch was measured by using reagents provided in a kit purchased from Megazyme International Ireland Ltd., County Wicklow Ireland. The kit included amyloglucosidase (12 mL, 3300 U/mL on soluble starch or 200 U/mL on ρ-nitrophenyl β-maltoside) at pH 4.5 and 40 °C, pancreatic α-amylase (pancreatin, 10 g, 3 ceralpha units/mg), glucose oxidase/peroxidase reagent buffer (GOPOD), which contains phosphate buffer (1 M, pH 7.4), ρ-hydroxybenzoic acid (0.22 M) and sodium azide (0.4% w/v). The GOPOD reagent enzymes include glucose oxidase (> 12,000 U) plus peroxidase (> 650 U) and 4-aminoantipyrine (80 mg), D-glucose standard solution (5 mL, 1.0 mg/mL) in 0.2% (w/v) benzoic acid. A RS control containing 52.7% resistant starch (dwb) content was also provided in the kit.

3.1.2. Solutions and Buffers

Solutions and buffers used for the project consisted of sodium maleate buffer (100 mM, pH 6.0) plus calcium chloride dihydrate (5 mM) and sodium azide (0.02% w/v), sodium acetate buffer (1.2 M, pH 3.8), sodium acetate buffer (100 mM, pH 4.5), potassium hydroxide solution (2 M), and aqueous ethanol (or IMS) (approximately 95% v/v). Dilute AMG (300 U/mL) was prepared using 2 mL of concentrated AMG solution (3300 U/mL) in 22 mL of 0.1 M sodium maleate buffer (0.1 M, pH6.0). The solution was divided into 5 mL aliquots and stored frozen in polypropylene containers between uses. The GOPOD buffer was prepared with reagent provided in the Megazyme kit by diluting
in 1 L of nano pure water and adding the GOPOD reagent enzyme, which was previously dissolved and quantitatively transferred into the solution. The solution was covered with aluminum foil to protect the enclosed solution from light. This solution was then divided into aliquots that were thawed only once during use. Pancreatic solution was prepared by suspending 1 g of the contents of pancreatic α-amylase in 100 mL of sodium maleate buffer (100 mM, pH 6.0) and stirred for 5 min on a magnetic stirrer. Dilute AMG (1 ml of 300 U/mL) was added and mixed well. The solution was centrifuged at 3000 rpm for 10 min (Beckman GS-6R centrifuge) and the supernatant was used immediately for extracting the resistant starch.

3.1.3. Sample Preparation

Processed foods included extruded ready-to-eat breakfast cereal, muffin, cookie, and nutritional bar, which were provided by Dr. Randy Wehling (Department of Food Science and Technology). The sports drink was prepared based on a proposed recipe. The formulations of each are shown in Table 3.1 to Table 3.5. The extruded cereal and cookie were blended in a Waring blender before RS extraction and analysis to obtain a smaller particles size. The granola bar was defatted with hexane for 2 hours, washed with water and dried in a vacuum oven to remove excess water prior to RS analysis. The muffin was defatted with hexane for 2 hours, and dried in vacuum oven to remove excess solvent prior to prebiotic extraction and analysis. All solid based food systems were homogenized to fine particles before analysis. All of the processed foods samples were dried in the convection oven to determine the moisture content according to AOAC Method 925.10.
### Table 3.1. Extruded ready-to-eat Breakfast Cereal Formulation

<table>
<thead>
<tr>
<th>Component</th>
<th>g/batch</th>
<th>Old formulation</th>
<th>New formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oat flour</td>
<td>1280</td>
<td>800</td>
<td></td>
</tr>
<tr>
<td>Corn flour</td>
<td>290</td>
<td>1010</td>
<td></td>
</tr>
<tr>
<td>Corn starch</td>
<td>240</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Sucrose (granulated table sugar)</td>
<td>120</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>Salt</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>15</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>15</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Prebiotic</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3.2. Muffin Formulation

<table>
<thead>
<tr>
<th>Component</th>
<th>g/batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-purpose flour (Bleached wheat flour, maltearley flour, niacin, iron, thiamin, mononitrate, riboflavin, folic acid)</td>
<td>250</td>
</tr>
<tr>
<td>Granulated sucrose</td>
<td>75</td>
</tr>
<tr>
<td>Baking powder (Baking soda, corn starch, sodium aluminium sulfate, calcium sulfate, monocalcium phosphate)</td>
<td>15</td>
</tr>
<tr>
<td>Salt</td>
<td>3.1</td>
</tr>
<tr>
<td>Eggs (whole, slightly beaten)</td>
<td>50</td>
</tr>
<tr>
<td>Milk (fluid)</td>
<td>200</td>
</tr>
<tr>
<td>Butter (melted)</td>
<td>75</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50</td>
</tr>
<tr>
<td>Prebiotic</td>
<td>7.5</td>
</tr>
</tbody>
</table>

### Table 3.3. Cookie Formulation

<table>
<thead>
<tr>
<th>Component</th>
<th>g/batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shortening</td>
<td>64</td>
</tr>
<tr>
<td>Sugar</td>
<td>130</td>
</tr>
<tr>
<td>Salt</td>
<td>2.1</td>
</tr>
<tr>
<td>Bicarbonate of Soda, USP</td>
<td>2.5</td>
</tr>
<tr>
<td>Dextrose solution (8.1 g dextrose hydrous, USP in 150ml water)</td>
<td>33</td>
</tr>
<tr>
<td>Distilled water</td>
<td>16</td>
</tr>
<tr>
<td>Flour 14% mb</td>
<td>225</td>
</tr>
</tbody>
</table>
### Table 3.4. Granola Bar Formulation

<table>
<thead>
<tr>
<th>Component</th>
<th>g/batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rolled oats (whole)</td>
<td>420</td>
</tr>
<tr>
<td>Granola cereal (whole grain rolled oats, evaporated cane juice, expeller pressed canola oil, defatted wheat germ, oat flour, brown rice syrup, molasses, salt, natural flavor, soy lecithin)</td>
<td>420</td>
</tr>
<tr>
<td>Margarine</td>
<td>50</td>
</tr>
<tr>
<td>Honey (clover)</td>
<td>350</td>
</tr>
<tr>
<td>Peanut butter (creamy style)</td>
<td>100</td>
</tr>
<tr>
<td>Sucrose (granulated table sugar)</td>
<td>50</td>
</tr>
<tr>
<td>Salt</td>
<td>5</td>
</tr>
<tr>
<td>Roasted peanuts (chopped)</td>
<td>75</td>
</tr>
<tr>
<td>Prebiotics</td>
<td>14.7</td>
</tr>
</tbody>
</table>

### Table 3.5. Sport Drink Formulation

<table>
<thead>
<tr>
<th>Component</th>
<th>g/10 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulated sucrose</td>
<td>250</td>
</tr>
<tr>
<td>Corn syrup solids</td>
<td>250</td>
</tr>
<tr>
<td>Citric acid</td>
<td>According to pH</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>10</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>1</td>
</tr>
<tr>
<td>Prebiotic - resistant starch</td>
<td>100</td>
</tr>
<tr>
<td>Red food color</td>
<td>Small amount</td>
</tr>
</tbody>
</table>
3.1.4. Resistant Starch Extraction and Analysis

Resistant starch tests were adapted for each matrix using Association of Official Analytical Chemists (AOAC) Official Method 2002.02 (AOAC Method, 2002.02) procedures as the reference protocol as described below.

