Lactose Polymerization to Polylactose: Furthering Our Understanding for Commercialization

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Dedication

To my parents, Lara and Joseph, for always being my biggest supporter and for reminding me that I can do anything if I put my mind to it and take it one step at a time.

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Abstract

Polylactose, soluble fiber that has been manufactured from a blend of lactose, glucose and citric acid using reactive extrusion, has been developed in our lab. A benchtop method for polylactose production was developed that allowed for the evaluation of formulation impact on the polymerization reaction and investigation of the use of permeate powder as the polylactose raw material. Water content and calcium phosphate concentration were the investigated formulation parameters. Elevated levels of water (22.86%, 28.57%, v/w) and calcium phosphate (0.928%, 1.856%, w/w) reduced the soluble fiber yield, meaning the polymerization reaction was inhibited. Polymerization of lactose in permeate was achieved on both a benchtop and pilot plant scale when using a blend of 90% permeate and 10% citric acid. This research also evaluated the development of a purification method for polylactose through filtration. Passing a 200 mg/mL solution of polylactose in water through a column packed with activated carbon, ion exchange resins composed of Amberlite and Ambersep and diatomaceous earth reduced the hydroxymethylfurfural (HMF) content to a level that was lower than the maximum level determined by the Joint FAO/WHO Expert Committee on Food Additives HMF limit in polydextrose, while increasing the fiber content and decreasing the citric acid content.

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1 Introduction

Consumption of dietary fibers and prebiotics has been linked with numerous health benefits, including reduced incidence of chronic disease, improved digestive health and reduced energy intake due to increased satiation (United States Department of Agriculture, 2015). Consumer's interest in dietary fiber and prebiotic-containing products has increased as more people are aware of the associated health benefits (Frost and Sullivan, 2012; Frost and Sullivan, 2017). To meet this demand, the food industry continues to develop more products containing fiber, contributing to the dramatic increase in the fiber market, from $211 M in 2010 to $512.3 M in 2017 (est.) (Frost and Sullivan, 2012; Mintel Group Ltd., 2016). In 2010, oligosaccharides accounted for the largest portion of total food fiber ingredients sold in the U.S. (27.6%), followed by inulin (20.8%) and wheat fiber (17.7%) (Frost and Sullivan, 2012). The global prebiotic ingredient market is also experiencing steady growth. The $1.35 billion market is projected to experience 5.4% growth over the next 5 years ending in a market size of $1.76 billion by 2021. GOS (galactooligosaccharides) dominate the prebiotic ingredient market globally because of their popularity in the infant nutrition market (Frost and Sullivan, 2017).

Carbohydrates with 3 to 10 sugars linked together are called oligosaccharides (Barreteau et al., 2006). The term used to describe the number of sugars that make up an oligosaccharide is "degree of polymerization" (DP). Oligosaccharides can be produced via the breakdown of polysaccharides or through a synthesis reaction, enzymatic or chemical, in which the hydroxyl group of an acceptor reacts with an anomeric center of a donor to form a glycosidic linkage (Bailey, 1965). Polymerization through a chemical synthesis occurs in the presence of acid and heat, while aldolase enzymes facilitate enzymatic synthesis (Collins and Ferrier, 1995; Barreteau et al., 2006). Commercial oligosaccharides such as GOS are produced through an enzymatic synthesis, while polydextrose is produced through a chemical synthesis (Rennhard, 1973; Gosling et al., 2010). Oligosaccharides can be classified as dietary fiber and a prebiotic (Chawla and

Patil, 2010). The FDA's formal definition for added dietary fiber states that the carbohydrate must be non-digestible and imparts a positive physiological effects on human health. This definition was introduced in May of 2016, impacting what carbohydrates can be classified as fiber, as well as the process required to establish an ingredient as a fiber (Food and Drug Administration, 2016$_a$). Prebiotic classification builds upon the dietary fiber definition by also requiring that the ingredient can be fermented by the intestinal microflora and promote growth and activity of intestinal microbes which benefit the human host (Roberfroid, 2007).

An alternative to the traditional chemical synthesis is reactive extrusion, a continuous process that uses a twin-screw extruder as the reactor. Polylactose, a polymerization product of lactose and glucose, is produced using this method (Tremaine et al., 2014). The heat applied during extrusion can cause non-enzymatic browning, influencing product appearance and quality. Flavor, aroma, and browning compounds can develop in the extruded products due to the Maillard reaction and caramelization (Heldman and Hartel, 1997$_a$). These reactions yield desirable attributes in some products; however, the intermediate compound hydroxymethylfurfural (HMF), which can also form during the reactions, is associated with negative health effects in humans (Nursten, 2005). Purification techniques, such as carbon filtration and chromatography, can be used to remove these browning compounds and refine the product (Riffer, 2000).

Increased popularity of dairy proteins and high protein, acid set, dairy products has left the dairy industry with an abundance of lactose-containing dairy co-products. Milk permeate and whey permeate contain over 75% lactose and delactosed permeate can contain a considerable amount of lactose based on the efficiency of the lactose harvesting process (Burrington et al., 2011). Acid whey, on the other hand, contains about 5% lactose in its liquid state and up to 70% in its dried state (American Dairy Products Institute, 2014; Sienkiwicz and Riedel, 1990). Permeate is currently used in a variety of food applications such as beverages and baked goods, unlike acid whey which is difficult to dry into a value added ingredient (Sienkiwicz and Riedel, 1990). Instead, acid whey is typically disposed of at the cost of the manufacturer (Elliot, 2013). Utilizing these

lactose-rich co-products as the input for oligosaccharide production would provide the dairy industry with a novel method to produce value-added ingredients from a waste stream.

There were two objectives for this project. The first was to develop a benchtop lactose polymerization method for use in pre-extrusion preliminary trials to better understand the impact of formulation on polymerization and to use this information to identify a successful reactive extrusion method to polymerize the lactose in permeate powder to oligosaccharides. The second objective was to develop a carbon filtration method to purify polylactose and reduce the HMF concentration to comply with the limit presented in the Joint FAO/WHO Expert Committee on Food Additives (JEFCA) polydextrose specification (0.1%).

2 Literature Review

2.1 Dietary Fiber

Prior to 2016, there were no U.S. regulations that standardized the definition of dietary fiber. Instead, various international and professional organizations established their own classifications. Most of these definitions stated that dietary fiber compounds originate from plant material and included nomenclature such as "non-digestible" and "resistant to digestion by human gastrointestinal enzymes" (Chawla and Patil, 2010). On May 27, 2016, the FDA published a proposed definition for dietary fiber in the Final Rule: Revision of the Nutrition and Supplement Facts Labels. Perhaps the most influential part of the new definition is the requirement for scientific evidence that demonstrates the physiological health benefit caused by consumption of the dietary fiber-containing food/ingredient. This definition could mean that the dietary fiber classification of several "added non-digestible carbohydrates" such as polydextrose, GOS and inulin is in question until the FDA confirms that there is sufficient scientific evidence that these carbohydrates have a beneficial physiological effect for humans (Food and Drug Administration, 2016$_a$; Food and Drug Administration, 2016$_d$).

Foods that are high in fiber include fruits, vegetables, whole grains and nuts (United States Department of Agriculture, 2015). The daily value for fiber is 25 g/day; however, most consumers do not achieve this recommendation (Food and Drug Administration, 2016$_b$; United States Department of Agriculture, 2015). Consumption of foods rich in dietary fiber can cause numerous health benefits including reduced low-density lipoprotein (LDL) cholesterol, reduced incidence of colon cancer and cardiovascular disease, and improved digestive regularity (Chawla and Patil, 2010; Food and Drug Administration, 2016$_d$).

The market for dietary fiber ingredients is in a period of growth with $285.4 million in sales in 2010 and an estimated $512.3 million in sales for 2017. Oligosaccharides, in particular, make up the largest section of the U.S. total food fiber sales (27.5%) (Frost and Sullivan, 2012). This increased market size is being driven by increased consumer

interest in healthy food options and continuous development of fiber-containing products (Frost and Sullivan, 2012; Mintel Group Ltd., 2016)

2.1.1 Definition

Before the FDA completed their final definition for dietary fiber in May of 2016, the main sources cited for a dietary fiber definition were the American Association of Cereal Chemists International (AACCI) and CODEX Alimentarius. The AACCI definition states:

> "Dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plants substances. Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation" (American Association of Cereal Chemists International, 2001).

The comprehensive definition published by CODEX in 2009 caused many other organizations such as Health Canada and the European Food Safety Authority (EFSA) to establish their own dietary fiber definitions (Jones, 2014). CODEX defines dietary fiber as:

> "Carbohydrate polymers with ten or more monomeric units, which are not hydrolysed by the endogenous enzymes in the small intestine of humans and belong to the following categories: edible carbohydrate polymers naturally occurring in the food as consumed, carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities or synthetic carbohydrate polymers which have been shown to have a

> physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities" (Joint FAO/WHO Food Standards Programme, 2010)

FDA published their definition of dietary fiber on May 27, 2016 in the Final Rule: Revision of the Nutrition and Supplement Facts Labels. This final rule defines dietary fiber as:

> "Non-digestible soluble and insoluble carbohydrates (with 3 or more monomeric units), and lignin that are intrinsic and intact in plants; isolated or synthetic non-digestible carbohydrates (with 3 or more monomeric units) determined by FDA to have physiological effects that are beneficial to human health" (Food and Drug Administration, 2016_a).

FDA's new definition of dietary fiber is similar to the CODEX in that it requires scientific evidence demonstrating that the dietary fiber benefits human health.

2.1.2 Prebiotics

Roberfroid, 2007 states that three criteria must be met in order for a carbohydrate to be considered a prebiotic. First, a prebiotic must be resistant to gastric acidity, human digestive enzymes and absorption in the gastrointestinal tract. Resistance to breakdown is critical so that the prebiotic reaches the large intestine intact for fermentation by microbes. Thus, the second and third criteria for a prebiotic are that it must be fermentable by the intestinal microflora and promote growth and activity of intestinal microbes which impart health benefits to the human host. Prebiotics have been shown to promote the growth of several intestinal bacteria that contribute to human health such as *Bifidobacteria*, *Enterobacteria*, *Clostridia* and *Lactobacilli*. In order for a carbohydrate to be considered a prebiotic, there should be substantial human intervention studies that demonstrate that all three prebiotic criteria are met (Roberfroid, 2007).

Dietary fibers have the ability to act as a prebiotic since they are metabolized by microbes in the human microflora which may promote health benefits in humans (Chawla and Patil, 2010). Inclusion of GOS in the diet increases growth of *Bifidobacteria* and *Lactobacilli* in the microbiota (Barreteau et al., 2006; Rodriguez-Colinas et al., 2013). Similarly, polydextrose is not digested in the upper digestive tract so it travels to the colon where fermentation by intestinal microflora can occur (Danisco Cultor America, 2002, Mead Johnson and Co., 2007).

The global prebiotic market is experiencing steady growth as consumers continue to learn about the health benefits associated with consumption of prebiotics. In 2016 the global market was worth $1.35 billion and there is 5.4% projected growth over the next 5 years, with the projected market in 2021 worth $1.76 billion. GOS dominates the global prebiotic market because of its use in the infant nutrition market, particularly in the Asia Pacific region and China (Frost and Sullivan, 2017).

2.1.3 Health benefits associated with fiber

The 2015-2020 Dietary Guidelines for Americans identified that consumption of fiber-containing foods leads to numerous health benefits for humans (United States Department of Agriculture, 2015). These health benefits span from reducing incidence of chronic disease, to improving digestive health and reducing energy intake by improving satiation. Epidemiological studies have reported both a reduction of colorectal cancer and no change in incidence for individuals with a diet containing dietary fiber (Fuchs et al., 1999; Hanson et al., 2011). Several mechanisms have been proposed that explain how fiber protects against colorectal cancer development, such as dilution of carcinogenic compounds due to increased stool bulk, fiber binds with carcinogenic compounds, and carcinogen production caused by bacterial breakdown of normal food components is limited due to a lower fecal pH (Slavin, 2001). Weight gain and obesity development has been prevented by increasing dietary fiber intake through whole grain consumption because these products provide more satiation than products containing refined grains (Liu et al., 2003). Consumption of dietary fiber has also been linked to improved laxative properties, increased stool bulk, and softening of fecal contents,

leading to overall improved digestive health (Gordon, 1989). Dietary fiber intake can have many physiological benefits, but under consumption is associated with greater risk of chronic diseases such as diabetes, atherosclerosis and obesity (Chawla and Patil, 2010).

In the United States, dietary fiber is under consumed by both men and women. Therefore, it is considered a nutrient of public health concern since low intake is associated with health problems (United States Department of Agriculture, 2015). While there is a gap between the current intake and recommended intake of dietary fiber, consumer's awareness of the health benefits associated with dietary fiber is increasing (Frost & Sullivan, 2012). Fiber is listed as the second most prominent food characteristic, behind protein, when consumers are purchasing "better-for-you" foods (Mintel Group Ltd., 2016). The majority of consumers associate dietary fiber consumption with improved digestive health (Mintel Group Ltd., 2012), but there is an opportunity to continue to promote the many benefits with fiber consumption, as well as the importance of regularly meeting the daily value for fiber (United States Department of Agriculture, 2015).

2.1.4 Fiber labeling changes

Publication of a formal dietary fiber definition by the FDA has altered which carbohydrates can be classified as dietary fiber on the nutrition and supplement labels. The dietary fiber status of non-digestible carbohydrates that are "intrinsic and intact" in plant-based foods such as fruits, vegetables, whole grains, legume and nuts is not impacted by this labeling change since they have already been shown to provide health benefits. Other foods that are unaffected by the labeling change are those that still contain "intrinsic and intact" non-digestible carbohydrates after processing, such as cereal bran, cocoa powder, flours and vegetable purees. The FDA has also concluded that there is sufficient evidence to support the dietary fiber status of seven "added" non-digestible carbohydrates: beta-glucan soluble fiber, psyllium husk, cellulose, guar gum, pectin, locust bean gum and hydroxypropylmethylcellulose (Food and Drug Administration, 2016$_c$; Food and Drug Administration, 2016$_d$).

The carbohydrates that are most affected by this labeling change are isolated or synthetic non-digestible carbohydrate ingredients. Currently, FDA is reviewing 26 of the most common fiber ingredients that fall into this classification to determine whether or not their consumption has a physiological benefit to humans. These ingredients include GOS, inulin, polydextrose, synthetic short chain fructooligosaccharides and xylooligosaccharides. At this time, the FDA has conducted a scientific review of published data on the health benefits associated with these carbohydrates and they are requesting comments to determine if there is enough data to support that the ingredients positively impact physiological health benefits to humans (Food and Drug Administration, 2016$_c$).

In order to get a new isolated or synthetic non-digestible carbohydrate approved for use as a dietary fiber, the manufacturer must submit a citizen's petition with scientific evidence proving that the ingredient contributes a physiological benefit to human health. The scientific evidence should include human intervention studies, which show the cause and effect relationship between addition of the carbohydrate to the diet and improved health. Data from animal and *in vitro* studies can only be included as supplemental evidence to propose a mechanism for how the carbohydrates are impacting human physiology (Food and Drug Administration, 2016$_d$).

2.2 Oligosaccharides

Oligosaccharides that are not digested by humans can be classified as dietary fiber and prebiotics (Meyer and Tungland, 2001). Depending on the reference, an oligosaccharide is defined as a carbohydrate with a degree of polymerization (DP) between 2 and 25 (Bailey, 1965; Barreteau et al., 2006; BeMiller and Huber, 2008). For the purposes of this paper, the IUB-IUPAC nomenclature for an oligosaccharide will be followed, which states that an oligosaccharide has a DP between 3 and 10 (Barreteau et al., 2006). Oligosaccharide characterization is based on the number of monosaccharides subunits: a trisaccharide contains three monosaccharides, a tetrasaccharide contains four monosaccharides, and so on (BeMiller and Huber, 2008). A majority of oligosaccharides are formed either through a synthesis reaction, chemical or enzymatic, or through

polysaccharide hydrolysis (Barreteau et al., 2006). Plant tissues often contain naturally occurring oligosaccharides, such as raffinose and stachyose. However, the only naturally occurring oligosaccharides in animals are galactooligosaccharides (GOS) found in mammalian milk (Bailey, 1965).

2.2.1 Polymerization reaction

Chemical synthesis of an oligosaccharide can occur via a condensation reaction in which a water molecule is removed following the combination of two monosaccharides (Bailey, 1965). Specifically, a hydroxyl group of a monosaccharide acceptor reacts with the anomeric center of a monosaccharide donor to form a glycosidic linkage. Additional monosaccharides are added to the newly formed disaccharide to build larger chains, which can then be characterized as oligosaccharides or polysaccharides (Figure 1) (Osborn and Khan, 2000). Several factors influence the final oligosaccharide structure including which hydroxyl group on the acceptor participates in the reaction and the structural form of the monosaccharide. The newly formed glycosidic bond also has two possible configurations, α and β (Bailey, 1965; Osborn and Khan, 2000). In order to achieve the desired configuration, environmental and stereochemical controls must be in place. Environmental controls include pH, temperature and solvent type, while stereochemical controls include addition of protecting groups and careful selection of the acceptor to ensure that the required hydroxyl group reacts (Osborn and Khan, 2000).

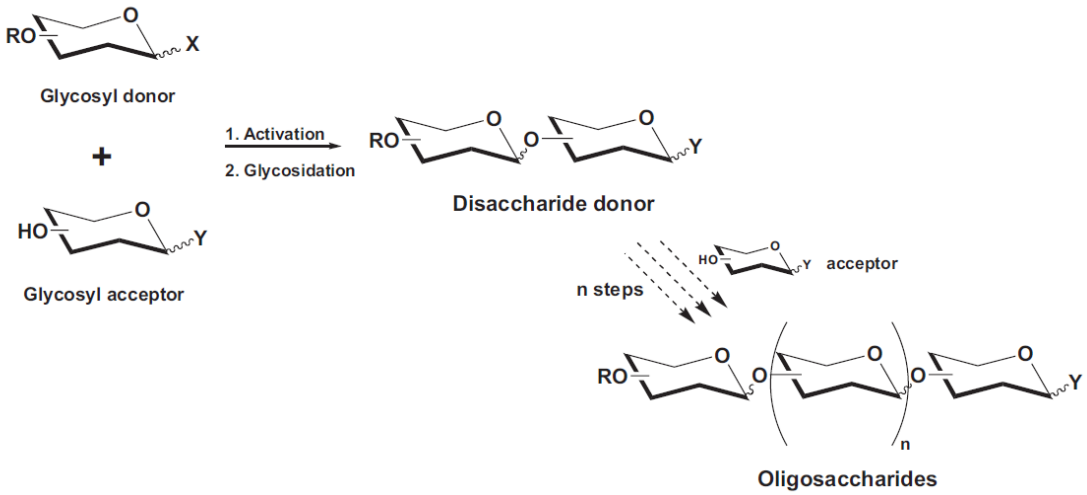

Figure 1. Polymerization Reaction Schematic (Barreteau et al., 2006)

2.2.1.1 Chemical synthesis

There are three possible pathways for carbohydrates that participate in this type of chemical reaction: hydrolysis, polymerization, or no reaction. Hydrolysis and polymerization are reversible equilibrium reactions which are influenced by the water content in the reaction system (Leuck, 1945). Certain parameters, specifically acid content and heat, can be manipulated to promote polymerization instead of hydrolysis (Collins and Ferrier, 1995). Additional conditions that influence the extent of acid-catalyzed chemical synthesis are pH, temperature, reaction duration and the sugar's structure (BeMiller and Huber, 2008).

Chemical synthesis of an oligosaccharide is unspecific, meaning a mix of α and β-glycosidic linkages can form in a variety of different spatial configurations (Bailey, 1965). The incorporation of specific glycosyl donors and acceptors with added protecting groups can be used to reduce the randomness of the reaction. To achieve a specific structure, the donor has all the hydroxyl groups except for one at its anomeric carbon protected with an acetate or benzoate ester, and the acceptor has all but one free hydroxyl group protected with similar esters (Osborn and Khan, 2000).

Commercial production of oligosaccharides using this type of synthesis is currently a challenge (Barreteau et al., 2006). The incorporation of multiple protection and

deprotection steps increases regio- and stereospecificity and yield, while increasing the reaction time and adding non-food grade chemicals that must be removed (Endo and Koizumi, 2000; Barreteau et al., 2006). Chemically synthesized oligosaccharides are manufactured using a batch process in the food industry. However, this method is associated with a long reaction time and high operating costs and the reactor can become corroded, causing manufacturers to prefer enzymatic synthesis over chemical synthesis (Hwang et al., 1997; Rastall and Gibson, 2002).

