Turning Low Value Whey Ingredients into Value Added Products

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Dedication

To my husband, for believing in me even when I doubted myself.

To my parents, for instilling in me the belief that I am capable of accomplishing anything I put my mind to.

To my sisters, for inspiring me to find a career that I am as passionate about as they are about theirs.

Abstract

Low value dairy products such as lactose and acid whey, pose a huge opportunity to be converted into value-added products. This research evaluated the effect of formula and processing conditions on the polymerization of lactose by reactive extrusion into indigestible oligomers. Three citric acid concentrations (2%, 4% and 6%) and two feed rates (15 kg/hr and 30 kg/hr) were evaluated for their effect on the polymerization of lactose. Samples extruded at 15 kg/hr resulted in a higher yield of indigestible oligomers (46.7-56.4%) than at 30 kg/hr (33.7-43.5%). A successful cleanup procedure for Greek yogurt acid whey was developed so that it could be concentrated and drum dried. Heat treating the acid whey resulted in a reduction of protein, fat, and moisture. Washing the condensed whey resulted in a reduction of lactic acid, protein, and a lighter colored drum dried product.

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1 Introduction

There are many byproducts of dairy processing which have the potential to be turned into value-added products. For example, whey from cheese making is now processed to create the valuable product whey protein. Big opportunities for value include the low value byproduct of whey permeate, lactose, and the byproduct of acidified dairy products, acid whey.

Lactose is considered a low value dairy ingredient, and is plentiful as a byproduct from the production of whey protein. Demand for fiber ingredients are high as the consumer market shifts towards a healthier eating mindset. If lactose could be converted into dietary fiber, it would be a value added product which would benefit dairy processors. Previous work performed by Tremaine, et al. (2014) shows that lactose can be polymerized into an indigestible substance through reactive extrusion. Processing conditions can greatly affect both the power and efficiency of production (Mottaz & Bruyas, 2001), as well as the composition of the final products produced via extrusion (Van Der Goot, Klaassens, & Janssen, 1997). The objective of the first part of this research was to investigate how extruder conditions can affect the final product from polymerized lactose. The goal was to balance the fiber yield, acidity, processing conditions and caramelization products. The product could be utilized as a feed product for animals or as a fiber additive in food applications.

Another problematic byproduct is acid whey. Greek yogurt production is booming as consumers become more concerned about eating healthy and adding protein to their diets. Greek yogurt is produced by separating whey from regular yogurt, which is referred to as acid whey because it has a higher quantity of acid in the whey than whey produced from renneted cheeses (sweet whey). Acid whey is composed of mostly water, with lactose as the most abundant solid (Zall, 1992). Current disposal methods for acid whey include anaerobic digestion, land farming and animal feed (Pesta, Meyer-Pittroff, & Russ, 2007;

Zall, 1992). There is not a large scale solution for the disposal of acid whey, and in fact dairy processors often must pay to dispose of the acid whey they produce. Acid whey is difficult to process due to stickiness from the lactic acid. Other dairy products which also have acid whey as a byproduct include cottage cheese, quark and cream cheese, and have the same disposal problem. The objective of the second part of this research is to develop a way to process acid whey using common industrial processing techniques so that it can be easily handled and useful in further applications, such as potentially to produce dietary fiber via reactive extrusion. This could create a value added product out of a substance which is currently considered waste.

2 Literature Review

2.1 Acid Whey

Acid whey is a byproduct which is produced when dairy products are made by coagulating the casein proteins in milk at their isoelectric pH, approximately 4.6 (Zall, 1992). In contrast, whey which is produced when dairy products are made by using enzymes such as rennet to coagulate milk is referred to as sweet whey (Zall, 1992). Sweet whey and acid whey are similar in composition, with a few main differences: pH, minerals (especially calcium) and protein content (Sienkiewicz & Riedel, 1990).

Acid whey has reported pHs from 4.5 to 5.3, with most centering around 4.6 because it is the isoelectric point for casein (Fox & McSweeney, 1998; Pesta et al., 2007; Sienkiewicz & Riedel, 1990). In comparison, analysis of sweet whey has reported pHs from 5.7 to 6.6 depending on what type of cheese was made and the processing conditions, with whey from Cheddar cheese in the range of 5.7 to 6.3 (Park, Bastian, Farkas, & Drake, 2014; Pesta et al., 2007; Sienkiewicz & Riedel, 1990). The pH of the whey will depend on a number of factors such as the type of product it is manufactured from, when and how fast the whey is cooled, as well as when and how it is heat treated.

In addition, sweet whey has a higher protein content (Fox, Guinee, Cogan, & McSweeney, 2000; Pesta et al., 2007; Sienkiewicz & Riedel, 1990). Acid whey is produced from acid coagulated dairy products such as quark, fromage frais, cottage cheese, cream cheese, and most notably in the U.S., Greek yogurt (Fox et al., 2000; Fox & McSweeney, 1998; Lucey & Singh, 2003; Sienkiewicz & Riedel, 1990). This method causes partial hydrolysis of whey proteins during the coagulation step, and results in more non-protein nitrogen in acid whey as compared to sweet whey

(Sienkiewicz & Riedel, 1990). Current disposal methods for acid whey are not meeting the needs for the large amounts of acid whey currently being produced. Therefore, there is a serious need for a solution for acid whey.

2.1.1 Greek Yogurt Production

Greek yogurt is made using the same process as regular yogurt, except for an additional step where water is removed from the milk gel via separation (Chandan & O'Rell, 2006a). This gives Greek yogurt its characteristic thick texture and concentrated protein, which consumers like (Desai, Shepard, & Drake, 2013). Greek yogurt can also be called strained or concentrated yogurt (Chandan & O'Rell, 2006a).

The main ingredient in yogurt is milk, which is standardized to a desired fat and solids content for the specific yogurt recipe (Chandan & O'Rell, 2006b). The standardized milk is pasteurized to kill human pathogens and other organisms that can compete with the desired cultures, and then it is homogenized to create an even fat distribution and minimize syneresis during incubation and storage (Bylund, 1995). Next, the milk is cooled to the inoculation temperature (40-45°C) so that cultures can be introduced (Bylund, 1995).

The standard of identity for yogurt states that by law in the United States, yogurt cultures must include *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (21 CFR § 131.200, 2014). During fermentation, these organisms produce lactic acid and various flavor molecules (Chandan & O'Rell, 2006b). The production of acid lowers the pH of the milk, which reduces the charge on the surface of the casein micelle and allows them to interact hydrophobically and coagulate (Fox & McSweeney, 1998; Horne, 2009; Lucey & Singh, 2003; Schulz-Collins & Senge, 2004). The isoelectric point of casein

micelles is around 4.6, at which point they approach neutral charge and hydrophobic interaction produces a casein network and forms a milk gel (Fox et al., 2000; Fox & McSweeney, 1998; Lucey & Singh, 2003). This creates the thicker texture of yogurt as compared to milk (Chandan & O'Rell, 2006a). Because milk for yogurt is pasteurized with an extended hold time, some whey proteins are denatured and interact with the casein network through disulfide linkages (Farkye, 2004; Schulz-Collins & Senge, 2004). This increases the thickness of the yogurt, as compared to utilizing milk which had not been heat treated for an extended time (Schulz-Collins & Senge, 2004), which therefore will also increase the yield when concentrating the gel to make Greek yogurt.

The fermentation of the starter cultures continues until the pH reaches 4.5-4.65, and then it is stopped by cooling down the yogurt to refrigeration temperature (Chandan & O'Rell, 2006a). At this point, plain yogurt has been produced from milk. Additional steps can involve adding flavoring or stirring, depending on the type of yogurt being produced (Chandan & O'Rell, 2006a).

Traditionally, Greek yogurt was produced by placing plain yogurt in cheese cloth to let excess whey drain out overnight (Chandan & O'Rell, 2006a). However, modern processing techniques have sped up this process by utilizing ultrafiltration and/or centrifugation to separate out excess whey to create the desired texture of Greek yogurt (Chandan & O'Rell, 2006a). During separation, the casein gel network is disrupted and the pores which previously trapped water and fat are disturbed, and whey is released from the yogurt (Lucey & Singh, 2003). The temperature of separation, heat treatment and gelation temperature all influence the amount of whey that can be separated from the yogurt (Lucey, Munro, & Singh, 1998; Lucey & Singh, 2003). Whey protein which is already denatured will stay in the yogurt matrix, however any whey protein still in its native state will separate out with the whey (Schulz-Collins & Senge, 2004).

Quark is an acidified dairy product which has a similar process to yogurt making. Quark manufacturers utilize additional whey protein in acid whey by heating it to 95°C to precipitate out additional whey protein, capturing it by using centrifugation, and then add it back into the milk for a subsequent batch of Quark (Schulz-Collins & Senge, 2004). The Westfalia process for producing Quark involves heat treating milk at 95°C to denature all whey proteins which increases the yield, but results in finer aggregates in the precipitate as compared with Quark which is made with milk pasteurized at 72°C (Schulz-Collins & Senge, 2004).

2.1.2 *Acid Whey Composition*

Acid whey is composed of mostly water, with solids ranging from 5.70% to 6.42% (Pesta et al., 2007; Sienkiewicz & Riedel, 1990). After water, lactose is the next most abundant material in acid whey at 4.40% to 4.70% (by weight), while ash (minerals) ranges from 0.60% to 0.80% and protein ranges from 0.30% to 0.62% (Fox et al., 2000; Pesta et al., 2007; Sienkiewicz & Riedel, 1990). Acid whey should have little fat, but can contain phospholipids (Fox & McSweeney, 1998). Therefore, lactose is the largest component of the solid material found in acid whey. The pH of acid whey can vary from 4.5 to 5.3, but is usually around 4.60 (Fox & McSweeney, 1998; Pesta et al., 2007; Sienkiewicz & Riedel, 1990).

2.1.3 *Acid Whey Disposal*

At this time, acid whey is seen as a waste product and must be disposed of. Acid whey is high in lactose and also contains protein, which are valuable products (Fox et al., 2000; Pesta et al., 2007; Sienkiewicz & Riedel, 1990). However, acid whey is difficult to process due to the stickiness caused by the lactic acid and often must be neutralized before it can be utilized (Sienkiewicz & Riedel, 1990). Sweet whey is widely available and utilized extensively as a source of lactose and whey protein

(Carr, Southward, & Creamer, 2003; Fox & McSweeney, 1998; Sienkiewicz & Riedel, 1990). Due to the difficulty of processing acid whey and the lower relative quantity of acid whey compared to sweet whey, acid whey has not been as extensively researched and utilized.

Acid whey cannot be simply discharged into a body of water because it has a large biological oxygen demand (Zall, 1992). A high biological oxygen demand is caused by microbiological organisms in water which digest the whey and use oxygen, which leads to a state of hypoxia, or oxygen depletion in the stream (Diaz & Breitburg, 2011). Fish and other marine animals must have dissolved oxygen in order to survive, and hypoxia has an obvious negative effect (Diaz & Breitburg, 2011). Because of its high biological oxygen demand, sewage treatments plants often charge an extra fee to dispose of acid whey (Arvanitoyannis & Kassaveti, 2008).

Several methods of disposal are currently available, including land application, alcoholic fermentation, and using it as animal feed (Pesta et al., 2007; Zall, 1992). Land application can be a good option, however the land can only take so much whey before it negatively affects the soil or there is so much acid whey that it washes into nearby bodies of water (Zall, 1992). Fermentation of acid whey can be done, however lactose fermenting organisms are not common and limited success has been seen with this disposal method (Pesta et al., 2007). In fact, greater success has been seen with sweet whey permeate from cheese making and so research has focused on this area (Pesta et al., 2007). Acid whey can be used to supplement animal feed for cows at rates of up to 12-15 liters of whey per 100 kg of body weight (Thivend, 1977). However, caution must be taken as there are some concerns with feeding large amounts of whey to ruminants. Ruminants require an adjustment period where a gradual increase in whey as feed is needed to avoid intestinal problems, their feed must be balanced to avoid an excess of lactic

acid production, and mineral imbalance must be monitored (Thivend, 1977). In addition, acid whey has been found to be less palatable to ruminants than sweet whey (Thivend, 1977).

2.2 Extrusion

Extrusion is a common industry process and refers to the process of forcing a product through a small opening (Kazemzadah, 2012). The main principle of extrusion, the Archimedes screw, dates back to the 3rd century BC and was devised by the ancient Greek mathematician Archimedes (Kazemzadah, 2012). Extrusion can be used with a variety of materials including plastic, concrete, metal, and food (Kazemzadah, 2012). Because of the high temperatures and pressures that can be achieved through extrusion, it is commonly used as a cooking process in the food industry (Riaz, 2000). Extrusion is used in many applications for food, such as ready to eat breakfast cereal, puffed snacks, pasta, confectionery, and textured protein (Berk, 2009). As a cooking method, extrusion has many advantages because it is a continuous process, including high throughput, energy efficiency, and automation (Riaz, 2000).

2.2.1 *Parts of an Extruder*

The simplest extruder design is a screw within a barrel which is used to force material through a small opening. Extruder design can vary in complexity, but the three main parts of an extruder are the screw(s), barrel and die.

2.2.1.1 Screw

A turning screw is the mechanism which is used to move the product through the extruder. The raised part of a screw is referred to as the flight, and is responsible for the conveying action of the screw (Riaz, 2000). The channel is the lower part of the screw which is in between the flights and is where the product is (Kazemzadah, 2012).

A screw has many different design elements which can include the diameter of the screw, pitch or angle of the flight, distance between flights, number of flights, shape of flights, and length of the screw (Kazemzadah, 2012). In addition, the screw can have different sections which perform different operations. The pitch of the screw flight can be varied to convey faster or slower, forward or reverse (Huber, 2000). The shape of the flight of the screw can be modified to provide greater mixing or kneading (Huber, 2000). A kneading block is formed by small "paddles" or "lobes" that when combine make a screw flight that changes in steps rather than being smooth like a traditional screw (Yacu, 2012). Reverse pitch elements are used to provide backwards flow. They increase the amount of fill in the barrel and increase the pressure (Huber, 2000). The kneading blocks and reverse elements both help facilitate the conversion of mechanical energy into heat, which results in higher temperatures and greater motor torque (Huber, 2000; Yacu, 2012). Twin-screw extruders can be easily modified by changing the type of screw elements and the order of screw elements to optimize screw design and processing conditions (Wagner, Mount, & Giles, 2014).

The ratio of the length of the screw to the diameter of the screw, or L/D ratio, is important when scaling from pilot size extruders to full scale extruders, and when comparing extruders from different manufacturers (Huber, 2000). In addition, the L/D ratio gives a relative idea of the energy input which will be required for production (Yacu, 2012).

An extruder can have a single screw, or two screws which fit together and are referred to as twin-screws (Riaz, 2000). Although single screw extruders are less expensive, twin-screw extruders have many advantages including an ability to handle materials with more moisture, a constant flow rate, and can

be utilized with a wide range of particle sizes of material (Riaz, 2000). Twin screws can rotate in the same direction or the opposite direction of each other and are referred to as co-rotating or counter-rotating, respectively (Kazemzadah, 2012). Co-rotating screws can provide much faster pumping than counter-rotating screws, however counter-rotating screws can produce much higher pressures than co-rotating (Riaz, 2000). Therefore, the type of rotation will depend on the desired processing conditions for each application. In addition, twin screws can be intermeshing which means that the two screws fit together precisely so that the flight of one screw matches up with the channel of the other screw (Riaz, 2000). Intermeshing screws provide excellent pumping and are self-cleaning, which means that the product is constantly cleaned from the screws (Riaz, 2000). Co-rotating, intermeshing twin screws are the most common type of extrusion used in the food industry (Kazemzadah, 2012).

2.2.1.2 BARREL

The barrel is the vessel in which the screw is housed. The barrel can incorporate heating or cooling elements by way of a jacket of steam, water, oil or cooling media, which help to control the temperature inside the extruder (Riaz, 2000). The clearance between the screw and the barrel can have a great impact on the finished product because it changes the flow pattern of material within the extruder (Yacu, 2012). The clearance can change based on screw design or as the barrel wears from usage over time (Yacu, 2012).

There are four main sections of the barrel which are characterized by the physical state of the product. They are the feeding zone, mixing zone, the final cooking zone, and the forming zone (Huber, 2000). In the feeding zone, the material is fed into the extruder and by decreasing the screw's flight

angle, it is compressed to remove excess air that comes in with the raw material (Huber, 2000). In this section the product is still in its raw form. In the next part of the barrel, the mixing zone, even more compression and mixing by shear force is achieved by decreasing the pitch and increasing the number of screw flights (Huber, 2000). In addition, kneading blocks can be used to provide additional mixing and reverse elements provide backwards flow (Huber, 2000). Reverse and forward elements are used in conjunction to facilitate mixing by combining backwards and forward flow (Yacu, 2012). During the mixing section, the raw materials begin to come together to form a cohesive mixture and the temperature of the product increases (Huber, 2000). In the next section, the final cooking zone, the highest temperature and pressure in the extruder are reached and the product undergoes maximum shear and compression (Huber, 2000). This last zone is where the product can experience great changes, such as a phase change to an amorphous solid or texturizing as seen in texturized vegetable protein production (Huber, 2000). The last section of a twin-screw extruder is the forming zone in which the product is conveyed out of the extruder and through a die to be formed into a final shape (Huber, 2000).

2.2.1.3 DIE

The die plate is the very last part of the extruder which provides the resistance against which the screw conveys the material (Huber, 2000). The die plate can have holes of any shape and is used to control the shape of the product. A cutter is generally used in conjunction with a die plate to control the length of the extruded product (Huber, 2000). As the product exits the extruder, it goes from the high pressure and high temperature environment inside the extruder to atmospheric conditions. This pressure drop can be increased by the decreasing number of holes in the die plate and/or decreasing the area of holes in the die plate (Yacu, 2012). A higher pressure

drop results in a longer retention time and higher mechanical energy, which leads to higher motor torque (Yacu, 2012). Some products, such as puffed cereal, utilize this pressure drop which results in rapid expansion and gives them their characteristic texture (Sevaston & Huber, 2000).

2.2.2 *Process Variables*

Extruder design and processing conditions have a large effect on the operation of the extruder. As such, many variable are monitored throughout the process, including specific mechanical energy, torque, feed rate and temperature. Another important process variable is the residence time of the material in the extruder. Extruder processing is a carefully controlled balance of all of these variables which results in optimal processing conditions.

2.2.2.1 SPECIFIC MECHANICAL ENERGY

The specific mechanical energy (SME) is the amount of power needed per unit weight of product extruded (Mottaz & Bruyas, 2001). SME can be calculated by taking the power supplied by the motor (kW) and dividing it by the feed rate of the material (kg/h) to get an SME in units of kW·h/kg (Yacu, 2012). SME generally shows the efficiency of the system because it shows the energy used per unit weight of product produced (Mottaz & Bruyas, 2001).

SME will increase when restriction to flow is increased (by either screw design or die design) or screw speed is increased (Yacu, 2012). SME will decrease when the barrel temperature is increased or feed flow rate is increased (Yacu, 2012).

2.2.2.2 TORQUE

In terms of the operation of an extruder, torque is power divided by angular velocity (Wagner et al., 2014). For an extruder, torque can be calculated by taking the power supplied by the motor (kW) divided by the rotational speed of the extruder (revolutions per minute) (Wagner et al., 2014). The units of torque are traditionally converted to units of force times length, such as N·m. The power supplied by the motor to the screw shaft is dependent on the torque and screw speed and is given by the equation Power = Screw Speed x Torque (Yacu, 2012). Torque, similarly to SME, shows how much energy is being used by the motor (Mottaz & Bruyas, 2001). The difference is that torque also depends on the rotational speed of the motor, and will change significantly depending upon the screw speed (Mottaz & Bruyas, 2001).