1. **Hydrolysis and solubilization of non-resistant starch:** A 100 ± 5 mg sample was accurately weighed directly into a screw cap glass tube (Corning culture tube; 10 × 125 mm). Pancreatic α-amylase (4 ml of 10 mg/mL) containing AMG (3 U/mL) was added to each tube. The tubes were tightly capped and mixed on a vortex mixer. The tubes were then attached vertically in a shaking water bath and were incubated at 37°C with continuous shaking (150 strokes/min) for exactly 16 hours (Lab–line instruments Inc. model no 3545). Figure 3.1 shows a flow diagram for the hydrolysis and solubilization of non-resistant starch.

2. **Purification of resistant starch pellet:** After 16 hours of incubation, the tubes were removed from the water bath and excess surface water was removed with paper towels. Ethanol (4 ml of 95% v/v) was added to the samples with vigorous stirring on a vortex mixer. The tubes were then centrifuged at 3000 rpm for 10 min. After centrifugation, the supernatants were carefully decanted and 2 mL of 50% ethanol was added to re-suspend the pellet. Following vigorous stirring on a vortex mixer, 6 mL of 50% ethanol was added and mixed. The samples were centrifuged again at 3000 rpm for 10 min. The supernatants were decanted and the suspension and centrifugation steps were repeated once more. Excess liquid from the pellet was removed by inversion of tubes on rack to air dry. Figure 3.1 shows a flow diagram for the purification of RS pellet.
3. **Measurement of resistant starch:** The RS extracted from the pellet was dissolved in 2 mL of 2 M KOH by vigorous stirring with a magnetic stirrer bar (8 × 12 mm) in an ice-water bath for approximately 20 min. Sodium acetate buffer (8 ml of 1.2 M pH 3.8) was added to each tube and the samples were stirred. Immediately, 0.1 mL of AMG (3300 U/mL) was added to each tube and the samples were mixed well prior to placing in a water bath at maintained at 50 °C. The samples were incubated for 30 min with intermittent mixing on a vortex mixer. For samples containing more than 10% RS content, dilution of the samples was needed. Therefore, the samples were quantitatively transferred to a 100 mL volumetric flask using a water wash bottle. The magnetic stirrer bar in the tube was retained with an external magnet while the solution from the tube was washed with water. The end volume was adjusted to 100 mL with nano pure water and the contents were mixed well. A 10 mL aliquot of the solution was centrifuged at 3000 rpm for 10 RS content. Direct centrifugation of the tubes was carried out at 3000 rpm for 10 min. For such samples, the final volume in the tube was approximately 10.1 mL; however, this volume would vary particularly if wet samples were analyzed and appropriate allowance for volume were made in the final calculations. Aliquots (0.1 ml) of either the diluted or undiluted supernatants were transferred in duplicate into glass test tubes (13 × 100 mm). The GOPOD reagent (3.0 ml) was added and incubated in a water bath at 50 °C for 20 min. The absorbance of each solution was measured at 510 nm against the reagent blank with a spectrophotometer (Beckman Coulter DU 800). Reagent blank solutions were prepared by mixing 0.1 mL of 100 mM sodium acetate buffer (pH 4.5) and 3.0 mL of GOPOD reagent. Standards were prepared by mixing D-glucose (1 mg/mL) with the appropriate amount of water to create a range of D-glucose standards.
0.1 mL of D-glucose standard (0.1 mL) was then mixed with 3 mL of GOPOD for measurement of the absorbance. Figure 3.2 shows flow diagram for the measurement of resistant starch.

### 3.1.5. Calculations

Resistant starch content (% dry weight basis) was calculated as follow:

\[
\text{Resistant starch (mg of RS/100 mg sample)} = \left( \frac{\text{mg/mL obtained from calibration curve}}{\text{mL final volume}} \right) \times \left( \frac{162}{180} \text{ factor to convert from free D-glucose, as determined, to anhydro-D-glucose as occurs in starch} \right) \times \left( \frac{100}{\text{moisture content of sample}} \right).
\]
Figure 3.1. Flow diagram for hydrolysis and solubilization of non-resistant starch and the purification of resistant starch pellet.
Figure 3.2. Flow diagram for the measurement of resistant starch.

Add 2mL of 2M KOH and stir 20 min in ice water bath over a magnetic stirrer.

Add 8mL of 1.2 M sodium acetate buffer and 0.1 mL of 1:3300 U/mL AMG.

For sample >10 % RS content; quantitatively transfer the contents to a 100 mL volumetric flask. Adjust to 100 mL with distilled water and mix well. Centrifuge an aliquot of the solution at 4000 rpm/min for 10 min.

Incubate samples in water bath at 50 °C for 30 min with intermittent mixing.

For sample <10 % RS content; directly centrifuge the tube at 4000 rpm/min for 10 min.

Transfer 0.1 mL aliquots supernatants into glass test tubes, add 3mL of GOPOD reagent and incubate at 50 °C for 20 min.

Prepare D-glucose standard by mixing 0.1 mL of D-glucose and 3.0 mL of GOPOD solution.

Measure the absorbance at 510 nm against a reagent blank.
3.2. Specific Aim 2: Validation of Extraction and Analysis Procedures

Methods to extract and analyze the samples were validated according to the statistical design presented in the United States Pharmacopia and the AOAC Peer verified methods; Manual for policies and procedures I (USPNF, 1995) (AOAC Method, 1998). Although many of these procedures have been AOAC certified, the methods were validated, and if needed optimized accordingly, to determine product specific method performance. As such, the following performance tests were completed, which are based upon:

3.2.1. Accuracy

Processed finished products that were prepared without supplemented RS were extracted / analyzed via the cited extraction / analysis methods described previously (S1) (AOAC Method, 1998). These control samples were then spiked with the resistant starch (RS₂) used to prepare the final processed foods at concentration of 1% (S2). The products were again extracted and analyzed according to the described methodologies. Method accuracy was thus accessed by determining % recoveries as follows: Percent (%) Recovery = (Conc. S2 - Conc. S1 / Known increment conc.) × 100. RS₂ standard, purchased from National Starch Food Innovation was used for determining the accuracy of resistant starch.
3.2.2. **Precision**

The relative standard deviation (% RSD) of individual results were determined by analyzing replicate samples of formulated product containing 1% spiked RS (n = 5-10) that were analyzed according to the conditions of the tests (AOAC Method, 1998).

3.2.3. **Linearity of calibration curves**

Standards (D-glucose) of varying concentrations (4-5 different concentrations) were determined and the responses were correlated vs. concentration. The regression curve, \( Y = mx + c \) was calculated by the method of least squares of the standard responses vs. concentration, where \( m \) is the slope of the line and \( c \) is the \( y \) intercept. The correlation coefficient was also determined (AOAC Method, 1998) to determine degree of linearity.

3.2.4. **Limit of detection**

Limit of detection was determined by calculating the mean value of the matrix blank response, i.e., the extracted unsupplement finished product, plus 2 standard deviation of the mean, expressed in analyte concentration for \( n > 5 \) replicate analyses. Limit of detection was corrected for recovery for methods with less than 100% recovery (AOAC Method, 1998).

3.2.5. **Limit of Quantitation**

In determining limit of quantitation, the mean value of the blank matrix response plus 10 standard deviations of the mean, expressed in analyte concentration was calculated for \( n > 5 \) replicate analyses.
3.2.6. **Specificity / selectivity**

Matrix blanks were analyzed to ensure that no interfering compounds were present or that a given carbohydrate is not indistinguishable from the corresponding standard material in the appropriate matrix (AOAC Method, 1998). According to the results generated from these studies, the extraction / analyses were used as cited and the data were analyzed / reported accounting for these performance characteristics. Alternatively, these methods were optimized further for given final product/ RS such that if % recoveries were lower than 50%, optimal procedures ratio of starting material to extraction solvent and the enzyme levels / reaction incubation times needed for the complete hydrolysis would be determined as initial steps in order to increase the percent recoveries.