Polydextrose is the main example of a commercial oligosaccharide that is produced through a chemical synthesis (Mead Johnson and Co., 2007). Xylooligosaccahrides can also be produced using a chemical synthesis, but enzymatic synthesis is favored in the industry (Vazquez et al., 2000).

2.2.1.2 Enzymatic synthesis

Enzymatic reactions yield oligosaccharides that are more specific, particularly in the type of glycosidic bond that is formed, α or β, and the location of the bond (Bailey, 1965; Osborn and Khan, 2000). The category of enzymes responsible for this type of synthesis is a group of over 30 lyases called aldolases. High substrate specificity and selectivity are characteristics of these enzymes, which facilitates reactions typically free of epimerization, racemization and rearrangements (Brito-Aria, 2007).

Glycosidases and glycosyltransferases are specific examples of aldolases used to facilitate the formation of the glycosidic linkage between a donor and acceptor in oligosaccharide synthesis. An alternative to a glycosyltransferase is a glycosylhydrolase which is more readily available, but less regio-specific (Barreteau et al., 2006). Dextransucrases and glucanotransferases are examples of commercially used glycosyltransferases and β-galactosidase is a glycosylhydrolase (Plou et al., 2002; Semenza and Aurrichio, 1989). Under the correct conditions this enzyme's function can be altered from causing hydrolysis to promoting synthesis of glycosidic bonds (Maugard et al., 2003).

Another advantage to leveraging enzyme's regiospecificity is that protecting groups do not need to be added, resulting in a more efficient synthesis and a reduction in the

amount of purification steps required post-polymerization. However, the use of enzymes does have several challenges including solubility issues in organic solvents needed for the reaction, heat and pH sensitivity during processing, in addition to high cost and availability of the enzymes (Osborn and Khan, 2000; Gosling et al., 2010).

A majority of synthetic oligosaccharides are produced using an enzymatic synthesis, such as GOS, fructo-oligosaccharides (FOS) and xylo-oligosaccharides (Environ International Corporation, 2000; Environ International Corporation, 2007; Shangdong Longlive Biotechnology, Ltd., 2013; Vazquez et al., 2000).

2.2.1.3 Lab-scale production of oligosaccharides

Chemical and enzymatic synthesis of oligosaccharides has been achieved on a lab-scale. Melt-polymerization of several sugar monomers was achieved by heating the sugars at 170°C for 5 hours under vacuum in a round bottom flask. Heating sugars, such as glucose, xylose, fucose and a glucose-galactose blend, resulted in the formation of oligosaccharides (Daines et al, 2015). A similar thermal polymerization procedure was also successfully used to create trisaccharides and a novel fructoglucan from a mixture of sucrose and citric acid (Manley-Harris and Richards, 1991; Manley-Harris and Richards, 1993).

Another technique that has been used for lab-scale oligosaccharide synthesis is microwave irradiation. Daines et al., 2015 found that heating a blend of lactose, sorbitol, citric acid and water in a microwave reactor yielded oligosaccharides with DP 2-6. However, the presence of glucose and galactose in the final product indicated that lactose hydrolysis was also occurring. These researchers hypothesized that the lactose was not directly polymerized; instead, the monomers were undergoing a melt polymerization to form oligosaccharides. Therefore, they developed a two stage process consisting of microwave hydrolysis of lactose followed by conventional melt polymerization to produce a material with a high polymeric content similar to commercial polydextrose.

Microwave irradiation has also been used for the enzymatic synthesis of GOS. Use of an immobilized enzyme with water and a hexanol co-solvent increased the selectivity for GOS synthesis by 217-fold, when compared to a conventional heating method that used

water and a free enzyme. During the conventional method, a reduction of GOS after 2 hours was reported, which was likely due to hydrolysis. Heating with the microwave method did not cause hydrolysis, meaning there was higher selectivity for the polymerization reaction, yielding a higher oligosaccharide content in the final product (Maugard et al., 2003).

2.2.2 Oligosaccharides produced from lactose or glucose

Oligosaccharides can be formed from a variety of different mono and disaccharides; however, glucose and lactose will be the focus for this literature review. GOS and polydextrose are two commercially available products that are produced from lactose and glucose, respectively. These oligosaccharides were both classified as a dietary fiber and prebiotic according to the old dietary fiber labeling regulations (Gosling et al., 2010; Mead Johnson and Co., 2007). Polylactose is a polymerization product of lactose and glucose that has been developed in the Schoenfuss lab in the Department of Food Science and Nutrition at the University of Minnesota – Twin Cities (Tremaine et al., 2014).

2.2.2.1 Galactooligosaccharides

Galactooligosaccharides (GOS) can be synthesized both naturally and through a conversion of lactose to oligosaccharides with varying degrees of polymerization. The trisaccharides, β(1→4)-galactosyllactose and β(1→6)- galactosyllactose are found in both human milk and commercially produced GOS (Schoterman, 2001). These oligosaccharides are considered to be prebiotics because they are not digested and they stimulate the growth of *Bifidobacterium* in humans, which leads to health benefits such as improved intestinal microflora and improved calcium absorption (Hughes and Hoover, 1995; Macfarlane et al., 2008; Vandenplas, 2002). GOS are often incorporated into infant formulas to mimic the benefits that human milk oligosaccharides impart in breast-feed infants, specifically antimicrobial and prebiotic activity (Angus et al., 2005; GTC Nutrition, 2009). The prebiotic classification of these oligosaccharides, coupled with their high heat and acid stability make them an attractive ingredient for use in food products (Schoterman, 2001).

In an industrial setting, these reactions typically occur in a batch reactor or in a continuous, ultrafiltration membrane reactor where the enzyme can be separated and used again (Gosling, et al., 2010).

2.2.2.1.1 GOS synthesis

During this synthesis of GOS, β-galactosidase, a glycosylhydrolase, synthesizes a transgalactosylation reaction (Gosling et al., 2010). While this enzyme is known for its ability to hydrolyze lactose, particularly in the manufacture of lactose-free dairy products, under the proper conditions it can also promote polymerization. In order for GOS synthesis to occur, the galactosyl acceptor must be another carbohydrate, instead of water which would promote lactose hydrolysis (Rodriguez-Colinas et al., 2012). Gosling et al., 2010 collected data from several studies on lactase enzymes used to synthesize GOS and reported that the ideal conditions for enzymatic synthesis are a pH range of 4.5 - 7.0, lactose concentration between 200 g/L and 600 g/L and a temperature range of 40-80°C. In addition, enzymes from different sources also have an impact on the final GOS yield and structure. Studies have shown that GOS synthesized with β-galactosidase from *Bacillus circulans* contain only β(1→4) linkages, while products synthesized from *Kluyveromyces lactis* sourced β-galactosidase contain only β(1→6) linkages. A typical optimized yield from enzymatic GOS synthesis is between 30% and 40% (w/w) (Gosling et al., 2010). One of the key factors to maximizing the GOS yield is to determine the ideal time to end the reaction so that the competition between hydrolysis (degradation) and transgalactosylation (synthesis) is minimized (Rodriguez-Colinas et al., 2012). If the reaction proceeds too long then hydrolysis becomes the dominating reaction and the lactose is broken down into glucose and galactose instead of forming oligosaccharides (Gosling et al, 2010).

2.2.2.1.2 Novel products with GOS

One unique method for GOS production leverages lactose removal in fluid milk through an enzymatic hydrolysis with β-galactosidase. As the lactose is broken down GOS can be formed, providing a unique opportunity for enhancing milk with a prebiotic.

β-galactosidases from *B.circulans* and *A.oryzae* yielded a maximum amount of GOS (4-6 g/L) at 40-50% of lactose conversion. This means that the milk enhanced with GOS would still contain about 50% of its initial lactose concentration. However, β-galactosidase from *K.lactis* produced the maximum amount of GOS (7.0 g/L) when about 95% of the lactose was converted. Since a majority of the lactose was converted, use of the *K.lactis* β-galactosidase could produce a beverage suitable for lactose intolerant consumers with an added dietary fiber and prebiotic benefit (Rodriguez-Colinas et al., 2014).

2.2.2.2 Polydextrose

Polydextrose was first invented by Pfizer Central Research in the late 1960s as a reduced calorie replacement for sugar and partial replacer for fat, flour and starch (Craig, 2001). In 1973 the technology for this ingredient was patented as U.S. Patent 3,766,176: "Polysaccharides and Their Preparation". This patent outlined the production of polydextrose and its use in several dietetic foods to impart the physical properties of natural sugars without adding nutritive value, replace starch, and prevent textural and overall quality changes (Rennhard, 1973).

This technology leveraged previous research on the production of glucose polymers in the presence of acid catalysts. The original glucose polymers produced with an acid catalyzed chemical synthesis required additional purification steps to remove inedible acid catalysts and separate the oligosaccharides from the aqueous or non-aqueous reaction media before they could be used as food ingredients (Mora, 1951). A melt condensation of glucose in the presence of edible organic acids and sorbitol is utilized to produce polydextrose (Rennhard, 1973). Citric acid or phosphoric acid are primarily used as the acid catalyst and cross-linkers for this reaction (Mead Johnson and Co., 2007). A vacuum was also incorporated into the reaction system to eliminate water produced during the condensation reaction and exclude air, in addition to minimizing decomposition and discoloration of the polydextrose by reducing the temperature required for the reaction. Combining glucose with sorbitol prior to the melt condensation improved processing in addition to color and taste. In this product sorbitol acts as a

plasticizer, which reduced the viscosity to improve processing and the final product, and it terminates the reaction to limit branching of the oligosaccharide (Rennhard, 1973). Impurities such as free glucose, sorbitol, levoglucosan (1,6-anhydro-D-glucose) and citric/phosphoric acid may be present in the polydextrose. After production, polydextrose can be neutralized with potassium hydroxide, reduced via hydrogenation with a transition metal catalyst or purified via decolorization and deionization (Joint FAO/WHO Expert Committee on Food Additives, 1998; 21 CFR 172.841, 2016).

Polydextrose is typically composed of 90 parts D-glucose and 10 parts sorbitol, with up to 1 part citric acid or 0.1 part phosphoric acid (Joint FAO/WHO Expert Committee on Food Additives, 1998; Danisco Cultor America, 2002, Mead Johnson and Co., 2007). The chemical synthesis causes random bonding between glucose-glucose and glucose-sorbitol, meaning the position of the glycosidic linkage is found in the β1-2, 1-3, 1-4, and 1-6 position (Figure 2) (Danisco Cultor America, 2002). An orientation of β1-6 is the most commonly found glycosidic linkage in polydextrose (Craig, 2001, Danisco Cultor America, 2002, Rennhard, 1973). A benefit of the random bonding is protection from enzyme activity and acid hydrolysis, particularly in the digestive tract (Danisco Cultor America, 2002, Mead Johnson and Co., 2007). Polydextrose has an average degree of polymerization of 12 and an average molecular weight of about 2,000 Da (Rennhard, 1973). Even though polydextrose contains a high enough degree of polymerization to be considered a polysaccharide, it does not analyze as a polysaccharide during precipitation with aqueous 80% ethanol (Craig, 2001). Because this oligosaccharides is not digested by humans and it stimulates the growth of *Bifidobacteria*, it is classified as a prebiotic (Jie et al., 2000; Probert et al., 2004).

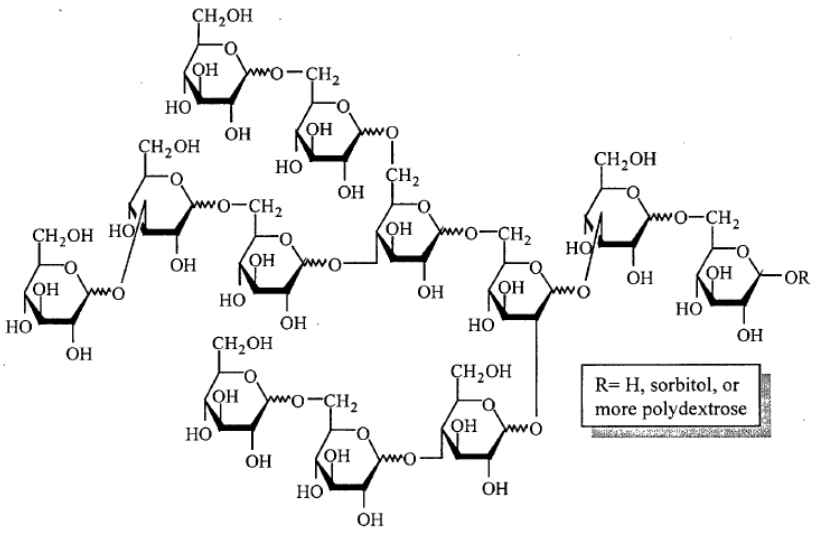

Figure 2. Polydextrose Structure (Danisco Coltor America, 2002)

Several approved uses for polydextrose include acting as a bulking aid, formulation aid, humectant and texturizer in all foods except meat, poultry, baby food and infant formula (21 CFR 172.841, 2016). Crunchiness in fried products and breakfast cereals is improved with polydextrose addition, as well as an increase in shelf life since the carbohydrate acts as a humectant (Danisco Cultor America, 2002). Polydextrose can also be added in combination with GOS as a prebiotic in infant formula (Mead Johnson Co., 2007) The Joint FAO/WHO Expert Committee on Food Additives (JECFA) determined in 1987 that polydextrose does not pose any health hazards to humans, meaning an acceptable daily intake level does not need to be specified (JECFA, 1987).

2.2.2.3 Polylactose

Hwang et al. 1997 first suggested the idea of utilizing an extruder as the reactor to facilitate polymerization of sugars. This group demonstrated that the high temperature, pressure and shear force from the extruder could be leveraged to yield a continuous method for polymerizing sugars. Tremaine et. al. 2014 built upon this study to develop polylactose, an extrusion polymerization product composed of lactose and glucose. The chemical synthesis of polylactose is catalyzed by citric acid. Various combinations of these three materials yielded between 37.1 and 59.8% (w/w, of starting material)

indigestible oligosaccharides, with over 90% of these oligomers being characterized as low molecular weight soluble dietary fiber through solubility analysis in 80% ethanol. The oligosaccharides produced in this study included trisaccharides, tetrasaccharides pentasaccharides and larger polymers. Formulation alterations impacted the polymerization yield; as the amount of citric acid catalyst increased, the yield of indigestible oligosaccharides also increased (Reid, 2015).

2.2.2.3.1 Factors affecting polymerization

Several factors related to the extrusion process have been identified that influence polymerization in polylactose, including temperature, feed rate and specific mechanical energy (SME). A higher fiber yield is achieved with a slower feed rate, which increases residence time and the SME, also described as the amount of shear that the product experiences. The temperature used influences the melting of the sugars and the browning of the product (Reid, 2015).

Formulation, specifically citric acid content and glucose addition, as well as the lactose source impact the production of polylactose. It was found that increasing the citric acid content increases the fiber yield in the product (Reid, 2015; Tremaine et al., 2014). Glucose addition improved processing of the polylactose by reducing the melting temperature of the sugar-acid blend and reducing the viscosity in the extruder. This viscosity change can be attributed to a phenomenon called deliquescence and the glucose monohydrate contributes additional moisture to the system (Tremaine et al., 2014). Crystalline lactose monohydrate has a higher melting temperature, 160 to 214°C, than glucose, 130 to 176°C (Lee et al., 2011; Raemy et al., 1983; Roos, 1993). The release of water during the condensation polymerization reaction, deliquescence and the added moisture from the glucose can help reduce the melting point of the crystalline sugars (Roos et al., 2013).

Experimentation has occurred with acid whey and permeate as the lactose source for polylactose extrusion; however, polymerization was not achieved. The product produced from the extrusion of acid whey with citric acid was black and it pitted the extruder, while the permeate-citric acid blend yielded a caramelized product without

polymerization. These dairy co-products are not a pure form of lactose, meaning that the other components such as protein and minerals could have an impact on the polymerization reaction.

2.2.2.3.2 Deliquescence

Water-solid interactions can significantly impact chemical and physical stability of food systems, influencing quality and shelf life. Deliquescence, a first-order phase conversion, can negatively impact food and pharmaceutical products, particularly powders containing water soluble crystalline compounds (Mauer and Taylor, 2010). Many food ingredients are deliquescent including crystalline sugars (sucrose, glucose, lactose and sorbitol), low molecular weight organic acids such as citric acid, and inorganic salts and vitamins (Salameh et al., 2006). At and above the deliquescence point, also known as critical relative humidity (RH_0), these deliquescent compounds interact with atmospheric water causing the surface of the solid particle to dissolve and form a saturated solution. Bulk water increases in the system as the water associated with the deliquescent material is released, which then dissolves the remainder of the crystal and surrounding crystals. Below the deliquescence point, minimal interaction occurs between water and the solid particle. The occurrence of deliquescence has a significant impact on colligative properties and can cause caking and processing challenges with powdered products. (Salameh et al., 2006; Mauer and Taylor, 2010).

When deliquescent compounds are combined, the deliquescence point is reduced. The number of total solutes in the saturated solution increases when more than one ingredient is present which drives the deliquescence point down (Allan et al., 2016). Many individual food ingredients, such as lactose and glucose, have a high critical relative humidity that is above 90% relative humidity at 25°C. Storage and processing conditions can be altered to ensure that a processing environment's relative humidity doesn't get that high. However, when ingredients are combined, a significant reduction in the critical relative humidity occurs. For example, a combination of glucose (91% RH_0 at 25°C) and citric acid (78% RH_0 at 25°C) reduces the relative humidity to 68% RH_0 at 25°C. This

humidity level is more feasible in manufacturing and storage, meaning caking and other quality problems may occur (Salameh et al., 2006).

With relation to polylactose production, the deliquescence point is likely lowered since three deliquescent materials are mixed. The addition of glucose to the lactose and citric acid blend was needed to improve flow through the extruder. By combining these three ingredients, the deliquescence point was likely lowered to a humidity level experienced during the extrusion process. Therefore, bulk water increased in the system which improved dissolution of the crystalline ingredients and reduced viscosity (Tremaine et al., 2014).

2.3 Alternative lactose sources for the polymerization reaction

An increased demand for high-protein dairy ingredients has simultaneously caused an increase in production of co-products such as milk permeate, whey permeate and delactosed permeate. These products are the waste stream from the manufacture of milk protein concentrate, whey protein concentrates and isolates and after lactose crystallization in whey and milk permeate, respectively. After the protein is removed, whey and milk permeates are left with over 76% lactose and despite its name, delactosed permeate can contain considerable amounts of lactose as well (Burrington et al., 2011).

Acid whey is another potential source of lactose for use in polymerization reactions. GOS have been produced from acid whey via an enzymatic synthesis with β-galactosidases from *A. oryzae* and *K. lactis* (Fischer and Kleinschmidt, 2015). Sweet whey permeate has also been used as a substrate for GOS production through enzymatic synthesis (Rustom et al., 1998; Corozo-Martinez et al., 2012).

Production of oligosaccharides from these coproducts and acid whey would provide the dairy industry with novel, alternative uses for the co-products that tap into the increasing fiber market. However, the composition of these substrates poses a challenge for oligosaccharide synthesis. All of the products contain elevated levels of minerals, in addition to protein and lactic acid in acid whey. The minerals in particular have impacted enzymatic synthesis of GOS because they can act as inhibitors or acceptors depending on which cations are present (Fischer and Kleinschmidt, 2015).

2.3.1 Acid whey

Production of several acid set dairy products, including Greek yogurt, cottage cheese and fresh cheeses such as quark, cream cheese and queso blanco, results in a waste product called acid whey (Sienkiweicz and Riedel, 1990, Walstra et al., 2006). The composition of acid whey differs from its counterpart sweet whey, most drastically in the ash and protein contents, and the liquid's pH (Gonzalez Siso, 1996; Fischer & Kleinschmidt, 2015). Acid whey's pH (4.6) is much more acidic than sweet whey (6.1) and it contains less protein. Calcium is also higher in acid whey because the colloidal calcium phosphate (CCP) inside the casein micelle solubilizes at the lower pH, causing it to leave the micelle and migrate to the acid whey (Wong et al., 1978; Sienkiweicz and Riedel, 1990; Zall, 1992; Pesta et al., 2007). Lactose content in liquid acid whey is comparable to the levels found in liquid sweet whey, about 4.5-5.0%. After the acid whey is dried, however, the lactose concentration can increase to over 50% (Sienkiweicz and Riedel, 1990).