Torque and SME will generally change in the same direction (either both increase or both decrease) when processing variables change but the exception is when screw speed or feed rate are changed (Yacu, 2012). Therefore, torque will increase when the restriction to flow is increased (by either screw design or die design) or feed flow rate is increased (Yacu, 2012). Torque will decrease when the barrel temperature is increased or when the screw speed is increased (Yacu, 2012).

2.2.2.3 TEMPERATURE

Energy input to the product is important because it provides the heat by which the cooking or melting process happens and increases the product temperature. Heat can be delivered to the product in three ways: conduction, convection and conversion (Kazemzadah, 2012). Conductive energy is provided by a heating jacket on the barrel, filled with water, oil or some type of conductive media, whereas convective heat could be provided by high

temperature steam injected into the product (Kazemzadah, 2012). Conversion energy is mechanical energy provided by the motor which is converted to heat through shear (Kazemzadah, 2012). Shear can be described as the work performed on the product which results in mixing and heat production (Riaz, 2000). All of these types of energy will provide heat to the product, and change the temperature profile throughout the extruder. This will in turn affect the melting temperature, and the finished product quality (Chessari & Sellahewa, 2001).

Temperature of the product inside the extruder will increase when resistance to flow is increased (either through screw or die design), barrel temperature is increased, or when screw speed is increased (Yacu, 2012). In contrast, the temperature of the product will decrease when the feed rate is increased (Yacu, 2012).

2.2.2.4 RESIDENCE TIME

The residence time represents the amount of time that the material spends in the extruder (Wagner et al., 2014). However, the residence time is actually a distribution because not all particles of material spend the same amount of time in the extruder (Wagner et al., 2014). The average residence time can be calculated by dividing the effective volume of the extruder by the volumetric flow rate (Wagner et al., 2014). However, in practice this is difficult to calculate because the effective volume of the extruder can change with screw design and complicated flow patterns.

The average residence time will increase with an extruder design that has more resistance to flow (either through screw design or through die design) (Wagner et al., 2014). The average residence time will decrease with an increase in feed rate (Yacu, 2012) or an increase in screw speed (Wagner et

al., 2014). As the barrel temperature is increased, the amount of material melted is increased, and/or the viscosity of material decreases, which in turn causes less resistance and decreases residence time (Wagner et al., 2014).

2.2.2.5 FEED RATE

The feed rate is an important factor in extrusion. For a starve-fed system, the throughput rate of the extruder is determined by the feed rate and not the screw speed (Wagner et al., 2014). This means that the barrel is not completely full of material, and increasing the flow rate will increase the total throughput of the extruder. If the screw speed is not high enough compared to the feed rate, the extruder will experience product buildup, which results in high torque (Wagner et al., 2014). A balance between the screw speed and feed rate must be achieved through careful monitoring throughout processing to ensure that the extruder will not exceed maximum torque, which has detrimental effects on equipment (Wagner et al., 2014).

2.3 Dietary Fiber

2.3.1 *Definition of Dietary Fiber*

The current definition of dietary fiber, as approved by the American Association of Cereal Chemists (AACC) Board of Directors is given as:

"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 plant substances. Dietary fiber promotes beneficial physiological effects including laxation and/or blood cholesterol attenuation, and/or blood glucose attenuation." (American Association of Cereal Chemists, 2000)

The current definition of dietary fiber as approved by Codex Alimentarius, which is an international set of regulations for food commonly used in Europe, is given as:

"Dietary fibre means carbohydrate polymers with a degree of polymerization (DP) not lower than 3; which are neither digested nor absorbed in the small intestine. Dietary fibre consists of one or more of: Edible carbohydrate polymers naturally occurring in the food as consumed, or carbohydrate polymers which have been obtained from food raw material by physical, enzymatic or chemical means, or of synthetic carbohydrate polymers. Dietary fibre generally has properties such as: Decreasing transit time and increasing stool bulk; Stimulating colonic fermentation; Reducing blood total and/or LDL cholesterol levels; or Reducing post-prandial blood glucose and/or insulin levels. Material considered as dietary fibre should have at least one of these properties." (Codex Alimentarius Committee of Nutrition and Foods for Special Dietary Uses, 2005)

Both definitions require that materials categorized as dietary fiber to be nondigestible by human enzymes in the small intestine, and require that they demonstrate some kind of physiological benefit to the host. In addition, both definitions also recognize synthesized dietary fiber as a source of dietary fiber; the AACC definition calls this out as "analogous carbohydrates" and the Codex definition includes "synthetic carbohydrate polymers."

2.3.2 Digestive Health

Long chain sugar polymers, such as oligosaccharides, cannot be absorbed by humans unless they are first broken down into monosaccharides by the digestion system (BeMiller, 2007). Important enzymes in the human digestion system include amylases, which break down starch or carbohydrates, and proteases, which

break down proteins (Cho, DeVries, & Prosky, 1997). Oligosaccharides may be completely digested, partially digested, or nondigestible which means that they can be caloric, partially caloric, or noncaloric, respectively (BeMiller, 2007). If the oligosaccharides are not fully digested in the small intestine, they move on to the large intestine where the natural microorganisms found in the large intestine break down the oligosaccharides into monosaccharides to be used as energy (BeMiller, 2007).

Prebiotics are defined as substances which "are not digested by the enzymes of the human small intestine but that provide beneficial physiological effects to the host by selectively stimulating the growth and/or bioactivity of beneficial microorganisms already resident in the large intestine (colon)" (BeMiller, 2007). Therefore, nondigestible oligosaccharides may be considered dietary fiber if they are proven to stimulate microorganisms in the large intestine. Prebiotics generally stimulate growth of *Bifidobacterium* and *Lactobacillus*, which are naturally found in the human large intestine (BeMiller, 2007).

The current recommended intake of dietary fiber for adults in the United States is 25 grams per day for women and 38 grams per day for men (U.S. Department of Agriculture & U.S. Department of Health and Human Services, 2010). Health benefits of increased dietary fiber include increased stool frequency, increased stool weight, suppressed pathogenic bacteria, improved calcium absorption, stimulation of *Bifidobacterium*, and reduces blood glucose and insulin levels after eating (Prosky, 2001).

2.3.3 *Dietary Fiber Analysis*

In the past, dietary fiber was measured by collecting the insoluble fraction of a solution of the material in 78% ethanol (Cho et al., 1997). However, soluble fibers such as inulin and oligofructose are soluble in this mixture and were not

traditionally counted as dietary fiber (Cho et al., 1997). However, the dietary fiber method was updated to include oligo- and polysaccharides which are soluble in 78% ethanol but are not digested by the enzymes found in the small intestine (Prosky, 2001).

2.3.3.1 AOAC Official Method 985.29

AOAC Official Method 985.29 was adopted worldwide in 1982 to measure dietary fiber (DeVries & Rader, 2005). This method digests the food using α-amylase, amyloglucosidase, and protease to break down starch and protein (AOAC International, 2013a). Then, four volumes of ethanol are added to create a 78% ethanol solution, in which some kinds of soluble fiber precipitate (AOAC International, 2013a). The solution is filtered to capture the soluble fiber, and then dried (AOAC International, 2013a). This residue is considered the total dietary fiber, after it is weighed and corrected for protein and ash (AOAC International, 2013a). Dietary fiber began to be thought of as the result obtained from AOAC Official Method 985.29 (DeVries & Rader, 2005). However, with the discovery of resistant starch, which acted physiologically as a dietary fiber, this mindset began to change. Undigested portions of resistant starch would not precipitate in 78% ethanol, and so after much discussion, a new method for dietary fiber that measured fiber which is soluble even in 78% ethanol was developed (DeVries & Rader, 2005).

2.3.3.2 AOAC Official Methods 2009.01 and 2011.25

AOAC Official Methods 2009.01 and 2011.25 quantify total dietary fiber in foods, including fiber which is soluble in 78% ethanol (DeVries & Rader, 2005). An enzyme kit including necessary reagents to perform methods 2009.01 and 2011.25 can be purchased from Megazyme International, and the procedure is outlined in the included booklet (Megazyme International,

2012b). Both methods start with digestion of the food by pancreatic α-amylase and amyloglucosidase to break down large carbohydrates, followed by digestion with protease to break down protein (AOAC International, 2013b, 2013c). Method 2009.01 then precipitates any water:alcohol insoluble dietary fiber (SDFP) with a solution of 78% ethanol (AOAC International, 2013b). This solutions is filtered to capture both the insoluble dietary fiber (IDF) and the SDFP (AOAC International, 2013b). Whereas the filtrate is concentrated, deionized, and then analyzed by high performance liquid chromatagrophy (HPLC) to quantify nonprecipitable water:alcohol soluble dietary fiber (SDFS) (AOAC International, 2013b).

In Method 2011.25, the digested sample is first filtered to capture IDF, and then SDFP is precipitated using 78% ethanol and filtered again to capture SDFP (AOAC International, 2013c). Both the IDF and SDFP residues are corrected for ash and protein (AOAC International, 2013c). Similar to Method 2009.01, the filtrate is concentrated, deionized, and analyzed by HPLC for SDFS (AOAC International, 2013c). The main difference between method 2011.25 and 2009.01 is that Method 2011.25 provides quantification for IDF and SDFP separately, while Method 2009.01 provides one amount for IDF and SDFP combined.

In order to quantify SDFS, an internal standard (D-sorbitol is recommended) is added to the solution directly after the digestion step (AOAC International, 2013b, 2013c). A known standard, such as a glucose ladder, is used to determine the demarcation point for carbohydrates which have a degree of polymerization (DP) which is greater than or equal to three (AOAC International, 2013b, 2013c). All carbohydrates which are greater than or equal to DP 3 are considered to be dietary fiber in AOAC Official Methods 2009.01 and 2011.25 (AOAC International, 2013b, 2013c).

2.3.3.3 CHALLENGES WITH AOAC OFFICIAL DIETARY FIBER METHODS

When testing a novel oligosaccharide for dietary fiber content, many challenges must be overcome. First, because it is a novel substance, there is no standard that can be purchased to achieve a valid response factor. The AOAC Official Method suggests glucose to calculate the response from oligosaccharides (AOAC International, 2013b, 2013c; Megazyme International, 2012b). However, because the structure of a novel oligosaccharide is unknown, it is also unknown if glucose will provide a similar response from the detector as the novel oligosaccharide.

In addition, the official method suggests that sorbitol be used as an internal standard (AOAC International, 2013b, 2013c; Megazyme International, 2012b). However, depending on the column used and constituents in the digested solution, sorbitol may co-elute with another molecule. A previous study on oligosaccharides produced from lactose via reactive extrusion found that sorbitol co-eluted with galactose on the column used, and so ribose was used as an internal standard (Tremaine, Reid, Tyl, & Schoenfuss, 2014).

One further obstacle with the dietary fiber method is that it allows for quantification of any carbohydrate with a degree of polymerization greater than or equal to three to be considered dietary fiber (AOAC International, 2013b, 2013c). Because the column will separate based on size, these molecules may have similar molecular weights as carbohydrates but may in fact be other products. With an oligosaccharide produced by reactive extrusion, there may be a multitude of carbohydrate products which vary in structure because of branching, but there can also be unknown products produced via caramelization or pyrolysis.

2.4 Oligosaccharides

Oligosaccharides are chains of sugars with at least three monosaccharide units and up to approximately ten monosaccharide units (Wrolstad, 2012). Sugars with more than ten monosaccharide units are usually considered polysaccharides (Cho et al., 1997). These definitions can vary and the term oligosaccharide can include anywhere from two to nineteen monosaccharide units, but in this paper oligosaccharide will refer to a sugar with between three and ten monosaccharide units.

2.4.1 *Condensation Polymerization Reactions*

Oligosaccharides can be formed when monosaccharide units are joined together via condensation reaction, where one water molecule is eliminated in order to form a bond (Bailey, 1965). This glycosidic bond can be formed between the hemiacetal form of a reducing sugar and any hydroxyl group on a second sugar, in the presence of an acid catalyst (Bailey, 1965) or with heat degradation such as in caramelization (Izydorczyk, 2005). Glycosidic bonds can also be formed via enzyme catalysts (Bailey, 1965). The resulting chain of sugars is characterized by degree of polymerization, which refers to the number of monomers chained together to form the oligosaccharide (Cho et al., 1997). For example, a chain of six monomers would be joined together by five condensation reactions and referred to as an oligosaccharide with a degree of polymerization of six.

2.4.1.1 ENZYMATIC

Oligosaccharides created by enzymatic reactions are much more specific than those created by chemical reactions because the enzymes themselves are designed to catalyze specific linkages between sugars (Bailey, 1965). Aldolases are enzymes which catalyze an addition of a ketone donor to an aldehyde acceptor to create a glycosidic linkage (Brito-Aria, 2007). However, the linkages are very specific and as a result there are over 30

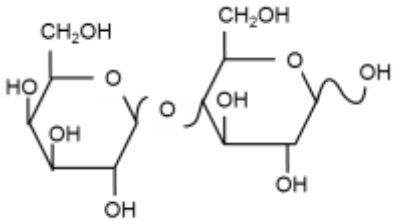
different aldolases which each catalyze a stereospecific reaction (Brito-Aria, 2007).

2.4.1.2 CHEMICAL

One way to create oligosaccharides is to heat sugars in the presence of an acid, which is referred to as acidic hydrolysis, which is a specific type of condensation polymerization (Brito-Aria, 2007). These sugars are then free to form glycosidic linkages and form oligosaccharide chains (BeMiller, 2007). This type of polymerization is seen in production of polydextrose (Craig, 2001) and caramel color (Izydorczyk, 2005). This reaction is not specific, and so it results in many types of linkages including α- and β- and involves random hydroxyl groups (Bailey, 1965). Because human enzymes can only break down specific linkages which are common in nature, chemically synthesized oligosaccharides are essentially nondigestible and noncaloric (BeMiller, 2007).

2.4.2 *Lactose*

Lactose occurs naturally in milk, and is found at levels of 4.5-4.8% in cow's milk (BeMiller & Whistler, 1996). Lactose is a disaccharide formed from the monomers glucose and galactose, which are small 6 carbon sugars (BeMiller & Whistler, 1996), as shown in Figure 1.



Galactose Glucose

Figure 1. Structure of lactose (BeMiller, 2007)

Because the lactose molecule has an exposed aldehyde group (on the glucose end), it is considered a reducing sugar, and can participate in acid catalyzed condensation reactions with hydroxyl groups on other sugars to form oligosaccharides (BeMiller, 2007; Wrolstad, 2012). In addition, other reducing sugars, which have exposed aldehyde or ketone groups, can react with any hydroxyl group on the lactose molecule to form an oligosaccharide. Because of the many ways that lactose molecules can form glycosidic linkages with other sugars, a random polymerization reaction with lactose would result in a large number of possible configurations of oligosaccharides.

2.4.3 *Commercial Oligosaccharides*

2.4.3.1 GALACTO-OLIGOSACCHARIDES

Galacto-oligosaccharides (GOS) are naturally found in human milk, particularly the trisaccharides β(1→4)-galactosyllactose and β(1→6)-galactosyllactose (Schoterman, 2001). GOS can also by synthesized from lactose molecules using an enzymatic process, which utilizes β-galactosidase (Gosling, Stevens, Barber, Kentish, & Gras, 2010; Schoterman, 2001). GOS

can either be produced using a continuous ultrafiltration reactor, or a batch reactor with subsequent membrane filtration for enzyme recovery (Gosling et al., 2010). GOS synthesis is usually performed at low temperatures (40°C – 60°C) to prevent enzyme denaturation (Martínez-Villaluenga, Cardelle-Cobas, Corzo, Olano, & Villamiel, 2008). Typical yields of GOS are between 18.1% and 52.5% when compared to the starting weight of lactose, depending on the type of enzyme and reaction conditions (Gosling et al., 2010).

The composition of GOS can vary widely in degree of polymerization (DP) and type of linkages (Schoterman, 2001). However, di-, tri- and up to hexasaccharides are most often seen (Gosling et al., 2010). Enzyme type, temperature, pH, substrate and enzyme concentration can all effect the yield and structure of GOS that is produced (Gosling et al., 2010; Martínez-Villaluenga et al., 2008). Enzymatically produced GOS usually is usually a linear structure of one or more galactose molecules linearly chained to a glucose molecule, however chains of all galactose molecules and branched structures with galactose and one glucose molecule are also known (Gosling et al., 2010).

GOS has been found to have the following benefits for human digestion: it does not promote tooth decay, improves calcium absorption, increases stool frequency, increases stool weight, and decreases risk of colon cancer (Prosky, 2001).

2.4.3.2 POLYDEXTROSE

Polydextrose is an example of a polymerized sugar synthesized by a chemical process (Craig, 2001). It is produced by condensation polymerization via acid catalyzation, which is accomplished in a batch process (Rennhard,

1973). Glucose and sorbitol are heated under vacuum in the presence of citric acid with reaction times reaching 8 hours (Rennhard, 1973). The vacuum removes water which drives the reaction towards polymerization, as water is a product of condensation polymerization (Bailey, 1965). Polydextrose has many uses, including as a bulking agent for sugar reduction, a humectant, an ingredient to reduce water migration, and to lower the freezing point of frozen desserts (BeMiller, 2007).

Polydextrose is a random polymer of glucose and sorbitol which is created using a food grade acid as a catalyst with heating under vacuum, and has an average degree of polymerization of 12 (Craig, 2001), with a DP of up to 20 (BeMiller, 2007). This is considerably larger than typical GOS molecules. The molecular weight of polydextrose can range from 162 to 20,000 Da, but an average weight is around 2000 Da (Craig, 2001). When polydextrose is produced, the time of the reaction and temperature must be carefully monitored to minimize formation of caramelization products and discoloration (Rennhard, 1973). Because it is produced chemically, polydextrose has random cross-linking and a variety of bonds within the molecule (Wrolstad, 2012), however most bonds are β(1→6) (Rennhard, 1973). An example of a possible polydextrose structure is shown in Figure 2.

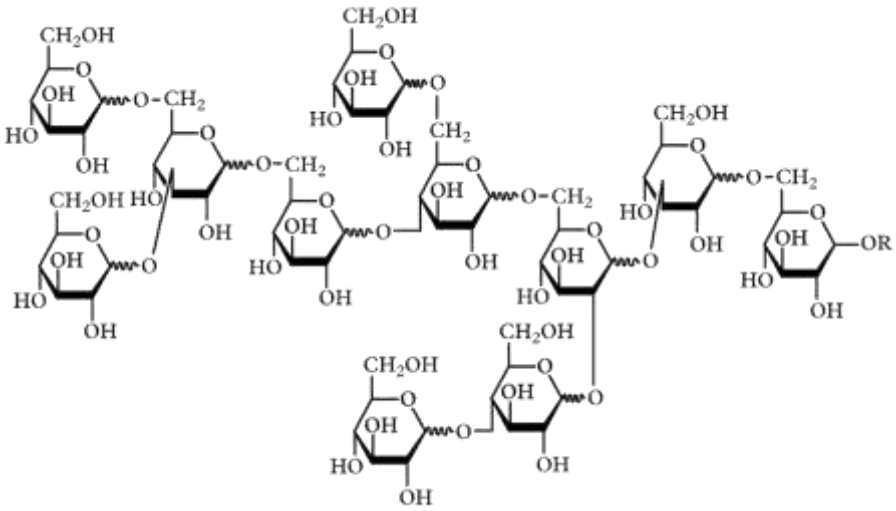


Figure 2. A possible structure of a polydextrose molecule (Craig, 2001).