3.3. **Specific Aim 3: Resistant Starch Content in Formulated Prototype Food Products Supplemented with 1% Resistant Starch.**

3.3.1. **Food Products Supplemented with 1% Resistant Starch**

Samples including muffin, extruded ready-to-eat breakfast cereal, cookie, and nutritional bar were supplemented with 1% resistant starch (RS₂) provided by Dr. Randy Wehling (Department of Food Science and Technology). Sports drink was supplemented with 1% RS based on proposed recipe. Each food products were defatted and dried according to the processed food formulations shown in Tables 3.1-3.5. Resistant starch levels in these samples were than determined using the Megazyme Resistant Starch Assay Procedure (AOAC Method, 2002.02). Percent recovery of different formulated prototype food products with 1% supplemented RS₂ was obtained and corrected based on the % recovery from the validation of these food products.
3.4. Specific Aim 4: Chemical Fate of Resistant Starch in Prototype Foods during Various Processing Treatments

3.4.1. Sports Drink Formulation

The pasteurized sports drink was prepared according to Table 3.5. All ingredients were mixed in a 20 L mixing tank with distilled water to a final volume of 10 L. To ensure quality sample collection and ease coil cleaning, sufficient amounts of red food coloring (Red Dye # 40) were added to the formula. A pH of 3.5 was adjusted accordingly for each batch using citric acid. The mixed ingredients were then stirred in the mixing tank while it was being pumped through a pipe to the heating coil in the Groen steam-jacketed kettle (Model No. TBD/7-40) by a pump with flow speed set at 3. The batch was heated to a minimum temperature of 175 °F using a Groen steam-jacketed kettle and a stainless steel coil. The drink product was hot-filled into PET bottles and allowed to cool. Upon cooling, the final sports drink products were stored at ambient temperatures prior to resistant starch analysis.

To determine the effects of pH and sweetener composition on prebiotic stability, the following treatments were implemented: 1) varied sucrose: HFCS ratios (1:2, 1:1, 2:1), and 2) pH values 3.0, 3.5, and 4.0. The experiment was completely randomized with a 3 x 3 factorial design. Each trial held constant sweetener ratio while adjusting the pH to 3.0, 3.5, and 4.0. Figure 3.3 shows the 3 x 3 factorial design.

3.4.2. Shelf Life Stability of Resistant Starch in Sports Drink

Resistant starch contents of the supplemented sports prepared with different sweetener composition were determined at month 0 and month 6 using Megazyme Resistant Starch Assay Procedure (AOAC Method, 2002.02).
3.4.3. Chemical Fate of Resistant Starch in Extruded ready-to-eat Breakfast Cereal

Extruded ready-to-eat breakfast cereal samples of different screw speed (120, 170, and 220 rpm) and temperature (110, 140, and 170 °C) were obtained from Dr. Randy Wehling (Department of Food Science and Technology). Cereal was extruded with a twin-conical screw laboratory extruder (C.W. Brabender Model 2003 GR-8). The extruder had a barrel diameter of 1.9 cm with a length: diameter ratio of 20:1. The mix was equilibrated overnight with appropriate additions of distilled water to obtain a final moisture content of 17% prior to extrusion. Trials run were conducted by Dr Wehling’s graduate student, Michelle Hoffman to determine optimum feed-mix moisture content, barrel temperature, and screw speed for cereal model expansion. The screw speed (170 rpm) and barrel temperature (140 °C) combination that provided optimum expansion was selected. A complete randomized design with variables two screw speeds (± 50 rpm from the optimum) and two temperatures (± 30 °C from the optimum) were conducted at each trial with the rest of extrusion parameters held constant. Resistant starch contents of extruded ready-to-eat breakfast cereals prepared under these conditions were determined using Megazyme Resistant Starch Assay Procedure (AOAC Method, 2002.02). Figure 3.4 shows the different variables of screw speeds and temperatures used while maintaining other extrusion parameters.
Figure 3.3. 3 x 3 factorial design.

Figure 3.4. Different variables of screw speeds and temperatures while maintaining other extrusion parameters.
Chapter 4. RESULTS AND DISCUSSION

Intake of novel foods that provide benefits above our daily nutritional requirement is increasing as consumers are becoming more concerned about maintaining a healthy lifestyle. Resistant starch is such a functional ingredient being studied for these attributes, especially in natural products containing high dietary fiber (Sanz, Salvador, & Fiszman, 2008b). Although the recommended intake of dietary fiber (> 1 year) is 19-38 g per day (Institute of Medicine, 2005), only 5% of Americans consume these quantities with intakes of 3-8 g/day being more common (Moshfegh, Goldman, & Cleveland, 2006; Murphy & Birkett, 2008). In contrast, developing countries consume diets high in dietary fiber (30-40 g/day), which may contribute in part to lower frequencies of metabolic diseases that are currently impacting Western society. For example, researchers have correlated higher starch intake by the Chinese and Indians to lower incidences of bowel cancer. As starch consumption decreases, bowel cancer also increases (Figure 4.1) with the United States having the lowest starch intake and the highest incidence of bowel cancer (Cassidy, Bingham, & Cummings, 1994). However, carbohydrate consumption is decreasing in many developing countries decreasing due to industrialization and higher earning power (Stephen, Sieber, Gerster, & Morgan, 1995). Similar to the United States, the European Union countries consume approximately 3-6 g/day of dietary fiber (Dyssler & Hoffmann, 1994) while dietary intake in Australia is slightly higher at 5-7 g/day (Baghurst, Baghurst, & Record, 2001) probably due to the commercial availability of top selling food products such as breads, baked goods and cereals high in RS.
Nonetheless based on the current fiber intake, it would require substantial dietary changes by all Western societies to achieve the recommended 20 g of fiber per day. A possible solution to this problem is to add RS to currently consumed products (Murphy, Douglass, & Birkett, 2008). Resistant starch is the preferred functional food ingredient compared to other types of dietary fiber due to its beneficial physical and physiological properties. Furthermore, RS is fermented slowly in the large bowel and is therefore more tolerable compared to other soluble fibers (Lunn & Buttriss, 2007).

A major obstacle when comparing RS containing food systems and thus a correlation with their health benefits is the discrepancy in the analytical tests applied and limitations of those methods used throughout the literature. The analytical method used to determine dietary fiber in the United States for labeling purposes does not adequately quantitate RS (Institute of Medicine, 2001). Different analytical procedures produce variable results in similar foods due the types of enzymes used, concentrations, sequence of application, and the conditions of experiments (Perera, Meda, & Tyler, 2010). Several colorimetric glucose assays that directly or indirectly measure RS have thus been developed and modified over the years by several authors (Englyst & Cummings, 1992); (Chung, Lim, & Lim, 2006); (Berry, 1986); (Muir & O'Dea, 1992);(Champ, Martin, Noah, & Gratas, 1999). Yet, these colorimetric glucose assays, which involve enzymatic hydrolysis of starch to determine RS content in food, are prone to inaccuracies in sample volume. The Megazyme RS assay procedures eliminated this variability by incorporating the total volume of RS after removal of digestible starch followed by measuring an aliquot of this sample via a colorimetric approach. The availability of the control starch in the Megazyme kit also has provided a point of reference for experimental errors. The
pitfall of the Megazyme procedure is that an enzyme is required for removing digestible starch generating high variability compared to gas-liquid chromatography where errors could be corrected by using an internal standard (Perera, Meda, & Tyler, 2010).

Nonetheless, the Megazyme procedure was used for this research project as it has undergone the rigorous approval process by the Association of Analytical Chemists (AOAC Method, 2002.02) and does not require complex instrumentation. The procedure was adapted and developed to monitor RS present in different types of products commonly consumed in the United States, including an extruded ready-to-eat breakfast cereal, a cookie, a muffin, a sport drink and a nutritional bar.
Figure 4.1. Correlation of starch intake (g/day) in different countries with incidence of bowel cancer (cases/100,000 year, age standardized).