An increase in production of acid-set dairy products, particularly Greek-style yogurts, has tremendously increased the volume of acid whey production in the U.S. In 2013, it was reported that New York State produced more than 550 million liters of acid whey annually, and production continues to increase with the soaring popularity of Greek yogurt (Elliot, 2013). Limited disposal methods are available for this abundance of acid whey due to its composition. Environmental disposal methods are hindered because of the high lactose content and low pH and further processing of acid whey into value added ingredients is also a challenge (Smithers, 2015). Some Greek yogurt manufacturers don't have the capabilities to dispose of the waste on their own and they would likely overpower the local wastewater treatment plant with the acid whey. Therefore, these manufacturers are being forced to pay farmers to take their acid whey for use as fertilizer or as a feed supplement, which reduces their profits (Charles, 2012, Elliot, 2013).

2.3.1.1 Acid set dairy product manufacturing

A variety of dairy products can be produced via an acid coagulation method including Greek yogurt, cottage cheese, quark and cream cheese. The acidifying agent can be

either a direct addition of acid or through lactic acid bacteria activity (Walstra et al., 2006). Insolubility of casein at a pH of 4.6 is the main mechanism governing this reaction (Lucey, 2013; O'Mahoney and Fox, 2013). As the pH decreases, the CCP leaves the casein micelle, neutralizing the negative charge that casein typically holds. Neutralization of the negative charge leads to a loss of steric stabilization causing aggregation of the casein micelles. The resulting gel is an irreversible gel of milk proteins (Lucey, 2009).

Greek yogurt, one of the main sources of acid whey production, utilizes an acid-set coagulation for gelation. Lactic acid production in this product occurs through thermophilic fermentation of glucose with the bacteria *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. In yogurt manufacturing, milk is first homogenized and pasteurized and then cooled prior to addition of the starter cultures. Depending on the type of yogurt, set yogurt or stirred yogurt, the product is then either packaged and incubated or incubated, stirred and then packaged (Walstra et al., 2006). Production of Greek-style yogurt requires a straining step to concentrate the solids and remove acid whey. Traditionally, the product was strained by placing the yogurt in a cloth bag and hanging it so that the acid whey could drain out. Today, the yogurt is typically concentrated using a centrifuge, quark separator, to achieve the higher solids percentage typical of Greek-style yogurt (Nsabimana et al., 2005).

2.3.1.2 Challenges with acid whey

Several technologies have been developed to convert sweet whey from a waste stream to a co-product of dairy manufacturing. The utilization of reverse osmosis and ultrafiltration membranes allows for the separation of whey into its main components: protein, lactose, and delactosed permeate (Pesta et al., 2007). Whey proteins are isolated by first concentrating the whey through evaporation, then separating the components through either microfiltration or ultrafiltration and lastly crystallizing the lactose (Pearce, 1992). Once the concentrated whey is spray dried, the most common resulting protein concentrates are either Whey Protein Concentrate (WPC), 35-80% total protein, or Whey Protein Isolate (WPI), 90% or more protein (Walstra et al., 2006). Lactose can also be

separated out from whey and converted to a value-added ingredient through crystallization, centrifugation and drying. These ingredients have a variety of uses in food products including providing added nutrient value and imparting functional properties (Morr, 1992; Holsinger, 1997; Walstra et al., 2006).

Acid whey, on the other hand, cannot be processed in the same manner due to its composition. Spray drying and roller drying are not effective for treatment of acid whey (Sienkiweicz and Riedel, 1990). Disposal of this by-product via environmental means is also prohibited due to its composition (Smithers, 2015). Therefore, manufacturers have been struggling to find appropriate disposal methods for vast amounts of acid whey in the current dairy supply chain.

2.3.1.3 Disposal

The high Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) associated with acid whey is the reason for the many disposal challenges. Acid whey has a BOD of 35,000 mg/L and a COD of 60,000 mg/L (Morr, 1992; Hale et al., 2003). These high numbers can be attributed to the high levels of lactose in the waste (Gonzalez Siso, 1996). Manufacturing facilities that are close to urban areas will either send waste to the municipal waste water treatment facility or utilize an on-site anaerobic digestion method. Whey has a waste strength that is 300 times stronger than typical municipal sewage, which not only makes it difficult to treat, but also increases the cost of disposal (Zall, 1992). The excess volumes of this waste, in combination with the high organic load, could overload a municipal facility if this disposal method was always used (Durham and Hourigan, 2007).

Other cheaper alternatives for disposal include natural applications such as spreading the whey on fields and incorporating it into animal feed. Spreading the whey on fields promotes plant growth due to its water and nutrient content (Morr, 1992). However, excess land application would adversely impact the soil's structure and salinity balance due to the dissolved salts and potential contaminants in the whey (Durham and Hourigan 2007). Over use for land applications could also lead to the production of run-off water. The high BOD of this waste steam would cause harm to the ecosystem if it were

incorporated into an aquatic environment. Microbes in the waterways use up the dissolved oxygen in the water to digest the large amounts of lactose found in the acid whey. This state of reduced dissolved oxygen in water, called hypoxia, is detrimental to most aquatic animal life (Diaz and Breitburg, 2011).

2.3.1.4 Animal feed

An alternative to acid whey disposal is to utilize it for use in livestock feed. Liquid and dried acid whey has been incorporated into pig and cattle feed and silage has been supplemented with acid whey as well (Marwaha and Kennedy, 1988; Smithers, 2015). Adding whey into the diet can enhance several nutritional benefits for the animals such as contributing protein and minerals such as calcium, phosphorus, sulfur, in addition to providing energy from the lactose (Sienkiewicz and Riedel, 1990).

While incorporation of acid whey in animal feed does reduce the volume that needs to be disposed, there are also several challenges. Transportation of liquid acid whey from the production facilities to the farm is expensive and production of dried acid whey is a challenge that increases the ingredient's cost (Sienkiewicz and Riedel, 1990). The high mineral content in acid whey limits the amount that can be incorporated into feed because it can cause gastrointestinal problems for dairy cattle, which then reduces milk production. In addition, far more acid whey is produced than can be used in feed so it does not solve the acid whey disposal problem. Therefore, the incorporation of acid whey into livestock feed is not an economically feasible option that would alleviate the problem of acid whey disposal (Marwaha and Kennedy, 1988, Sienkiewicz and Riedel, 1990).

2.3.1.5 Stickiness with drying

A majority of dairy co-products are dried via spray drying or roller drying to manufacture a powdered ingredient. Production of an acid whey powder is very challenging because the product becomes sticky during drying, which reduces drying efficiency and produces a product with low shelf stability (Sienkiewicz and Riedel, 1990).

Research focused on explaining the stickiness and hygroscopicity of delactosed permeate identified that composition had a large impact. As with acid whey, delactosed permeate (DLP) contains lactic acid and minerals, which have been shown to impact drying unit operations and stability after drying. The Peleg model was used to evaluate water absorption and the Peleg constants K_1 and K_2 were specifically monitored to measure initial sorption rate (moisture uptake) and sorption capacity (moisture adsorption), respectively (Liang et al., 2009; Bund and Hartel, 2010). Liang et al., 2009 found that an increase in both lactic acid and minerals caused a decrease in the rate constants which means initial sorption rate and maximum sorption capacity increased. Potassium, chloride and sodium were identified as the minerals having the biggest influence on sorption behavior. Lactose alone did not have a large impact on sorption, yet the amount of total sugars influenced uptake of water. This suggests that sugars besides lactose impact sorption (Liang et al., 2009). Addition of whey protein isolate to the DLP decreased the amount of moisture adsorption, by contributing to an increase in both rate constants. It is hypothesized that addition of the protein reduced the lactic acid and minerals' ability to interact with water (Bund and Hartel, 2010).

Glass transition temperature is one of the main factors governing moisture sorption and it impacts drying and powder quality post-drying. Lactic acid has a glass transition less than -50°C which negatively impacts drying (Liang et al., 2009). Conversely, proteins have a higher glass transition temperature, so adding protein to delactosed permeate can improve drying characteristics by increasing the product's glass transition temperature (Bund and Hartel, 2010). Stickiness associated with drying acid whey, is therefore likely due to the presence of lactic acid and minerals. The low protein content in acid whey is likely not sufficient to help offset the very low glass transition temperature of lactic acid, resulting in a hydroscopic product that is hard to dry and has low stability post-drying.

2.3.2 Permeate

Membrane filtration of whey and milk yields whey protein concentrates and isolates and milk protein concentrates, respectively. Once the protein is removed, the remaining

product is called whey permeate and milk permeate (Burrington et al, 2011). These products contain a minimum of 76% lactose, 2-7% protein and milk minerals and organic acids (United States Dairy Export Council, 2015). After filtration, the permeate is then spray dried to create permeate powder (Morr, 1992).

Whey permeate, also called deproteinized whey or modified whey, is known to have good solubility and an appealing dairy flavor (Burrington et al., 2011; United States Diary Export Council, 2015). Milk permeate has an even cleaner flavor profile since it does not go through the cheese making process which introduces flavors such as acid and bitterness (United States Diary Export Council, 2015).

2.3.2.1 Delactosed permeate

Lactose can be harvested from milk or whey permeate through a series of crystallizing and decanting steps for use as a food ingredient. After the lactose is removed, the co-product is called delactosed permeate (Burrington et al. 2011). This removal process is not completely efficient, meaning delactosed permeate actually contains a considerable amount of lactose. Milk minerals, organic acids, and other residual sugars, are also found in delactosed permeate. The high moisture content (~60%), coupled with the low glass transition temperature of lactic acid make delactosed permeate very hygroscopic and difficult to dry (Bund and Hartel, 2010; Liang et al., 2009). Since the product is so difficult to process, it is typically just used in animal feed or spread on fields as a fertilizer (Vembu and Rathinam, 1997).

Composition of delactosed permeate varies greatly between manufacturers due to the differences in the whey and milk permeate manufacturing, which include variances in the cheese and whey manufacturing processes, milk composition variability, and differences in lactose refining procedures (Liang et al, 2009).

2.3.2.2 Permeate and delactosed permeate food applications

Permeate and sweet whey are compositionally similar so permeate can be used as a substitute when building the solids content of a food product or when whey or lactose are being used. These co-products have been used in beverage applications, in addition to

confectionary products, dips and sauces. Permeate, in particular, can enhance the brown color of baked goods because the high levels of lactose can participate in the Maillard reaction (Frankowski et al., 2014; U.S. Dairy Export Council, 2015).

Both permeate and delactosed permeate have shown promise as salt substitutes. These co-products contain NaCl and KCl which contribute a salty taste and lactic and orotic acid which enhance salty perception (Frankowski et al., 2014). Since delactosed permeate undergoes more concentration and separation unit operations than permeate, it contains a higher amount of minerals and organic acids. The elevated levels of these components increase the salty taste perception of delactosed permeate. In a sensory evaluation conducted by Smith et al., 2016, the salt in a cream of broccoli soup was replaced with milk, Cheddar whey and Mozzarella whey permeate and delactosed permeate. While the panelists preferred the salt-containing control, the soup with the milk, Cheddar whey and Mozzarella whey permeate were liked more than the no-salt control, and the soup with delactosed permeate was at parity with the no-salt control. The panelists indicated that these samples were also saltier than the no-salt control (Smith et al., 2016).

The U.S. Dairy Export Council reported that only 20% of permeate produced in the U.S. is used for food applications (Burrington et al., 2011), meaning there is a large opportunity to identify novel uses for permeate and delactosed permeate.

2.4 Non-enzymatic browning reactions occurring during extrusion of sugars

Browning of foods during processing and storage can have both a desirable and undesirable impact. Some products rely on the flavor, aroma and color from non-enzymatic browning, such as coffee, bread and caramel candies, while other product's quality can deteriorate with browning, such as milk powder and dehydrated fruits. The extent of these reactions is governed by the food's composition as well as processing and storage conditions (BeMiller and Huber, 2008; Sikorski et al., 2008). Nonenzymatic browning includes caramelization of sugars and the Maillard reaction which produce browning pigments called melanoidins. Depending on a food's composition and the

processing parameters, it is possible to undergo both reactions in one product (Sikorski et al., 2008).

2.4.1 Caramelization

Development of browning color, aroma and flavor compounds, without free-amine containing compounds is referred to as caramelization (Hodge, 1953). Sucrose is one of the most common sugars that participates in a caramelization reaction, but other reducing sugars can also be a reactant. Various acids and salts are added to influence what kind of caramel color is produced (Nursten, 2005). Three reactions have been identified as the main reactions in caramelization – 1,2-enolization, dehydration to furfurals and fission (Feather and Harris, 1973). The resulting compounds are a complex mixture of polymeric material, with an unidentified structure. Increasing conditions such as temperature and pH increases the extent of caramelization (BeMiller and Huber, 2008).

2.4.1.1 Caramel Colors

The standard of identity for caramel colors states:

> "The color additive caramel is the dark-brown liquid of solid material resulting from the carefully controlled heat treatment of the following food-grade carbohydrates: dextrose, invert sugar, lactose, malt syrup, molasses, starch hydrolysates and fractions thereof, and sucrose" (21CFR73.85, 2016).

Specific food-grade acids, alkalis and salts are also identified for use in caramel color production, which include acetic acid, citric acid, ammonium, potassium and sodium hydroxide and ammonium, sodium and potassium carbonate (21CFR73.85, 2016).

According to the Joint FAO/WHO Expert Committee on Food Additives, 2006 the four classes of caramel color (I to IV) are differentiated by the acids, alkalis and salts that are used during the heating of a food-grade sugar. Both Class I (plain caramel or caustic caramel) and Class II (caustic sulfite caramel) utilize sodium hydroxide or other acids and bases, while Class II also includes a sulfite addition in manufacturing. Carbohydrates are heated in the presence of ammonium ions in the production of Class

III (ammonium caramel) and Class IV (sulfite ammonium caramel), with the manufacturing of Class IV also including sulfites. Acids or bases can also be incorporated when producing Class III and Class IV caramel colors (Joint FAO/WHO Expert Committee on Food Additives, 2006). The browning compounds in Class III and IV caramel colors are believed to be similar to the melanoidins produced by the Maillard reaction since ammonium is used in the manufacture. Processing differences for caramel colors impacts the pH and the mixture of browning compounds, thereby impacting the end uses of the product. Class III caramel colors have a solution pH between 4.2 and 4.8 and it is commonly used in bakery products and syrups, while Class IV caramel colors are more acidic (pH 2 – 4.5) and they are best known for use in soft drinks and other acidic beverages (European Food Safety Authority, 2011_b; Nursten, 2005; BeMiller and Huber, 2008)

Caramel colors have come under scrutiny because of the presence of 4-methylimidazole (4-MEI), a browning compound formed during production that has been shown to increase lung tumor occurrence in mice. Both the Food and Drug Administration (FDA) and European Food Safety Authority (EFSA) had assessed the risk of 4-MEI in food and have determined that this compound is not in high enough quantities in foods to pose a risk to human health (European Food Safety Authority, 2011_b; Food and Drug Administration, 2014).

2.4.2 Maillard reaction

The Maillard reaction is an intricate reaction that causes the production of browning, aroma and flavor compounds in food products during processing at elevated temperatures and throughout extended storage. Reaction conditions, type of reactants and the occurrence of several intermediate pathways contribute to the complexity of the reaction and its products (BeMiller and Huber, 2008).

Three reactants are required for the Maillard reaction to proceed - a reducing sugar, a free amine group and water (Nursten, 2005). Examples of reducing sugars include glucose, fructose, ribose, lactose and maltose. The amine can be in the form of proteins,

peptides or amino acids and the ε-amino group found in the lysine residue is considered to be the main reactive group of the protein (Sikorski et al., 2008).

This complex reaction can be divided into three distinct stages– initial stage, intermediate stage and final stage (Hodge, 1953; Sikorski et al., 2008). Hodge, 1953 described the Maillard reaction in detail. The initial stage begins with a dehydration reaction between the reducing sugar and the amino group. An unstable Schiff base is formed that is in equilibrium with its cyclized form, a n-substituted glycosylamine. When the reducing sugar is an aldose, the next step is the Amadori rearrangement, an irreversible, acid catalyzed reaction. The intermediate stage begins with Amadori compounds undergoing additional decomposition reactions to form 1-deoxyosone, 3-deoxyosone and 4-deoxyosne, with 3-deoxyosone being the most abundant intermediate compound. Ketoses proceed through the Heyns rearrangement which produces 2-amino-2-deoxyaldoses. Furfurals and reductones are then formed through dehydration in acidic conditions and in neutral/alkaline conditions, respectively. These sugar degradation products then react with α-amino acids through the Strecker degradation forming aldehydes, ammonia and carbon dioxide from the amino acid oxidation. The final stage of Maillard browning causes the development of flavor, aroma and browning compounds. Aldehydes formed from the Stecker degradation react with amine derivatives to form aroma compounds. Heterocyclic amines called melanoidins are formed through an aldehyde-amine condensation. These polymeric compounds are considered to be the main browning pigments resulting from Maillard browning and they can also form flavor compounds, including pyridines, pyrazines and pyrroles (Hodge, 1953; Nursten, 2005, Sikorski et al., 2008).

Besides influencing a food product's taste and appearance, Maillard browning can also impact nutritional quality. During the Amadori rearrangement and Amadori compound formation, the lysine becomes bound in the Amadori compound, inhibiting the nutritional availability of an essential amino acid (Nursten, 2005).

2.4.2.1 Impact of processing conditions on the Maillard reaction

Several factors influence the occurrence of the Maillard reaction, as well as impacting the mixture of browning compounds that result. Temperature, time at the reaction temperature, pH, reducing sugar and amino acid presence, water activity (a_w) and water content all effect the reaction (BeMiller and Huber, 2008).

Addition of sulfur dioxide inhibits the Maillard reaction in dehydrated fruit and vegetable products. During the reaction, sulfur dioxide interacts with carbonyl intermediates, which prevents the interaction of sugars and amino acids required for the reaction (Nursten, 2005).

2.4.3 Hydroxymethylfurfural (HMF)

Furfurals are intermediate compounds produced during nonenzymatic browning, particularly caramelization and Maillard browning, which polymerize to form browning compounds. 5-hydroxymethyl-2-furalaldehyde, commonly known as hydroxymethylfurfural (HMF) is a furan produced during processing and storage conditions at elevated temperatures that is associated with potential health concerns for humans (Nursten, 2005). Measurement of HMF is one method that the food industry utilizes to evaluate the extent of the Maillard reaction during processing (Morales, 2009).

HMF is produced during the Maillard reaction by the dehydration of 3-deoxyosone (3-deoxyhexosulose), an Amadori product (Figure 3). In the presence of heat, slightly acidic conditions, pH > 5, and an amine-containing compound, melanoidins can be formed from HMF and other furfurals (Figure 4) (BeMiller and Huber, 2008; Hodge, 1967). During caramelization, HMF is produced during the dehydration of hexoses, through an intermediate of 3-deoxyosone (Nursten, 2005). Processing conditions such as temperature, pH, and water activity impact HMF formation during both the Maillard reaction and caramelization (Morales, 2009).

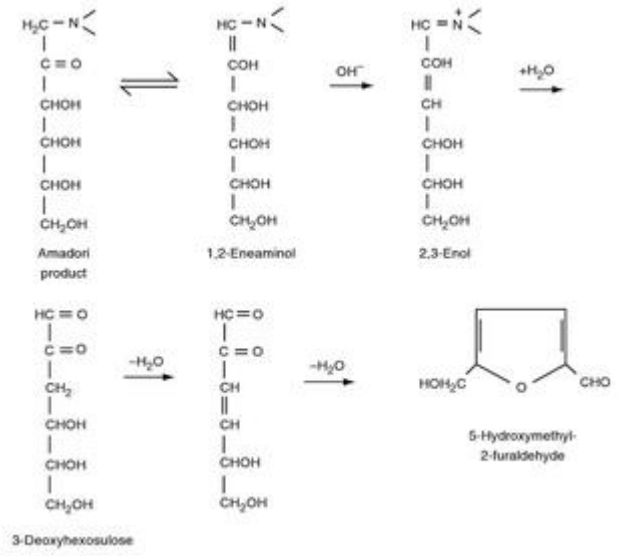

Figure 3. HMF Formation from the Amadori product (BeMiller and Huber, 2008)

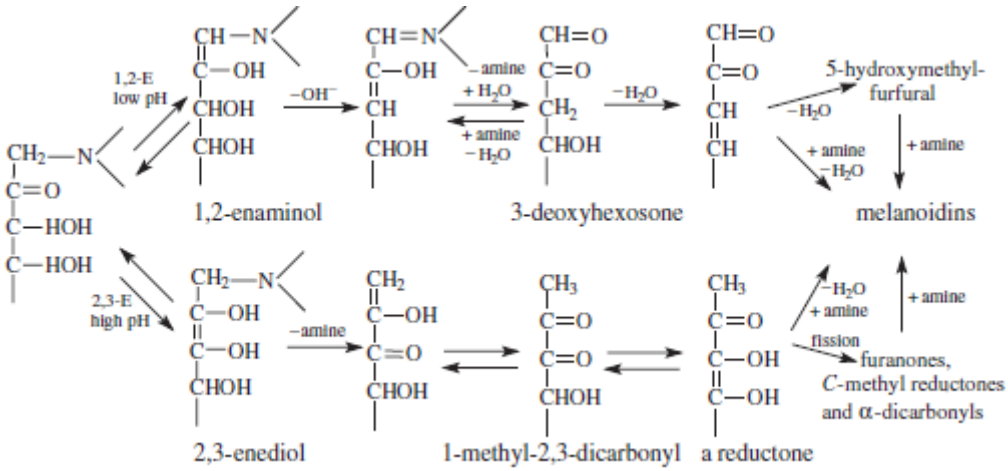

Figure 4. Formation of HMF in the Maillard Reaction (Hodge, 1967)

Production of HMF in milk products occurs through two different processes. The aldehyde group of lactose and lysine-rich proteins in milk can undergo the Maillard reaction and form the Amadori product lactulosyllysine. After 1,2-enolization of this compound, 3-deoxyosone is formed, which then further proceeds to form HMF (O'Brien and Morrisey, 1989). The Lobry de Bruyn-Alberda van Eksenstein transformation is the second process that yields HMF in milk products through lactose isomerization and degradation (Ames, 1992).