Human enzymes can only digest approximately 10% of the bonds found in polydextrose and so the other 90% acts as dietary fiber in regards to human digestion and additionally, it is considered a prebiotic (BeMiller, 2007; Craig, 2001; Wrolstad, 2012). The physiological benefits of ingesting polydextrose include lower faecal pH, increased stool weight, reduced transit time, increased *Lactobacillus* and *Bifidobacterium* in the small intestine, and a decrease in pathogenic bacteria (Craig, 2001).

2.4.4 *Methods of Production*

Oligosaccharides can be created by either chemical or enzymatic reactions, and through batch or continuous processes.

2.4.4.1 Batch Reaction

Condensation polymerization by acid catalyzation of sugars can be accomplished using a batch reaction, which can have long reaction times from 8 hours up to 120 hours and are usually performed under vacuum

(Manley-Harris & Richards, 1994; Mora & Pacsu, 1955). A well-known commercial product, polydextrose, is produced with a batch reaction from glucose in the presence of citric acid with temperatures of 160°C and batch times around 8 hours (Rennhard, 1973). Citric acid has been shown to be the preferred acid in condensation reactions and acid hydrolysis of sugars because it is an edible acid which performs well (Manley-Harris & Richards, 1991; Rennhard, 1973).

2.4.4.2 REACTIVE EXTRUSION

Polymerization under acidic conditions can also be accomplished with an extruder (Antrim, Barresi, McPherson, & Wang, 2005; Hwang, Kim, & Kim, 1997; Tremaine et al., 2014). Extruders have been shown to have greater efficiency than batch reactions to produce similar products (Xie, Yu, Liu, & Chen, 2006).

Hwang et al. (1997) successfully showed both glucose and lactose, in the presence of citric acid, could be polymerized using an extruder as a reaction vessel with insoluble fiber yields ranging between 0.72% and 35.43%. Tremaine et al. (2014) further showed that lactose when combined with glucose and citric acid, and processed in a twin-screw extruder under high shear conditions resulted in soluble indigestible oligosaccharide yields between 37.1% and 59.8%. The product was further characterized and confirmed with mass spectrometry that oligosaccharides with a degree of polymerization of 3 and greater were created (Tremaine et al., 2014).

A group at Grain Processing Corporation has shown that oligosaccharides can be produced using maltodextrin and a short chain sugar, such as glucose, in the presence of citric acid using an extruder (Antrim et al., 2005). This

group also found that a higher citric acid concentration resulted in more indigestible product (Antrim, Barresi, & McPherson, 2006).

Work using reactive extrusion with starch and citric acid has also shown that highly cross-linked products are formed (Menzel et al., 2013; Olivato, Grossmann, Yamashita, Eiras, & Pessan, 2012; Olsson et al., 2013; O'Brien & Wang, 2009; Shi et al., 2007; Van Der Goot et al., 1997). An increase in citric acid content in starch extrusion been shown to increase cross-linking by esterification reactions (Menzel et al., 2013; Olivato et al., 2012; Shi et al., 2007), increase the molecular weight of products formed (Van Der Goot et al., 1997), and increase the amount of dietary fiber (Unlu & Faller, 1998). An increase in shear stress within an extruder has also been shown to increase cross-linking (O'Brien & Wang, 2009). In addition, increased citric acid can lead to a decrease in shear viscosity of the product inside the extruder (Jiugao, Ning, & Xiaofei, 2005; Ning, Jiugao, & Chunmei, 2007) and act as a plasticizer by breaking down larger sugars into smaller molecules (Liu, Xie, Yu, Chen, & Li, 2009).

2.5 Browning Reactions

Brown color in food can be caused by a variety of reactions which can be separated into two main groups, enzymatic and non-enzymatic. Enzymatic browning reactions involve an enzyme, such as polyphenol oxidase (Mesquita & Queiroz, 2013; Wrolstad, 2012). The most common non-enyzmatic browning reactions are caused by Maillard reaction and caramelization (Nursten, 2005). Because the nature of the research in this thesis does not encompass enzymatic browning, only the Maillard reaction and caramelization will be discussed. The Maillard reaction and caramelization often proceed under the same conditions, and some of their reaction pathways are similar and overlapping (Nursten, 2005).

2.5.1 *Maillard Reaction*

The Maillard reaction is a complex reaction which involves the production of brown color and flavor from reducing sugars and free amines (BeMiller & Whistler, 1996). Reducing sugars are defined as sugars which contain a hemiacetal or hemiketal group, which will rearrange to form an aldehyde group in their open chain form which is reactive (Wrolstad, 2012). Free amine groups come from amino acids, which can be part of a protein chain, peptide or in a free amino acid (Nursten, 2005). The Maillard reaction provides the desired flavors in coffee, baked bread, and roasted meat but can also be responsible for unwanted burnt taste (Eskin, Ho, & Shahidi, 2013). There are many reactions and pathways within the Maillard reaction group, which results in hundreds of possible products, depending on the starting reactants (Nursten, 2005). These reactions include sugar-amine condensation, Amadori rearrangement, sugar dehydration, sugar fragmentation, Strecker degradation, adol condensation, and aldehyde-amine condensation (Nursten, 2005). Over 50 volatile compounds were identified in a Maillard reaction between glycine and glucose, including carbonyls, furans, pyrroles, pyridines, pyrazines, and oxazoles (Ames, Guy, & Kipping, 2001).

The Maillard reaction is favored at a basic pH, but can also happen at an acidic pH (Eskin et al., 2013). A low pH favors a 1,2 enolization of the sugar, whereas a high pH favors the 2,3 enolization, which can influence the composition of reaction products (Nursten, 2005). Specifically for Maillard reactions within an extruder, the effect of pH on the reaction products is temperature dependent. One study looking at a Maillard reaction between glycine and glucose found that the total volatile compounds increased with decreasing pH at 180°C and increased with increasing pH at 120°C (Ames et al., 2001). However, in most cases an increase in pH to alkaline range will show an increase in browning (Wrolstad, 2012).

An increase in temperature increases the rate of reaction for the Maillard reaction, as is the case with many chemical reactions (Eskin et al., 2013). It has been shown that the total amount of volatile compounds in a glycine and glucose system increases with an increase in temperature when measured at various temperatures between 100°C and 300°C (Tehrani, Kersiene, Adams, Venskutonis, & De Kimpe, 2002). In addition, Tehrani et. al (2002) found that the composition of volatiles was also temperature dependent. Moisture also affects the Maillard reaction and too little water will inhibit the reaction by preventing mobility of the reactants, while too much water will dilute the reactants and also inhibit the reaction (Nursten, 2005).

2.5.2 *Caramelization*

Caramelization refers to a reaction at elevated temperatures between carbohydrates, or sugars (BeMiller & Whistler, 1996). Caramelization, like the Maillard reaction, is a complex reaction with many products. In fact, caramelization of glucose at 250°C with no other compounds present, resulted in over 100 volatile compounds (Nursten, 2005). Caramelization contributes flavor and color to many cooked foods, including bread, roasted meat, and caramel color (BeMiller & Whistler, 1996).

The basic pathway of caramelization is that sugars degrade by thermal dehydration through enolization, which creates many smaller reactive molecules which then combine to form polymerized molecules which have a brown color and characteristic flavor (Wrolstad, 2012). Caramelization can proceed under both basic or acidic conditions, however the reaction is better catalyzed at a basic pH (Eskin et al., 2013). Similar to the Maillard reaction, low pH favors 1,2 enolization compared to 2,3 enolization at a higher pH (Eskin et al., 2013). There are four classes of caramel color which are prepared under different conditions,

and used as food colorants. Class I caramel is also called plain caramel and is created by heating a sugar with an acid or a base, but without ammonium or sulfite ions (BeMiller, 2007).

When subjecting simple sugars to high temperature in the presence of acid, many complex reactions can occur and a vast array of products are formed (Eskin et al., 2013). Caramel can be formed from heating monosaccharides, and has been characterized to show that they contain oligosaccharides with a degree of polymerization of up to 6, hydration and dehydration products of the starting material, and formation of aromatic compounds through dehydration, even when the starting product was a disaccharide or monosaccharide (Golon & Kuhnert, 2013). The brown pigment seen in caramelization is from a class of compounds referred to as melanoidins and caramels, which are complex polymeric molecules (Moss, 2002).

2.5.3 *Methods to Analyze Color*

Color is one part of the visual perception of an object and can change depending on three major components: the object itself, the light source, and the observer (Choudhury, 2014). The type of light source, or illuminant, can change the perception of color by the observer. Many standard light sources have been identified, including bright daylight, overcast daylight, fluorescent lamps, and tungsten lamps, but the standard light source used in color measurement for food is D65 which refers to a specific type of midday daylight which has a color temperature of 6500°K and includes ultraviolet light (MacDougall, 2002). The degrees of visual field refers to how the human eye perceives color and which types of light-detecting receptors are utilized (MacDougall, 2002). The 10° observer field is most commonly used to measure color because it is more representative of human vision (Joshi & Brimelow, 2002).

A color space is an attempt to define a color numerically, and represents a position in a three dimensional representation of color (Choudhury, 2014). Common color spaces include the Commission international de l'éclairage (CIE) or International Commission on Illumination LAB, Hunter *L, a, b*, CIELUV, CIEU*V*W*, and RGB (Choudhury, 2014). CIELAB and Hunter *L, a, b* are the most common color spaces used in the food industry (MacDougall, 2002). Both color scales define *L, a, b* in similar ways, but the difference is that Hunter coordinates utilize a square-root transformation of color data compared to the cube-root transformation utilized by CIELAB color space (Choudhury, 2014). *L is* the amount of white versus black, with a maximum of 100 (white) and minimum of 0 (black), a positive *a* represents red and negative *a* represents green, and a positive *b* represents yellow and a negative *b* represents blue (Choudhury, 2014).

Because color perception can change with light source and degrees of visual field, the reflectance curve is often used because it is independent of these factors (Choudhury, 2014). A reflectance spectrum in the visible light range (360-700 nm) can be taken and then converted to any color space, illuminant and observer combination for comparison (Choudhury, 2014). Therefore, to directly compare color coordinates, it is important that the color space, illuminant type and observer viewing field are the same (Joshi & Brimelow, 2002).

Both the Maillard reaction and caramelization produce brown pigment, which can be measured in the visible spectrum between 360 and 420 nm (Nursten, 2005). These brown molecules are referred to as melanoidins and caramels, and are complex polymeric molecules (Moss, 2002). Melanoidins have very complex structures due to the many different pathways and intermediates of the Maillard reaction, and can have molecular weights of up to 100,000 (BeMiller, 2007). Because there are so many different structures of melanoidins, there is a range of wavelengths at which they show strong absorbance, but one wavelength must

consistently be used to compare products (Wrolstad, 2012). Absorbance at 420 nm has been used in many studies to identify brown pigment caused by Maillard or caramelization (Ajandouz, Desseaux, Tazi, & Puigserver, 2008; Ajandouz, Tchiakpe, Ore, Benajiba, & Puigserver, 2001; Carabasa-Giribet & Ibarz-Ribas, 2000; Kroh, 1994; Wrolstad, 2012).

3 Lactose Extrusion

3.1 Introduction

Lactose, a disaccharide sugar which occurs naturally in milk (BeMiller & Whistler, 1996), is often considered a low value product that can be isolated in the production of more valuable whey proteins. Polymerizing lactose into a mixture of oligomers via twin-screw extrusion could produce a value added product, if it can be classified as dietary fiber. Dietary fiber can be used as a bulking agent to replace sugar in reduced sugar application or used as added fiber.

Polymerization of sugars utilizing heat and acid has been studied and characterized in many studies (Manley-Harris & Richards, 1993; Manley-Harris & Richards, 1994; Mora & Pacsu, 1955). Additionally, the production of a widely available commercial fiber product, polydextrose, is based on the concept of condensation polymerization via acidic hydrolysis (Antrim et al., 2005; Antrim et al., 2006; Rennhard, 1973).

Extrusion has been investigated as a means to facilitate thermal polymerization of glucose and lactose with the addition of citric acid (Hwang et al., 1997; Hwang, Kim, & Kim, 1998). Work previously completed in the Schoenfuss lab shows that indigestible oligomers, which can potentially be categorized as dietary fiber, can be produced from lactose using a citric acid catalyst with yields between 37.1% and 59.8% (Tremaine et al., 2014). This study showed promising results with regard to extruder operation with the addition of 20% glucose, and a higher yield of indigestible oligomers at 2% citric acid when compared to 1% citric acid (Tremaine et al., 2014). Because an increase in citric acid content has been associated with increased cross-linking (Menzel et al., 2013; O'Brien & Wang, 2009; Van Der Goot et al., 1997), we hypothesized that a further increase in citric acid would result in higher yields of indigestible oligomers. Extruder processing parameters have been

shown to affect the rate of polymerization in reactive extrusion (Van Der Goot et al., 1997), as well as influence the efficiency of the extruder (Mottaz & Bruyas, 2001). An increase in shear stress within the extruder has also been shown to increase cross-linking (O'Brien & Wang, 2009). Hwang et al. (1997, 1998) found success in polymerizing glucose with feed rates of 60-65 kg/hr. Research has shown that increasing the feed rate when it is low compared to the screw speed will decrease the average residence time and result in a decrease in chemical conversion; however an increase feed rate when the system is already at a high feed rate compared to screw speed will not change the chemical conversion significantly (Zhu & Jaluria, 2001). We hypothesized that an increase in feed rate would result in better extruder efficiency, due to expected decreases in torque and specific mechanical energy, while still producing desirable reaction conditions for polymerization.

This study evaluates citric acid concentrations of 2%, 4% and 6% with 20% glucose and the remaining weight percentage made up of lactose to examine the effects of citric acid levels on the yield of indigestible oligomers. In addition, this study also evaluates the effect of feed rate on yield and extruder efficiency by testing both 15 kg/hr and 30 kg/hr feed rates using the described formulas.

3.2 Materials and Methods

3.2.1 *Materials*

Refined edible fine grind lactose (Davisco Foods International, Inc., Eden Prarie, MN, USA), dextrose (Roquette Granulated XX Dri Sweet, Roquette America, Inc. Geneva, IL, USA) and citric acid (Gadot Biochemical Industries Ltd., Harifa Bay, Israel) were used in all extrusion trials.

3.2.2 *Extrusion*

3.2.2.1 EXTRUDER SCREW DESIGN

All experiments were done using a Buhler 44 mm co-rotating twin-screw extruder DNDL 44 with a length to diameter ratio of 28 (Bühler AG, Uzwil, Switzerland). The screw design and processing conditions are based on those described in previous experiments done at the University of Minnesota (Tremaine et al., 2014). 39 screw elements of varying pitch angles were used including 9 kneading block elements (6 forward and 3 reverse), 20 forward conveying elements and 10 reverse conveying elements. The design included many reverse elements in order to maximize time that the product spent in the extruder to achieve greater reaction time, as well as more efficient conversion of mechanical energy to heat which resulted in higher temperatures. The full screw design is shown in Figure 3.

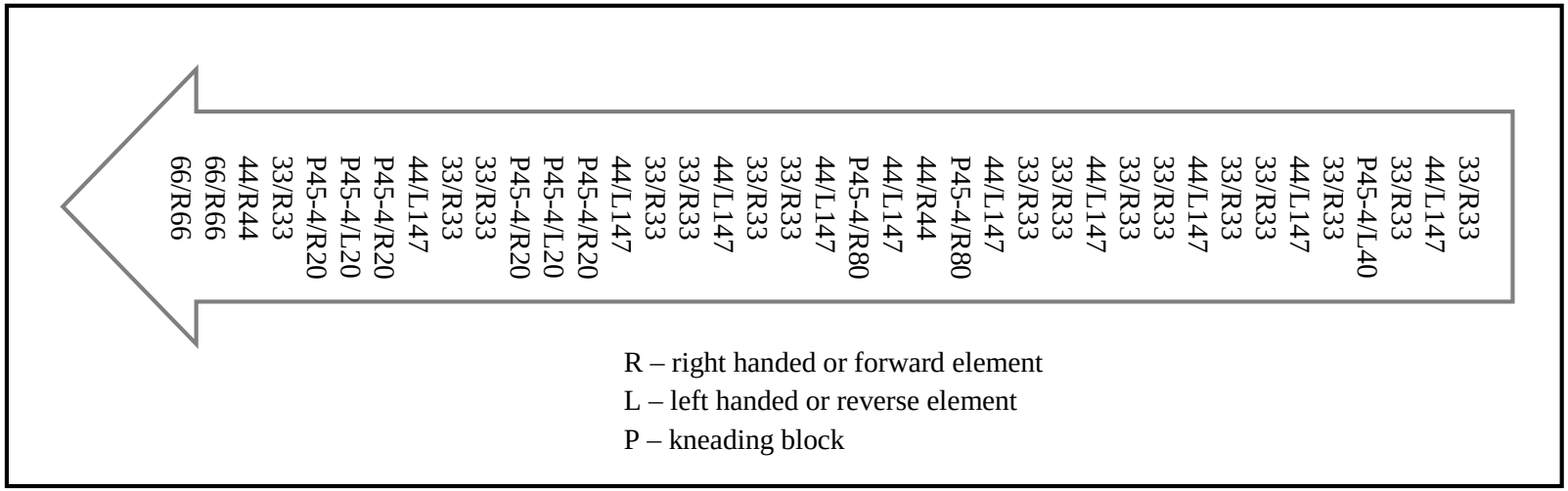


Figure 3. Screw design for a Buhler 44 mm twin-screw extruder used to polymerize lactose.

3.2.2.2 Extruder Processing Conditions

The barrel zones had different heating temperatures as described from the feeder inlet to outlet: barrel zones #2 and #3 were set at 238°C, zone #4 was set at 238°C, zones #5 and #6 were set at 238°C and there was no heating in zone #7. A heat transfer control system model H47212DT (Mokon, Buffalo, NY, USA) was used to maintain the temperature for each barrel's oil heating jacket. No die plate was used at the outlet of the extruder. The feed rate into the extruder was controlled by a K-Tron Soder K-ML-KT20 loss-in-weight feeder (K-Tron Ltd., Niderlenz, Switzerland). The screw speed for all trials was held constant at 250 rpm.

3.2.3 *Experimental Design*

Each batch consisted of 20% w/w glucose, while the amount of citric acid was varied at three levels: 2%, 4%, and 6% w/w. The remaining percentage of the batch was lactose. These three formulas were extruded at two feed rates, 15 kg/hr and 30 kg/hr in duplicate. The order of formulas was randomized within each replicate set.

3.2.3.1 Materials Preparation

Lactose, glucose and citric acid were mixed in 14 kg batches using a ribbon blender model IMS-1 (Bepex International LLC, Minneapolis, MN, USA) for two minutes in the forward direction followed by two minutes in the reverse direction.

3.2.3.2 Product Treatment

The extruded product was a hot melted caramel-like substance which varied in color from light tan to dark brown. Upon cooling, it hardened into a glass. The product was collected directly from the extruder outlet on stainless steel

trays and allowed to cool to room temperature. It was ground with a coffee grinder and stored in glass jars at room temperature until further analysis was completed.

3.2.4 *Process Responses*

Process conditions for each batch were monitored to ensure that the extruder reached steady state. After steady state was achieved, three readings of each process response were taken while product was collected for approximately 5-10 minutes.

3.2.4.1 SPECIFIC MECHANICAL ENERGY

Specific mechanical energy (SME) was calculated by the automation software by measuring the power utilized by the motor and dividing by the feed rate to calculate the energy per kg of product fed to the extruder.

3.2.4.2 TORQUE

Torque was calculated by the automation software by measuring the power utilized by the motor and dividing by the screw speed to calculate the force times length (N·m), or torque, of the motor.