Source: (Cassidy, Bingham, & Cummings, 1994).

4.1.1. Resistant starch (RS<sub>2</sub>)

Resistant starch Hi-maizeTM 260 from National Starch (RS<sub>2</sub>) was chosen as RS standard for this research project. As the reagents and RS containing control can be purchased as a kit from Megazyme, the ability of these supplies to detect RS<sub>2</sub> at the levels added to the food products was initially determined by applying the assay to known amounts (based on gravimetric data) of the purified standard. Typical method performance (validation) criteria were then evaluated by using the kit as recommended by the manufacturer with slight modifications (Table 4.1). Most notably, a calibration curve using a range of external standards was always prepared to monitor the final glucose content (refer to Figure 3.2 and Figure 4.2). This step differed from the original procedure based on only one point to ensure that the final glucose levels of subsequent food based samples fell within the linear range of the calibration curve. The linearity of the calibration curve was determined based on glucose standards ranging from 0.1-1.0 mg/mL, which resulted in a regression curve of \( Y = 0.9918x + 0.0057 \) and a correlation coefficient of, \( R^2 = 0.9995 \). As shown in forthcoming Tables for the food systems (Specific Aim 2), the high degree of linearity stayed consistent throughout the duration of the study. Using this calibration curve, the detection and quantitation limits were determined with blank samples and converted to RS levels using the calculations cited in the Materials and Methods section. These values resulted in substantially lower detection (0.0013%) and quantitation (0.0048%) needed to assay the final products.
Table 4.1. Performance characteristic of the Megazyme resistant starch assay procedure as applied to the RS₂ standard.

<table>
<thead>
<tr>
<th>Performance Characteristic</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Linearity (regression analysis)</strong></td>
<td></td>
</tr>
<tr>
<td>D-glucose</td>
<td>Y = 0.9918x + 0.0057</td>
</tr>
<tr>
<td></td>
<td>( r^2 = 0.9995 )</td>
</tr>
<tr>
<td><em><em>Precision (RSD % based on SEM</em>)</em>*</td>
<td></td>
</tr>
<tr>
<td>RS₂ Standard: 1% (n = 3)</td>
<td>18.79</td>
</tr>
<tr>
<td>RS₂ Standard: 2% (n = 3)</td>
<td>14.21</td>
</tr>
<tr>
<td>RS₂ Standard: 3% (n = 3)</td>
<td>11.41</td>
</tr>
<tr>
<td>Control starch (n = 31)</td>
<td>6.08</td>
</tr>
<tr>
<td><strong>Detection limit (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Blank (n = 30)</td>
<td>0.0013</td>
</tr>
<tr>
<td><strong>Quantitation limit (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Blank (n = 30)</td>
<td>0.0048</td>
</tr>
<tr>
<td><strong>Accuracy/specificity (%)</strong></td>
<td></td>
</tr>
<tr>
<td>RS₂ Standard: 1% (n = 3)</td>
<td>146.39</td>
</tr>
<tr>
<td>RS₂ Standard: 2% (n = 3)</td>
<td>115.85</td>
</tr>
<tr>
<td>RS₂ Standard: 3% (n = 3)</td>
<td>99.63</td>
</tr>
<tr>
<td>Control starch (n = 31)</td>
<td>150.03</td>
</tr>
</tbody>
</table>

*SEM: Standard Error of the Mean

Figure 4.2. D-glucose calibration curve.
Method precision was then determined by analyzing the relative standard deviation (% RSD) of 1%, 2%, and 3% (w/v) RS\textsubscript{2} standards (n = 3). As shown in Table 4.1, precision increased with increasing concentrations of RS\textsubscript{2} but was substantially higher compared to the control starch (6.1% for 52.5 g / 100 g of RS in the starch). RS levels in the control starch were much higher than the quantitation limit of the assay and probably contributed to the low % RSD. Also, the sample size for control starch samples (n = 31) was higher compared to RS\textsubscript{2} samples potentially affecting the variability between the samples.

Accuracy was determined for the 1%, 2%, and 3% RS\textsubscript{2} standards (n = 3) resulting in 146.4, 115.8, and 99.6 % recovery, respectively. Considering that the recovery for the 1% RS\textsubscript{2} standard was higher compared to the other two samples, these results are mostly likely due to the low amounts of RS\textsubscript{2} standard being tested. The % recovery for control starch (n = 31) was also higher (150.95) than expected. Although we are uncertain as to why this occurred, the control was consistently higher throughout the project. One contributing experiment procedure could be due to the shaking process during sample incubation. The recommendation for this assay is to attach the samples horizontally with continuous shaking (200 strokes/min) at 37 °C for 16 hr. Because leakage occurring using this approach, the samples were attached vertically in the water bath. The possible outcome from this modification was that the non-resistant starch in the sample was not digested fully during the first 16 hr incubation resulting in higher final RS values. As a result, the control samples (n = 3) were always assayed and a correction factor applied to the test samples based on these results. It was also determined that 3-5 trials completed on separate days for each of 3-6 analyses were required to adequately
qualify the assay for food matrices due to the higher variability and lower accuracy of the method when applied to a 1% RS₂ standard.

4.1.2. Extruded ready-to-eat breakfast cereal

Extrusion processes that involve heat and moisture in manufacturing cereal products destroys the food matrix and both RS₁ and RS₂ availability in the food (Alsaffar, 2011). This process increases the susceptibility of starch to enzyme digestibility as the starch granules undergo gelatinization. The subsequent process of cooling the cereal product during tempering and storing may lead to RS₃ formation due to retrogradation of the starch. In most cases, RS content naturally occurring in breakfast cereals were not high enough to exert beneficial physiological effects (Alsaffar, 2011). Supplementing RS to extruded ready-to-eat breakfast cereal may increase consumers’ intake to the recommended levels as cereal grains and products are the major contributors to carbohydrate intake (Table 4.2) (Alsaffar, 2011). Furthermore, there are no current studies focusing on supplemented RS in breakfast cereal let alone the chemical fate of the RS in this matrix.

Validation of the assay for an extruded cereal was determined for accuracy (Table 4.3) by analyzing 1% of RS₂ standard spiked into the base cereal formula (without supplemented RS₂ during processes), which served as the blank. Although the spiked RS₂ had not been subjected to the processing event, the spiking step allowed for the development of optimal extraction procedures in the presence of this matrix. This approach to determine method accuracy in not typically applied to food based ingredients and thus has not been reported for method development of any prebiotic. Rather the quantity of prebiotic is determined after the processing event (Perera, Meda, & Tyler,
making it difficult to determine if any detrimental effects resulted from the process or the inability of the assay to extract / detect the analyte of interest. As a result, we applied the procedures mandated by the Food Drug and Administration for the pharmaceutical industry where the analyte of interest must be spiked into the matrix complex to determine accuracy (USPNF, 1995). It is even more critical to evaluate the accuracy of an RS assay via the spiked recovery method due to the complexity of the different food systems and the unique properties of different RS types. For example, the formulation of extruded ready-to-eat breakfast cereal contains higher soluble starches (oat and corn starches) that might account for part of the RS if these starches are not hydrolyzed completely. Additionally, the Megazyme RS assay is able to detect RS$_1$, RS$_2$, and RS$_3$ but not RS$_4$ in food products as determined by other studies completed in our laboratory. This might be due to the synthetic sources of the RS$_4$, which have been chemically modified and cross-bonded that leads to reduced digestibility in vitro.
### Table 4.2. Resistant starch content of some breakfast cereal product.