The presence of HMF in foods can occur either by direct-heat treatment (coffee, bread and dried fruits) or through inclusion of ingredients containing HMF (caramel colors in sodas, beer and other alcohols) (Morales, 2009).

2.4.3.1 Limits in food

Regulations on the amount of HMF allowed in food products varies by food product and by country. CODEX Alimentarius established an upper limit of 40 mg HMF/kg honey as a method for evaluating adulteration with invert sugar and excess heat-treatment. When the product is over-heated in an effort to minimize crystallization during shelf life and supplemented with invert sugar, the HMF content in honey dramatically increases (Morales, 2009).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has also established a limit of HMF in polydextose of 0.1% (Joint FAO/WHO Expert Committee on Food Additives, 1998). While there are no specifications for HMF in the United States, companies have adopted the international JECFA standard for their products. Mead Johnson and Co. abided by the JEFCA HMF limit for their product, a combination of polydextrose and GOS in infant formula, as seen in their 2007 GRAS submission.

2.4.3.2 Health concerns

Elevated levels of HMF can cause discomfort including eye, upper respiratory tract, and skin irritation. The activity of HMF in the human body is influenced by its structure and reactive sites (Morales, 2009).

Studies have identified HMF as a potentially genotoxic and carcinogenic compound for humans (Bruce et al., 1993; Lee et al., 1995; Svendsen et al., 2009). *In vitro* studies have demonstrated the genotoxicity of HMF; however, *in vivo* studies in rodents have not supported the claim that HMF is genotoxic and carcinogenic (European Food Safety Authority, $2011_{a,b}$). Overall, the conflicting results related to HMF health concerns inhibit the substantiation of the carcinogenic or genotoxic impact of HMF (Capuano and Fogliano, 2011; European Food Safety Authority, 2011_{b}).

2.4.3.3 Measurement of HMF

Several different analytical techniques have been used to measure HMF including colorimetric, spectrophotometric, and chromatographic methods. The colorimetric analysis was the first published method for HMF measurement. This method was developed for use in dairies and included using a 2-thiobarbituric acid (TBA) reaction product to bind with HMF to indicate the HMF content in the sample (Keeney and Bassette, 1959). In current colorimetric analysis of HMF, resorcinol, aniline p-toluidine and TBA are used to bind to HMF. The reaction is then measured in the visible range using a spectrophotometer. While the colorimetric assays are fairly simple to complete, there are problems with interference of other compounds. When data from a colorimetric and chromatographic assay were compared, up to 70% of the HMF from the colorimetric assay was from interfering compounds (Morales et al., 1996).

Spectrophotometric methods are similar to the colorimetric methods, but they are not measured in the visible range. Various studies have measured the absorbance of HMF-containing solutions at 284 nm and 336 nm (White, 1979; Truzzi et al., 2012). The specification for polydextrose published by JECFA also used a spectrophotometric method at 283 nm for HMF quantification (Joint FAO/WHO Expert Committee on Food Additives, 1998). The spectrophotometric assay would suffer from the same interference problem as the colorimetric method (Morales et al., 1996).

Several studies have shown that chromatographic methods are more accurate than the spectrophotometric measurements since there is not interference with non-HMF compounds that absorb at the set absorbance maxima (Zappala et al., 2005, Morales, 2009). The difference in analysis results is even more apparent for products containing very low levels of HMF (Truzzi et al., 2012). HMF and other furfurals can be individually identified by chromatographic methods by using internal standards. While this analysis occurs at a similar absorbance as the spectrophotometric method, the ability to identify specific compounds ensures that only HMF is being measured. An average absorbance used for chromatographic analysis is 280 nm, with the HMF maxima being

284 nm and the furfural maxima is 277 nm (Morales et al., 1997; Morales, 2009; Truzzi et al., 2012).

2.4.4 Purification technologies

Manufacturing of several products such as gas, water, sugar, molasses, and corn syrup utilize purification and decolorization technologies. These processes leverage the use of adsorbents, including granulated activated charcoal, powdered activated charcoal, bone charcoal, ion-exchange resins and chromatography to remove impurities and color compounds (Abram and Ramage, 1979; Field and Benecke, 2000). Adsorbents bind colorants and other compounds through a process of adsorption; thereby removing them from the solution (Ibarz et al., 2012). Carbon filters have been a staple in the purification of waste water for reuse and drinking water purification (Kuo et al., 1998). The food industry also utilizes carbon filtration, in addition to chromatography, for decolorization and purification of fruit juices, honey, sugar, molasses and maple syrups (Marsh and Rodriguez-Reinoso, 2006).

2.4.4.1 Activated carbon

The original adsorbent used by the sugar industry for secondary decolorization after affination and clarification was bone charcoal. This material, made from ground, de-greased cattle bones, has good adsorbent and buffering properties and can remove ash (Abram and Ramage, 1979). Today, some sugar refining operations continue to use bone charcoal, but many new facilities have moved to the use of activated charcoal because of its enhanced decolorizing power and lower cost. Activated carbon is produced by heating powdered carbon to 600-700°C in a reducing atmosphere, followed by steam activation at 900-1000°C. This process yields a granular carbon with both a high degree of porosity and randomly distributed pores in a variety of shapes and sizes (Bansal et al., 1988; Field and Benecke, 2000). Several properties of activated carbon make it an ideal adsorbent, such as large surface area with high surface reactivity, and an accessible internal structure which enhances adsorption rate (Bansal et al., 1988).

Activated carbon is a good general adsorbent, meaning it is not specific for one specific type of colorant. Both phenolic and flavonoid colorants originating from the sugar cane plant and process-induced browning compounds from the Maillard reaction and caramelization are removed by carbon filtration (Field and Benecke, 2000). The use of carbon with a high surface area is ideal for removing colorants since they are adsorbed as a monomolecular layer, meaning a large surface are is needed. Polymeric colorants, such as melanoidins, bind well to carbon since the color compound can bind at multiple sites (Riffer, 2000).

Research has been conducted on the use of agricultural by-products to manufacture activated carbon, since the coal used for producing granular activated carbon is a non-renewable resource. Sugar cane residues left over after extracting the juice, sugar beet pulp and pecan shells have all been used to produce activated carbons with similar decolorization properties as commercial granular activated carbon. However, the properties of the carbons generated from the by-products are greatly influenced by the binder used and the activation method (Ahmedna et al., 1997; Ahmedna et al., 2000; Mudoga et al., 2008; Pendyal et al., 1999)

2.4.4.2 Carbon filtration

Carbon filtration on an industrial scale occurs through either a fixed-bed or a pulsed-bed system. In both these systems, the carbon is housed in an adsorption column. The solution to be purified, called liquor, is simply passed through a stationary column in fixed-bed filtration. In pulsed-bed filtration, however, the carbon and liquor are moving in a counter current manner. As the carbon is spent, new carbon enters through the top of the column and the liquor comes in the bottom of the column (Field and Benecke, 2000). Porosity of the carbon reduces over time as liquor is added to the column, decreasing its effectiveness (Riffer, 2000). The size of the column, number of columns, and residence time in the column are all dependent on the color load of the liquor and the facility's processing capabilities (Field and Benecke, 2000).

2.4.4.3 Chromatography

Another technique for separating out components from a mixture is chromatography. This technology utilizes a column packed with an adsorbent that interacts with a solution as it passed through. The pore size of the adsorbent dictates which compounds pass through and which do not, altering the residence time of compounds. Therefore, compounds elute at different times so they can be separated from the mixture (Ismail and Nielsen, 2010). This analytical method is more selective than carbon filtration in regards to what compounds are removed and when (Riffer, 2000)

Chromatography has been utilized for the separation of sugar and color compounds from molasses and for concentration of high fructose corn syrup after glucose isomerase isomerization (Visuri and Klibanov, 1986; Wu et al., 1999; Hatano et al., 2009). Size exclusion chromatography has specifically been used to fractionate the various sugars in GOS and to separate out oligosaccharides in honey (Hernandez et al., 2009; Sanz et al., 2005). Ion exchange chromatography has also been a popular technique to separate out carbohydrates for purification since the method has the ability to separate carbohydrates with a very similar size (Nobre et al., 2015).

3 Polymerization of Lactose to Soluble Fiber through Microwave Polymerization and Reactive Extrusion

3.1 Introduction

Oligosaccharides, carbohydrates with a degree of polymerization between 2 and 10, account for a large percentage of the U.S. food fibers market (Frost and Sullivan, 2012). These ingredients are attractive to the food industry because of their prebiotic and dietary fiber classification and they generally do not adversely affect flavor and mouthfeel, unlike insoluble fibers. Soluble fiber, such as oligosaccharides, have a low viscosity and can be used specifically to influence the texture and rheology of a product (Tungland and Meyer, 2002). Fiber-containing foods are becoming increasingly popular as consumers learn of the health benefits associated with fiber, such as colon cancer prevention, improved digestion, and obesity prevention through improved satiation (Mintel Group Ltd., 2016; United States Department of Agriculture, 2015).

Chemical synthesis of oligosaccharides has been accomplished on benchtop, pilot plant and commercial scales. Polydextrose, a commercially available oligosaccharide, is produced through a melt polymerization of glucose, sorbitol and citric acid under vacuum (Rennhard, 1973; Tungland and Meyer, 2002) and polylactose has been produced through pilot plant scale reactive extrusion of lactose, glucose and citric acid. (Tremaine et al., 2014). A traditional acid-catalyzed melt polymerization under vacuum has been used to synthesize oligomers on a benchtop scale by various researchers. Several sugars have been used as the building blocks for these oligomers including sucrose, glucose, galactose, xylose, a mixture of glucose and galactose and a mixture of fucose and galactose (Daines et al., 2015; Manley-Harris and Richards, 1991; Manley-Harris and Richards, 1993). Acid catalyzed polymerization with a microwave reaction system, another benchtop method, has successfully produced polydextrose that is comparable to the commercial product (Wang et al., 2014).

Direct polymerization of lactose to oligomers through both the traditional melt polymerization method and a microwave reactor method has not been reported to be

successful. To achieve a product with a high proportion of oligomers and a low proportion of monomers, one study used phosphoric acid to hydrolyze lactose to glucose and galactose in a microwave, and then the monomers went through a traditional melt polymerization process under vacuum (Daines et al., 2015).

The development of a microwave reactor method to polymerize lactose to oligomers without a pre-hydrolysis step would allow for hypothesis driven research prior to reactive extrusion. The impact of formulation on the polymerization reaction is not well understood. Previous reactive extrusion trials with a lactose-rich permeate powder did not yield oligosaccharides. The main differences between the polylactose raw materials and permeate, are the presence of milk minerals, nitrogen and water (Sienkiewicz and Riedel, 1990). Establishing an understanding of the impact of formulation using a benchtop method, such as a microwave reactor, could allow for the production of novel oligosaccharides from dairy co-products.

We successfully developed a microwave reactor method for the polymerization of lactose and glucose to polylactose. Since the polymerization of lactose in permeate powder was not successful during extrusion trials, we hypothesized that the presence of other components such as milk minerals and moisture influenced the reaction. We expected that the oligomer yield would be decreased with increasing water and milk mineral content. Our objectives were to use the developed microwave reactor method to investigate the impact of formulation on lactose polymerization and use this information to identify a successful reactive extrusion method to polymerize the lactose in permeate powder to oligosaccharides.

3.2 Materials and Methods

The materials and methods are described in this section. The extended methods can be found in Section A.1 of the Appendix.

3.2.1 Materials

α-lactose monohydrate, dextrose, citric acid, calcium phosphate (Sigma Aldrich, St. Louis, MO, USA) and IdaPro permeate powder (Idaho Milk Products, Jerome, ID, USA)

were used in the microwave reaction system trials. IdaPro permeate powder (Idaho Milk Products) and citric acid (Jungbunzlauer, Basel, Switzerland) were used for extrusion of the permeate–acid blend.

3.2.2 Sample preparation for microwave polymerization of lactose

Seven grams of an α-lactose monohydrate (74%), dextrose (20%) and citric acid (6%) blend and 1 mL of reverse osmosis water were added to a 110 mL MARSXpress Plus Teflon microwave reactor vessel (CEM Corporation, Matthews, NC, USA). The vessel was closed and the product equilibrated for 24 hours. During experimentation with permeate, 7 grams of the permeate-acid blend and 1 mL of reverse osmosis water was added to the MARSXpress Plus Teflon microwave reactor vessel.

3.2.3 Microwave polymerization of lactose

After 24 hours, the vessels were placed in a Mars 6 microwave reactor (CEM Corporation) and the heating profile used was a 14 minute ramp time to 140°C at 1800W. Once the heating ramp time was complete, the product was immediately removed from the vessels and cooled.

3.2.4 Reactive extrusion of a permeate and citric acid blend

Permeate powder (90%) and citric acid (10%) were mixed in 14 kg batches using an IMS-1 ribbon blender (Bepex International LLC, Minneapolis, MN, USA) in a forward and reverse direction for 2 minutes each. The extrusion set-up for the Bühler 44 mm co-rotating twin-screw extruder DNDL 44 with a length to diameter ratio of 28 (Bühler AG, Uzwil, Switzerland) is described in Tremaine et al., 2014. The flow rate used for this blend of powders was 15 kg/hr. After extrusion, the polymerization product was collected and cooled on metal trays.

3.2.5 Chemical analysis

Dietary fiber

The integrated total dietary fiber assay procedure item number K-INTDF 02/15 (Megazyme International, Bray, Ireland) was used to quantify the amount of low

molecular weight soluble dietary fiber (LMWSDF) formed during the polymerization reaction. This method is based on AOAC Method 2009.01 with minor alterations. D-ribose (Sigma Aldrich) was used as the internal standard for high performance liquid chromatography (HPLC) analysis. All HPLC analysis used a Transgenomics CHO-411 column (Omaha, NE, USA) and a Sedex 85 LT low temperature evaporative light scattering detector (ELSD –LT) (Shimadzu Corporation). The HPLC conditions used were a column temperature of 80°C, flow rate of 0.3 mL/min and a double distilled water mobile phase. The ELSD nebulizer temperature was set at 40°C and the nitrogen pressure was 250 kPa.

Lactose and glucose

Lactose and glucose in the polymerization products were quantified using the lactose/sucrose/D-glucose enzymatic assay procedure item number: K-LACSU 09/14 (Megazyme International). A Shimadzu UV-1800 spectrophotometer was used for measuring absorbance as per the method (Shimadzu Corporation, Kyoto, Japan).

pH

The method used to measure the polymerization product pH was adapted from a standard method for measuring the pH of whey powder (Case et al., 1985). A solution of 3.25 grams of ground polymerization product was diluted with 50 mL RO water and the pH was measured with a pH probe (Accumet Basic AB15, Fischer Scientific, Pittsburgh, PA, USA).

3.2.6 Statistical analysis

The values presented are the means of quadruplicate determinations. All data were analyzed with univariate analysis of variance (ANOVA) coupled with a Tukey Honest Significant Difference (HSD) test using R Studio Version 0.99.484 (R Development Core Team, 2017).

3.3 Results and Discussion

3.3.1 Impact of water content on lactose polymerization

The maximum LMWSDF yield was achieved with a 18.57% water addition and the yield decreased at higher water addition levels (Table 1). In polydextrose and glucose-based oligosaccharide microwave-assisted polymerization studies, a peak water addition value was identified. A phosphoric acid catalyzed, microwave polymerization of polydextrose was reported to require 10% (v/w) water addition for maximum oligosaccharide production, while the reported peak water addition was 30% (v/w) for a glucose oxidase and peroxidase catalyzed microwave polymerization of glucose (Li et al., 2006; Wang et al., 2014). Water acts as the initiator for the microwave-assisted reaction, meaning it helps the reactants to absorb microwave energy (Wang et a., 2014). A dipole is needed for a solvent to absorb microwave energy, meaning polar solvents, such as water, are ideal for microwave-assisted reactions (Larhed and Hallberg, 2001). Li et al., 2006, concluded that low initiator quantities can't absorb the microwave energy effectively when polymerizing glucose to oligosaccharides. Therefore, small water additions, such as the 1.43% and 3.57% used in the present study were not sufficient to promote optimal polymerization.

Environmental conditions, such as water content, greatly impact the lactose polymerization reaction. In order to form a glycosidic linkage, the anomeric center of a donor reacts with a hydroxyl group on the acceptor. When water is present it can act as the acceptor instead of the sugar, causing hydrolysis of lactose instead of polymerization (Mahoney, 1998; Oman, 2016). With regards to our experiment, the polymerization reaction was reduced at the highest water addition level; however, lactose hydrolysis did not occur. The increase in the residual lactose content with decreasing LMWDF, demonstrates that the sugar is not being polymerized and lactose is not breaking down to its monomers. Li et al., 2006 hypothesized that during microwave heating the water volatilizes quickly, which prevents it from acting as an acceptor in the polymerization reaction.

Table 1. Impact of water content on a lactose-glucose-citric acid blend polymerized with a microwave-assisted method[1]

Water Added (% v/w)	LMWSDF (%)[2]	Residual Lactose (%)	Residual Glucose (%)
1.43	18.49 ± 3.35^{a}	2.73 ± 1.20^{a}	1.37 ± 0.76^{a}
3.57	20.56 ± 1.64^{ab}	2.49 ± 0.33^{a}	1.79 ± 0.28^{a}
7.14	27.56 ± 1.92^{bc}	3.13 ± 0.98^{a}	2.96 ± 0.81^{a}
14.29	29.06 ± 2.23^{c}	12.16 ± 1.68^{bc}	4.48 ± 0.99^{a}
18.57	31.26 ± 0.60^{c}	10.54 ± 1.75^{b}	8.07 ± 0.86^{b}
22.86	$17.14 \pm 1.04a^{d}$	17.86 ± 0.51^{cd}	12.18 ± 0.39^{c}
28.57	10.43 ± 0.72^{d}	20.69 ± 2.45^{d}	12.66 ± 0.47^{c}

[1] Values are means ± one standard deviation (N = 4), a-d Means without a common superscript letter within the same column are significantly different ($p<0.05$)
[2] Low molecular weight soluble fiber

3.3.2 Impact of calcium phosphate concentration on lactose polymerization

Calcium phosphate addition of 0.9280% and 1.856% imitates the calcium content of dairy co-products such as acid whey and permeate powder (Sienkiewicz and Riedel, 1990; Wong et al., 1978). At the highest calcium phosphate addition, polymerization was reduced, as shown by the decrease in LMWSDF and increase in residual lactose and glucose (Table 2).

The presence of cations and pH changes have been shown to impact the enzymatic synthesis of GOS by altering the enzyme's activity (Fischer and Kleinschmidt, 2015; Madani et al., 1999). The impact of ions on polymerization through a chemical synthesis has been researched in the field of polymer science. Surfactants, ionic and non-ionic, are incorporated into the reaction system for emulsion polymerization of waterborne resins, such as synthetic rubbers, coatings and adhesives, to stabilize the oil-water interface (Chern, 2006). The pH of the emulsion did not impact the rate of polymerization, rather the type of surfactant used influenced the conversion of monomers to polymers and the rate of polymerization (Niranjan et al., 2011). Cationic surfactants have been shown to decrease the polymerization reaction, while anionic surfactants have promoted polymerization (Niranjan et al., 2011; Patra et al., 2004). The inhibition caused by cationic surfactants is attributed to electrostatic repulsion (Patra et el., 2004). Mineral-containing fillers utilized for the polymerization of styrene have also been shown to have

an inhibitory effect on the rate of polymerization (Kucher et al., 1989). Addition of calcium phosphate to a blend of lactose and glucose prior to polymerization could cause an inhibitory effect similar to the ion effect seen in polymer polymerization. While pH did not impact polymerization for the waterborne resins described in the study of Niranjan et al., 2011, polymerization of sugars is impacted by pH changes (Collins and Ferrier, 1995). Therefore, the increase in pH with increasing calcium phosphate (Table 2) also may have inhibited the polymerization of lactose to LMWSDF.