3.2.4.3 TEMPERATURE

Product temperature was measured using a temperature probe inserted into the flowing product in the extruder barrel. The temperature was measured at 30.5, 48.3, 110.5, and 119.4 cm from the feeder end, as well as at the exit.

3.2.5 *Chemical and Physical Analysis*

3.2.5.1 COLOR

A solution of 2.00 ± 0.05 grams of extrudate diluted to 50 mL with reverse osmosis water was used to analyze color. This solution was analyzed for Hunter *L*, *a*, and *b* values using a spectral scan in reflectance mode from 360-700 nm in 1nm increments at medium speed on a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) using UV/PC Optional color analysis software version 3.10. A D65 illuminant and 10° observer viewing angle were used to calculate Hunter *L*, *a*, and *b* values.

Absorption at 420 nm, commonly used to identify brown pigment from the Maillard and caramelization reactions, was measured on a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) using UV Probe 2.43 software. The same concentration as the Hunter *L*, *a*, *b* analysis was used.

3.2.5.2 LACTOSE AND GLUCOSE

Lactose and glucose contents of the extrudate product after extrusion were determined using an enzymatic test kit from Megazyme (Lactose/Sucrose/D-Glucose Kit, Megazyme, Bray, Ireland). The kit is based on approved methods by the American Association of Cereal Chemists to measure glucose and lactose (American Association of Cereal Chemists, 1983).

3.2.5.3 MOISTURE

Moisture content was measured of both the raw mix and extrudate product using the Karl Fischer method. 0.6-1.0 grams of sample, depending on the expected moisture content, was mixed with 25 g of > 99% methanol (Sigma-

Aldrich, St. Louis, MO, USA) and shaken for 18 hours in airtight glass vials fitted with rubber septums to facilitate extraction of moisture. The moisture was then measured using a Karl Fischer titration apparatus (Aquatest-CMA #02-901-11, Photovolt Instruments Inc., Minneapolis, MN, USA) prepared with Karl Fischer generator solution (Photovolt Instruments Inc., Minneapolis, MN, USA) and Karl Fischer pyridine-free vessel solution (Photovolt Instruments Inc., Minneapolis, MN, USA). This method is based on the method for Karl Fischer specifically for dairy products with low levels of moisture per Method 15.117 as described in the Standard Methods for the Examination of Dairy Products (Hooi et al., 2004).

3.2.5.4 pH

The pH of the both the raw mixes and extruded products were measured by diluting 6.5 grams to 100 mL with RO water with a pH probe (Accumet Basic AB15, Fischer Scientific, Pittsburgh, PA, USA), as described in Method 15.021 in the Standard Methods for the Examination of Dairy Products (Hooi et al., 2004).

3.2.5.5 Titratable Acidity

Titratable acidity of both the raw mixes and the extrudates were measured by titrating between 0.7 and 2.0 grams of extrudate (depending on the expected acidity) with 0.1 M NaOH (Sigma-Aldrich, St. Louis, MO, USA) to an endpoint of pH 8.2 with an autotitrator (Mettler Toledo T50, Mettler-Toledo, Columbus, OH). The titratable acidity is expressed as % citric acid by weight.

3.2.5.6 DIETARY FIBER

Indigestible material, or dietary fiber, in the extrudate products was determined using the integrated total dietary fiber assay procedure (Megazyme, Bray, Ireland) AOAC Method 2009.01 with a few modifications (AOAC International, 2013b; Megazyme International, 2012a; Tremaine et al., 2014). D-Ribose (Sigma-Aldrich, St. Louis, MO, USA) was determined to be a suitable internal standard instead of sorbitol based on previous research (Tremaine et al., 2014). Low molecular weight indigestible compounds were quantified on a Transgenomics CHO-411 column (Omaha, NE, USA) incubated at 80°C. An eluent of double distilled degassed water at 0.3 mL per minute was used with this column. For detection, an evaporative light scattering detector (ELSD) (Shimadzu Corporation, Kyoto, Japan) was used with a temperature of 40°C and a nitrogen pressure of 250 kPa. A column performance check standard oligosaccharide, Phenomonex ALO-3038 (Phenomonex, Torrance, CA, USA), was run analyzed using the same conditions to determine the demarcation point between disaccharides and carbohydrates with a degree of polymerization of 3 and higher.

3.2.6 *Statistical Analysis*

The values presented are the means of either duplicate or triplicate determinations. Results from replicate extrusion runs are combined. All data was analyzed using univariate analysis of variance (ANOVA) followed by pairwise comparisons using Tukey honestly significant difference in R version 3.1.0 (R Development Core Team, 2008).

3.3 Results

3.3.1 *Process Responses*

Table 1 shows the influence that citric acid content and feed rate had on process responses specific mechanical energy (SME) and torque. It can be seen that the feed rate greatly affected both the SME and torque, and that the citric acid content also influenced the process responses.

Table 1. The influence of citric acid content and feed rate on process responses for lactose extrusion formulas.

Formula			Feed Rate	Specific Mechanical Energy	Motor Torque
Glucose [1] (%, w/w)	**Citric Acid [1] (%, w/w)**	**Lactose [1] (%, w/w)**	**kg/hr**	**W·h/kg [2]**	**N·m [2]**
20	2	78	15	113 ± 2[d]	61 ± 1[b]
20	4	76	15	105 ± 1[c]	58 ± 1[a]
20	6	74	15	103 ± 4[c]	56 ± 2[a]
20	2	78	30	91 ± 1[b]	101 ± 1[d]
20	4	76	30	86 ± 2[a]	94 ± 2[c]
20	6	74	30	82 ± 1[a]	92 ± 1[c]

1. Percentage expressed as weight by weight on a wet basis (moisture in initial product included)
2. ± one standard deviation (N = 6)
a-d. Means sharing the same superscript are no significantly different from each other ($p>0.05$)

An increase in feed rate from 15kg/hr to 30 kg/hr resulted in a decrease in SME from 92-101 to 103-113 W·h/kg, which is an average decrease of 21 W·h/kg. SME decreased with increasing citric acid content for both the 15 kg/hr group (113 to 103 W·h/kg) and the 30 kg/hr group (91 to 82 W·h/kg) (Table 1).

An increase in feed rate from 15kg/hr to 30 kg/hr resulted in an increase in torque from 56-61 to 92-101 N·m, which is an average increase of 37 N·m. As the citric acid content increased, a small decrease in torque was seen for both the 15 kg/hr group (61-56 N·m) and the 30 kg/hr group (101-92 N·m).

The influence of citric acid content and feed rate on the temperatures of the product is shown in Table 2.

Table 2. The influence of citric acid content and feed rate on product temperatures for lactose extrusion formulas.

Formula[1]			Feed Rate	Product Temperature[2]				
Glucose (%, w/w)	Citric Acid (%, w/w)	Lactose (%, w/w)	kg/hr	30.5 cm	48.3 cm	110.5 cm	119.4 cm	Outlet
				° C	° C	° C	° C	° C
20	2	78	15	193.3 ± 0.4^{e}	205.7 ± 0.7^{d}	183.5 ± 0.7^{bc}	176.6 ± 1.3^{a}	167.0 ± 1.8^{a}
20	4	76	15	191.6 ± 0.4^{d}	204.9 ± 0.3^{d}	184.2 ± 0.3^{c}	179.2 ± 0.4^{bc}	169.3 ± 0.8^{a}
20	6	74	15	190.6 ± 0.3^{d}	204.0 ± 0.6^{c}	183.1 ± 1.8^{abc}	178.4 ± 2.2^{abc}	167.0 ± 2.6^{a}
20	2	78	30	189.0 ± 0.2^{c}	201.0 ± 0.4^{b}	183.6 ± 0.2^{bc}	180.4 ± 0.5^{c}	169.0 ± 1.4^{a}
20	4	76	30	187.6 ± 0.3^{b}	199.8 ± 0.5^{a}	182.2 ± 0.7^{ab}	179.0 ± 0.6^{bc}	168.5 ± 1.0^{a}
20	6	74	30	185.9 ± 1.6^{a}	199.3 ± 0.3^{a}	181.6 ± 0.5^{a}	177.6 ± 0.7^{ab}	166.5 ± 1.7^{a}

1. Percentage expressed as weight by weight on a wet basis (moisture in initial product included)

2. Product temperatures were taken along the barrel as measured from the feeder end. ± one standard deviation shown (N = 6)

a-e. Means sharing the same superscript are no significantly different from each other ($p>0.05$)

It can be seen in Table 2 that there was no effect on the outlet temperature, and that feed rate and citric acid content had a small effect on product temperatures along the barrel.

There were no clear differences between temperatures with regards to citric acid content. Temperatures recorded closer to the barrel outlet were not different between feed rates. However, temperatures recorded at 30.5 and 48.3 cm from the feeder end showed that the 30 kg/hr sample reached a significantly lower temperature when compared to the 15 kg/hr sample measured at the same location. All samples reached a maximum temperature at 48.3 cm from the feeder end, which varied between 199.3 to 205.7°C.

3.3.2 *Color Analysis*

Table 3 shows the effect of citric acid content and feed rate on color measurements, including Hunter *L, a, b* values, and the absorbance at 420 nm. Significant differences between the two feed rates were seen, and some differences with change in citric acid concentration were seen, particularly within the 15 kg/hr feed rate group (Table 3).

Table 3. The influence of citric acid content and feed rate on color measurements for lactose extrusion products.

Formula[1]			Feed Rate	Browning	Color[2]		
Glucose (%, w/w)	**Citric Acid (%, w/w)**	**Lactose (%, w/w)**	**kg/hr**	**Absorbance at 420 nm**	**Hunter *L***	**Hunter *a***	**Hunter *b***
20	2	78	15	0.554 ± 0.095^{b}	83.84 ± 0.51^{b}	-7.05 ± 0.17^{a}	27.23 ± 2.67^{b}
20	4	76	15	0.654 ± 0.003^{c}	83.59 ± 0.34^{b}	-7.26 ± 0.08^{a}	30.01 ± 0.22^{c}
20	6	74	15	0.934 ± 0.092^{d}	80.99 ± 1.23^{a}	-6.19 ± 0.68^{bc}	35.03 ± 1.23^{d}
20	2	78	30	0.194 ± 0.004^{a}	87.41 ± 0.35^{c}	-6.06 ± 0.16^{b}	14.83 ± 0.33^{a}
20	4	76	30	0.195 ± 0.009^{a}	88.50 ± 0.56^{cd}	-6.45 ± 0.15^{bc}	15.21 ± 0.05^{a}
20	6	74	30	0.200 ± 0.014^{a}	89.17 ± 0.21^{d}	-6.74 ± 0.16^{ac}	15.91 ± 0.66^{a}

1. Percentage expressed as weight by weight on a wet basis (moisture in initial product included)

2. ± one standard deviation (N = 6)

a-d. Means sharing the same superscript are no significantly different from each other ($p>0.05$)

As shown in Table 3, the absorbance of the 30 kg/hr feed rate samples (0.194-0.200) was significantly lower than the absorbance of the 15 kg/hr feed rate samples (0.554-0.934), showing that significantly more browning occurred in the lower feed rate samples.

There were no differences within the 30 kg/hr feed rate group for absorbance at 420 nm, however the 15 kg/hr feed rate group showed that browning significantly increased with increasing citric acid content. Samples at 2%, 4%, and 6% citric acid content showed absorbances of 0.554, 0.654, and 0.934, respectively.

The color of the lactose extrusion products was measured using the Hunter *L, a, b* color space in which *L* describes pure white as a value of 100 and pure black as a value of 0 (Choudhury, 2014). The lactose extrudates showed significance with regards to Hunter *L* value between the 15 kg/hr and 30 kg/hr feed rate groups. The 30 kg/hr feed rate samples were more white with Hunter *L* values from 87.41-89.17 while the 15 kg/hr feed rate samples had values from 80.99-83.84. Within the 15 kg/hr feed rate group, the 6% citric acid sample was significantly more black than the 2% or 4% citric acid samples.

A positive Hunter *a* value indicates redness while a negative value indicates greenness (Choudhury, 2014). All samples showed negative Hunter *a* values close to zero, ranging from -7.26 to -6.06. These values are dependent upon the light source, and because they are close to zero it can be concluded that the samples were not strongly red nor green. No differences were seen between groups or within sample groups.

A positive Hunter *b* value indicates yellowness while a negative value indicates blueness (Choudhury, 2014). All samples showed positive *b* values, with the 30 kg/hr feed rate samples being significantly lower (14.83 to 15.91) than the 15 kg/hr

feed rate samples (27.23 to 35.03). This shows that all samples were yellow, and that the 30 kg/hr samples were significantly less yellow.

There were no differences between the Hunter *b* values in the 30 kg/hr feed rate group. However, the 15 kg/hr feed rate group showed a significant increase in Hunter *b* value as acid content increased, with the 2%, 4%, and 6% citric acid groups showing Hunter *b* values of 27.23, 30.01, and 35.03, respectively.

3.3.3 *Chemical Analysis*

Table 4 shows the influence of citric acid content and feed rate on the moisture, pH and titratable acidity of lactose extrusion products, while Table 5 shows the effect of citric acid content and feed rate on the lactose, glucose, and low molecular weight soluble fiber (LMWSF) content of the lactose extrudates.

Table 4. The influence of citric acid content and feed rate on moisture, pH and titratable acidity for lactose extrusion products and raw mixes.

Formula[1]			Feed Rate	Moisture[2]		pH[2]		Titratable Acidity[2]	
Glucose (%, w/w)	Citric Acid (%, w/w)	Lactose (%, w/w)	kg/hr	Raw Mix (%, w/w)	Extrudate (%, w/w)	Raw Mix	Extrudate	Raw Mix (%, w/w as citric acid)	Extrudate (%, w/w as citric acid)
20	2	78	15	3.67 ± 0.28^{a}	0.52 ± 0.14^{a}	2.67 ± 0.07^{b}	2.89 ± 0.02^{d}	2.04 ± 0.43^{a}	1.55 ± 0.01^{a}
			30		0.60 ± 0.14^{a}		2.89 ± 0.04^{d}		1.72 ± 0.01^{a}
20	4	76	15	3.98 ± 0.37^{a}	0.62 ± 0.13^{ab}	2.58 ± 0.01^{a}	2.77 ± 0.04^{b}	4.39 ± 0.98^{b}	3.01 ± 0.01^{b}
			30		0.80 ± 0.24^{ab}		2.72 ± 0.04^{bc}		3.51 ± 0.03^{c}
20	6	74	15	3.39 ± 0.45^{a}	0.51 ± 0.20^{a}	2.56 ± 0.02^{a}	2.66 ± 0.05^{c}	5.64 ± 0.39^{c}	4.72 ± 0.23^{d}
			30		0.96 ± 0.25^{b}		2.57 ± 0.02^{a}		5.38 ± 0.11^{e}

1. Percentage expressed as weight by weight on a wet basis (moisture in initial product included)

2. ± one standard deviation (N = 6)

a-e. Means sharing the same superscript are no significantly different from each other ($p>0.05$)

As shown in Table 4, there were no differences between the moisture content of the raw mixes. Therefore, any difference in moisture content is due to changes in the extrusion process or lactose formula. However, there were no significant differences between lactose extrudates.

The raw mix with 2% citric acid had a significantly higher pH (2.67) than either the 4% or 6% citric acid formulas (2.58, 2.56). The 2% citric acid lactose extrusion products showed a significantly higher pH than the other formulas (Table 4). There was no significance between the 4% and 6% citric acid lactose extrudates, however a general trend of a decrease in pH with an increase citric acid content was observed. No differences between feed rate groups were seen.

Table 4 shows that, as expected, the raw mixes significantly increased in TA (reported on a weight by weight basis as citric acid) as the amount of citric acid in the raw mix was increased.

TA was lowest for the 2% citric acid products (1.55% for the 15 kg/hr samples and 1.72% for the 30 kg/hr samples), and then increased significantly with increasing acid content with 3.01-3.51% for 4% citric acid formulas and 4.72-5.38% for 6% citric acid formulas.

There was no difference between feed rate groups for the 2% citric acid samples. However, the 4% and 6% citric acid formulas showed significantly lower TA for the 15 kg/hr feed rate samples (3.01% and 4.72% for 4% and 6% citric acid) when compared to the 30 kg/hr feed rate samples (3.51% and 5.38% for 4% and 6% citric acid).

Table 5. The influence of citric acid content and feed rate on lactose, glucose and low molecular weight soluble fiber content of lactose extrusion products.

Formula[1]			Feed Rate	Low molecular weight soluble fiber[2,3]	Lactose[3]	Glucose[3]	Percentage Original Lactose Remaining[3,4]	Percentage Original Glucose Remaining[3,4]
Glucose (%, w/w)	Citric Acid (%, w/w)	Lactose (%, w/w)	kg/hr	%, w/w	%, w/w	%, w/w	%	%
20	2	78	15	53.0 ± 7.2^{cd}	16.3 ± 1.1^{d}	3.2 ± 0.1^{c}	21.0 ± 1.4^{c}	16.6 ± 0.4^{c}
20	4	76	15	56.4 ± 7.2^{d}	6.3 ± 0.7^{b}	2.4 ± 0.1^{b}	8.4 ± 0.9^{b}	12.2 ± 0.4^{b}
20	6	74	15	46.7 ± 3.1^{bcd}	3.0 ± 0.2^{a}	1.7 ± 0.1^{a}	4.1 ± 0.2^{a}	8.8 ± 0.6^{a}
20	2	78	30	33.7 ± 2.3^{a}	34.2 ± 1.9^{f}	3.8 ± 0.2^{d}	44.1 ± 2.4^{e}	19.8 ± 1.2^{e}
20	4	76	30	43.5 ± 3.9^{abc}	21.5 ± 0.7^{e}	3.7 ± 0.1^{d}	28.6 ± 0.9^{d}	19.1 ± 0.6^{de}
20	6	74	30	38.5 ± 13.6^{ab}	14.1 ± 0.8^{c}	3.4 ± 0.1^{c}	19.3 ± 1.0^{c}	18.0 ± 0.4^{d}

1. Percentage expressed as weight by weight on a wet basis (moisture in initial product included)

2. Tested per AOAC Method 2009.01. This analysis was performed by Dr. Catrin Tyl.

3. ± one standard deviation (N = 6 for lactose and glucose, N = 4 for dietary fiber)

4. The component (%, w/w dry basis) in the extrusion product was compared to the component (%, w/w dry basis) in the raw mix to obtain the percentage remaining in the final product

a-e. Means sharing the same superscript are no significantly different from each other (p>0.05)

All products had different lactose contents which ranged from 3.0% to 34.2% on a weight by weight basis (Table 5). However, because of the differing amounts of lactose in the raw mix (74% to 78%), it is important to analyze the percentage of lactose which remains in the sample compared to the starting lactose content. The 15 kg/hr feed rate sample group had significantly lower percentages of the original lactose remaining (4.1%-21.0%) than the 30 kg/hr group (19.3%-44.1%). With an increase in acid from 2% to 6%, the percentage of original lactose remaining increased significantly. The 15 kg/hr feed rate samples went from 21.0% of original lactose remaining with a 2% citric acid formula to only 4.1% at a 6% citric acid formula, while the original lactose remaining in the 30 kg/hr feed rate samples went from 44.1% to 19.3% at 4% and 6% citric acid, respectively.

All glucose contents were low (1.7%-3.7%) when compared to lactose (3.0%-34.2%). Because all formulas started with 20% glucose, this shows a high rate of conversion. When examining the percentage of original glucose when compared to the original amount of glucose in the raw mix, the 15 kg/hr feed rate group shows significantly less remaining glucose than the 30 kg/hr feed rate group with the 15 kg/hr group at 8.8-16.6% remaining glucose while the 30 kg/hr group had 18.0-19.8% remaining glucose. When analyzing the effect of citric acid, the effects were more pronounced in the 15 kg/hr feed rate group, with the percentage of original glucose remaining going from 16.6% at a 2% citric acid level to 8.8% at a 6% citric acid level.