<table>
<thead>
<tr>
<th>Breakfast cereal</th>
<th>RS (g/100g food, as eaten)</th>
<th>Method of RS analysis</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>All bran</td>
<td>1.10</td>
<td>Englyst method</td>
<td>Englyst et al. (1996)</td>
</tr>
<tr>
<td>Porridge oats</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weetabix</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oat bran</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rice crispies</td>
<td>2.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muesli</td>
<td>1.30</td>
<td>Englyst method</td>
<td>Englyst et al. (2007)</td>
</tr>
<tr>
<td>Shredded wheat</td>
<td>1.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn flakes</td>
<td>3.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All bran</td>
<td>0.10</td>
<td>Englyst method</td>
<td>Englyst &amp; Cummings (1987)</td>
</tr>
<tr>
<td>Fruit filled cereal bar</td>
<td>2.30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Source: (Alsaffar, 2011).
Table 4.3: Performance characteristic of the Megazyme resistant starch assay procedure-extruded ready-to-eat breakfast cereal.

<table>
<thead>
<tr>
<th>Performance characteristic</th>
<th>Results (without optimization)</th>
<th>Results (with optimization^)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Linearity (regression analysis)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-glucose</td>
<td>Y = 0.9918x+0.0057, R² = 0.9995</td>
<td>Y = 0.9918x+0.0057, R² = 0.9995</td>
</tr>
<tr>
<td><strong>Absolute RS Levels (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extruded cereal base formula (n = 6-8)</td>
<td>0.17</td>
<td>0.24</td>
</tr>
<tr>
<td>Extruded cereal with 1% RS (n = 9)</td>
<td>0.33</td>
<td>1.09</td>
</tr>
<tr>
<td><em><em>Precision (RSD % based on SEM</em>)</em>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extruded cereal base formula (n = 6-8)</td>
<td>13.85</td>
<td>7.05</td>
</tr>
<tr>
<td>Extruded cereal with 1% RS (n = 9)</td>
<td>15.29</td>
<td>3.71</td>
</tr>
<tr>
<td><strong>Detection limit (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extruded cereal base formula (n = 6-8)</td>
<td>0.25</td>
<td>0.30</td>
</tr>
<tr>
<td><strong>Quantitation limit (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extruded cereal base formula (n = 6-8)</td>
<td>0.56</td>
<td>0.53</td>
</tr>
<tr>
<td><strong>Accuracy/specificity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extruded cereal with 1% RS (n = 9)</td>
<td>16.69</td>
<td>85.26</td>
</tr>
</tbody>
</table>

^† With individual points eliminated via Grubb's outlier tests.
^† With per day average points eliminated via ANOVA test (p < 0.05) using LSD.
^*SEM: Standard Error of the Mean.
^ New formulation of extruded-ready-to-eat breakfast cereal was prepared.
Using the formulated product shown in Table 3.1, the % accuracy was fairly low (16.69%) suggesting incomplete recovery of the RS$_2$ from the matrix. To ensure that the starch was completely broken down during the extraction phase of the assay, different amounts of pancreatic α-amylase (4, 6, and 8 mL) and incubation times (8, 16, 20, and 32 hr) were studied while the final incubation time (30, 60, and 120 min) and amount of AMG (0.1-2.7 mL) were also tested to ensure complete solubilization of the isolated RS$_2$ and breakdown to glucose. However, these modifications did not significantly affect % recovery (data not shown).

A new formulation of the extruded ready-to-eat breakfast cereal prototype was ultimately prepared (Table 3.1), which consisted of adjusting the oat flour and corn flour to produce a higher puffing quality to the final process. This change in formulation drastically affected the % recovery (85.26%) of the assay. The % RSD based on standard error of mean (SEM) for the base formula and for the spiked sample also decreased from 13.85 to 7.05% and from 15.29 to 3.71%, respectively. Additionally, the detection and quantitation limits were respectively 0.30% and 0.53%. Both values are lower than that needed to consistently quantitate the spiked 1% RS$_2$ sample (absolute mean value of 1.09%) for this matrix (Table 4.3). The improved ability of the assay to monitor the new formulation was probably due to the decreased amount of oat flour, which contains higher β-glucan levels. β-glucan is naturally present in grains such as oats, barley, yeast, bacteria, algae, and mushroom and are non-digestible in the body. The β-glucan in oat flour may have interfered with the extraction/solubility of the RS assay.
4.1.3. **Muffin**

A muffin matrix supplemented with various levels of RS and β-glucan has been used to study the effects on postprandial plasma glucose and insulin in women (Behall, Hallfrisch, Scholfield, & Liljeberg-Elmstahl, 2006). However, no other studies on the chemical fate of RS in baked goods have been reported. Therefore, a significant gap of information exists in this area especially in light of the potential impacts that Maillard browning may have on the biological and functional activity of RS. Furthermore, the Megazyme assay has been applied to cereal starches, beans, and corn flakes (McCleary & Monaghan, 2002) but muffin matrices have been neglected. As the muffin is a completely different matrix containing higher levels of moisture, fat, and protein compared to cereals, the RS assay was adapted and validated for the formulation shown in Table 3.2. Spiking the matrix with 1% RS\textsubscript{2} initially resulted in low % recoveries (28.12%). It was hypothesized that moisture was interfering with accurate extraction or detection of the RS\textsubscript{2} by preventing homogenous distribution of the spiked into the matrix, i.e., sticking to the sides of the container, blender, etc. It was also determined that defatting the muffin prior to analysis was a critical step in preventing amylose-lipid interaction that may influence the reliability of the RS content. The amylose-lipid complexes could naturally present in starch or formed upon gelatinization of starch in high lipid containing foods (Alsaffar, 2011). This complex could reduce detectable RS in food as less amylose is available for the formation of double helices and RS (Sajilata, Singhal, & Kulkarni, 2006). Eerlingen et al. (1994) showed that wheat starch containing contained reduced RS levels. These hypotheses were tested by spiking 1% of RS\textsubscript{2} into a dried sample, followed by removing the fat by hexane phase extraction. The method was
then applied to the extracted sample as directed by the assay and the fat/moisture was corrected for in the final calculations. Additionally, 0.9 mL of AMG was used instead of 0.1 mL of AMG to ensure complete breakdown of the isolated RS2. These modifications resulted in an increase in the background noise of the base formula (4.01%) and the 1% spiked samples (4.67%) (Table 4.4). Percent recovery also increased (65.75%) but with a concomitant increase in the detection limit (1.44 % to 4.44%) and the quantitation limit (1.86% to 6.14%). As the absolute value of the spiked sample is slightly higher than the detection limit but much lower than the quantitation limit, the assay as developed is able to detect 1% RS2 in muffin matrix but cannot reliably quantitate this prebiotic. Yet, precision remained low with only slight increases with sample handling modifications as the base formula % RSD increased from 2.26 to 3.06% while 1% RS2 samples increased from 3.29 to 3.75%.

Additional method development did not improve any of these characteristics, e.g., adding a protease step to the method. Because the encapsulation of starch by protein molecules could occur, especially in food high in protein, (Englyst & Cummings, 1992); (Muir & O'Dea, 1992); (Goñi, Garcia-Diz, Mañas, & Saura-Calixto, 1996); (Åkerberg, Liljeberg, Granfeldt, Drews, & Björck, 1998); (Eerlingen, Crombez, & Delcour, 1993a) pepsin or other protease pretreatments have been introduced in RS assay protocols to increase amylolytic enzymes accessibility to starch granules (Holm, Bjorch, Drews, & Asp, 1986). Protease pretreatment was not included in the final assay method because preliminary data showed that the additional method characteristics were not affected (data not shown). Higher precision and accuracy may occur for muffin samples
containing higher RS levels as the Megazyem kit was designed for analyzing samples containing more than 2% w/w RS.
Table 4.4: Performance characteristic of the Megazyme resistant starch assay procedure - muffin.