Table 2. Impact of calcium phosphate on a sugar-acid blend polymerized with a microwave-assisted method[1]

Calcium Phosphate Content (%, w/w)	LMWSDF (%)[2]	Residual Lactose (%)	Residual Glucose (%)	pH
0	29.06 ± 2.23^{a}	12.16 ± 1.68^{a}	4.48 ± 0.99^{a}	2.68 ± 0.03^{a}
0.1824	32.24 ± 2.47^{b}	12.29 ± 0.73^{a}	7.57 ± 0.31^{a}	2.76 ± 0.01^{b}
0.3650	30.07 ± 3.48^{ab}	31.56 ± 7.93^{a}	12.52 ± 1.98^{b}	2.76 ± 0.02^{b}
0.4563	28.47 ± 0.93^{ab}	15.33 ± 5.92^{a}	7.78 ± 2.23^{a}	2.79 ± 0.01^{b}
0.9280	19.56 ± 4.95^{ac}	29.79 ± 0.93^{a}	11.31 ± 0.23^{b}	2.86 ± 0.02^{c}
1.856	14.48 ± 0.65^{c}	32.21 ± 6.77^{a}	12.36 ± 1.32^{b}	3.00 ± 0.01^{d}

[1] Values are means ± one standard deviation (N = 4), a-d Means without a common superscript letter within the same column are significantly different ($p<0.05$)
[2] Low molecular weight soluble fiber

3.3.3 Increasing citric acid concentration to improve dietary fiber yield

The citric acid content was increased for the sugar-acid blend containing the highest calcium phosphate concentration and the permeate-acid blend to investigate if the dietary fiber yield could be increased. The permeate powder had an ash content of 7.37 ± 0.98 %; therefore, a reduction in polymerization due to the impact of minerals, such as calcium, was expected. A citric acid content of 8 and 10% increased the LMWSDF for the sugar-acid blend and the permeate-blend. The reduction in lactose and glucose also demonstrates that the monomers are being polymerized to dietary fiber (Table 3 and 4).

Citric acid acts as a catalyst and cross-linker during the polymerization reaction and it becomes covalently bonded in the carbohydrate through esterification (Daines et al., 2015; Olsson et al., 2013; Rennhard, 1973). For the microwave polymerization of

polydextrose and a glucose based oligosaccharide without sorbitol, a peak catalyst value, 1.2% phosphoric acid and 10% glucose oxidase and peroxidase, respectively, was determined that led to the maximum oligosaccharide yield (Li et al., 2006; Wang et al., 2014). Lower levels of the citric acid catalyst yielded lower levels of dietary fiber in our study (Table 3 and 4). This result is similar to what has been observed in the microwave polymerization studies, as well as scaled-up production of polydextrose and polylactose (Li et al., 2006; Rennhard, 1973; Tremaine et al., 2014; Wang et al., 2014)

Table 3. Impact of citric acid on a sugar-acid blend with elevated calcium phosphate[1]

Calcium Phosphate Content (%, w/w)	Citric Acid Content (%)	LMWSDF (%)[2]	Residual Lactose (%)	Residual Glucose (%)	pH
1.856	6	14.48 ± 0.65^{a}	32.21 ± 6.77^{a}	12.36 ± 1.32^{a}	3.00 ± 0.01^{a}
1.856	8	20.15 ± 0.78^{b}	20.14 ± 3.44^{a}	9.61 ± 1.28^{a}	2.94 ± 0.01^{ab}
1.856	10	19.29 ± 1.05^{b}	20.50 ± 1.00^{a}	8.89 ± 0.56^{a}	2.89 ± 0.02^{b}

[1] Values are means ± one standard deviation (N = 4), a-b Means without a common superscript letter within the same column are significantly different ($p<0.05$)
[2] Low molecular weight soluble fiber

Table 4. Composition of the permeate-acid blend microwave polymerization product[1]

Formula					
Permeate (%)	Citric Acid Content (%)	LMWSDF (%)[2]	Residual Lactose (%)	Residual Glucose (%)	pH
94	6	2.20 ± 0.24^{a}	54.32 ± 4.72^{a}	1.53 ± 0.18^{a}	3.89 ± 0.03^{a}
92	8	3.11 ± 0.54^{ab}	36.58 ± 3.66^{ab}	2.11 ± 0.41^{a}	3.68 ± 0.02^{b}
90	10	3.93 ± 0.21^{b}	31.77 ± 3.61^{b}	2.18 ± 0.18^{a}	3.56 ± 0.03^{c}

[1] Values are means ± one standard deviation (N = 4), a-c Means without a common superscript letter within the same column are significantly different ($p<0.05$)
[2] Low molecular weight soluble fiber

3.3.4 Reactive extrusion of a permeate acid-blend

The composition of the permeate-acid product produced through reactive extrusion matched the product produced from the microwave polymerization method. The sugar-acid blend formulation used for this reactive extrusion trial yielded successful

polymerization of the sugars to LMWSDF (Table 5) unlike previous trials. Citric acid content was higher than previous trials; therefore the increased acid catalyst likely promoted increased polymerization (Li et al., 2006; Wang et al., 2014).

During reactive extrusion the permeate-acid blend polymerization product developed a dark brown color, which is darker than the amber color of polylactose. Browning has occurred during the polymerization reaction in both benchtop polymerization and reactive extrusion of sugars (Manley-Harris and Richards, 1993; Tremaine et al., 2014). Color development is attributed to caramelization in the sugar-acid blends containing pure sugars (Buera, 1987_a), while browning in the permeate-acid blend can be caused by simultaneous caramelization and Maillard browning (Saltmarch, 1981). Caramelization has been observed for lactose and glucose, with lactose caramelizing at a faster rate than glucose (Buera et al., 1987_a). Lactose has been shown to participate in the Maillard reaction, in addition to caramelization, at a lower pH, pH 5.0, as demonstrated by a lactose-glycine model system (Buera, 1987_b). Quality deterioration of whey powders, a product similar in composition to permeate powder, has been widely attributed to non-enzymatic browning during storage (Labuza and Saltmarch, 1981). Whey powder, as well as permeate powder, contains lactose, a reducing sugar, and protein high in lysine, meaning the Maillard reaction can occur under the right conditions (Saltmarch, 1981). Since the rate of Maillard browning is increased with decreasing pH due to the acid catalysis of the sugar group, particularly at increased temperature, (Dattatreya et al., 2006), Maillard browning most likely occurred during reactive extrusion.

Browning was observed for the citric acid catalyzed reactive extrusion of polylactose (Tremaine et al., 2014). At low levels of citric acid, 1-2%, the concentration did not impact browning (Tremaine et al., 2014); however, a 6% citric acid concentration for the formula extruded at a 15 kg/hr feed rate browned more than a formula with 2 and 4% citric acid (Reid, 2015). Therefore, the elevated citric acid level used for the reactive extrusion of a permeate-acid blend, coupled with the dry blend's composition likely caused the darker brown color.

Table 5. Composition of the permeate-acid blend reactive extrusion product[1]

LMWSDF (%)[2]	Residual Lactose (%)	Residual Glucose (%)
4.25 ± 0.23	24.75 ± 0.25	0.93 ± 0.09

[1] Values are means ± one standard deviation (N = 4)
[2] Low molecular weight soluble fiber

3.4 Conclusion

It was determined that formulation impacts the polymerization of lactose to soluble fiber. Elevated water and calcium phosphate contents caused a reduction in the polymerization yield during microwave polymerization of a lactose, glucose and citric acid blend. Increasing the citric acid catalyst from 6% to 8 and 10% increased the dietary fiber yield because of the higher catalyst concentration. Utilization of a higher citric acid content for benchtop polymerization and reactive extrusion of a permeate-acid blend enabled polymerization of lactose to soluble fiber, unlike previous trials. The product produced through reactive extrusion was a darker brown than polylactose. Further optimization of the reactive extrusion of a permeate-acid blend could provide the food industry with a unique soluble fiber that is produced from a dairy co-product.

4 Purification of Polylactose Using Activated Carbon

4.1 Introduction

The occurrence of browning reactions during processing can have a positive impact on flavor and aroma development in products such as coffee and cocoa, yet it can cause quality deterioration during storage for other products such as whey powders (Martins et al., 2001; Saltmarch, 1981). Some compounds produced during browning reactions are also reported to be toxic to humans (Nursten, 2005). Non-enzymatic browning occurs during the polymerization of sugars to oligosaccharides in benchtop, pilot plant and commercial scale processes (GTC Nutrition, 2009; Manley-Harris and Richards, 1993; Manley-Harris and Richards, 1994; Tremaine et al., 2014). The raw material composition and the manufacturing conditions impacts whether Maillard browning, caramelization or a combination of both reactions occurs (Sikorski et al., 2008). Maillard browning is a condensation reaction that occurs in the presence of a reducing sugar, a compound containing a free, primary amine group, and water to produce melanoidins, brown nitrogen-containing polymers and co-polymers (Hodge, 1953). Caramelization, on the other hand, is the decomposition of sugars to a complex mixture of browning compounds without amine involvement (Buera et al., 1987$_b$; Golon and Kuhnert, 2012; Nursten, 2005).

Many intermediate compounds are formed during Maillard browning and caramelization. 5-Hydroxymethyl-2-furalaldehyde, commonly known as hydroxymethylfurfural (HMF) is a furan that is produced by the dehydration of 3-deoxyosone in Maillard browning, and the dehydration of hexoses in caramelization (Morales, 2009). HMF has been associated with adverse health effects for humans, including genotoxicity, upper respiratory tract irritation and skin irritation (European Food Safety Authority, 2011; Morales, 2009). Because of these health concerns, regulations have been established for HMF limits in food. Codex Alimentarius has established a limit of 40 mg HMF/kg honey for determining if adulteration or excessive heat treatment has occurred for honey (Morales, 2009) and the Joint FAO/WHO Expert

Committee on Food Additives (JECFA) has also established a 0.1% limit of HMF for polydextose (Joint FAO/WHO Expert Committee on Food Additives, 1998). There is not a formal limit for HMF in the United States; however some companies have adopted the 0.1% limit for their products (Mead Johnson and Co., 2007).

Carbon filtration can be used as a purification step for removing unwanted components, such as mono- and disaccharides in oligosaccharide ingredients (Hernandez et al., 2009; Nobre et al., 2015). Columns filled with carbon and Celite (diatomaceous earth) (Morales et al., 2006; Hernandez et al., 2009; Whistler and Durso, 1950), filtration methods, treatment with yeast to remove monosaccharides, and size exclusion chromatography (Hernandez et al., 2009; Nobre et al., 2015; Sanz et al., 2005) have all been used to remove individual sugars from mixtures of carbohydrates. These purification techniques are critical for the production of ingredients for the food and pharmaceutical industry because the presence of mono- and di-saccharides influences the prebiotic and caloric value of the ingredient (Nobre et al., 2015).

Another use for these purification methods in the food industry, particularly sugar and molasses manufacturing, is decolorization (Riffer, 2000). Activated carbon is commonly used because it is a cost efficient, successful decolorizing and purification adsorbent (Hernandez et al., 2009; Nobre et al., 2015). The high surface area of the activated carbon is achieved by heating powdered carbon to 600-700°C in a reducing atmosphere, followed by steam activation at 900-1000°C, which makes the pores in the carbon (Field and Benecke, 2000). Pore size distribution and pore volume can be modified by altering the activation procedure (Kuhn and Filho, 2010). The carbon's adsorption capacity, which dictates the amount of color removed, is attributed to its total surface area, internal pore structure, and functional groups that are on the pore's surface (Bansal et al., 1988). Browning compounds from the Maillard reaction and caramelization have been removed through carbon filtration (Field and Benecke, 2000). Commercial GOS and polydextrose utilize carbon filtration, as well as membrane filtration and ion exchange techniques for purification and decolorization (GTC Nutrition 2009; Mead Johnson and Co., 2007).

Polylactose, a product produced from reactive extrusion of a lactose, glucose, and citric acid dry blend contained a HMF content that was above the JECFA limit. We hypothesized that carbon filtration could act as both a decolorization and purification step to remove HMF and increase the fiber content of polylactose. The objective for this study was to develop a carbon filtration method to purify polylactose and reduce the HMF concentration to comply with the limit presented in the JEFCA polydextrose specification (0.1%).

4.2 Materials and Methods

The materials and methods are described in this section. The extended methods can be found in Sections A.2 and A.3 of the Appendix.

4.2.1 Materials

Refined edible fine grind lactose, >99% purity (Davisco Foods International, Inc., Eden Prairie, MN, USA), dextrose monohydrate (Roquette America, Inc., Geneva, IL, USA) and citric acid anhydrous (Jungbunzlauer, Basel, Switzerland) were used for the reactive extrusion of polylactose. The carbon filtration columns were filled with diatomaceous earth (Fisher Scientific Education, Nazareth, PA, USA), Amberlite FPA OH^- and Ambersep 200 H^+ (Megazyme International, Bray, Ireland) and NORIT GAC 1240 Plus activated carbon (Cabot Norit Americas, Inc., Pryor, OK, USA).

4.2.2 Polylactose reactive extrusion

A blend of lactose (74%), dextrose (20%) and citric acid (4%) was mixed in 14 kg batches using an IMS-1 ribbon blender (Bepex International LLC, Minneapolis, MN, USA) in a forward and reverse direction for 2 minutes each. The extrusion set-up for the Bühler 44 mm co-rotating twin-screw extruder DNDL 44 with a length to diameter ratio of 28 (Bühler AG, Uzwil, Switzerland) is described in Tremaine et al., 2014. The flow rate used for this product was 15 kg/hr. After extrusion, the polymerization product was collected and cooled on metal trays.

4.2.3 Small scale benchtop carbon filtration

A Bio-Rad 20 mL disposable polypropylene column (Hercules, CA, USA) was filled with 0.5 g of diatomaceous earth (Fisher Scientific) and 5 g of NORIT GAC 1240 PLUS granular activated carbon (Cabon Norit Americas Inc.) and then rinsed with 20 mL of reverse osmosis water. Two mL of a 100 mg/mL solution of polylactose in reverse osmosis water was added to the column with 2 mL of reverse osmosis water. Twenty mL of reverse osmosis water was used to rinse the column. All samples eluted at a rate of approximately 1 mL/min.

4.2.4 Large scale benchtop carbon filtration

A glass column (~2463 cm^3) was filled with 15 g of diatomaceous earth (Fisher Scientific) and then 400 g of Cabot NORIT GAC 1240 PLUS granular activated carbon (Cabon Norit Americas Inc.) was added on top. When preparing a mixed-bed column, an ion exchange resin consisting of 50 g of Amberlite FPA 53 $OH^-$and 50 g of Ambersep 200 H^+ (Megazyme International) was added between the diatomaceous earth and the activated carbon layers. The column was rinsed with 3000 mL of reverse osmosis water and then 800 mL of a 200 mg/mL solution of polylactose in water was added to the column with 200 mL of reverse osmosis water. 1000 mL of reverse osmosis water was used to rinse the column. All samples eluted at a rate of approximately 3 mL/min.

4.2.5 Spray drying polylactose

An APV Anhydro Type I spray dryer (SPX FLOW, Inc., Charlotte, NC, USA) with an APV CF-100 atomizer (SPX FLOW, Inc.) was used to spray dry the carbon filtration eluent. The spray drying conditions were inlet temperature, 185°C; outlet temperature, 90°C; flow rate, 220 mL/min; atomizer, 24,000 rpm.

4.2.6 Chemical analysis

Citric acid

The citric acid content of polylactose was measured using the citric acid (citrate) assay manual assay procedure item number K-CITR 11/14 (Megazyme International). A

Shimadzu UV-1800 spectrophotometer was used for measuring absorbance as per the method (Shimadzu Corporation, Kyoto, Japan).

Dietary fiber

The integrated total dietary fiber assay procedure item number K-INTDF 02/15 (Megazyme International) was used to quantify the amount of non-digestible oligomers in polylactose. This method is based on AOAC Method 2009.01 with minor alterations. D-ribose (Sigma Aldrich) was used as the internal standard for high performance liquid chromatography (HPLC) analysis. All HPLC analysis used a Transgenomics CHO-411 column (Omaha, NE, USA) and a Sedex 85 LT low temperature evaporative light scattering detector (ELSD –LT) (Shimadzu Corporation). The HPLC conditions used were a column temperature of 80°C, flow rate of 0.3 mL/min and a double distilled water mobile phase. The ELSD nebulizer temperature was set at 40°C and the nitrogen pressure was 250 kPa.

Lactose and glucose

Lactose and glucose were quantified in polylactose using the lactose/sucrose/D-glucose enzymatic assay procedure item number: K-LACSU 09/14 (Megazyme International). A Shimadzu UV-1800 spectrophotometer was used for measuring absorbance as per the method (Shimadzu Corporation).

4.2.7 Color analysis

Absorption at 420 nm

The absorbance at 420 nm was used to measure the amount of brown pigments in polylactose. A 100 mg/mL polylactose in reverse osmosis water solution was measured using the Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation).

Hydroxymethylfurfural (HMF) Quantification

HMF was quantified using a method adapted from Truzzi et al., 2012. Polylactose samples were diluted in double distilled water to a concentration of 100 mg/mL and

filtered through a 0.45 μm syringe filter. The sample was injected into a Shimadzu LC-2010 HT system with a UV-Vis detector (Shimadzu Corporation). All HPLC analyses used an YMC Pack ODS-AM column (YMC CO. Ltd., Kyoto, Japan). The conditions used were an isocratic mobile phase, water- methanol 95 + 5v/v; flow rate, 0.8 mL/min; injection volume, 20 μL; column temperature 30°C; detection, 285 nm. The standard curve for polylactose HMF quantification (r^2 = 0.998) used 5 standard solutions of HMF in double distilled water (0.05 mg/mL, 0.075 mg/mL, 0.1 mg/mL, 0.15 mg/mL and 0.2 mg/mL) and the standard curve for the carbon filtered polylactose HMF quantification (r^2 = 0.998) also used 5 HMF standards (9.38 x10^{-5} mg/mL, 1.88 x 10^{-4} mg/mL, 3.75 x 10^{-4} mg/mL, 7.50 x 10^{-4} mg/mL and 1.50 x 10^{-3} mg/mL).

Hunter L, a, b

The same solution as the absorption at 420 nm was used for the Hunter *L, a, b* analysis. This solution was analyzed using the Shimadzu UV-1800 spectrophotometer in spectral mode from 360 nm to 700 nm in 1 nm increments at medium speed (Shimadzu Corporation). UV/PC Optional color analysis software version 3.10 was used to obtain the Hunter *L, a, b* values. A D65 illuminant and 10° observer viewing angle were used.

4.2.8 Statistical analysis

The values presented are the means of quadruplicate determinations. All data were analyzed with univariate analysis of variance (ANOVA) coupled with a Tukey Honest Significant Difference (HSD) test using R Studio Version 0.99.484. The color data for the polylactose pre- and post-spray drying was also analyzed using a t-test (R Development Core Team, 2017).

4.3 Results and Discussion

4.3.1 Compositional analysis of carbon filtered polylactose

All of the carbon filtration methods increased the dietary fiber content of polylactose, while reducing the citric acid content. The lactose content increased when using the large

scale benchtop methods as the polylactose was further purified and the glucose content was unchanged (Table 6).

Previous studies using carbon filtration for the purification of fructooligosaccharides (FOS) and GOS found that mono- and di-saccharides such as glucose, sucrose, fructose and lactose could be removed by using an ethanol solution for desorption (Hernandez, 2009; Kuhn and Filho, 2010; Nobre et al., 2012). Kuhn and Filho, 2010 found that desorption of sugars from carbon was not possible with pure water, rather an ethanol solution was required. Removal of mono- and disaccharides from carbon columns has been achieved with 0.1-10% (v/v) ethanol solution and oligosaccharides in honey and FOS have been removed using 15-50% (v/v) ethanol solutions (Hernandez et al., 2009; Kuhn and Filho, 2010; Nobre et al., 2012; Sanz et al., 2005; Swallow and Low, 1990; Whistler and Durso, 1950). Monosaccharide removal has been reported to be as high as 93% (w/w) for a fermentation broth filtered through activated carbon (Nobre et al., 2012). In this present study it was found that elution of both LMWSDF and mono- and disaccharides was possible with pure water. Previous trials with ethanol adsorption and desorption did not improve the purification process (data not shown). The reduced adsorption of mono- and disaccharides to the activated carbon filtration method used in this study can be attributed to the type of carbon used and the affinity of the -CH groups of the sugars to bind to the carbon (Nobre et al., 2015). The smaller sugars may have been able to pass through the pores of the carbon, which led to elution with the rinse water, instead of adsorption to the carbon.