Lactose extrusion products were analyzed per AOAC Method 2009.01, which employs digestive enzymes to simulate human digestion in order to measure the soluble and insoluble fiber content of a food substance. The lactose extrudates were almost all lower molecular weight soluble fiber (LMWSF) with insignificant amounts of insoluble fiber observed. As a part of AOAC Method 2009.01,

pigment is removed through filtering and does not contribute to indigestible material.

The 15 kg/hr sample at 2% citric acid had higher LMWSF (53.0%) than the same formula at 30 kg/hr (33.7%). The 4% citric acid formula also had higher LMWSF at 15 kg/hr (56.4%) compared to 30 kg/hr (43.5%). The 6% citric acid formula showed no difference between feed rates. The LMWSF yield was not different within the 15 kg/hr feed rate group or within the 30 kg/hr feed rate group. However, the LMWSF yield of the 2% and 6% citric acid formulas extruded at 30 kg/hr were lower than all formulas extruded at 15 kg/hr.

Chromatograms of the extrusion products prepared according to AOAC Method 2009.01 utilizing high performance liquid chromatography (HPLC) and an evaporative light scattering detector (ELSD) comparing the effect of the acid for the 15 kg/hr feed rate and the 30 kg/hr feed rate are shown in Figure 4 and Figure 5, respectively.

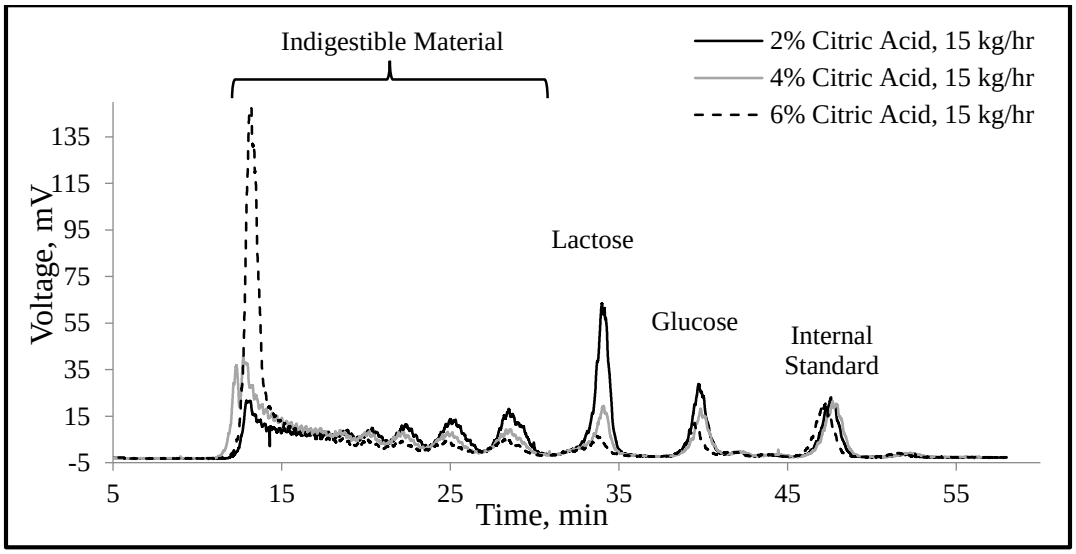


Figure 4. Influence of citric acid content on the composition of lactose extrudates extruded at 15 kg/hr shown by high performance liquid chromatography (HPLC) with an evaporative light scattering detector (ELSD). Samples were prepared according to AOAC Method 2009.01 for dietary fiber analysis.

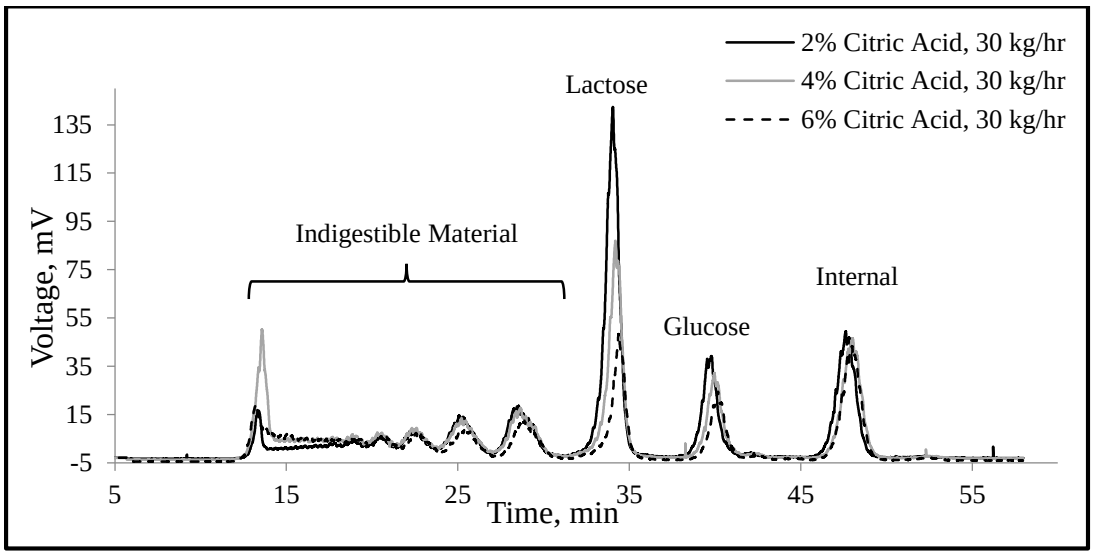


Figure 5. Influence of citric acid content on the composition of lactose extrudates extruded at 30 kg/hr shown by high performance liquid chromatography (HPLC) with an evaporative light scattering detector (ELSD). Samples were prepared according to AOAC Method 2009.01 for dietary fiber analysis.

All of the extrudates show some polymerization, as evidenced by the peaks that elute before lactose, which have a DP greater than or equal to 3.

3.4 Discussion

3.4.1 *Effect of Feed Rate*

More LMWSF was seen in the 2% and 4% citric acid samples extruded at the 15 kg/hr feed rate than the same samples extruded at 30 kg/hr (Table 5). In addition, less of the original amount of glucose and lactose remained in the samples for the 15 kg/hr feed rate when compared to the 30 kg/hr feed rate. An increase in feed rate results in a shorter residence time for the material within the extruder (Ainsworth, Ibanoglu, & Hayes, 1997; Gao, Walsh, Bigio, Briber, & Wetzel, 1999), which can also lead to a decrease in chemical conversion (Zhu & Jaluria, 2001). A longer residence time would also cause an increase in shear which is shown by an increase in SME (Yacu, 2012). Shear has also been shown to increase cross-linking which results in more polymerization (O'Brien & Wang, 2009). A decrease in feed rate would allow for more chemical conversion through residence time and shear, which would increase LMWSF and result in less remaining glucose and lactose.

When the feed rate was increased from 15 to 30 kg/hr, the SME decreased, which showed there was a greater operating efficiency for the extruder at this speed. Temperatures recorded at 48.3 cm from the feeder end showed that the 15 kg/hr sample reached a higher maximum temperature (204.0-205.7°C) than the samples extruded at 30 kg/hr (199.3-201.0°C). A longer residence time at a lower feed rate can allow for more conversion of mechanical energy to heat through shear as explained by Kazemzadah (2012).

The samples extruded at 15 kg/hr had a lower TA than those at 30 kg/hr, which could be due to increased decomposition of citric acid since this treatment achieved higher temperatures. Decomposition of citric acid has been reported to occur at temperatures between 202 and 222°C (Wyrzykowski, Hebanowska, Nowak-Wiczk, Makowski, & Chmurzyński, 2011). In addition, citric acid has been shown to be incorporated into the final product through esterification (Olivato et al., 2012; Shi et al., 2007). The lower feed rate generally had higher dietary fiber, and had greater loss of lactose. This demonstrates that greater chemical conversion of lactose, whether to LMWSF or other products, occurred which resulted in less free acid in the final product produced at the lower feed rate.

The absorbance at 420 nm, which is a commonly accepted method to measure brown pigment produced from caramelization (Ajandouz et al., 2001; Carabasa-Giribet & Ibarz-Ribas, 2000; Kroh, 1994; Wrolstad, 2012), was higher for the samples extruded at 15 kg/hr than those at 30 kg/hr. The samples in the 15 kg/hr group had a longer residence time and reached a higher maximum temperature. A longer residence time would result in more participation in reactions such as caramelization and polymerization (Ajandouz et al., 2001), and the product would experience more shear. However, higher temperatures experienced by the samples extruded at 15 kg/hr can also cause an increase in caramelization products (Ajandouz et al., 2008), which explains the darker color seen for the 15 kg/hr samples. In addition, caramelization can also result in polymerized products with a DP of up to 6 (Golon & Kuhnert, 2013), which could also contribute to the increase in LMWSF at a lower feed rate. These results suggest that a higher feed rate will reduce LMWSF and caramelization products, but that a balance to maximize LMWSF and minimize browning from caramelization may be possible at a higher feed rate.

3.4.2 *Effect of Citric Acid*

There was no difference in LMWSF with respect to acid concentration, which is most likely due to the high variability seen between extrusion replicates. The TA of the extruded samples increased and pH decreased with an increase in citric acid in the raw mix, which shows that the samples were more acidic. The change in TA and pH can be attributed to the increase in citric acid in the raw mix.

Less lactose and glucose remained as the amount of citric acid increased, indicating that more lactose and glucose were participating in chemical reactions which converted them from their original form. An increase in citric acid can cause an increase in cross-linking (Menzel et al., 2013; Olsson et al., 2013; O'Brien & Wang, 2009) and an increase in caramelization (Ajandouz et al., 2008), which could both account for a reduction in lactose and glucose. Brown pigment, as measured by absorbance at 420 nm, increased with citric acid content for the 15 kg/hr feed rate samples, but was not different between the 30 kg/hr feed rate samples. These results are also supported by *Hunter L, a, b* analysis, which showed that the products were darker and more yellow as citric acid content increased. An increase in acid can lead to more caramelization (Ajandouz et al., 2001), which results in many complex products, including oligomers with a reported DP of up to 6 (Golon & Kuhnert, 2013). An increase in caramelization would explain both the decrease in lactose and glucose and the increase in brown pigment seen. Higher than optimal levels of acid have been found to produce excess color and off flavors in polydextrose polymerization (Shah, Craig, Morrill, & Wuesthoff, 1998), which suggests that more acid can encourage participation in other reactions with undesirable products.

SME and torque both decreased with increasing citric acid content. Because the feed rate and screw speed were held constant, this indicates that the change was

caused by a decrease in resistance within the extruder (Yacu, 2012) which could be explained by the citric acid acting as a plasticizer. This could occur due to the moisture associated with citric acid that is released during heating (Barbooti & Al-Sammerrai, 1986), or citric acid could catalyze the condensation polymerization reaction which would result in free water molecules which plasticized the material in the extruder (Pushpadass et al., 2009). As water was released, the humidity within the extruder would increase, causing the lactose, glucose and citric acid to reach their deliquiescence relative humidities and release water (Kwok, Mauer, & Taylor, 2010; Peng, Chow, & Chan, 2001), further decreasing the viscosity. As citric acid percentage increased, a decrease in torque was seen, which has been shown to correlate with a decrease in viscosity (Akdogan, 1996).

3.5 Conclusion

This study found that a higher feed rate (30 kg/hr compared to 15 kg/hr) results in better extrusion efficiency and resulted in lower maximum product temperatures. Although a higher feed rate resulted in lower yields of LMWSF, it also produced less brown pigment and higher acidity. These results indicate that a higher feed rate could lead to reduced brown pigment, while still yielding LMWSF through reactive extrusion. This study also found that a higher level of citric acid (6%), resulted in better extrusion efficiency. However, an increase in acid also resulted in an increase in browning and acidity in the final product. Citric acid catalyzes both the production of polymerized material and undesired brown pigment, therefore a careful balance must be achieved.

The LMWSF analyzed in this study contains oligomers and other unknown compounds, and more work is currently being done to characterize these compounds. In addition, the beneficial effects of this indigestible product in the digestive systems of humans should also be evaluated. This work could lead to the commercialization of a value-added food ingredient produced from lactose via twin-screw extrusion.

4 Acid Whey

4.1 Introduction

Acid whey is produced as a byproduct of dairy products produced by the acidified coagulation of milk, such as quark, fromage frais, cottage cheese, cream cheese, and Greek yogurt (Fox et al., 2000; Zall, 1992). Greek yogurt is produced by separating whey from regular yogurt (Chandan & O'Rell, 2006a), which results in its characteristic thick texture. Difficulty has been experienced in processing acid whey due to stickiness caused by the lactic acid (Sienkiewicz & Ridel, 1990). Lactose and protein from sweet whey (produced through renneted dairy products) is already highly utilized and widely available (Carr et al., 2003; Fox & McSweeney, 1998; Sienkiewicz & Ridel, 1990). Due to the difficulty of processing and a relatively lower abundance when compared to sweet whey, acid whey has not been as widely investigated to process into value-added products.

Currently, the few options to dispose of acid whey include land application, alcoholic fermentation, and using it as animal feed (Pesta et al., 2007; Zall, 1992). Dairy processors must pay to have acid whey disposed of, due to its high biological oxygen demand (BOD) (Zall, 1992). A high BOD leads to a state of hypoxia, or oxygen depletion in streams or bodies of water, which negatively affects marine life (Diaz & Breitburg, 2011). 150 million gallons of acid whey were produced in the Northeast region of the United States in 2012 (Elliott, 2013) and production continues to expand. Therefore, there are great opportunities to both produce value added products from acid whey, and solve environmental issues created by disposal issues.

Acid whey is high in lactose, and also contains protein (Fox et al., 2000; Pesta et al., 2007; Sienkiewicz & Ridel, 1990); either of which could be utilized if they could be isolated or concentrated from acid whey. Acid whey is difficult to dry due to the

stickiness caused by the lactic acid and often must be neutralized before it can be utilized (Sienkiewicz & Ridel, 1990). It has been demonstrated that lactose can be polymerized into an indigestible product via extrusion, which could potentially be classified as dietary fiber (Tremaine et al., 2014). Commercially dried acid whey is produced by a few companies. Extrusion of this product in an attempt to polymerize the lactose contained within it was peformed in a preliminary study as part of this research, but did not yield good results (data not shown). A dry, crumbly, black substance was produced when utilizing the same extrusion conditions as in Tremaine et al. (2014). The amount of acid in the product also created noxious fumes and pitted the hardened steel components of the extruder. Therefore, the objective of this research was to develop a cleanup method for acid whey in order to utilize the lactose component in later extrusion trials.

We hypothesized, due to observations during the extrusion of commercial acid whey, that the lactic acid and the protein were the main obstacles to processing. The acid in the product caused noxious fumes and pitted the hardened steel components of the extruder. In addition, the product was black which could have been caused by protein degradation. Therefore, processing methods to reduce the acid and protein were investigated. Another objective of this research was to use processing equipment and processes for this cleanup which are commonly used industrially.

Lactose solubility decreases with temperature and results in lactose crystallization (Fox & McSweeney, 1998). Lactose crystallized from sweet whey is purified with water and sold commercially (Sienkiewicz & Ridel, 1990). This study evaluates processing steps such as heat treatment, condensing, crystallization, washing and drum drying of acid whey produced by Greek yogurt. The effect of processing on the composition of the drum dried product was evaluated to determine if it can be utilized in value-added food ingredient processes.

4.2 Materials and Methods

4.2.1 *Materials*

Grade A whole milk (Land O' Lakes, Arden Hills, MN, USA) and a mixed strain frozen culture consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (YFL702, Danisco, Copenhagen, Denmark) were used to make each batch of Greek yogurt.

4.2.2 *Acid Whey Preparation*

4.2.2.1 YOGURT MAKING

34 kg of Grade A whole milk was pasteurized by heating to 82°C in a can pasteurizer and maintaining that temperature for 30 minutes. The milk was then cooled to 43°C and 16 grams of culture was added. The pH of the yogurt was monitored until it reached 4.60. The yogurt was then refrigerated at 2-4°C for two to three days, until further treatment could be performed.

4.2.2.2 ACID WHEY SEPARATION AND CLARIFICATION

The yogurt was warmed to room temperature and then run through a Westfalia SB7 desludging centrifuge (GEA Westfalia Separator Group, Düsseldorf, Germany) run at 9200 rpm. The supernatant (the acid whey portion) was retained. The acid whey obtained from one batch of yogurt was condensed in a glass evaporator operated at approximately 55°C at a vacuum of 638 mm Hg. The starting weight of acid whey was 13.9 kg at 5.63% total solids. The acid whey was condensed until the total solids reached 61.25%. Approximately 1.1 kg of condensed acid whey was obtained.

A second batch of yogurt was made and the acid whey was separated as previously described. The acid whey was then heat treated at 82°C in a can pasteurizer for 30 minutes to precipitate whey proteins. The acid whey was then run through a Westfalia SB7 desludging centrifuge (GEA Westfalia Separator Group, Düsseldorf, Germany) at 9200 rpm to separate any precipitated protein. The supernatant (acid whey portion) was retained. The starting weight of the acid whey was 19.2 kg at 5.25% total solids. The acid whey was condensed until the total solids reached 65.55%. Approximately 1.5 kg of condensed acid whey was obtained.

Both batches of condensed acid whey were refrigerated for 3 days, which resulted in lactose crystallization due to the decrease in solubility of lactose. After refrigeration, the portions of condensed acid whey were stored frozen for at least one month, until further experimentation was performed.

4.2.2.3 Acid Removal

The condensed whey which was not heat treated, and the condensed whey which was heat treated were each separated into three portions of approximately 250 g. The first portion was not treated and kept as is.

The second portion was washed by mixing a 2:1 ratio of product with reverse osmosis (RO) water. The mixture was centrifuged at 1000x *g* for 3 minutes at 23°C on a Beckman-Coulter Avanti J-E centrifuge (Beckman Coulter Inc., Brea, CA, USA) with a JA-14 rotor and 250 mL centrifuge bottles. The supernatant was discarded and the precipitate was retained.

The third portion was washed with RO water using the same method as previously described. The retained precipitate was again washed with RO water, using a 2:1 ratio of product to water and mixing thoroughly. It was

centrifuged under the same conditions, and the precipitate was retained. Approximately 250 g, 100 g, and 50 g were obtained for the condensed acid whey which was not washed, washed once, and washed twice, respectively. Additional washing resulted in lost product through the centrifugation process. The precipitates were stored frozen until further experimentation was completed.

4.2.2.4 DRUM DRYING

A 6" pilot scale twin drum dryer (Buflovak LLC, Buffalo, NY, USA) set at 4 rpm and heated to 250°C was used to dry the condensed acid whey with various washing treatments. Approximately 200 g, 100 g, and 50 g of condensed whey which was not washed, washed once, and washed twice (respectively) were drum dried by adding about 10 grams at a time to the drum dryer. This resulted in approximately 125 g, 40 g, and 25 g of dried product for the not washed, washed once, and washed twice samples, respectively.

4.2.3 *Chemical and Physical Analysis*

4.2.3.1 COLOR

A solution of 2.00 ± 0.05 grams of dried whey was diluted to 50 mL with reverse osmosis water to analyze color. The solution was analyzed for Hunter *L*, *a*, and *b* values using a spectral scan in reflectance mode from 360-700 nm in 1nm increments at medium speed on a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) using UV/PC Optional color analysis software 3.10. A D65 illuminant and 10° observer viewing angle were used to calculate Hunter *L*, *a*, and *b* values.