<table>
<thead>
<tr>
<th>Performance characteristic</th>
<th>Results (without optimization)</th>
<th>Results (with optimization&lt;sup&gt;^&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Linearity (regression analysis)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-glucose</td>
<td>( Y = 0.9918x+0.0057, )</td>
<td>( Y = 0.9918x+0.0057, )</td>
</tr>
<tr>
<td></td>
<td>( R^2 = 0.9995 )</td>
<td>( R^2 = 0.9995 )</td>
</tr>
<tr>
<td><strong>Absolute values (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muffin base formula (n = 6-23)</td>
<td>1.33</td>
<td>4.01</td>
</tr>
<tr>
<td>Muffin Spiked formula (n = 6-23)</td>
<td>1.62</td>
<td>4.67</td>
</tr>
<tr>
<td><em><em>Precision (RSD % based on SEM</em>)</em>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muffin base formula (n = 6-23)</td>
<td>2.26</td>
<td>3.06</td>
</tr>
<tr>
<td>Muffin with 1% RS (n = 6-23)</td>
<td>3.29</td>
<td>3.75</td>
</tr>
<tr>
<td><strong>Detection limit (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muffin base formula (n = 6-23)</td>
<td>1.44</td>
<td>4.44</td>
</tr>
<tr>
<td><strong>Quantitation limit (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muffin base formula (n = 6-23)</td>
<td>1.86</td>
<td>6.14</td>
</tr>
<tr>
<td><strong>Accuracy/specificity (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muffin with 1% RS (n = 6-23)</td>
<td>28.12</td>
<td>65.75</td>
</tr>
</tbody>
</table>

<sup>†</sup>With individual points eliminated via Grubb’s outlier tests.
<sup>‡</sup>With per day average points eliminated via ANOVA test (p < 0.05) using LSD.
*SEM: Standard Error of the Mean.
^ Fat removal by hexane phase.
4.1.4. Cookie

Incorporating RS into a cookie matrix may be a popular means to increase fiber intake while maintaining overall sensory qualities. A study conducted by National Starch and Chemical Company showed that 40% total dietary fiber (TDF-RS) produced a tender, shortbread like texture cookie with a richer butter flavor (Waring, 2011). A cookie based matrix is expected to contain higher RS<sub>2</sub> levels compared to a muffin and extruded breakfast cereal, as cookies are baked in a limited water system with dry heat applied, thereby preventing starch granules from gelatinizing. Despite the potential benefits of RS fortified cookies, no studies have been conducted to date examining the chemical fate under typical cooking operations.

The initial hurdle for testing the cookie matrix (Table 3.3) was obtaining a well-pelletted solid after the first enzymatic reaction. At this point in the test, the RS<sub>2</sub> is insoluble and dispersed throughout the solid material. To ensure high % recoveries, all the solid material was needed to solubilize the RS. This problem was remediated by increasing the centrifuge speed (3000 to 4000 rpm) and the time (10 min to 20 min). Due to this simple step, recoveries of 1% spiked RS<sub>2</sub> increased from 25.71% to 77.71% (Table 4.5). In an effort to increase the % recovery, the final digested sample was decreased from 10 to 5 mL to concentrate the glucose resulting in a recovery of 103.96%. These additional steps also produced lower detection limits (3.01% to 2.68%) and quantitation limits (15.05% to 6.41%). Yet, the absolute value of the spiked samples (2.79%) was slightly higher than the detection limit but much lower than the quantitation limit. The % RSD for cookie base formula before and after optimization was similar at respective values of 14.22% and 15.41% but variability increased for the 1% RS<sub>2</sub> after optimization.
(from 3.66% to 9.03%), which may be due to the additional steps to increase recovery and to assaying the spiked RS at a level below the quantitation limit. More optimization of the assay for cookie matrix could be conducted to improve method variability and detection / quantitation limits, i.e., notably increasing the number of sample replicates but at added cost of sample analysis.
Table 4.5: Performance characteristic of the Megazyme resistant starch assay procedure - cookie

<table>
<thead>
<tr>
<th>Performance characteristic</th>
<th>Results without optimization</th>
<th>Results with optimization^</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Linearity (regression analysis)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-glucose</td>
<td>Y= 0.9918x+0.0057, R² = 0.9995</td>
<td>Y= 0.9918x+0.0057, R² = 0.9995</td>
</tr>
<tr>
<td><strong>Absolute values (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cookie base formula (n = 8-12)</td>
<td>2.39</td>
<td>1.75</td>
</tr>
<tr>
<td>Cookie with 1% RS (n = 8-12)</td>
<td>3.25</td>
<td>2.79</td>
</tr>
<tr>
<td><em><em>Precision (RSD % based on SEM</em>)</em>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cookie base formula (n = 8-12)</td>
<td>14.22</td>
<td>15.41</td>
</tr>
<tr>
<td>Cookie with 1% RS (n = 8-12)</td>
<td>3.66</td>
<td>9.03</td>
</tr>
<tr>
<td><strong>Detection limit (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cookie base formula (n = 8-12)</td>
<td>3.01</td>
<td>2.68</td>
</tr>
<tr>
<td><strong>Quantitation limit (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cookie base formula (n = 8-12)</td>
<td>15.05</td>
<td>6.41</td>
</tr>
<tr>
<td><strong>Accuracy/specificity (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cookie with 1% RS (n = 8-12)</td>
<td>77.71</td>
<td>103.96</td>
</tr>
</tbody>
</table>

†With individual points eliminated via Grubb’s outlier tests.
†With per day average points eliminated via ANOVA test (p < 0.05) using Tukey HSD.
*SEM: Standard Error of the Mean.
^Centrifugation speed and time after first enzymatic step was doubled to ensure complete pelleting and end volume was adjusted to a more concentrated solution for detection.
4.1.5. Granola bar

A granola bar was selected for this study as it is another popularly consumed US snack and its formulation (Table 3.4) differed from the other food products. As the granola bar was neither baked nor processed, it was hypothesized that RS$_2$ would be more stable but a fat removal step was needed to prevent amylase-lipid interactions.

It must be emphasized that different ingredients in the granola, such as roasted chopped peanuts and whole rolled oats, together with the higher viscosity of honey and peanut butter affected our ability to homogenously disperse the RS$_2$ spike, thereby requiring extra mixing than was used for the other products. The granola bar was then defatted with hexane, washed with water and dried under vacuum to remove excess water. Applying these steps resulted in a recovery of 73.30% (Table 4.6). As the absolute value of the spiked sample (1.01%) was higher than both detection limit and quantitation limit, the assay as developed was able to detect and reliably quantitate 1% RS$_2$ in the granola bar matrix. The % RSD for granola bar base formula was 8.26% and for 1% RS$_2$ spiked samples 4.85%. The higher variability obtained for the granola bar base formula was probably due to the lower RS content available for detection.
Table 4.6. Performance characteristic of the Megazyme resistant starch assay procedure - granola bar

<table>
<thead>
<tr>
<th>Performance characteristic</th>
<th>Results with optimization^</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Linearity (regression analysis)</strong></td>
<td></td>
</tr>
<tr>
<td>D-glucose</td>
<td>Y = 0.9918x+0.0057,</td>
</tr>
<tr>
<td></td>
<td>R² = 0.9995</td>
</tr>
<tr>
<td><strong>Absolute values (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Granola bar base formula (n = 14)</td>
<td>0.28</td>
</tr>
<tr>
<td>Granola bar with 1% RS (n = 16)</td>
<td>1.01</td>
</tr>
<tr>
<td><strong>Precision (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Granola bar base formula (n = 14)</td>
<td>8.26</td>
</tr>
<tr>
<td>Granola bar with 1% RS (n = 16)</td>
<td>4.85</td>
</tr>
<tr>
<td><strong>Detection limit (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Granola bar base formula (n = 14)</td>
<td>0.36</td>
</tr>
<tr>
<td><strong>Quantitation limit (mg/100 mg of sample)</strong></td>
<td></td>
</tr>
<tr>
<td>Granola bar base formula (n = 14)</td>
<td>0.68</td>
</tr>
<tr>
<td><strong>Accuracy/specificity (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Granola bar with 1% RS (n = 16)</td>
<td>73.30</td>
</tr>
</tbody>
</table>

^ Fat removal by hexane phase.