Neutralization of oligosaccharides has been previously carried out with a solvent pH adjustment or addition of carbonates of potassium, sodium, calcium or magnesium for polydextrose (Torres, 1986; Rennhard, 1973). The removal of citric acid through carbon filtration, as shown in this study (Table 6), would eliminate the need for a separate neutralization step for oligosaccharides produced through an acid-catalyzed polymerization reaction.

Table 6. Compositional analysis of polylactose and polylactose that was treated with three carbon filtration methods[1]

Sample	LMWSDF (%)[2]	Lactose (%)	Glucose (%)	Citric Acid (%)
Polylactose	35.75 ± 0.93[a]	16.63 ± 0.92[a]	5.82 ± 0.48[a]	2.02 ± 0.17[a]
Carbon Filtered Polylactose (Small Scale Benchtop Method)	55.62 ± 2.08[b]	12.73 ± 0.94[b]	8.51 ± 0.40[b]	0.22 ± 0.17[b]
Carbon Filtered Polylactose (Large Scale Benchtop Method)	47.99 ± 2.05[c]	16.89 ± 0.53[a]	5.35 ± 0.09[a]	0.03 ± 0.05[b]
Mixed Bed Carbon Filtered Polylactose	50.13 ± 1.16[c]	21.92 ± 0.66[c]	6.27 ± 0.22[a]	0.05 ± 0.06 [b]

[1] Values are means ± one standard deviation (N = 4), a-c Means without a common superscript letter within the same column are significantly different ($p<0.05$)
[2] Low molecular weight soluble fiber

4.3.2 Impact of ion exchange resin addition to carbon filtration system

Caramelization reaction products consist of a complex mixture of hexose oligomers, oligomer dehydration and hydration products, fractionation products and colored aromatic compounds. The hexose oligomers are polymerization products of glucose, with a maximum degree of polymerization of 6 (Golon and Kuhnert, 2012). These caramelization products could be represented by the large first peak on the chromatogram for undigested carbon filtered polylactose (green chromatogram) (Figure 5). This peak was removed during the Megazyme Integrated Dietary Fiber Assay which includes deionization before HPLC analysis using an Amberlite/Ambersep ion exchange resin. The inclusion of an ion exchange resin in the carbon filtration method removed these proposed caramelization products, as demonstrated by the undigested carbon + ion exchange resin filtered spray dried polylactose chromatogram (Figure 5). Similar carbohydrates can be separated by ion exchange resins (Nobre et al., 2015); therefore, the inclusion of the Amberlite/Ambersep resin separated the caramelization products out, while maintaining the LMWSDF content.

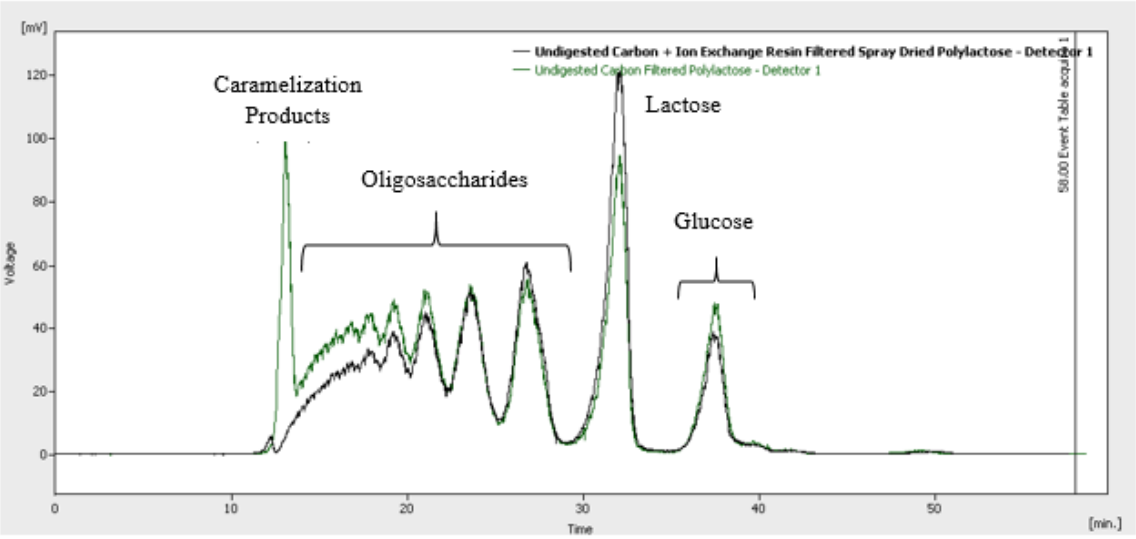

Figure 5. HPLC chromatogram comparing polylactose that was passed through a column containing carbon (green) and a mixed-bed column (black).

4.3.3 Decolorization of polylactose through carbon filtration

Carbon filtration reduced the HMF content of polylactose by over 99.6% and reduced the absorbance at 420nm by over 77% (Table 7). A study conducted by Carabasa et al., 1995 measured the change in HMF for clarified fruit juices after decolorization with activated carbon. HMF content was reduced with increasing contact time and increasing temperature. They found that after 50 minutes of contact with the activated carbon at a temperature of 20°C, HMF was reduced by 40%. It was also reported in this study that HMF adsorbed to the carbon before other dark color components in the fruit juices. Our study shows that the decreasing HMF content corresponds to a decrease in the absorbance at 420 nm. This relationship was also observed for the purification of fruit juices by carbon filtration (Carabasa et al., 1995).

The absorbance at 420 nm has been used to measure the amount of brown pigments from Maillard browning and caramelization in a product (Ajandouz et al., 2001 Ajandouz et al., 2008; Kroh, 1994). This measurement is widely used for evaluating the extent of decolorization after treatment with an adsorbent (Ahmeda et al., 1997; Ahmeda et al., 2000; Carabasa et al., 1995; Mudoga et al., 2008; Pendyal et al., 1990). A comparison of the absorbance at 420 nm of a sugar syrup before and after carbon filtration has been used to measure the percent molasses color removed. It was reported that commerical activated carbon removed 55-75% of the molasses color from a molasses solution (Pendyal et al., 1990). Other studies have found that commerical activated carbons can remove over 90% of the color for molasses and over 45% of the color for raw sugar (Ahmeda et al., 1997; Ahmeda et al., 2000; Mudoga et al., 2008). The successful decolorization activity of activated carbon is attributed to the melanoidin removal ability of the adsorbent. At low melanoidin concentrations, 5 g/L, 99.9% of the color was reportedly removed and at a higher concentration, 40 g/L, over 70% of the color was removed (Satyawali and Balakrishnan, 2007).

Hunter *L*,*a*,*b* values are a color space that defines color numerically and indicates the degree of lightness (*L*) with 0 representing black and 100 representing white, the red-green color of the product (*a*), and the yellow-blue color of the product (*b*) (Choudhury,

2014). The increase in the *L* value for the carbon filtered polylactose (Table 7) represents the change from the original light brown color to a white powder post filtration and drying (Figure 6). Polylactose filtered with the large benchtop method and the pre-spray drying product from the mixed bed carbon filtration method were both freeze dried before color analysis. When comparing the two filtration methods, the *L* value was similar, but the absorbance at 420 nm was lower for the large scale method than the mixed bed method. An increase in lightness, as measured by the Hunter *L*,*a*,*b* scale, was observed for apple juice that was purified using an adsorbent resin. As the adsorption of color compounds to the resin increased, the lightness of the product also increased (Albert et al., 2012). The present study demonstrates this as well. The incorporation of an adsorbent resin to the carbon column further increased in the lightness of the product (*L*), compard to the product filtered with just activated carbon (Table 7).

Table 7. Impact of carbon filtration and spray drying on polylactose color[1]

Sample	HMF (%)[2]	Abs (420 nm)	*L*	*a*	*b*
Polylactose	0.090 ± 0.005^{a}	0.363 ± 0.013^{a}	86.17 ± 0.68^{a}	-7.13 ± 0.13^{a}	22.10 ± 0.17^{a}
Carbon Filtered Polylactose (Large Benchtop Method)	N.D.	0.041 ± 0.003^{b}	91.89 ± 0.13^{b}	-5.70 ± 0.05^{b}	7.55 ± 0.13^{b}
Mixed Bed Carbon Filtered Polylactose Before Spray Drying	$0.000149 \pm 0.00^{A,b}$	0.082 ± 0.005^{c}	$92.60 \pm 0.41^{A,b}$	-5.47 ± 0.05^{b}	7.09 ± 0.13^{bc}
Mixed Bed Carbon Filtered Spray Dried Polylactose	$0.000355 \pm 0.00^{B,b}$	0.077 ± 0.003^{c}	$93.25 \pm 0.15^{B,b}$	-5.66 ± 0.03^{b}	7.01 ± 0.11^{c}

[1] Values are means ± one standard deviation (N = 4), a-c Means without a common superscript letter within the same column are significantly different ($p<0.05$), A-B Means without a common superscript letter within the same column are significantly different ($p<0.05$)

[2] Hydroxymethylfurfural

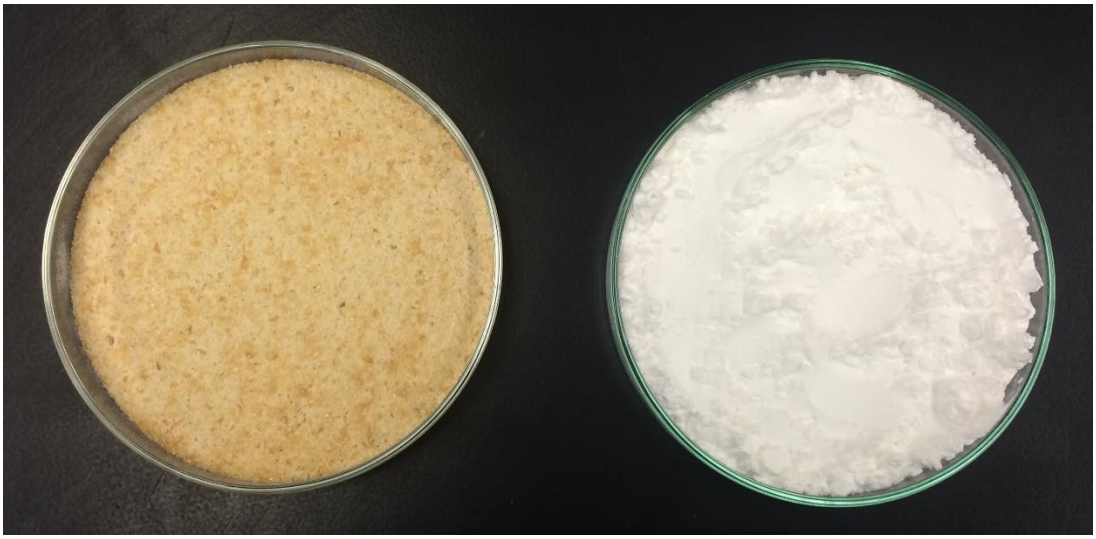

Figure 6. Color comparison of polylactose before (left) and after (right) carbon filtration

4.3.4 Impact of spray drying on the color and HMF content of carbon filtered polylactose

Spray drying is a common unit operation for the production of milk, whey, lactose and cheese powders. This unit operation is considered to be a gentle heating step because of the evaporative cooling effect that occurs. As the water is removed from the droplet, the moisture loss offsets the transfer of heat from the air into the droplet, preventing the product from experiencing temperatures above the wet-bulb temperature of the air (usually below 50°C). The likelihood of thermal degradation is reduced since the air has cooled by the time the dry product comes in contact with it (Heldman and Hartel, 1997_b). Despite the gentle heating of the spray drying process, non-enzymatic browning occurred during spray drying of the polylactose, as evidenced by the increase in HMF content and decrease in the lightness of the product (*L*) (Table 7). Previous research has found that spray drying lactose results in an increase in HMF (Koshy et al., 1965). While the HMF did increase during spray drying, the reduction in the HMF content when compared to the original polylactose product was still 99.6%.

4.4 Conclusion

Purification of polylactose using a carbon filtration system resulted in an increase in LMWSDF and lactose, while decreasing the HMF and citric acid contents. The HMF

content was reduced to levels well below the JEFCA limit for polydextrose (0.1%) and the brown color was successfully removed from the product. Carbon filtration also removed the oligomers that are believed to be a reaction product of caramelization. While the HMF content was increased with spray drying, there was still a 99.6% reduction in HMF content from non-filtered polylactose. The development of this successful carbon filtration method is critical for the continued progress towards commercializing polylactose.

5 Concluding Remarks and Next Steps

A benchtop method for lactose polymerization to soluble fiber was successfully developed using a Mars 6 microwave reaction system. This research demonstrates that the sugar-acid blend formulation is a critical parameter to consider in order to optimize dietary fiber yield. An increase in water content up to 18.57% (v/w) increased dietary fiber yield, while water additions above this level resulted in reduced fiber yield. The addition of calcium phosphate, a compound present in whey permeate, also caused a reduced dietary fiber yield. Successful polymerization of lactose in permeate powder on a benchtop and pilot plant scale indicates that this co-product could be used as a raw material for polylactose production; however, the yield is much lower than when lactose alone is processed. The dairy industry could use this technology to produce a novel ingredient from a current waste stream.

Purification of polylactose using activated carbon, ion exchange resins and diatomaceous earth resulted in a product with increased dietary fiber and lactose and reduced amounts of HMF and citric acid. The filtered product was also decolorized by this purification method. This research establishes that filtration can yield polylactose that meets the JEFCA standards, while increasing the purity of the product.

Research is currently being conducted on the filter purified, spray dried polylactose to determine if polylactose has benefits that allow for a dietary fiber classification. The animal study will assess the efficacy of polylactose in the prevention of colon cancer, diabeters, obesity, non-alcoholic fatty liver disease and metabolic syndrome. It will also assess the effect of polylactose on the microbiome of the dietary induced obese rats to determine if it could potentially be classified as a prebiotic. Additional next steps for the project include investigating the impact of residual protein in dairy co-products on lactose polymerization and optimization of the extrusion process for a permeate-acid blend. Increased residence time in the twin-screw extruder, lower temperature, and alteration of the acid, protein and mineral concentration may help increase the dietary fiber yield.

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7 Appendix

A.1 Extended Methods for Benchtop Polymerization of Sugars to Oligosaccharides

A.1.1 Production of polymerization products in the Mars 6 microwave reactor

Objective: The purpose of this method is to produce polymerization products on a benchtop scale before scaling up to the extruder. The amount and type of sugar added, acid concentration and water addition amount can be adjusted.

Materials:
α-Lactose Monohydrate
Glucose
Permeate powder
Citric Acid
Reverse osmosis water

Equipment:
Blender (Kitchen-Aid)
Mars 6 Microwave Digestion System (CEM Corporation)
8 MARSXpress Plus Teflon Vessels (CEM Corporation)
8 Vessel Stoppers (CEM Corporation)
8 Vessel Caps (CEM Corporation)
Pipettor
Cap tightener
Plastic bag
Test tube rack
Aluminum weighing dish

Procedure:
1. Prepare the powder blend by blending the dry ingredients in the blender for 5 minutes. Stop the blender after each minute and scrape down the edges.
2. Add 7 grams of powder to each vessel and add 1 mL of reverse osmosis water to each vessel.
3. Put the stopper and cap on the vessel and tighten until the cap clicks twice. Store the vessels upright in a plastic bag for 24 hours.
4. Place the vessels in the inner circle of the carousel and push down so that they are fully in the sleeves.
5. Set up the Mars 6 Program under Classic Method.
    - Temperature - 140°C
    - Ramp time - 14 minutes
    - Hold time - 0.1 seconds

- Cool down - 0 minutes

6. Place the carousel in the Mars 6 and start the program.
7. Once the program is complete, remove the carousel from the Mars 6, and carefully open each tube. Remove the product and place it in an aluminum weighing dish to cool.

A.1.2 Reactive extrusion of a permeate-acid blend

Objective: The purpose of this method is to produce an oligosaccharide from the lactose in permeate powder using reactive extrusion.

Materials:
Permeate powder
Citric Acid

Equipment:
Scale
Plastic bucket
Ribbon blender (IMS-1)
Plastic bag
Spatula
Bühler 44 mm co-rotating twin-screw extruder
Loss-in-weight feeder (K-Tron Soder K-ML-KT20)
Heat transfer control system (model H47212DT)
Metal Trays
Wire drying rack

Procedure:
Sugar-acid blend preparation

1. Weigh ingredients required for a 30 lb batch of a 90% permeate and 10% citric acid blend into a large bucket and then pour into the ribbon blender.
2. Close both the grated cover and the top cover of the ribbon blender. Mix for 2 minutes in both forward and reverse directions. Press stop and let the blades come to a complete stop before changing directions.
3. Open the top cover of the ribbon blender and continue mixing in either forward or reverse direction to get the powder out of the ribbon blender.
4. Slowly open the trap door at the bottom of the ribbon blender and pour the mix into a plastic bag. To make sure the powder gets into the bag, take the top of the bag and wrap it around the exit of the ribbon blender. Make sure to carefully control the flow through the trap door.
5. With the ribbon blender off, use a spatula to scrape the remaining mix into the bag.

Extruder set-up

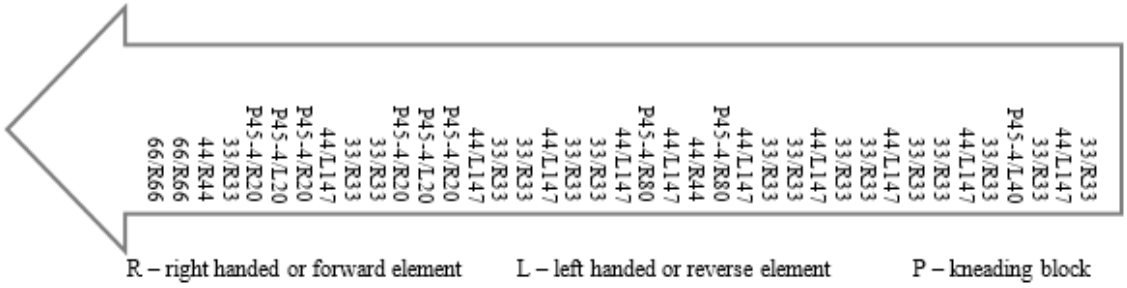

Figure A1. Screw design for a Buhler 44 mm twin-screw extruder used to polymerize lactose.

1. The pilot plant staff will start up the extruder by pouring the feed into the K-Tron extruder feeder, warming up the barrels, rotating the screws, and slowly increasing the feed rate.
    Extrusion Conditions:
        i. Barrel zones #2, #3, #4, #5, #6 - 238°C
        ii. Barrel zone #7 - no heating
            1. Temperature maintained by heat transfer control system model H47212DT
        iii. Feed rate - 15 kg/hr
            1. Feed rate controlled by K-Tron Soder K-ML-KT20 loss-in-weight feeder
            2. Screw speed – 250 rpm
        iv. Die plate - none
2. Once the conditions have been reached and the extruder has reached steady state with regards to the die temperature, die pressure, and motor torque, begin to collect the samples.
3. Record the extrusion operation data on the operation data sheet at the beginning and towards the end of every sample collection.

Sample collection from extruder

1. As the product comes off the extruder, allow it to drop on a metal tray in a single layer.
2. Once the tray is full, move the tray to a drying rack so the product can cool and place a new tray below the extruder.

A.1.3 Dietary fiber

Objective: The purpose of this experiment is to measure the amount of total dietary fiber in the polymerization products, which is an indication of how much polymerization occurred.