Absorption at 420 nm was measured on a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) using UV Probe 2.43 software. The concentration of the solution was adjusted as needed to obtain an absorbance between 0.1 and 1.5. All absorbances were corrected based on concentration for final comparison.

4.2.3.2 LACTOSE AND GLUCOSE

Lactose and glucose contents of the condensed acid whey before and after drum drying were determined using an enzymatic test kit from Megazyme (Lactose/Sucrose/D-Glucose Kit, Megazyme, Bray, Ireland).

4.2.3.3 TOTAL SOLIDS

Total solids content of the condensed whey before and after drum drying was determined by vacuum oven as per Method 15.086 in the Standard Methods for the Examination of Dairy Products (Hooi et al., 2004). 1.0-1.5 grams of sample were placed in pre-dried aluminum pans and dried in a vacuum oven at 65°C at a pressure of -86 to -96 kPa with a dried air flow rate of approximately 117 mL/min. The samples were dried for 16 hours, allowed to cool to room temperature in a desiccator, and the difference in weight was recorded as moisture. The condensed whey samples prior to drum drying were pre-dried using a steam table for 30 minutes before drying in the vacuum oven.

4.2.3.4 ASH

Ash was determined by combustion in a muffle furnace. One to 1.5 grams of sample was placed into pre-ashed crucibles. The crucibles were ashed at 550°C for 12 hours. Samples were cooled to below 100°C before removing

and placed in a desiccator to cool to room temperature. The weight of the remaining material was recorded as ash.

4.2.3.5 PROTEIN

Protein content was determined using a Dumas protein analysis system (Buchi DuMaster D-480, Buchi, Flawil, Switzerland). 250 mg of sample was analyzed in duplicate. Before analysis, the acid whey samples were pre-dried using a steam table for 30 minutes to condense the samples so that they were within the detection limit of the Dumas analyzer and protein concentration was adjusted accordingly.

4.2.3.6 FAT

Fat was measured by Mojonnier using three extractions as described in Method 15.086 in the Standard Methods for the Examination of Dairy Products (Hooi et al., 2004). 1.00 grams of dried condensed acid whey and 10.00 grams of liquid acid whey were analyzed for each sample.

4.2.3.7 PH

The pH of the drum dried acid whey products was measured by diluting 6.5 grams to 100 mL with RO water and measuring with a pH meter (Accumet Basic AB15, Fischer Scientific, Pittsburgh, PA, USA).

4.2.3.8 TITRATABLE ACIDITY

Titratable acidity of the drum dried condensed acid whey products was measured by titrating between 0.7 and 2.0 grams of extrudate (depending on the expected acidity) with 0.1 M NaOH (Sigma Aldrich, St. Louis, MO, USA) to an endpoint of pH 8.2 with an autotitrator (Mettler Toledo T50,

Mettler Toledo, Columbus, OH, USA). The titratable acidity was expressed as % lactic acid by weight.

4.2.3.9 High Performance Liquid Chromatography (HPLC) to Determine Degree of Polymerization (DP) of Carbohydrates

HPLC analysis was conducted to determine if drum drying resulted in polymerization of the sugars in the whey. Drum dried acid whey (0.5 g) along with an internal standard of ribose (0.05 g), was diluted to 5 mL using RO water. The solution was filtered through a 0.45 μm syringe filter prior to analysis. HPLC analysis was performed using a Transgenomics CHO-411 column (Omaha, NE, USA) with detection by low temperature evaporative light scattering detection (ELSD-LT) (Shimadzu Corporation, Kyoto, Japan). The ELSD nebulizer was set to 40°C and the nitrogen pressure was 250 kPa. The column temperature was maintained at 80°C. The mobile phase was double distilled degassed water at a flow rate of 0.3 mL per min.

A column performance check standard oligosaccharide, Phenomonex ALO-3038 (Phenomonex, Torrance, CA, USA), was analyzed using the same conditions to determine the demarcation point between disaccharides and carbohydrates with a degree of polymerization of 3 and higher.

4.2.4 *Statistical Analysis*

The values presented are the means of either duplicate or triplicate determinations. All data was analyzed using univariate analysis of variance (ANOVA) followed by pairwise comparisons using Tukey honestly significant difference in R version 3.1.0 (R Development Core Team, 2008).

4.3 Results

4.3.1 Acid Whey Composition

The composition of acid whey produced by centrifuging two yogurt batches are shown in Table 7. The fat and protein content of the original milk is shown in Table 6. One batch of acid whey was heat treated to precipitate additional proteins. One batch of whey was processed without heat treatment to determine if the effects of heat treating were beneficial enough to warrant the extra cost of processing.

Table 6. Fat and protein content of milk prior to processing.

Replicate	Fat[1]	Protein[1]
	%, w/w	%, w/w
Rep 1	3.75	2.99
Rep 2	3.85	3.12

1. Analysis performed by Eurofins DQCI via Mid-IR (Mounds View, MN, USA).

Table 7. Composition of liquid acid whey prior to condensing.

Heat Treatment		Lactose[1]	Glucose[1]	Total Solids[1]	Fat[1]	Protein[1]	Titratable Acidity[1]	pH[1]
		%, w/w	%, w/w	%, w/w	%, w/w	%, w/w	%, w/w as lactic acid	
None	Rep 1	2.23 ± 0.09^{a}	0.03 ± 0.00^{b}	5.63 ± 0.04^{b}	1.15 ± 0.01^{b}	0.06 ± 0.01^{a}	0.34 ± 0.00^{a}	4.33 ± 0.01^{a}
Prior to heat treatment	Rep 2	3.26 ± 0.01^{c}	0.01 ± 0.00^{a}	6.27 ± 0.01^{c}	0.31 ± 0.02^{a}	0.49 ± 0.01^{c}	0.46 ± 0.00^{c}	4.37 ± 0.05^{a}
After heat treatment[2]	Rep 2	2.89 ± 0.04^{b}	0.01 ± 0.01^{a}	5.25 ± 0.03^{a}	0.24 ± 0.06^{a}	0.16 ± 0.01^{b}	0.38 ± 0.00^{b}	4.32 ± 0.07^{a}

1. ± one standard deviation (N = 3)

2. Rep 2 was heat treated at 82°C for 30 minutes and then centrifuged to remove precipitated protein.

a-c. Means sharing the same superscript are not significantly different from each other ($p>0.05$)

As shown in Table 6, replicates 1 and 2 varied in fat and protein content, but were within the expected ranges for cow's milk (Boland, 2003; Fox et al., 2000; Fox & McSweeney, 1998).

Comparing the acid whey produced in Rep 1 to Rep 2 (both before heat treatment), variability in the sugar composition was evident. Rep 1 had a lower lactose content (2.23% vs. 3.26%), higher glucose content (0.03% vs. 0.01%), lower total solids (5.63% vs. 6.27%), higher fat (1.15% vs. 0.31%), lower protein (0.06% vs. 0.49%), and lower TA (0.34% vs. 0.46%), when compared to Rep 2 before heat treatment. No differences in pH measurements between samples were observed.

A portion of the Rep 2 acid whey was heat treated to precipitate whey proteins. When comparing Rep 2 before and after heat treatment, a decrease in lactose content (3.26% vs. 2.89%), a decrease in total solids moisture (6.27% vs. 5.35%), a decrease in protein (0.49% vs. 0.16%) and a decrease in TA (0.46% vs. 0.38%) was observed. No differences in glucose, fat, or pH were seen.

The differences between the batches of liquid whey seem small, however the next step in the process was to condense the batches by over 11 times by removing water, which emphasizes these differences greatly.

4.3.2 Condensed Acid Whey Composition

Prior to condensing, the acid whey was mostly transparent with a slight milky white to yellow opaqueness. After condensing, both products were a bright yellow color, with Rep 1 (not heat treated) having a milkier appearance. The texture was a thick pudding-like consistency, with the heat treated condensed whey appearing more granular than the not heat treated condensed whey.

Portions of the condensed acid whey samples were then washed with reverse osmosis water in an attempt to remove lactic acid from the crystallized lactose and protein. Both sets of condensed acid whey were divided into three portions: one was left untreated, one was washed once, and the last portion was washed twice. Each time the product was washed, the mixture was centrifuged and excess water in the supernatant was discarded. As the amount of washing increased, the condensed whey appeared less yellow and more white.

4.3.3 Drum Dried Acid Whey

The portions of condensed acid whey with different washing treatments were drum dried. During drum drying, the products varied widely in processing ability, as well as the consistency and color of the final products. A qualitative description is given in Table 8. Total solids, protein, lactose, glucose, ash, and fat were analyzed for the drum dried acid whey and are presented in Table 9. The total solids of all samples dramatically increased after drum drying with the evaporation of water, and ranged from 93.57% to 98.17.

Table 8. Qualitative description of drum dried acid whey.

Treatment		Processing Description	Qualitative Description	Appearance (after grinding)
Heat Treatment	**Wash**			
Heat treated (Replicate 2)	None	Very sticky, stuck to drum dryer blade, caramel-consistency	Dark brown, large glassy pieces	
	Once	Slightly sticky, flaked off of drum dryer into small clumps	Light tan powder, some clumps	
	Twice	Dried nicely, flaked off the drum dryer into a powder	White powder, few clumps	
No heat treatment (Replicate 1)	None	Very sticky, stuck to drum dryer blade, caused oil buildup on drums, caramel like consistency,	Brown, large pieces, pliable	
	Once	Somewhat sticky, built up on drum dryer blade, caused some oil buildup, crumbly consistency	Dark tan, large clumps with some powder	
	Twice	Slightly sticky, flaked off of drum dryer into small clumps, slight oil buildup	Light tan powder with some clumps	

Table 9. Composition of drum dried condensed acid whey.

Treatment		Total Solids[1]	Protein[1]	Lactose[1]	Glucose[1]	Ash[1]	Fat[1]
Heat Treatment	**Wash**	**%, w/w**	**%, w/w**	**%, w/w**	**%, w/w**	**%, w/w**	**%, w/w**
Heat treated (Replicate 2)	None	95.07 ± 0.09^{b}	4.33 ± 0.05^{d}	41.0 ± 0.8^{b}	0.2 ± 0.0^{a}	12.57 ± 0.00^{f}	1.26 ± 0.06^{a}
	Once	98.17 ± 0.20^{e}	1.14 ± 0.06^{b}	69.7 ± 1.4^{d}	0.2 ± 0.0^{ab}	6.32 ± 0.06^{c}	1.19 ± 0.31^{a}
	Twice	97.59 ± 0.04^{de}	0.33 ± 0.01^{a}	76.2 ± 0.9^{e}	0.1 ± 0.0^{b}	2.93 ± 0.04^{a}	0.67 ± 0.05^{a}
No heat treatment (Replicate 1)	None	93.57 ± 0.47^{a}	4.69 ± 0.08^{e}	37.1 ± 0.6^{a}	0.7 ± 0.0^{c}	9.91 ± 0.03^{e}	16.51 ± 0.23^{b}
	Once	95.76 ± 0.09^{c}	4.22 ± 0.04^{d}	44.7 ± 0.5^{c}	0.8 ± 0.0^{c}	7.74 ± 0.01^{d}	21.54 ± 0.66^{c}
	Twice	97.25 ± 0.06^{d}	4.01 ± 0.02^{c}	45.0 ± 0.2^{c}	0.8 ± 0.1^{c}	5.69 ± 0.03^{b}	22.58 ± 0.24^{d}

1. ± one standard deviation (N = 3 except N = 2 for protein)

a-c. Means sharing the same superscript are no significantly different from each other ($p>0.05$)

After drum drying, the samples from whey which was heat treated had more total solids (95.07%-98.17%) when compared to the samples which were not heat treated (93.57%-97.25%).

The heat treated samples showed an increase in total solids between no washing and some washing, but no difference was seen between one and two washings. However, the samples which were not heat treated showed an increase in total solids from no washing to two washings.

The protein content of the heat treated samples was lower (0.33%-4.33%) than the samples which were not heat treated (4.01%-4.69%) (Table 9). The protein content decreased with washing treatment for the acid whey with and without heat treatment, however the heat treated whey showed a much larger drop in protein content as washing treatment increased, going from 4.22% down to 0.33%.

The heat treated acid whey had significantly more lactose (41.0%-76.2%) than the whey which was not heat treated (37.1%-45.0%) (Table 9). When looking at the effect of washing, both groups showed that more washing effectively increased lactose concentration. However, the effect was more pronounced for the heat treated samples.

After drum drying, the glucose became more concentrated for the group which was not heat treated (0.7%-0.8%), but stayed the same for the heat treated whey samples (0.1%-0.2%) (Table 9).

The heat treated samples showed a higher ash content (2.93%-12.57%) as compared to the samples which were not heat treated (5.69%-9.91%) as shown in Table 9. Ash content decreased with washing treatment as the heat treated dried acid whey went from 12.57% with no washing to 2.93% after two washings. The

whey which was not heat treated showed a similar pattern where the ash content went from 9.91% with no washing to 5.69% with two washings.

The fat content of the heat treated samples after drum drying ranged from 0.67% to 1.26% compared to the samples which were not heat treated at 16.51% to 22.58% (Table 9). Fat content was not different between the samples in the heat treated group, and all had low fat content (0.67%-1.26%) when compared to the group which was not heat treated (16.51%-22.58%). The samples within the group without heat treatment showed an increase in fat content with washing treatment going from 16.51% fat with no washing to 22.58% fat with two washings.

pH and titratable acidity were measured in the condensed acid whey after drying and the results are presented in Table 10.

Table 10. pH and titratable acidity of drum dried condensed acid whey.

Treatment		**Titratable Acidity[1]**	**pH[1]**
Heat Treatment	**Wash**	**%, w/w as lactic acid**	
Heat treated (Replicate 2)	None	7.35 ± 0.20^{f}	4.51 ± 0.01^{b}
	Once	3.05 ± 0.09^{c}	4.77 ± 0.01^{e}
	Twice	1.11 ± 0.02^{a}	4.88 ± 0.01^{f}
No heat treatment (Replicate 1)	None	6.05 ± 0.08^{e}	4.40 ± 0.01^{a}
	Once	4.15 ± 0.04^{d}	4.54 ± 0.02^{c}
	Twice	2.44 ± 0.07^{b}	4.68 ± 0.02^{d}

1. ± one standard deviation (N = 3)
a-c. Means sharing the same superscript are no significantly different from each other ($p>0.05$)

All samples had an acidic pH and values ranged from 4.40 to 4.88. The heat treated samples showed a higher pH than the samples which were not heat treated.

In regards to the washing treatment, the pH increased with the amount of washing within both the heat treated and not heat treated groups.

Titratable acidity (TA) was calculated as lactic acid and ranged from 1.11% to 7.35% for all samples (Table 10).

For the samples which were not washed, the heat treated sample had a higher titratable acidity (7.35%) when compared to the sample which was not heat treated (6.05%). However, for the samples which were washed once and washed twice, the opposite pattern was observed where the samples which were not heat treated had more acidity than the heat treated samples. In addition, the heat treated samples saw a greater decrease in TA with washing.

Color measurements were taken to quantify the extent of these reactions and the data is presented in Table 11.

Table 11. Color measurements for drum dried condensed acid whey.

Treatment		Browning	Color		
Heat Treatment	**Wash**	**Absorbance at 420 nm**	**Hunter *L* [1]**	**Hunter *a* [1]**	**Hunter *b* [1]**
Heat treated (Replicate 2)	None	0.626 ± 0.016^{b}	13.52 ± 0.47^{c}	8.74 ± 0.25^{d}	9.28 ± 0.32^{c}
	Once	0.131 ± 0.001^{a}	41.94 ± 0.39^{d}	1.24 ± 0.12^{c}	15.72 ± 0.05^{d}
	Twice	0.080 ± 0.001^{a}	53.82 ± 1.35^{e}	-0.09 ± 0.20^{a}	15.73 ± 0.28^{d}
No heat treatment (Replicate 1)	None	1.301 ± 0.015^{e}	3.68 ± 0.16^{a}	0.39 ± 0.09^{b}	2.14 ± 0.03^{b}
	Once	1.153 ± 0.023^{d}	4.51 ± 0.11^{ab}	-0.01 ± 0.01^{a}	1.93 ± 0.04^{ab}
	Twice	0.878 ± 0.078^{c}	5.71 ± 0.28^{b}	-0.13 ± 0.01^{a}	1.57 ± 0.08^{a}

1. ± one standard deviation (N = 3)

a-c. Means sharing the same superscript are no significantly different from each other ($p>0.05$)

A higher absorbance at 420 nm was seen with the whey which was not heat treated (0.878-1.301) when compared to the heat treated whey samples (0.080-0.626). This indicates that more yellow-brown products were present in the whey which was not heat treated. When looking at the effect of washing, the absorbance at 420 nm decreased with an increase in the amount of washing.

The Hunter *L* value represents pure white at a value of 100 and pure black at a value of 0 (Choudhury, 2014). The heat treated group was more white than the whey samples which were not heat treated, which means the whey which was not heat treated was darker. The whey samples which were not heat treated were not significantly different from each other with *L* values ranging from 3.68 to 5.71, while the heat treated samples were significantly whiter as the amount of washing increased with values from 13.52 to 53.82.

A positive Hunter *a* value indicates the redness of a sample while a negative *a* value indicates greenness (Choudhury, 2014). All samples which were not heat treated were close to zero with values from -0.13 to 0.39, which indicates they were neither red nor green. The heat treated samples showed a decrease in redness as the amount of washing increased.

A positive Hunter *b* value indicates yellowness while a negative *b* value indicates blueness (Choudhury, 2014). All samples showed positive *b* values, however the heat treated whey samples were more yellow (9.28-15.73) than the whey samples which were not heat treated. For the heat treated group, washing once or twice increased the yellowness of the sample when compared to not washing the sample at all. For the whey samples which were not heat treated, no difference between washing treatments was seen.

Chromatograms of the drum dried acid whey were run to determine if polymerization of the lactose occurred during drum drying are shown in Figure 6 and Figure 7.

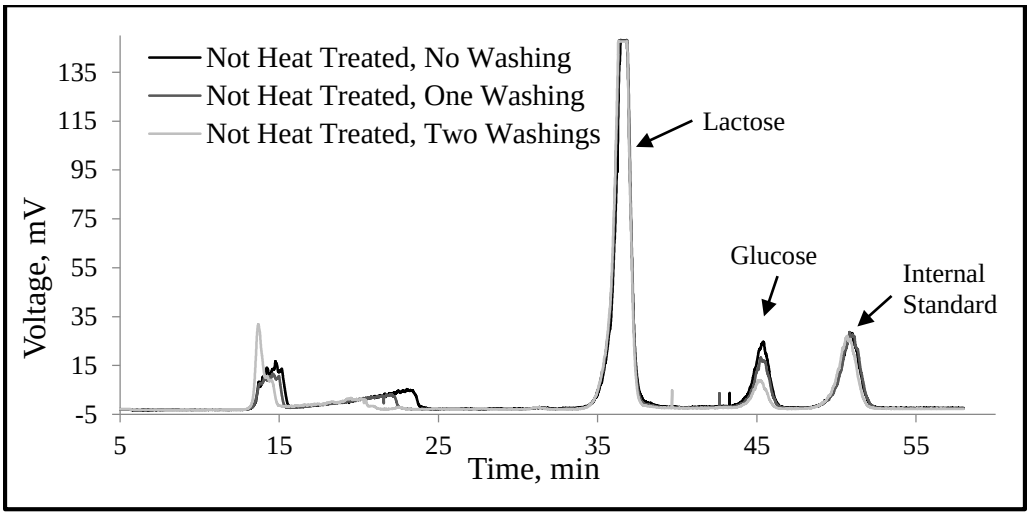


Figure 6. Influence of washing with water on the composition of condensed and drum dried unpasteurized acid whey shown by high performance liquid chromatography (HPLC) with an evaporating light scattering detector (ELSD).