†With individual points eliminated via Grubb’s outlier tests.
†With per day average points eliminated via ANOVA test (p < 0.05) using LSD.
*SEM: Standard Error of the Mean.
4.1.6.  Sport Drink

The development of new energy as sports drinks has risen rapidly in the 21st century (Just drinks, 2007) with marketing targeted mainly at the Generation Y population. Although RS is expected to be an excellent functional ingredient for thickened, opaque health beverages where insoluble fiber is desired (Sajilata, Singhal, & Kulkarni, 2006), the sensory properties and acceptability of sports drink fortified with insoluble RS could be a marketing challenge. Nonetheless, the health benefitting properties of this prebiotic may overcome this issue. The assay as developed was therefore applied to a sports drink matrix (Table 3.5). No pretreatment and extraction steps were needed as the percent recovery of the 1% RS spiked sample was 97.42 with a % RSD of only 1.53 (Table 4.7). The detection / quantitation limit were also well below that needed to reliably assay a 1% RS containing drink.
Table 4.7. Performance characteristic of the Megazyme resistant starch assay procedure - sports drink

<table>
<thead>
<tr>
<th>Performance characteristic</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Linearity (regression analysis)</strong></td>
<td></td>
</tr>
<tr>
<td>D-glucose</td>
<td>$Y = 0.9918x + 0.0057$, $r^2 = 0.9995$</td>
</tr>
<tr>
<td><em><em>Precision (RSD % based on SEM</em>)</em>*</td>
<td></td>
</tr>
<tr>
<td>Sports drink with 1% RS ($n = 9$)</td>
<td>1.53</td>
</tr>
<tr>
<td><strong>Detection limit (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Sports drink base formula ($n = 8$)</td>
<td>0.0013</td>
</tr>
<tr>
<td><strong>Quantitation limit (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Sports drink base formula ($n = 8$)</td>
<td>0.0048</td>
</tr>
<tr>
<td><strong>Accuracy/specificity</strong></td>
<td></td>
</tr>
<tr>
<td>Sports drink with 1% RS ($n = 9$)</td>
<td>97.42</td>
</tr>
</tbody>
</table>

†With individual points eliminated via Grubb’s outlier tests.
‡With per day average points eliminated via ANOVA test ($p < 0.05$) using Tukey HSD.
*SEM: Standard Error of the Mean.
4.2. Specific Aim 3: Determination of Resistant Starch Content in Formulated Prototype Food Products Supplemented with 1% Resistant Starch.

Published data remain limited about the stability of RS during food processes such as baking, extrusion, pasteurization, high temperature heating, low pH condition, etc., despite the food industry’s interest in supplement products. However, studies have shown that RS present in legumes, potatoes, and bananas are affected by processing and storage conditions. Muir & O’Dea, (1992) showed that cooked oats and firm bananas had lower RS content compared to the uncooked oats and bananas whereas grounded rice had lower RS content compared to whole rice. Pressure cooking and boiling increased the amount of RS in rice and waxy amaranth starch but roasting, extrusion cooking, frying, and drum drying resulted in reduced RS content in rice and waxy amaranth starch (Parachu & Kulkarni, 1997).

The commercial form of RS₂, Hi-maize, was developed to be resistant to mild food processing and include Novelose® 240 (National Starch and Chemical Company), Amylomaize VII (Cerestar Inc), and Hi-maizeTM 260 (National Starch Food Innovation). High-amylose maize starches have high gelatinization temperatures (154 °C to 171 °C) where the swelling of starch molecules occurs in the presence of water and the granules are then completely disrupted. This temperature is considered high for conventional cooking and is not often reached (Sajilata, Singhal, & Kulkarni, 2006).

The objective of specific aim 3 was to determine the RS content in formulated prototype food products containing 1% supplemented RS₂, including extruded ready-to-eat breakfast cereal, muffin, cookie, granola bar, and sports drink after different food processes. The muffin was prepared and kept in the freezer at -20 °C whereas the
extruded cereal, cookie, and granola bar were prepared and kept at ambient temperature prior to analysis. The sports drink, which contained 5% carbohydrate (equal amount of sucrose and high fructose corn syrup) was adjusted to pH 3.5 and was pasteurized at 79 °C. The assay as adapted for each matrix (Specific Aim 1) was applied to the supplemented products and correction factors determined from the % recovery studies were applied to the final results.

As shown in Table 4.8 and Figure 4.3, the sports drink had a recovery of ~ 96.1% indicating that pasteurization did not significantly affect the stability of RS in this matrix. RS₂ has a higher gelatinization temperature than was reached by pasteurizing the sports drink at 79 °C. Both the muffin and cookies had slightly lower % recoveries at 91.0% and 86.8%, respectively. The supplemented muffin also had been stored in the freezer (-20 °C) for some time before analysis, which may have caused structural changes to the RS. According to Niba (2003), baked goods such as breads and muffins that contain retrograded starch (RS₃) are susceptible to starch restructuring with storage. In addition, RS content in cornbread was reported to decrease after 7 days of storage at 20, 4, and -20 °C. The starch damage of this cornbread increased after 7 days of storage and was especially high in cornbread stored at -20 °C (Niba, 2003).

The granola bar was prepared without any other processing conditions and stored at ambient temperature but only resulted in 70.0% recovery. One possible explanation for these low recoveries was that the RS₂ ingredient was not be homogenously mixed with the other ingredients during the preparation. A similar effect was determined during method validation necessitating thorough mixing to ensure high % recovery.
Lastly, the ready-to-eat breakfast cereal was formulated with 1% RS$_2$ and then extruded at optimum screw speed and temperature of 170 rpm and 140 °C, respectively. The final product was stored in ambient temperature prior to analysis.

Analysis of the final RS$_2$ levels resulted in a 241.9% recovery, which was more than expected. Considering that the higher gelatinization temperature of RS$_2$ was reached during the extrusion process, the 1% supplemented RS$_2$ may have undergone gelatinization followed by retrogradation during the storage period. Another contributing factor for the high % recovery could be due to the ingredients in extruded cereal, such as oat flour and corn flour that may have also undergone gelatinization followed by retrogradation. Starch typically hydrates at temperatures ranging from 40 to 120 °C. Different starch granules, source of starch and its amylose content gelatinize at different temperatures in the presence of water. Upon cooling, starch undergoes retrogradation process where the starch molecules start to re-associate and form tightly packed structures that are stabilized with hydrogen bonding. This form of starch is thermally stable and resistant to amylase which is known as RS$_3$ (Haralamu, 2000) and are present in cereal products.
Table 4.8. Processing effect on resistant starch contents of different formulated prototype food products spiked with 1 % RS₂ (unit: %, dry weight basis)

<table>
<thead>
<tr>
<th>Sample</th>
<th>RS content of food products after processing</th>
<th>1 % supplemented RS₂</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Base formula</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extruded ready-to-eat</td>
<td>0.24±0.02*</td>
<td>2.30±0.33</td>
<td>241.49±31</td>
</tr>
<tr>
<td>breakfast cereal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muffin</td>
<td>4.01±0.12</td>
<td>4.61±0.26</td>
<td>90.95±14</td>
</tr>
<tr>
<td>Cookie</td>
<td>1.75±0.27</td>
<td>2.65±0.51</td>
<td>86.84±24</td>
</tr>
<tr>
<td>Granola bar</td>
<td>0.28±0.02</td>
<td>0.79±0.14</td>
<td>69.96±12</td>
</tr>
<tr>
<td>Sports drink</td>
<td>-</td>
<td>93.81±2.20</td>
<td>96.10±2.20</td>
</tr>
</tbody>
</table>

*SEM: Standard Error of the Mean. x SEM, n = 8-23.  
†With individual points eliminated via Grubb’s outlier tests.  
‡With per day average points eliminated via ANOVA test (P < 0.05) using Tukey HSD.