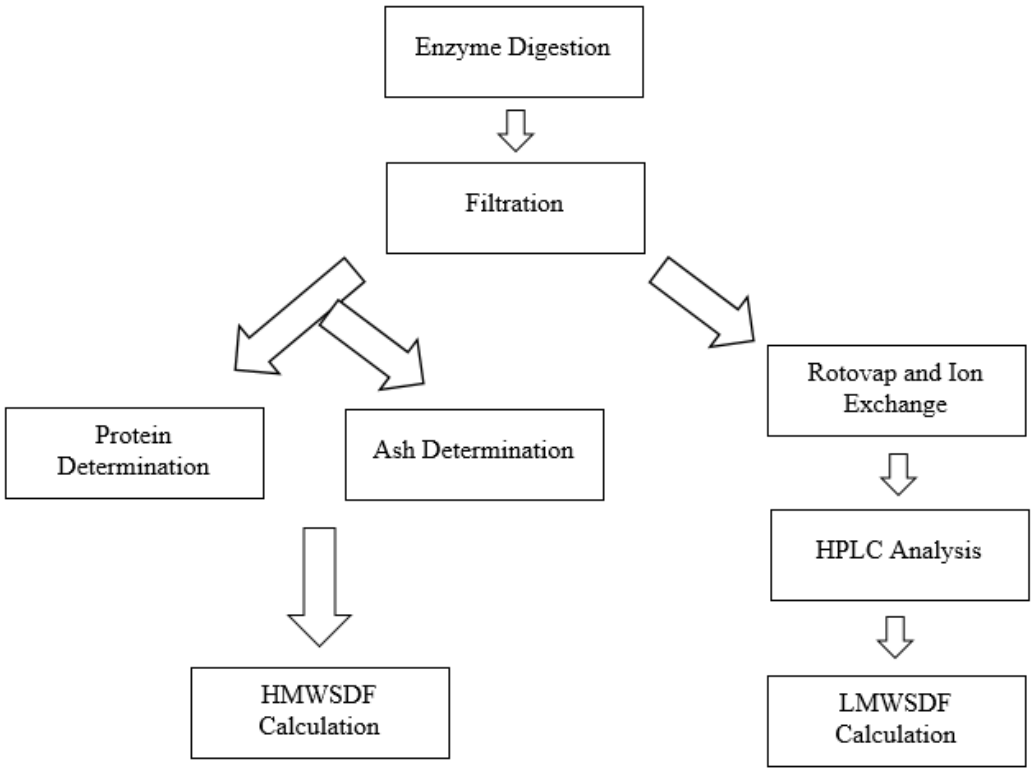

Figure A2. Dietary fiber assay schematic

Digestion:

Materials:

Integrated Total Dietary Fiber Kit (Megazyme)

- Bottle 1: Concentrated pancreatic α-amylase; 2g, 150,000 Ceralpha Units/g. Stable for > 5 years when stored dry at -20°C.
- Bottle 2: Amyloglucosidase (AMG)(20mL, 3300 Units/mL). Stable for > 3 years at 4°C.
- Bottle 3: Purified protease (10mL, 350 tyrosine units/mL). Stable for > 3 years at 4°C.

Maleic acid
Distilled water
Calcium chloride dihydrate
Tris buffer salt

Glacial acetic acid
Sodium hydroxide
Ground polymerization product
Ribose

Equipment:
Beaker (2000 mL)
Volumetric flask (1L, 2L)
Stir bar
Stir plate
pH meter with calibration standards, 4.0, 7.0, 10.0 (Accumet AB15/15+)
250 mL plastic bottle with a cap
Shaking water bath for 37°C incubation
Non-shaking water bath for 60°C and 95-100°C incubation
Pipettor

Procedure:
Reagent preparation

1. 4 M NaOH
    a. Prepare the solution by dissolving NaOH in distilled water to achieve a concentration of 160 g/L.
2. Sodium maleate buffer (50mM, pH 6.0 plus 2 mM $CaCl_2$)
    a. Calibrate the pH meter.
    b. Dissolve 11.6g of maleic acid in 1600 mL of distilled water.
    c. Adjust the pH to 6.0 with 4M NaOH.
    d. Add 0.6 g of calcium chloride dihydrate and adjust the volume to 2 L.
3. Pancreatic α-amylase (50 Units/mL)/AMG (3.4 Units/mL)
    a. Dissolve 0.10 g of purified porcine pancreatic α-amylase in 290 mL of sodium maleate buffer immediately before use.
    b. Stir for 5 minutes and add 0.3 mL of AMG.
4. 0.75 Tris base solution
    a. Add 90.8g of Tris buffer salt to approximately 800 mL of distilled water and dissolve.
    b. Adjust the volume to 2L using a volumetric flask.
5. 2M Acetic acid solution
    a. Add 115 mL of glacial acetic acid to a 1L volumetric flask.
    b. Dilute to 1L with distilled water.

Enzyme digestion procedure

1. Weigh Sample - Weigh 1.000 ± 0.005g of sample, in duplicate and quantitatively transfer the sample to a 250 mL bottle.
2. Enzyme Addition - Wet the sample with about 1 mL of ethanol and add 40 mL of the pancreatic α-amylase/AMG solution to the bottle.
3. Incubation - Incubate the samples for exactly 16 hours in a shaking water bath set to 37°C and 150 rpm.
4. pH adjustment to approximately 8.2
    a. After the 16 hours, remove the bottles from the water bath and add 3.0 mL of the 0.75 M Tris base solution to stop the reaction.
    b. Re-cap the bottles and incubate the bottles in a non-shaking water bath for 20 minutes at 95-100°C. Shake the bottles every 5 minutes.
5. Cool - Remove the samples from the water bath and cool to approximately 60 °C.
6. Protease Treatment
    a. Using a positive displacement pipettor, add 0.1 mL of the protease solution to each bottle.
    b. Incubate the bottles at 60°C for 20 minutes.
7. pH adjustment - Remove the bottles from the water bath and add 4.0 mL of 2 M acetic acid to each bottle to achieve a final pH of about 4.3. Swirl the bottle to mix the solution well.
8. Internal Standard – Add 100 mg of ribose to each bottle and mix well.
    a. At this point the solution can be refrigerated if not proceeding to the filtration step immediately.
9. Continue to the filtration step.

Filtration

Materials:

Digested samples in 250mL bottles
Ethanol, 95% v/v
Distilled water
Ethanol, 78% v/v
Celite, acid washed
Acetone

Equipment:

Volumetric flask (1 L)
Fritted crucibles: 50 mL, pore size coarse: 40-60 μm
Drying oven
Dessicator
Analytical balance
Transfer pipette
Graduated cylinder (10 mL, 25 mL)

Vacuum pump
Rubber fitting for vacuum filter flask
Vacuum filter flask (250 mL, 500 mL)
Muffle oven

Reagent preparation:

1. Ethanol, 78% v/v
    a. Add 821 mL of 95% (v/v) ethanol to a 1L volumetric flask.
    b. Bring to volume with distilled water.

Crucible preparation:

1. Add approximately 1.0 g of Celite to each crucible.
2. Dry the crucibles in a drying oven set to 130°C for at least one hour (to constant weight) or overnight.
3. Remove the crucibles from the oven once at constant weight and place in a dessicator to cool for at least one hour.
4. After cooled, record the weight of the crucible + Celite.

Procedure:

1. Pre-heat ~200 mL of 95% (v/v) ethanol to 60°C.
2. Precipitation
    a. Add 192 mL of 95% 60°C (v/v) ethanol at to the 250 mL containing the digested samples.
        i. If the samples have been refrigerated between digestion and filtration, pre-heat the sample to 60°C.
    b. Swirl the contents of the bottle and allow the precipitate to form for 60 minutes at room temperature.
3. Filtration Setup
    a. Lightly tap the Celite into an even layer and place the crucible on a rubber fitting on top of a 500 mL filter flask. Turn on the vacuum.
    b. Using a transfer pipette, wet and redistribute the Celite in the crucible. Add about 5 mL of 78% (v/v) ethanol by tracing the inside edge of the crucible to wet the outer edge of the Celite. Slowly add an additional 10 mL of ethanol with a graduated cylinder to wet all of the Celite.
4. Filtration
    a. Slowly pour the digested sample into the crucible so that it passes through the Celite.
    b. Quantitatively transfer any remaining sample to the crucible with ~ 15 mL of 78% (v/v) ethanol.
        i. Keep this filtrate for further analysis. Divide the filtrate in half so that one sample is used for Low Molecular Weight Soluble Dietary

Fiber (LMWSDF) quantification and one sample is saved if analysis needs to be repeated.

5. Wash
    a. Move the crucible and rubber fitting to a 250 mL filter flask before washing the Celite.
    b. Wash the residue with two 15 mL quantities of 78% (v/v) ethanol, then two 15 mL quantities of 95% (v/v) ethanol and finally two 15 mL portions of acetone.
        i. This filtrate does not need to be kept for further analysis.
6. Dry crucibles
    a. Place the crucibles in a drying oven and dry overnight at 105°C.
    b. Cool the crucibles for at least 1 hour and weigh the crucibles containing the residue and Celite.
        i. Since the samples were run in duplicate, the residue from one crucible is used for protein analysis and the other is used for ash.

Ash determination

Materials:

Crucible containing residue and Celite

Equipment:

Muffle oven
Analytical balance
Dessicator

Procedure:

1. Ash the crucible containing the Celite and residue at 525°C for 5 hours.
2. Allow the crucibles to cool and then allow them to cool to room temperature in a dessicator.
3. Weigh the crucible.

Calculation:

% Ash

$$\%\ Ash = \left(\frac{Weight\ of\ Ashed\ Crucible+Celite+Residue\ (g) - Weight\ Crucible+Celite(g)}{Weight\ of\ residue\ (g)}\right) * 100$$

Ash (mg)

$$Ash\ (mg) = Mass\ of\ residue\ (g) * \frac{\%\ Ash}{100} * \frac{1000\ mg}{1\ g}$$

Protein determination (Kjeldahl)

Materials:

Crucibles with residue
Weigh paper
Soy protein isolate (positive control)
Kjeldahl tablets
Sulfuric acid, 95-98%
Distilled water
32% NaOH
4% Boric acid
0.1 N HCl
0.2 Methyl red

Equipment:

Volumetric flask (1L)
Metal spatula
Weigh boat
Kjeldahl digestion block (Büchi)
Kjeldahl distillation unit (Büchi)
250 mL Erlenmayer flask
10 mL buret
Ring stand
Buret clamp

Reagent preparation:

1. 32% Sodium Hydroxide
    a. Add 320g of sodium hydroxide tablets to a 1L volumetric flask. Bring to volume with distilled water.
    b. Dissolve the sodium hydroxide. Add more water to bring to volume if needed.

Procedure:

1. Transfer the residue from the crucible to a piece of weigh paper using a metal spatula. Fold the paper tightly around the sample and store in a labeled weigh boat.
    a. Store the samples in a dessicator if not using immediately.
2. Add the sample and weigh paper bundle to the Kjeldahl flask.
    a. Run 3 blanks with just weigh paper and 2-3 positive control using soy protein isolate.
3. Begin the digestion stop by adding two Kjeldahl tablets to ach flask and 10 mL of sulfuric acid.
4. Digest at 420°C for 90 minutes.

5. Allow the samples to cool and remove the flasks from the digestion block.
6. Once cooled, add 50 mL of distilled water.
7. Distillation
    a. Add 50 mL of boric acid and two drops of methyl red to a 250 mL Erlenmeyer flask.
    b. Place a Kjeldahl flask and one prepared Erlenmeyer flask in the Buchi distillation unit.
    c. Add 150 mL of sodium hydroxide to the Kjeldahl flask.
    d. Distill the sample into the Erlenmeyer flask.
8. Titration
    a. Titrate a blank sample with HCl to a pink endpoint.
    b. Titrate the samples with HCl to match the pink color achieved with the blank.
9. Calculate the protein content using a factor of 6.25.

Calculation:

<u>% Nitrogen</u>

$$\%\ Nitrogen = \frac{\left(N\ HCl\left(\frac{mol\ HCl}{1000\ mL}\right) * \left(HCl\ titrated\ for\ sample\ (mL) - HCl\ titrated\ for\ blank\ (mL)\right) * \frac{14\ g\ N}{mol}\right)}{(Sample\ mass\ (g) * 1000)} * 100$$

<u>% Protein</u>

$$\%\ Protein = \%Nitrogen * 6.25$$

<u>Protein (mg)</u>

$$Protein\ (mg) = Mass\ of\ residue\ (g) * \frac{\%\ Protein}{100} * \frac{1000\ mg}{1\ g}$$

<u>HPLC analysis preparation</u>

Materials:

Filtrate
Amberlite FPA 53 (OH^-) (Megazyme International)
Ambersep 200 (H^+) (Megazyme International)
Double distilled water (DDW)

Equipment:

Round bottom flask (250 mL)
Rotovap (Büchi)
Beaker (600mL)
Weigh boat
Disposable column (Bio-Rad)

Glass vacuum manifold (Supelco)
Glass test tube
Graduated cylinder (25 mL)
Vacuum pump
Pipettor
Centrifuge tube (50 mL)
Parafilm
Freeze dryer
Freeze dryer flask
Vortex mixer
5 mL disposable syringe
0.45 μm syringe filter
HPLC vial with cap and septum

Procedure:

1. Evaporation
    a. Transfer half of the sample's filtrate to a 250 mL round bottom flask.
    b. Place the flask on a Rotovap and evaporate to dryness under vacuum at 60°C.
    c. Evaporate for about 30 minutes until about 7 mL of sample is left.
2. Sample deionization
    a. Combine 4 g of Amberlite and 4 g Ambersep in a beaker.
    b. Add the 8 g of the ion exchange resin to a Bio-Rad disposable column.
    c. Place the column on top of the glass vacuum manifold and place a test tube below the column in the manifold.
    d. Add 20 mL of DDW to the column and open the valve to allow the water to elute.
    e. Apply vacuum to ensure that all the water is eluted.
    f. Replace the test tubes with clean test tubes before deionization and close the valves.
    g. Add 2 mL of filtrate and then 2 mL of DDW to the column and allow it to percolate in the resin.
    h. Add 10 mL of DDW to the column and open the valves so that the sample elutes at a rate of about 1.0 mL per minute.
    i. Transfer the eluent to a centrifuge tube. Add an additional 10 mL of DDW to the column.
    j. Turn on the vacuum to remove any remaining eluent and add it to the centrifuge tube.
3. Freeze drying
    a. Add Parafilm to the top of the centrifuge tube and poke 3 holes in it.
    b. Freeze the sample on an angle so that the sample is on a bias.

c. Once frozen, place the centrifuge tubes in a freeze dryer flask and freeze dry until dryness.

4. Microfiltration of the deionized sample
    a. Solubilize the freeze dried sample using 2 mL of DDW.
    b. Vortex the sample until fully dissolved.
    c. Transfer the sample to a 5 mL disposable syringe with a 0.45 μm syringe filter.
    d. Filter the sample into an HPLC vial.

HPLC analysis for LMWSDF determination

Materials:

HPLC vials containing microfiltered sample
Glucose
Ribose
Double Distilled Water (DDW)

Equipment:

Volumetric flask (100mL)
5 mL disposable syringe
0.45 μm syringe filter
HPLC vial with cap and septum
High Performance Liquid Chromatography (HPLC) system (Beckman Coulter) with Evaporative Light Scanning Detector (ELSD) (Shimadzu SEDEX Model 85-LT LT-ELSD)

Procedure:

1. Response factor sample preparation
    a. Weigh 0.1, 0.2, and 0.3 grams of glucose into three separate 100 mL volumetric flasks.
    b. Add 0.2 grams of ribose into each flask.
    c. Dilute to volume with DDW.
    d. Transfer the sample to a 5 mL disposable syringe with a 0.45 μm syringe filter.
    e. Filter the sample into an HPLC vial.
2. HPLC – ELSD set-up
    a. Set up with equipment to meet the following conditions:
        - Reversed phase
        - Mobile phase – DDW
        - Sample injection volume – 20 μL
        - Column – Transgenomics CARBO-Sep CHO-411
        - Column temperature - 80°C

- Flow rate – 0.3 mL/min
- ELSD Detection Conditions - 40°C and 350 kPa

b. Turn on all the equipment and allow the column heater and ELSD to come to temperature (~20 minutes).

3. Run samples
   a. Run each response factor sample and record the chromatogram.
   b. Run each dietary fiber sample and record the chromatogram.
4. Peak area determination
   a. For the response factor sample measure the peak area of the D-glucose and the ribose internal standard.
   b. For the dietary fiber sample measure the peak area of the LMSWDF (all peaks before lactose which has a DP greater than or equal to 3) and the peak area of the ribose.

Calculations:

High Molecular Weight Soluble Dietary Fiber (HMWSDF):

Blank Calculation:

$$Blank\ (mg) = \frac{Residue\ mass\ of\ blank\ 1\ (mg) + residue\ mass\ of\ blank\ 2\ (mg)}{2} - Mass\ of\ protein\ from\ Blank\ 1\ (mg) - Mass\ of\ ash\ from\ Blank\ 2\ (mg)$$

HMWSDF:

$$HMWDF\left(\frac{mg}{100g}\right) = \frac{mass\ of\ residue\ (mg) - mass\ of\ ash\ (mg) - mass\ of\ protein\ (mg) - blank\ (mg)}{weight\ of\ sample\ (g)} \times 100\ g$$

%HMWSDF:

$$HMWDF\% = HMWDF\left(\frac{mg}{100g}\right) \times \frac{1\ mg}{1000\ mg}$$

Response Factor:

Figure A3. Peak area ratio:mass ratio for glucose:ribose used to calculate the response factor

Response Factor Calculation:

$$Response\ Factor = 1/slope$$

LMWSDF Calculation:

$$LMWSDF\left(\frac{mg}{100g}\right) = \frac{\left(\left(Response\ factor * \frac{Mass\ of\ internal\ standard\ (mg)}{mL}\right) * \left(\frac{LMWSDF\ Peak\ Area}{Internal\ Standard\ Peak\ Area}\right) * 100\right)}{Mass\ of\ sample\ (g)}$$

$$LMWSDF\% = LMWSDF\left(\frac{mg}{100g}\right) \times \frac{1\ g}{1000\ mg}$$

A.1.4 Lactose and glucose

Objective: The purpose of this experiment is to measure both lactose and glucose in raw materials before polymerization and residual lactose and glucose post polymerization.

Materials:

Megazyme Lactose Sucrose/D-Glucose Kit

- Bottle 2: β-Galactosidase (lactase; *A.niger*, 1 mL) suspension. Stable for > 2 years at 4°C.

- Bottle 3: GOPOD Reagent Buffer. Buffer (50 mL, pH 7.4), p-hydroxybenzoic acid and sodium azide (0.095% w/v). Stable for > 4 years at 4°C.
- Bottle 4: GOPOD Reagent Enzymes. Glucose oxidase plus peroxidase and 4-aminoantipyrine. Freeze-dried powder. Stable for > 5 years at 4°C.
- Bottle 5: D-Glucose standard solution (5mL, 1.0 mg/mL) in 0.2% benzoic acid. Stable for > 5 years at room temperature.

Glacial acetic acid
1M sodium hydroxide
Distilled water
Ground polymerization product

Equipment:
pH probe with calibration standards, 4.0, 7.0, 10.0 (Accumet AB 15/15+)
50 and 1000 mL volumetric flasks with glass stoppers
15 mL Centrifuge tubes
Aluminum foil
125 mL plastic bottle
Weigh boats
Analytical balance
Small glass test tubes with screw caps
Test tube rack
Positive displacement pipettor
Water bath
Vortex mixer
Cuvettes
Shimadzu UV-1800 spectrophotometer set at 510nm

Procedure:
Reagent preparation
1. Sodium acetate buffer preparation (50mM, pH 4.5)
    a. Calibrate the pH meter with the standards, 4.0, 7.0, 10.0
    b. Add 2.9 mL of glacial acetic acid to 900 mL of distilled water.
    c. Adjust the pH to 4.5 by adding 1M (4g/100 mL) sodium hydroxide solution.
    d. Bring the solution to volume with RO water in a 1 L volumetric flask.
    e. Store the buffer at 4°C.
2. Solution 2
    a. Combine the contents of Bottle 2 in 19 mL of sodium acetate buffer.
    b. Create 5 mL aliquots of the solution using centrifuge tubes. Store frozen between use. Stable for >2 years at -20°C.
3. Solution 3
    a. Dilute bottle 3 to 1 L with distilled water.
4. GOPOD Reagent

a. Add ~10 mL of solution 3 to bottle 4 to dissolve.
b. Quantitatively transfer this solution back into the bottle containing the 1 L Solution 3.
c. Cover the bottle with aluminum foil to protect it from the light. Aliquot the solution into 125 mL plastic bottles, cover the bottles with aluminum foil and then place the bottles in the freezer to store. Stable for ~3 months at 2-5°C or >12 months at -20°C

Assay procedure:

1. Prepare the sample solution by dissolving polymerization product in distilled water and bring to volume using a 50 mL volumetric flask. The concentration of lactose needed in the solution is 1 mg/mL – 3 mg/mL (want an absorbance > 0.1).
2. Prepare the blank in duplicate by adding 0.4 mL of distilled water to a glass test tube.
3. Prepare the glucose standard in duplicate by combining 0.2 mL of the D-glucose standard solution with 0.3 mL distilled water.
4. Pipette 0.2 mL of the sample extract into 4 test tubes. Add 0.2 mL of sodium acetate buffer to 2 of the test tubes (Glucose determination) and add 0.2 mL of β-Galactosidase to the remaining 2 test tubes (Lactose determination):
5. Incubate all the test tubes, including the reagent blanks and glucose standards in a 50°C water bath for 20 minutes.
6. Add 3.0 mL of GOPOD Reagent to all of the tubes and vortex the sample. Incubate the tubes at 50°C for 20 minutes.
7. Vortex the solution and add to a cuvette.
8. Measure all the absorbances at 510 nm against the reagent blank.