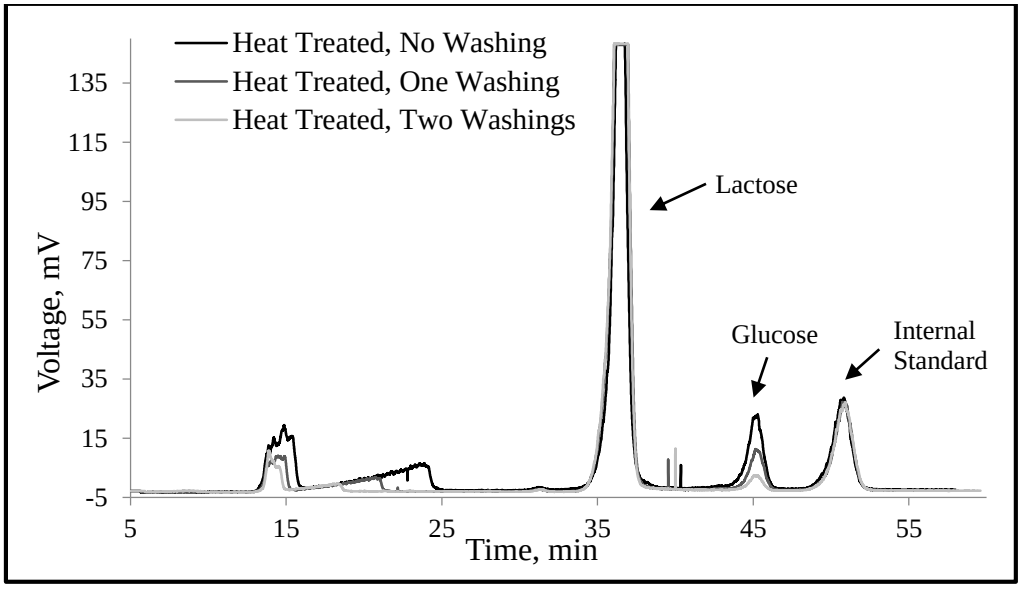


Figure 7. Influence of washing with water on the composition of condensed and drum dried pasteurized acid whey shown by high performance liquid chromatography (HPLC) with an evaporating light scattering detector (ELSD).

Both Figure 6 and Figure 7 show that there is a substantial amount of lactose, and some glucose in all samples. When comparing the chromatograms to the oligosaccharide standard (data not shown) we can see that no tri-saccharides or tetra-saccharides were formed. However, there are peaks of unknown composition which elute first off of the column, which could contain polymerized material or other components in the dried products that are soluble and pass through the syringe filter (such as peptides). The chromatograms were very similar in regards to heat treated and not heat treated samples, however the washing treatment seems to have had an effect on the early eluting compounds.

4.4 Discussion

4.4.1 Differences in Acid Whey Replicates

Comparing the acid whey produced in Rep 1 to Rep 2 (both before heat treatment), variability in the sugar composition was evident (Table 7). Acid whey from yogurt produced by Rep 1 had a lower lactose content, lower total solids, lower protein, and a lower titratable acidity (TA) when compared to Rep 2. These differences could be attributed to the fermentation action of the starter cultures which generate lactic acid by metabolizing lactose (Chandan & O'Rell, 2006a; Fox et al., 2000; Fox & McSweeney, 1998), or could be due to differences in the composition of the milk itself.

The differences in whey composition could also be due to differences in the protein gel strength of the yogurt which could affect whey removal. A protein gel is formed when the pH of the milk is reduced by the starter culture producing lactic acid (Chandan & O'Rell, 2006a). As the pH decreases it approaches the isoelectric point of casein micelles, which causes them to form a gel network (Fox et al., 2000). The gel strength can depend on factors such as the heat treatment conditions, milk protein content, incubation temperature, ending pH, and cooling

conditions (Lucey, 2009; Lucey & Singh, 2003). When the gel is broken during separation of the acid whey, the pores which previously trapped water are disrupted, which allows whey to escape, and depending on the strength of the gel, differing amount of fat and protein may leave the gel matrix with the whey during separation (Lucey & Singh, 2003).

After heat treatment of the Rep 2 whey, the TA was reduced to 0.38% which was similar to the acidity of Rep 1 whey. Proteins with acidic amino acid residues can contribute to TA, and so the drop in TA could be explained by proteins which were aggregated by the heat treatment at acidic conditions (Singh & Havea, 2003) and were removed by the centrifugation step utilized.

4.4.2 Effect of Heat Treatment on Acid Whey

The intent of heat treating was to remove whey proteins by heat precipitation in the presence of the lactic acid inherent in the whey, similar to ricotta and Queso Blanco production (Farkye, 2004). The heat treated acid whey was then centrifuged to remove precipitated protein.

The heat treated acid whey successfully showed a reduction in protein content. Acid whey usually has a low pH and is generally between 4.5 and 5.3 (Fox & McSweeney, 1998; Pesta et al., 2007; Sienkiewicz & Riedel, 1990). The isoelectric point is 5.2 for α-lactalbumin, 4.8 for α-lactalbumin (Fox, 2009) and 5.4 for bovine serum albumin (Shi, Zhou, & Sun, 2005), which are all close to the pH of acid whey. A protein near its isoelectric point experiences reduced electrostatic repulsions (Fox, 2009; Singh & Havea, 2003). Heat treatment causes the protein to unfold, exposing hydrophobic amino acid residues, and further encourages hydrophobic interaction between whey proteins (Edwards, Creamer, & Jameson, 2009; Lucey, 2009; Singh & Havea, 2003). In our study, heat treating the whey followed by centrifugation successfully reduced the protein content compared to

unheated whey, as expected. It also had the beneficial effect of reducing the fat content.

Elevated temperatures have been shown to increase interaction between whey proteins and milk fat globules (Corredig & Dalgleish, 1996; Dalgleish & Banks, 1991; Guyomarc'h, Law, & Dalgleish, 2003). This interaction is likely caused the fat to aggregate with the protein and be removed during centrifugation after heat treatment of the acid whey. Whey samples which were not heat treated also had higher moisture, in addition to protein and fat. The increase in water could be attributed to the water binding properties of whey protein (Berlin, Kliman, Anderson, & Pallansch, 1973) as well as the interaction between water and the phospholipids found in whey (Fox & McSweeney, 1998).

These differences in the condensed whey composition resulted in differences in the finished drum dried products. Heat treated whey had a much lighter color (when washed) as measured by the Hunter *L* values, and less brown pigment when measured by absorbance at 420 nm. Because both protein and sugars were present in the acid whey, the heat induced generation of both Maillard and caramelization products are possible (BeMiller & Whistler, 1996). The higher color levels in the non-heat treated whey could be due to the increased protein content of the whey, which could increase Maillard reaction products; or the darker color could also be due to the higher acidity shown by TA, which would mean increased caramelization products (Ajandouz et al., 2008).

4.4.3 *Effect of Washing Condensed Acid Whey*

The intent of the washing treatment was to remove lactic acid and protein. The amount of lactic acid has a large effect upon the taste and functionality of final products, as well as how it performs during processing as it can cause stickiness of

the product, as seen in the spray drying of whey (Modler & Emmons, 1978; Saffari & Langrish, 2014). The pH increased, the TA decreased, and the ash content decreased with the amount of washing within both the heat treated and not heat treated groups (Table 10, Table 9). This indicates that the washing treatment was effective at reducing acidity through the removal of lactic acid in water and removing minerals through solubility in water.

The protein content decreased with washing treatment for both groups of acid whey with and without heat treatment, however the heat treated whey showed a much larger drop in protein content as washing treatment increased (Table 9). The heat treatment followed by centrifugation caused a decrease in protein due to precipitation of a portion of the whey proteins. Any remaining protein (measured as % nitrogen) in the acid whey would likely be casein proteolytic products from the starter culture activity (Sienkiewicz & Riedel, 1990), and any remaining whey proteins which did not precipitate. These soluble proteins would be more easily removed with water, accounting for the higher drop in protein with washing for the heat treated samples.

Fat content was not different between the samples in the heat treated group, and all samples had low fat content compared to the whey which was not heat treated. The samples within the group that was not heat treated showed an increase in fat content with washing treatment indicating that washing did not remove fat. As discussed previously, the denatured protein in the heat treated acid whey would have exposed hydrophobic residues (Singh & Havea, 2003), which would more freely interact with fat molecules (Dalgleish & Banks, 1991; Guyomarc'h et al., 2003), and could have trapped both fat and protein in the precipitate and allowed its removal during centrifugation. Because the heat treated samples contained less protein, there was less interaction with fat, and possibly some fat was able to separate into the supernatant during the washing of that treatment, as evidenced by

a lower fat content after the 2nd wash. The fat in the whey samples which was not heat treated was further concentrated as the washing effect effectively removed protein and acidity, and resulted in higher total solids.

When looking at the effect of washing, the absorbance at 420 nm decreased and the samples were lighter (higher L values) with an increase in the amount of washing (Table 11). The condensed whey samples which were washed had less protein and lower acidity than those samples which were not washed. This would be expected to result in a decrease in Maillard products because both free amine groups and reducing sugars are required in the reaction (BeMiller & Whistler, 1996). Additionally, a decrease in acidity would result in a decrease in caramelization (Ajandouz et al., 2008). The changes in chemistry due to washing therefore explain the color difference seen for the different treatments.

Both the heat treated and not heat treated groups showed that more washing effectively increased lactose concentration. However, the effect was more pronounced for the heat treated samples. Impurities have been shown to interfere with the crystallization of lactose (Wong & Hartel, 2014), which could explain the higher lactose content of the washed heat treated acid whey. The results indicate that although some lactose may be lost during the washing process, a larger fraction of other components is removed, successfully concentrating the lactose. The removal of acid and fat are important due to the difficulty seen in spray drying products high in lactic acid (Modler & Emmons, 1978; Saffari & Langrish, 2014) and high in fat (Langrish, Marquez, & Kota, 2006). Therefore we can conclude that the washing treatment did not cause excessive loss of lactose, and in fact was successful in concentrating it for the heat treated samples.

4.5 Conclusion

This study showed that acid whey which is heat treated and centrifuged to clarify

prior to condensing resulted in a reduction of protein, fat, and moisture. Heat treatment of the whey also resulted in a lighter colored drum dried product. Washing the condensed acid whey with water prior to drum drying resulted in a reduction of acidity and protein, which resulted in an increase in lactose concentration. The condensed whey which was washed also had a lighter color after drum drying, due to a decrease in caramelization and Maillard products. These results indicate that a process which involves heat treating acid whey and clarifying it by centrifugation, combined with washing it with water after condensing and crystallizing, could result in a product with decreased protein, fat, and lactic acid. A reduction in lactic acid and fat would allow for easier processing by drum drying or spray drying, due to a decrease in stickiness. This would allow for a concentrated lactose powder to be manufactured from acid whey that could be used in other processes. Further work to refine this process and investigate value added uses for dried acid whey created with this process should be explored.

5 Conclusions and Recommendations

Lactose and acid whey are both considered low value dairy products. This research shows great promise for producing value added products out of both, which would benefit the dairy industry greatly.

This research demonstrated that lactose can be successfully polymerized utilizing extrusion into a product which has the potential to be classified as the valuable product, dietary fiber. Processing conditions and citric acid addition rates can be varied to create a desirable product with acceptable yield and a reduced amount of caramelization. This product can potentially be used as a food ingredient for either humans or animals. It is recommended to further this work, that different temperatures be evaluated for their effect on the yield and composition of the lactose product. In addition, further characterization of the product is necessary. Studies which can demonstrate a beneficial effect for a human or animal digestive system should also be done, which will add additional value to the product.

Acid whey has become an increasingly difficult problem with the recent increase in Greek yogurt production. This research has outlined a simple cleanup process which results in an easy to handle dry product high in lactose and protein. This product can be easily transported, and further processed into a value-added product. It is recommended that further trials with a heat treated and clarified dried acid whey be conducted, and extrusion investigated as a way to polymerize the high lactose content into an indigestible fiber-like product.

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6 Appendix A: Extended Methods

6.1 Extended Methods for Product Analysis

6.1.1 *Titratable Acidity*

Objective

The purpose of this experiment was to measure the titratable acidity of the extrudates to determine how formulation alterations affected titratable acidity.

Materials and Equipment

- Ground extrudates and processed acid whey
- Autotitrator (Mettler T50) with Mettler-Toledo DG115-SC pH probe
- Sample cups
- 0.1M NaOH (Alfa Aesar)
- Reverse osmosis (RO) water
- 100 mL beaker
- Analytical balance

Procedure

1. Calibrate the pH meter with the calibration standards 4.0 and 7.0.
2. Weigh the appropriate amount of extrudate into a sample cup, analyzing each sample in triplicate.
3. Weigh out sample so that at least 1.0 mL of titrant per sample is used.

Sample Name	Weight of Extrudate
6% Citric Acid	0.7 g
4% Citric Acid	1.0 g
2% Citric Acid	2.0 g

Condensed Acid Whey	0.4 - 0.6 g
Acid Whey	5.0 g

1. Weigh out 50 grams of water (50 mL) into a small beaker (100 mL) and transfer to the sample cup.
2. Make sure that the sample is fully dissolved before beginning titration.
3. Perform the titration with 0.1 M NaOH.
4. Record the volume of titrant needed to reach a pH of 8.2 (phenolphthalein indicator point).
5. Rinse the electrode with RO water between samples and blot dry.
6. Titrate two blanks using 50.0 grams of water.
7. Convert the extrudate and raw mix to percent citric acid and the acid whey to percent lactic acid.

Calculation of Citric Acid Percentage

1. Conversion from mL 0.1 M NaOH to grams of citric acid.$(\text{mLNaOH-mLNaOHinBlanks}) \times \frac{\text{L}}{1000\text{mL}} \times \frac{0.1\text{molNaOH}}{\text{L}} \times \frac{1\text{molcitricacid}}{3\text{molNaOH}} \times \frac{192.124\text{gcitricacid}}{\text{molcitricacid}} = \text{gcitricacid}$

$$(mL\ NaOH - mL\ NaOH\ in\ Blanks) \times \frac{L}{1000\ mL} \times \frac{0.1\ mol\ NaOH}{L} \times \frac{1\ mol\ citric\ acid}{3\ mol\ NaOH} \times \frac{192.124\ g\ citric\ acid}{mol\ citric\ acid} = g\ citric\ acid$$

2. Grams of citric acid to percentage of citric acid.

$$\frac{g\ citric\ acid}{g\ sample} \times 100 = \%\ citric\ acid\ (w/w)$$

Calculation of Citric Acid Percentage

1. Conversion from mL 0.1 M NaOH to grams of lactic acid.

$$(mL\ NaOH - mL\ NaOH\ in\ Blanks) \times \frac{L}{1000\ mL} \times \frac{0.1\ mol\ NaOH}{L} \times \frac{1\ mol\ lactic\ acid}{1\ mol\ NaOH} \times \frac{90.08\ g\ lactic\ acid}{mol\ lactic\ acid} = g\ lactic\ acid$$

2. Grams of lactic acid to percentage of lactic acid.

$$\frac{g\ lactic\ acid}{g\ sample} \times 100 = \%\ lactic\ acid\ (w/w)$$

6.1.2 *Lactose and Glucose*

Objective

The purpose of this experiment was to measure the amount of lactose in the products. These results will allow us to determine how much lactose is in the products before and after processing.

Materials and Equipment

- Megazyme Lactose Sucrose/D-Glucose Kit (Megazyme, K-LACSU) containing the following:
    - Bottle 2: β-Galactosidase (lactase; A. *niger*, 1 mL) suspension. (Stable for > 2 years at 4°C.)
    - Bottle 3: Glucose oxidase/peroxidase (GOPOD) Reagent Buffer. Buffer (48 mL, pH 7.4), *p*-hydroxybenzoic acid and sodium azide (0.4% 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 -20°C.)
    - Bottle 5: D-Glucose standard solution (5 mL, 1.0 mg/mL) in 0.2% (w/v) benzoic acid.
      (Stable for >5 years at room temperature.)
- Glacial acetic acid (Fisher Scientific)

- 1M sodium hydroxide (Sigma Aldrich)
- Volumetric flasks and stoppers
- Ground extrudates and processed acid whey
- Reverse osmosis water
- Aluminum foil
- Pipettes and tips
- Weigh boats
- 50°C water bath (Caliper Life Sciences TurboVap LV – only water bath functionality used)
- Beckman DU 640B spectrophotometer
- Test tubes
- Cuvettes
- Polypropylene tubes
- Vortexer

Sample Preparation

1. Prepare sample solutions of each extrudate of approximately 2-3 mg/mL concentration.
2. Use 3 mg/mL for the 15 kg/hr feed rate samples and 2 mg/mL 30kg/hr feed rate samples to account for the difference in lactose concentration. (Absorbances should be above 0.1 for all readings.)

Reagent Preparation

1. Sodium acetate buffer (Buffer 1)
    a. Combine 2.9 mL of glacial acetic acid (1.05 g/mL) with 900 mL of reverse osmosis water in a 1L beaker.
    b. Adjust the pH to 4.5 using 1 M (4 g/100 mL) sodium hydroxide.
    c. Transfer to a 1L volumetric flask.
    d. Adjust the volume to 1 L using reverse osmosis buffer.

2. β-galactosidase solution (Solution 2)
    a. Combine the contents of Bottle 2 (β-galactosidase suspension) with 19 mL of Buffer 1 (sodium acetate buffer prepared as stated above). (Note that the 19mL of buffer will not fit into Bottle 2, and so this must be done in a separate container.)
    b. Divide into 5 mL aliquots and store frozen in polypropylene tubes between uses. (Stable for 2 years at -20°C)
3. GOPOD Reagent Buffer (Solution 3)
    a. Add the contents of Bottle 3 to a 1 L flask.
    b. Adjust the volume to 1 L with DD water.
4. Glucose Determination Reagent (GOPOD reagent)
    a. Dissolve the contents of Bottle 4 (GOPOD reagent enzymes) in 20 mL of Solution 3.
    b. Quantitatively transfer the entire contents back into the flask containing Solution 3.
    c. Cover with aluminum foil to protect from light.
    d. Divide reagent that will not be used within 3 months into 200 mL aliquots and freeze in polypropylene containers. Do not freeze/thaw more than once. (Stable for ~3 months at 2-5°C or >12 months at -20°C.)
5. Use the contents of Bottle 5 (D-Glucose standard) as supplied. (Stable for >5 years at room temperature.)
6. Bottles 1 and 6 are not needed for determining lactose and glucose content.

Procedure

1. Prepare the reagent blank with 0.4 mL distilled water.
2. Prepare the glucose control (in duplicate) by combining 0.1 mL of the D-glucose standard solution and 0.3 mL of distilled water.

3. Pipette 0.2 mL of the sample solution into six test tubes. Add 0.2 mL of water to three of the test tubes (A). Add 0.2 mL of β-galactosidase to the remaining three test tubes (C). Repeat for all samples to be tested.
4. Vortex all test tubes. Incubate all tubes, including blanks and glucose control, at 50°C for 20 minutes.
5. Add 3.0 mL of GOPOD reagent to all tubes. Vortex all test tubes. Incubate at 50°C for 20 minutes.
6. Vortex all test tubes. Transfer to cuvettes and measure all absorbances at 510 nm against the reagent blank.