Figure 4.3. Percent recovery of 1% supplemented RS₂ in different formulated prototype food products after processing.

Results are shown as x SEM for 8-23 analyses.
4.3. Specific Aim 4: Chemical Fate of Resistant Starch in Prototype Foods during Various Processing Treatments as Applied to Extruded Ready-to-eat Breakfast Cereal and a Sports Drink.

4.3.1. Sports drink

To determine the effects of pH and sweetener composition on RS stability, the following treatments were implemented 1) varied sucrose: high fructose corn syrup (HFCS) ratios (1:2, 1:1, and 2:1), 2) and pH values (3.0, 3.5, and 4.0). The experiment was completely randomized with a split-box plot design. Each trial was held at constant sweetener ratio while varying the pH level of the sports drink by adding citric acid. RS\textsubscript{2} (1%) was added into the sports drink prior to pasteurization at 79 °C. To ascertain the shelf life of RS in sports drink, i.e., whether RS is stable in low pH beverages, the different formulated sports drink were measured for their RS content at month 0 and month 6. The sports drink was stored at ambient temperature (20 °C) to mimic the conditions typically used to distribute a sports drink.

There was no significant difference in RS levels for the sports drink (pH 3, and 3.5) stored for 6 months that contained equal composition of sucrose (S) and HFCS (Figure 4.4a). A slight decrease occurred for the pH 4 formulation at 0 and 6 month, which could be due to the sugar composition, but more likely caused during initial RS supplementation. However, a significant reduction in RS (~10%) resulted over time for the 1:2 sucrose: HFCS sports drink at each pH (Figure 4.4b). The RS levels for the 2:1 sucrose: HFCS pH 4 formulations were also significantly lower over the 6 month period but RS was not affected during storage when the drink was formulated at pH 3.0 or 3.5 (Figure 4.4c). These results indicated that the combination of sugars and pH parameters
affect the shelf life of RS containing sports drink. More studies are needed with extended time periods and storage conditions that are typically used for a sports drink.
Results are shown as $\bar{x} \pm$ SEM (analyzed 3-9 times). Bars with different letters are statistically different ($p>0.05$) using Tukey HSD test.

Figure 4.4. RS content (%) of sports drink at month 0 and month 6 for pH 3.0, 3.5, and 4.0. a.) 1S:1H, b.) 1S:2H, c.) 2S:1H. (S: Sucrose, H: High fructose sugar).
4.3.2. Extruded Ready-to-eat Breakfast Cereal

Studies have shown that chemical changes occur to the starch molecules when pure wheat starch, high-amylose-corn starch, corn starch, and potato starch are extruded leading to formation of RS (Faraj, Vasanthan, & Hoover, 2004); (Kim & Lee, 1998); (Unlu & Faller, 1998). These chemical changes include the gelatinization of starch molecules, cross-linking of proteins, and the generation of flavors in the extruded products (Riha, Hwang, Karwe, Hartman, & Ho, 1996). As stated previously, the retrogradation process that follows the gelatinization of starch molecules can also produce RS$_3$ (Faraj, Vasanthan, & Hoover, 2004). As such, there are several parameters that might affect the formation of RS in extruded cereal grain starches such as feed moisture (FM), barrel temperature, screw speed, and post-extrusion storage temperature. Kim et al. (1998) showed that the RS content of raw pastry wheat flour increased significantly with increased feed moisture (20, 40, and 60 %), and storage period (0, 7, and 14 days) but the RS values were not significantly correlated to the screw speeds (150, 200, and 250). A study conducted by Shin et al. (2002) showed that extrusion of corn starch at 110 °C barrel temperature, 150 rpm screw speed, and approximately 30% moisture yielded the highest amount of RS (14.2-15.5%). According to Faraj et al. (2004) extruded cereal grain based foods that containing low RS amounts (0-0.6%) can be optimized by post-extrusion conditions, which included storage temperature and period.

To evaluate the chemical stability of 1% supplemented RS$_2$ (Hi-maize™ 260) in extruded ready-to-eat cereal, barrel temperatures (± 30 °C from the optimum) and screw speeds (± 50 rpm from the optimum) were varied during the extrusion process. Each trial was held at optimum temperature while varying these parameters. The highest RS
content was obtained in extruded ready-to-eat breakfast cereal extruded at optimum
temperature (140 °C) and screw speed (170 rpm) (Figure 4.5 and Figure 4.6). The lowest
temperature (110 °C) resulted in RS content of 1.89% while the optimum temperature
(140 °C) produced 2.30% RS, which was probably caused by starch retrograding to RS.<
The cereal extruded at highest temperature (170 °C) contained the lowest RS content
(1.48%). This high temperature might depolymerize the amylopectin in starch, breaking
down the starch to smaller molecules. As a result the starch molecules, which include RS
in extruded cereal, would not retrograde further at the elevated temperature. The
optimum screw speed (170 rpm) gave the highest amount of RS (2.30%) in the extruded
cereal while the cereal extruded with the highest screw speed (220 rpm) contained the
lowest amounts of RS (1.94%). The high sheer from screw speed could disrupt the starch
molecules hence retrogradation upon heating would be disrupted. Even though a trend is
evident in RS content for the different process parameters, the mean values are not
statistically different (Figure 4.5 and Figure 4.6). More studies are required with more
process replicates and higher supplemented levels to determine the significance of these
processing events on RS.
Figure 4.5. Resistant starch content (%) in extruded cereal with different screw speeds (rpm) at optimum temperature (140 °C).

Results are shown as $\bar{x} \pm$ SEM (analyzed 15-20 times). Bars with different letters are statistically different (p>0.05) using Tukey HSD test.

Figure 4.6. Resistant starch content (%) in extruded cereal with different temperatures (°C) at optimum screw speed (170 rpm).

Results are shown as $\bar{x} \pm$ SEM (analyzed 15-20 times). Bars with different letters are statistically different (p>0.05) using Tukey HSD test.
CONCLUSIONS

Supplementation of RS into popular consumed processed food is a probable solution for increasing intake of this prebiotic. Due to the complexity of such systems, the Megazyme assay was adapted and validated for measuring RS in extruded cereal, muffin, cookie, granola bar, and sports drink. Application of the validated method to 1% supplemented processed foods showed processing effects, especially for the extruded cereal. RS was also significantly affected when the process was changed in terms of sports drink formulation (sweetener composition and pH) but only non-significant trends resulted when changing the extrusion parameters (barrel temperatures and screw speeds) for the cereal. In general, a more robust and reliable method (Megazyme RS assay) has to be applied on any RS supplemented processed food in determining the chemical fate of RS under different processing treatments.

In this research, the accuracy of the RS assay was determined via a spiked recovery method, which is not a typical step for food based ingredients and thus has not been reported in literature for method development of any prebiotic. Therefore, future analysis of RS content in food products should be validated for each product due to their complex matrices. Based on these studies, however, it was determined that optimization of the method is necessary to increase accuracy and other method characteristics for most of the food matrices.
REFERENCES


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