Calculations:

Conversion from absorbance to µg based on glucose:

$$F = \frac{100\ \mu g\ of\ glucose}{average\ absorbance\ for\ glucose\ standards\ (100\ \mu g\ of\ glucose)}$$

D-Glucose %, (w/w):

$$\frac{Absorbance\ of A}{0.2\ mL\ (sample\ volume)} \times F\ \frac{\mu g}{absorbance} \times \frac{1\ mg}{1000\ \mu g} \times \frac{mL\ of\ volume}{mg\ of\ sample} \times 100$$

Lactose % (w/w):

$$\frac{Absorbance\ of\ C - Aborbance\ of\ A}{0.2\ mL\ (sample\ volume)} \times F\ \frac{\mu g}{absorbance} \times \frac{1\ g}{1000\ \mu g} \times \frac{mL\ of\ volume}{mg\ of\ sample} \times \frac{342\ g\ lactose}{180\ g\ glucose} \times 100$$

A.1.5 pH

Adapted from: Case, R.A.., Bradley Jr., R.L., and Williams, R.R. 1985. Chemical and Physical Methods. P. 333 in: Standard Methods for the Examination of Dairy Products. Fifteenth ed. Richardson, G.H., ed. American Public Health Association, Washington, D.C.

Objective: The purpose was to measure the pH of the polymerization products to understand if formulation induced pH changes impacts polymerization.

Materials:
Ground polymerization products
Distilled water

Equipment:
25 mL beaker
50 mL volumetric flask
Small stir bar
Stir plate
pH probe with calibration standards, 4.0, 7.0, 10.0 (Accumet AB15/15+)

Procedure:

1. Add 3.25 g of ground polymerization product to a 25 mL beaker.
2. Add ~ 25 mL of distilled water to the beaker and a small stir bar. Stir the solution on a stir plate until the ground product is completely dissolved.
3. Quantitatively transfer the solution to a 50 mL volumetric flask and bring to volume with RO water.
4. Calibrate the pH probe with the 4.0, 7.0 and 10.0 calibration standards.
5. Pour ~ 25 mL of solution into another small glass beaker.
6. Measure the pH of the sample. Rinse the probe between samples with distilled water and blot dry with a Kim wipe.

A.2 Extended Methods for Carbon Filtration of Polylactose

A.2.1 Reactive extrusion of polylactose

Objective: The purpose of this method is to produce pilot plant scale polylactose to be carbon filtered through the benchtop and scaled-up carbon filtration methods.

Materials:
Lactose
Glucose
Citric Acid

Equipment:
Scale
Plastic bucket
Ribbon blender (IMS-1)
Plastic bag
Spatula
Bühler 44 mm co-rotating twin-screw extruder
Loss-in-weight feeder (K-Tron Soder K-ML-KT20)
Heat transfer control system (model H47212DT)
Metal Trays
Wire drying rack

Procedure:
Sugar-acid blend preparation

1. Weigh ingredients required for a 30 lb batch into a large bucket and then pour into the ribbon blender. The polylactose dry mix had a composition of 74% lactose, 20% glucose and 4% citric acid.
2. Close both the grated cover and the top cover of the ribbon blender. Mix for 2 minutes in both forward and reverse directions. Press stop and let the blades come to a complete stop before changing directions.
3. Open the top cover of the ribbon blender and continue mixing in either forward or reverse direction to get the powder out of the ribbon blender.
4. Slowly open the trap door at the bottom of the ribbon blender and pour the mix into a plastic bag. To make sure the powder gets into the bag, take the top of the bag and wrap it around the exit of the ribbon blender. Make sure to carefully control the flow through the trap door.
5. With the ribbon blender off, use a spatula to scrape the remaining mix into the bag.

Extruder set-up shown in Appendix A.1.2

1. The pilot plant staff will start up the extruder by pouring the feed into the K-Tron extruder feeder, warming up the barrels, rotating the screws, and slowly increasing the feed rate.
    a. Extrusion Conditions
        i. Barrel zones #2, #3, #4, #5, #6 - 238°C
        ii. Barrel zone #7 - no heating
            1. Temperature maintained by heat transfer control system model H47212DT
        iii. Feed rate - 15 kg/hr
            1. Feed rate controlled by K-Tron Soder K-ML-KT20 loss-in-weight feeder
            2. Screw speed – 250 rpm
        iv. Die plate - none
2. Once the conditions have been reached and the extruder has reached steady state with regards to the die temperature, die pressure, and motor torque, begin to collect the samples.
3. Record the extrusion operation data on the operation data sheet at the beginning and towards the end of every sample collection.

Sample collection from extruder

1. As the product comes off the extruder, allow it to drop on a metal tray in a single layer.
2. Once the tray is full, move the tray to a drying rack so the product can cool and place a new tray below the extruder.

A.2.2 Small scale benchtop carbon filtration

Objective: The objective of this experiment is to develop a method for carbon filtration.

Materials:

NORIT GAC 1240 Plus granular activated carbon (Cabot Norit Americas, Inc.)
Diatomaceous earth
Ground polylactose
RO water

Equipment:

Analytical balance
Beaker
Stir Bar
Stir plate
Disposable column (Bio-Rad)
Glass vacuum manifold (Supelco)
Glass test tubes

Graduated cylinder (10 mL)
Pipettor

Procedure:

1. Prepare a 100 mg/mL solution of polylactose by dissolving the product in RO water. 2 mL of solution will be added to each column.
2. Prepare the carbon filtration column by adding 0.5 g of diatomaceous earth to the bottom of the column followed by 5 g of granular activated carbon.
3. Place the columns on the manifold and put a glass test tube underneath the column.
4. Rinse the column with 20 mL of RO water.
5. Discard the wash water and replace the test tube with clean test tubes.
6. Add 2 mL of the polylactose solution to the column, followed by 2 mL of RO water. Allow this to percolate in the carbon.
7. Add 20 mL of additional water to the column and elute the solution at a rate of ~1 mL/min

A.2.3 Scaled up benchtop carbon filtration

Objective: The purpose of this experiment is to develop a carbon filtration method that will be used for scaled up polylactose purification prior to an animal study.

Materials:

NORIT GAC 1240 Plus granular activated carbon (Cabot Norit Americas, Inc.)
Diatomaceous earth
Amberlite FPA 53 (OH^-) (Megazyme)
Ambersep 200 (H^+) (Megazyme)
Ground polylactose
RO water

Equipment:

Analytical balance
Beaker
Stir Bar
Stir plate
Funnel
Glass column
Glass bottle (1 L)
Erlenmeyer flask (2 L)
Graduated cylinder (500 mL)

Procedure:

1. Prepare a 200 mg/mL solution of polylactose by dissolving the product in RO water. 800 mL of solution will be added to each column.
2. Prepare the column by adding 15 g of diatomaceous earth, followed by 400 g of granular activated carbon.
3. If preparing a mixed-bed column, mix 50g of Amberlite with 50g of Ambersep.
    a. Prepare the column by adding 15 g of diatomaceous earth, followed by the 100g of the Amberlite and Ambersep mixture, and finally add 400 g of granular activated carbon.
4. Place a glass bottle below the column and rinse with 3000 mL of RO water.
5. Replace the glass bottle with an Erlenmeyer flask before filtering the polylactose solution.
6. Add the 800 mL of the polylactose solution to the column in addition to 200 mL of RO water.
7. Begin to elute the solution at a rate of about 3 mL/min. Add an additional 1000 mL of RO water to the column.

A.2.4 Spray drying of carbon filtered polylactose

Objective: The purpose of this experiment is to develop a carbon filtration method that will be used for scaled up polylactose purification prior to an animal study.

Materials:

Carbon filtration eluent

Equipment:

APV Anhydro Type I spray dryer with an APV CF-100 atomizer
Rubber mallet

Procedure:

1. The pilot plant staff will set-up the spray dryer so that it warms up. The conditions used are:
    - Inlet temperature - 185°C
    - Outlet temperature - 90°C
    - Flow rate – 220 mL/min
    - Atomizer – 24,000 rpm
2. Run the carbon filtration through the spray dryer until all of the product is dried.
3. Occasionally tap the spray dryer with the rubber mallet to loosen the powder from the sides of the dryer

A.3 Extended Methods for Analysis of Polylactose and Carbon Filtered Polylactose

A.3.1 Hydroxymethylfurfural quantification

Objective: The objective of this experiment is to determine the HMF content of polylactose.

Materials:
HPLC grade methanol
Hydroxymethylfurfural standard
Double distilled water
Ground polylactose

Equipment:
Volumetric flask
Analytical balance
Shimadzu LC-2010C HT
YMC Pack ODS-AM C18 Column
HPLC vials and caps

Procedure:
Mobile Phase Preparation

1. Add 50 mL of HPLC grade methanol to a 1L volumetric flask. Bring to volume with double distilled water.

Standard Curve Generation

1. Prepare 5 concentrations of hydroxymethylfurfural in double distilled water for the polylactose standard curve (0.05 mg/mL, 0.075 mg/mL, 0.1 mg/mL, 0.15 mg/mL and 0.2 mg/mL).
2. Prepare 5 concentrations of hydroxymethylfurfural in double distilled water for the carbon filtered polylactose standard curve (9.38×10^{-5} mg/mL, 1.88×10^{-4} mg/mL, 3.75×10^{-4} mg/mL, 7.50×10^{-4} mg/mL and 1.50×10^{-3} mg/mL).
3. Prepare each solution for injection by transferring the sample to a 5 mL disposable syringe with a 0.45 μm syringe filter.
4. Filter the sample into an HPLC vial.
5. Set up the HPLC with the following conditions:
    - Reversed phase
    - Mobile phase – 95% DDW 5% Methanol
    - Sample injection volume – 20 μL
    - Column – YMC Pack ODS-AM C18 Column, 250 x 4.6 mm I.D.
    - Column temperature - 30°C

- Flow rate – 0.8 mL/min
- Absorbance – 285 nm
- Duration – 35 min

6. Run each standard.
7. Plot the peak area vs. the HMF concentration to generate the standard curve (Figure)

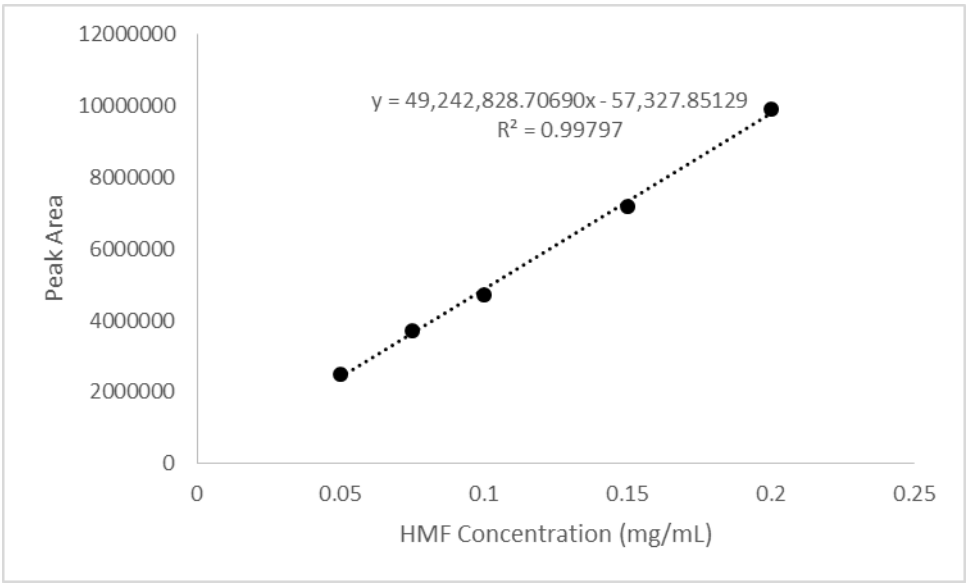

Figure A4. Standard curve for polylactose HMF quantification

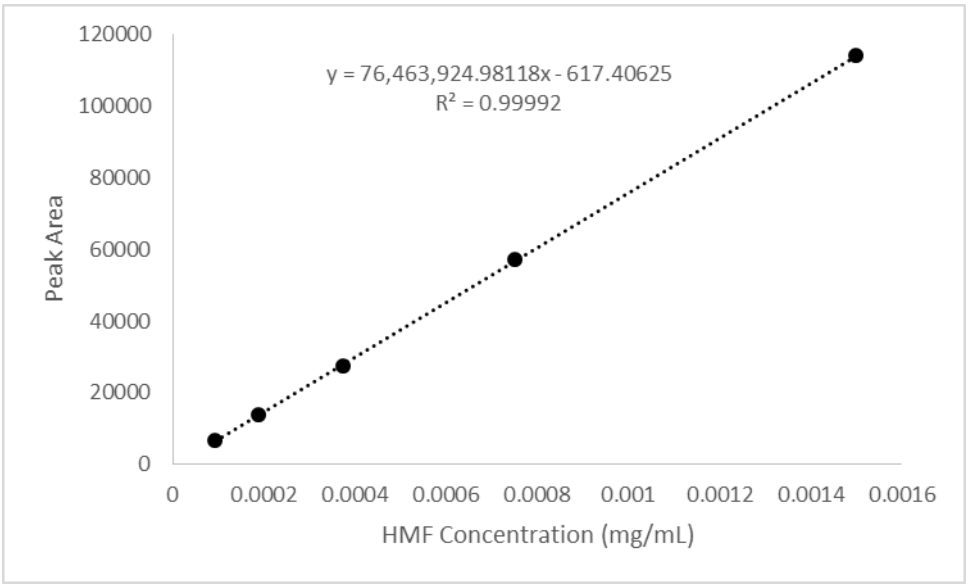

Figure A5. Standard curve for carbon filtered polylactose HMF quantification

HMF Quantification

1. Prepare a 100 mg/mL solution of polylactose in DDW.
2. Prepare each solution for injection by transferring the sample to a 5 mL disposable syringe with a 0.45 μm syringe filter.
3. Filter the sample into an HPLC vial.
4. Run the sample in triplicate using the HPLC conditions outlined in the Standard Curve procedure.

$$HMF\ (\%) = \frac{Sample\ Peak\ Area - Slope}{Intercept}$$

A.3.2 Color (Absorbance at 420 nm)

Objective: The objective of this experiment is to determine the amount of browning of the polylactose.

Materials:
Ground polylactose
RO Water

Equipment:
Volumetric flask (50 mL)
Cuvette
Shimadzu UV-1800 spectrophotometer set at 420 nm with UV Probe 2.43 software

Procedure:

1. Prepare the sample solution by dissolving 5 g of polylactose in distilled water and bring to volume using a 50 mL volumetric flask.
2. Transfer the solution to a plastic cuvette.
3. Measure the absorbance of the sample at 420 nm.
4. Repeat in triplicate for each sample.

A.3.3 Color (Hunter L, a, b)

Objective: The purpose of this experiment is to measure the color of polylactose.

Materials:
Sample solution used for the absorbance at 420 nm measurement

Equipment:
Cuvette

Shimadzu UV-1800 spectrophotometer set at 420 nm with UV Probe 2.43 software
Color Analysis Software (UV/PC version 3.10)

Procedure:

1. Using the cuvette with the sample from the absorbance at 420 nm analysis measure the Hunter L, a, b values.
2. Measure a spectrum in reflectance mode from 360 – 700 nm in 1 nm increments at medium speed.
3. Convert to Hunter L, a, b values using color analysis software. Use a D65 illuminant (standard daylight) and a 10° observer viewing angle.
4. Repeat in triplicate for each sample.

A.3.4 Citric acid

Objective: The purpose of this experiment is to measure the amount of citric acid in polylactose and carbon filtered polylactose.

Materials:

Citric Acid (Citrate) Assay (Megazyme)

- Bottle 1: Buffer (40 mL, pH 7.5) plus sodium azide (0.02%) as a preservative. Stable for > 2 years at 4°C.
- Bottle 2: NADH plus Polyvinylpyrollidone (PVP). Stable for > 5 years at -20°C.
- Bottle 3: L-malate dehydrogenase plus D-lactate dehydrogenase (L-MDH/D-LDH), 1.5 mL. Stable for > 2 years at 4°C.
- Bottle 4: Citrate lyase lyophilisate. Stable for > 5 years at -20°C.

RO Water
Ground or freeze-dried polylactose

Equipment:

Graduated cylinder (25 mL)
Beaker (100 mL)
Polypropylene tubes (25 mL)
Volumetric flask (100 mL)
Small glass test tubes with screw caps
Test tube rack
Positive displacement pipettor
Vortex Mixer
Cuvettes
Parafilm
Shimadzu UV-1800 spectrophotometer set at 510nm with UV Probe 2.43 software

Procedure:

Reagent Preparation

1. Solution 1
    a. Use the contents of Bottle 1 as supplied.
2. Solution 2
    a. Dissolve the contents of Bottle 2 in 16 mL of distilled water. Stable for > 1 year at 4°C or stable for > 2 years at -20°C. Divide the solution into appropriately sized aliquots and store in polypropylene tubes.
3. Solution 3
    a. Use the contents of Bottle 3 as supplied. Before opening for the first time, shake the bottle to incorporate any enzyme that may have settled on the rubber stopper.
4. Solution 4
    a. Add 0.55 mL of of RO water to Bottle 4 and dissolve the contents of Bottle 4. Stable for 4 weeks at 4°C or > 6 months at -20°C.

Assay Procedure

1. Prepare the sample solution by dissolving 0.125g of polylactose in distilled water and bring to volume using a 100 mL volumetric flask. The concentration of citric acid in each cuvette needs to be 1.0 – 100 μg.
2. Pipette the following solutions into small glass test tubes:

	Blank	**Sample**
RO Water	2.00 mL	1.80 mL
Sample solution	-	0.20 mL
Solution 1 (buffer)	0.50 mL	0.50 mL
Solution 2 (NADH/PVP)	0.20 mL	0.20 mL
Suspension 3 (L-MDH/D-LDH)	0.02 mL	0.02 mL

Table A1. Citric acid assay protocol

3. Vortex the solution. After approximately 4 minutes, vortex the solution and transfer the solution to a cuvette.
4. Measure the absorbances at 340 nm against the water. This absorbance is A_1.
5. Add 0.02 mL of Solution 4 to the cuvette containing the blank and the cuvette containing the sample.
6. Cover the cuvette with Parafilm and carefully invert the cuvette to mix the solution.
7. After approximately 5 minutes, invert the cuvette again.
8. Measure the absorbances at 340 nm against the water. This absorbance is A_2.

Calculations:

$$\Delta A_{citric\ acid} = \left(A_{1\ sample} - A_{2\ sample}\right) - \left(A_{1\ blank} - A_{2\ blank}\right)$$

$$Citric\ acid\ concentration = \frac{V\ (mL)\ x\ MW\ \left(\frac{g}{mol}\right)}{\varepsilon\ (1\ x\ mol^{-1}\ x\ cm^{-1})\ x\ d\ (cm)\ x\ v\ (mL)}\ x\ \Delta A_{citric\ acid}$$

V = final volume
MW = molecular weigt of citric acid
ε = extinction coefficient of NADH at 340 nm = 6300 (1 x mol^{-1} x cm^{-1})
d = light path
v = sample volume

$$Citric\ acid\ concentration = \frac{2.74\ (mL)\ x\ 192.1\ \left(\frac{g}{mol}\right)}{6300\ (1\ x\ mol^{-1}\ x\ cm^{-1})\ x\ 1.0\ (cm)\ x\ 0.20\ (mL)}\ x\ \Delta A_{citric\ acid}$$

$$Citric\ acid\ concentration = 0.4177\ x\ \Delta A_{citric\ acid}$$

$$Citric\ Acid\ Content\ (\%) = \frac{citric\ acid\ concentration\ \left(\frac{g}{L\ sample\ solution}\right)}{weight\ sample\ \left(\frac{g}{L\ sample\ solution}\right)}$$