Calculation of Glucose and Lactose

1. Conversion from absorbance to µg based on glucose

$$F = \frac{100\ \mu g\ of\ glucose}{absorbance\ for\ 100\ \mu g\ of\ glucose}$$

2. Conversion to % Glucose (w/w)

$$\frac{Absorbance\ of\ C}{0.2\ mL\ (sample\ volume)} \times F\ \frac{\mu g}{absorbance} \times \frac{1\ g}{10^6\ \mu g} \times \frac{mL\ of\ volume}{g\ of\ extrudate\ (original\ concentration)} \times 100$$

3. Conversion to % Lactose (w/w)

$$\frac{Absorbance\ of\ C - Aborbance\ of\ A}{0.2\ mL\ (sample\ volume)} \times F\ \frac{\mu g}{absorbance} \times \frac{1\ g}{10^6\ \mu g} \times \frac{mL\ of\ volume}{g\ of\ extrudate\ (original\ concentration)} \times \frac{342\ g\ lactose}{180\ g\ glucose} \times 100$$

6.1.3 *Color Analysis*

Objective

The purpose of this experiment was to measure the color of the products. The absorbance at 420 nm was used to determine browning and Hunter L, a, and b values were used to determine the color as the eyes perceive it.

Materials and Equipment

- Ground extrudates and processed acid whey
- Reverse osmosis (RO) water
- Volumetric flasks
- Cuvettes
- Spectrophotometer (Shimadzu UV-1800) with UVProbe 2.43 software
- Color Analysis Software (UV/PC version 3.10)

Procedure

1. Prepare sample solutions of each product.
    a. Weight 2.00 grams of sample into a 50 mL volumetric flask.
    b. Dilute to volume with RO water.
    This concentration was chosen to get an absorbance less than 1.5 but close to 1.0 for all samples. Concentrations may need to be adjusted to obtain an absorbance between 0.1 and 1.3, and absorbances adjusted accordingly.
    c. Transfer the solution to a plastic cuvette.
2. Measure the absorbance.
    a. Measure the absorbance of each sample at 420 nm.
    b. Measure the Hunter L, a, b, values.
        i. Measure a spectrum in reflectance mode from 360-700 nm in 1 nm increments at medium speed.
        ii. Convert to Hunter L, a, b, values using color analysis software.

Use a D65 illuminant (standard daylight) and a 10° observer viewing angle.

3. Repeat in triplicate for each sample.

6.1.4 pH

Objective

The purpose of this experiment was to measure the pH of the products and raw mixes to determine how formulation alterations and processing affected pH.

Materials and Equipment

- Ground extrudates and processed acid whey
- pH probe with calibration standards 4.0, 7.0 and 10.0
- Small glass beakers
- 100 mL volumetric flasks
- Reverse osmosis (RO) water
- Glass stir rods

Procedure

1. Calibrate the pH meter with the calibration standards 4.0 and 7.0.
2. Weigh 6.5 g of crushed sample into a small glass beaker, analyzing each sample in triplicate.
3. Add ~40mL of RO water into the small glass beaker containing the sample.
4. Stir the sample and water with a stir rod until well mixed.
   (This may take 5-10 minutes.)
5. Transfer the dissolved sample, quantitatively, to a 100 mL volumetric flask.
6. Make up the volume with RO water. Mix well.
7. Pour a small amount of the sample into a small glass beaker for pH measurement.

8. Determine the pH of the samples. Rinse the electrode with RO water in between measurements and blot dry with a Kim wipe.

6.1.5 *Degree of Polymerization*

Objective

The purpose of this experiment was to examine the extrudates to see if polymerization occurred, and the degree of polymerization. The polymerization between conditions (particle size, feed rate, acid level) were compared to identify differences.

Materials and Equipment

- Ground extrudates
- Double distilled (DD) water
- Ribose
- Disposable plastic syringe
- 0.45 μm filter
- High performance liquid chromatography (HPLC) system with evaporative light scattering detector (ELSD) with 100 μL sample loop
- Sodium form ligand-exchange column with 4% cross linked styrene divinylbenzene Polymer (Transgenomics CHO-411) (7.8 x 300 mm stainless steel)
- Column performance check standard oligosaccharide (Phenomonex ALO-3038)

Sample Preparation

1. Prepare sample solutions of each extrudate
    a. Weight 0.5 g of sample and 0.05 g of ribose into a small beaker.
    b. Dissolve with ~3 mL of DD water.

c. Transfer quantitatively to a 5 mL volumetric flask and dilute to volume with DD water.

2. Microfiltration of the sample
    a. Fit a 0.45 um filter onto a small disposable plastic syringe. Filter ~1.5 mL of the extrudate and ribose solution through the filter into an HPLC vial.

Procedure

1. Prepare the HPLC and ELSD detector.
    a. Set the following conditions:
        - Elution type: isocratic
        - Mobile phase: double distilled water
        - Flow rate: 0.3 mL/min
        - Column temperature: 80 °C
        - Run time: 60 minutes
        - Collection time: 55 minutes
        - Sample injection volume: 10 µL
        - Detection: ELSD (40 °C, 250 kPa, Gain: 5)
2. Allow to equilibrate for 40-60 minutes.
3. Run each sample and record the chromatogram.
4. Run the performance check standard to determine the time that glucose, lactose, and other polymerized sugars will elute.

6.2 Extended Methods for Analysis of Extrusion Products

6.2.1 *Extrusion of Lactose*

Objective

Previous research by the Schoenfuss lab has shown that lactose can be polymerized in a twin screw extruder with a citric acid catalyst. The purpose of

this experiment was to extrude lactose with varying levels of citric acid to observe the effect on the yield of oligosaccharides. The resulting product(s) from this extrusion will be further analyzed for characteristics such as dietary fiber content, moisture, color, etc.

Materials and Equipment

- Lactose, refined edible fine grind
- Lactose, refined edible
- Glucose
- Citric acid
- Scale
- Ribbon blender
- Large plastic bags for collection of prepared mixes
- Large buckets
- Spatula
- Buhler 44mm extruder and screws
- Gloves
- Stainless steel trays
- Stainless steel buckets
- Silicone spray
- Storage containers, glass jars and PP containers
- Spice grinder

Experimental Design

Three formulas of varying citric acid concentrations (2%, 4%, and 6% citric acid w/w) will be tested. There will be 20% w/w glucose in each formula, as it was shown in previous trials to lower the melting temperature and allow the extrusion to run more smoothly. The remaining percentage of each formula will be lactose.

The order of extrusion for the formulas will be randomized. Each formula will be extruded in triplicate over a period of two days.

Procedure

1. Blend the raw mixes.
    a. Weigh the ingredients required for a 30 lb batch into a large bucket and then pour into the ribbon blender. The batch formulas are shown below.

    Make sure that the trap door is closed at the bottom of the ribbon blender.

    Make an extra 10 lbs of formula for the first batch of the day for start-up of the extruder.

Extrusion Batch Formulas

Batch Number	Sample ID	Ingredient amount (lbs)/30 lb batch		
		Lactose	Glucose	Citric acid
1	20G 6CA Extr 1	22.2	6	1.8
2	20G 2CA Extr 1	23.4	6	0.6
3	20G 4CA Extr 1	22.8	6	1.2
4	20G 2CA Extr 2	23.4	6	0.6
5	20G 4CA Extr 2	22.8	6	1.2
6	20G 6CA Extr 2	22.2	6	1.8

    b. Close both the grated cover and the top cover of the ribbon blender when all ingredients are in the ribbon blender.
    c. Mix for 2 minutes in both forward and reverse directions. Press stop and let the blades come to a complete stop before changing directions.

d. Open the top cover of the ribbon blender and continue mixing in the reverse direction (essential for the mixing to occur in order to get the mix out of the ribbon blender).
e. Open a bag and set inside a large bucket—do not fold the top part of the bag down.
f. Slowly open the trap door at the bottom of the ribbon blender and pour the mix into the bag that is sitting in the bucket. To direct mix 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.
g. With the ribbon blender off, use a spatula to scrape the remaining mix into the bag.
h. Collect some of the dry mix and store in a PP container for future analysis.

2. Repeat step 1 for the remaining formulas.
3. Start up the extruder.
    a. Allow the extrusion staff to start up the extruder. This will include pouring the feed into the K-Tron extruder feeder, warming up the barrels, rotating the screws, and slowly increasing the dry feed rate. The dry feed rate is increased to the set feed rate as the barrels warm up and the product begins to melt (the % torque will need to be closely monitored).
4. Collect the product.
    a. Once the product has fully melted and the extruder has reached steady state with regards to the die temperature, die pressure, and motor torque, begin to collect the samples on stainless steel trays. Make sure to wear gloves and be careful because the product is molten hot.

(Use a large stainless steel bucket sprayed with silicone to collect excess waste product during startup and changeover, which will not be collected for analysis.)

b. Record the extrusion operation data on the operation data sheet four times during sample collection, approximately once every two minutes.

c. Allow the product to cool in the stainless steel trays and then break up the product so that it can be temporarily stored.

5. Grind the sample into a fine powder using a spice grinder and store in an airtight glass container at room temperature until further analysis is conducted.

6.2.2 *Moisture by Karl Fisher*

Objective

The purpose of this experiment was to measure the amount of moisture in the extrudates, and starting materials.

Materials and Equipment

- Ground extrudates
- Raw mixes
- Methanol, ACS grade
- Glass vials with caps and septums
- Syringe and rubber stopper
- Karl Fisher apparatus prepared with Karl Fisher reagents
- Analytical balance
- Rotary shaker

Procedure

1. Prepare sample for extraction.
    a. Add 1.00 ± 0.01 grams of sample into a glass vial, record weight.
    b. Add 25.0 ± 0.5 grams of methanol, record weight.
    c. Tightly close vials with caps with septum inserts.
    d. Prepare three blanks with methanol only, no sample.
2. Place all vials, including blanks, on a rotary shaker for 18 hours to extract the moisture from the sample.
    a. Stop the shaker after 18 hours and allow solids to settle for 1 hour.
3. Turn on the Karl Fisher titration machine.
    a. Calibrate the machine, and ready it for use.
4. Measure moisture by Karl Fisher titration.
    a. Record the weight of the empty syringe plus rubber stopper.
    b. Draw up approximately 1.0 mL of extract, without disturbing the sediment.
    c. Quickly stopper the syringe and record the weight of the syringe plus sample plus rubber stopper.
    d. Quickly remove the stopper, insert the syringe into the Karl Fisher vessel, and insert the sample. Start the titration.
    e. Remove the syringe and quickly stopper it. Record the weight of the empty syringe plus rubber stopper.
    f. Record the amount of moisture (R Value in ug) titrated by the Karl Fisher instrument.
5. Titrate the blanks in the same way.

Calculation of Moisture Content

1. Calculate the average MF (mg water per mg methanol) from the blank samples.

$$MF = \frac{\frac{R\ Value\ (ug)\ from\ blanks}{1000}}{mg\ of\ Methanol}$$

2. Calculate the total water in the sample contributed from the both methanol and the sample.

$$Total\ mg\ water\ in\ the\ sample = \frac{R\ Value\ (ug)}{1000} \times \frac{g\ methanol\ in\ vial}{g\ of\ extract\ injected}$$

3. Calculate the water contributed by the methanol.

$$mg\ water\ contributed\ by\ methanol = g\ metanol\ in\ vial \times average\ MF\ from\ blanks$$

Calculate the water content on a weight by weight basis.

$$\%\ Moisture\ (w/w) = \frac{total\ mg\ water\ in\ the\ sample - water\ contributed\ by\ methanol}{initial\ weight\ of\ sample} \times 100$$

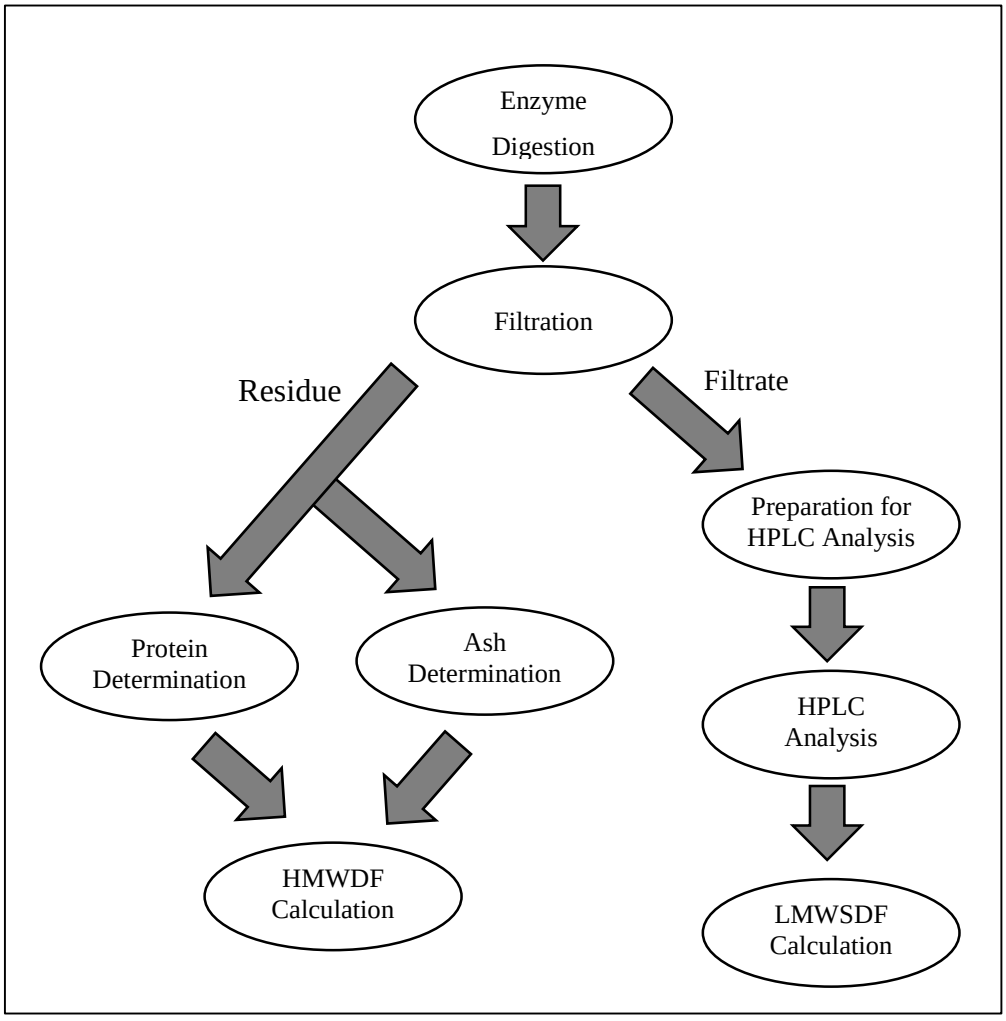
6.2.3 *Dietary Fiber*

Objective

The purpose of this experiment was to measure the amount of dietary in the extrudates. These results will allow us to determine how much dietary fiber was created in each sample during the extrusion process.

Overview of Process

The assay consists of six main steps: enzyme digestion, filtration, low molecular weight soluble dietary fiber (LMWSDF) determination, ash determination, protein determination, and high molecular weight dietary fiber (HMWDF) calculation. The process is shown by the flow diagram below.



Enzyme Digestion

Materials and Equipment

- Powdered extrudates
- Ethanol, 95% v/v
- Megazyme Integrated Total Dietary Fibre Kit (Megazyme, K-INTDF) containing the following:
    - Bottle 1: Concentrated pancreatic α-amylase (E-PANAA) (2 g, 150,000 Ceralpha Units/g).

(Stable for > 5 years stored dry at -20 °C.)

- Bottle 2: Amyloglucosidase (E-AMGDF) (6 mL, 3300 Units/mL).
  (Stable for > 3 years at 4 °C.)
- Bottle 3: Purified protease (E-BSPRT) (10 mL, 350 tyrosine unit/mL).
  (Stable for > 3 years at -20 °C.)

- Maleic acid (Sigma M0375)
- Reverse osmosis (RO) water
- Sodium hydroxide, 4M
- Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)
- Sodium azide (Sigma S8032)
- Volumetric flasks (2 L, 1 L, 500 mL, 100 mL)
- Beakers for mixing
- Trizma base (Sigma T1503)
- Glacial acetic acid
- Sodium azide (Sigma S8032)
- D-ribose (Sigma R7500)
- Analytical balance
- 600 mL beakers
- Aluminum foil
- Shaking water bath for 37 °C and 60 °C incubations
- Non-shaking water bath for 95-100 °C incubation
- Pipettes and tips
- Thermometer
- Positive displacement dispenser

Reagent Preparation

1. Sodium maleate buffer (50 mM, pH 6.0 plus 2mM $CaCl_2$ and 0.02% sodium azide)

   (Stable for > 1 year at 4°C.)

   a. Dissolve 11.6 g of maleic acid in 1600 mL of RO water.
   b. Adjust the pH to 6.0 with 4 M (160 g/L) NaOH.
   c. Add 0.6 g of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$).
   d. Add 0.4 g of sodium azide. Do not add sodium azide until the pH has been adjusted or poisonous gas could be released.
   e. Adjust the volume to 2L.

2. Pancreatic α-amylase/AMG

   (Stable for > 1 year at room temperature.)

   a. Dissolve 0.10 g of purified porcine pancreatic α-amylase in 290 mL of sodium maleate buffer.
   b. Stir for 5 min on a stir plate.
   c. Add 0.3 mL of AMG.
   d. Stir for 1 additional minute
   e. Store on ice if solution is not used immediately.

3. Trizma base

   (Stable for > 1 year at room temperature.)

   a. Dissolve 90.8 g of Trizma base to approximately 800 mL of RO water.
   b. Adjust the volume to 1 L.

4. 2M Acetic Acid

   (Stable for > 1 year at room temperature.)

   a. Add 115 mL of glacial acetic acid to a 1 L volumetric flask.
   b. Dilute to 1 L with RO water.\

Procedure

1. Blanks

a. Run two blanks along with samples to measure any contributions that the reagents add to the protein and ash content of the residue.

2. Weigh samples.
    a. Weigh 1.00 ± 0.05 g of sample, in duplicate, into 600 mL beakers.
    b. Addition of pancreatic α-amylase/AMG enzymes.
    c. Add 1.0 mL of ethanol to each beaker.
    d. Add 40 mL of pancreatic α-amylase/AMG mixture to each beaker.
    e. Cover each beaker with aluminum foil.
3. Incubation with pancreatic α-amylase/AMG
    a. Transfer the covered beakers to a shaking incubation bath.
    b. Incubate the beakers at 37 °C at a motor setting of 4 in a shaking water bath for exactly 16 hours.
    c. Inactivation of α-amylase and AMG
    d. Remove all samples from the shaking water bath and increase the temperature of the bath to 60 °C.
    e. Immediately add 3.0 mL of 0.75 M Trizma base solution to stop the reaction.
    f. Incubate the sample beakers at 95-100 °C in a non-shaking water bath for 20 minutes. Shake by hand every 5 minutes.
    g. Ensure that the final temperature of the sample is > 90 °C. (Checking one sample is adequate.)
4. Cool samples.
    a. Remove all samples from the 95 °C water bath (use gloves).
    b. Cool to approximately 60 °C.
5. Addition and incubation with protease.
    a. Add 0.1 mL of protease solution with a positive displacement dispenser (solutions is viscous).

b. Incubate in a water bath at 60 °C for 30 minutes. If the water bath is not up to temperature, pour some of the 95-100 °C water into the water bath.

6. pH Adjustment
    a. Add 4.0 mL of 2 M acetic acid to each beaker and swirl to mix. (This gives a final pH of approximately 4.3)
    b. Addition of internal standard
    c. Add 0.10 grams of ribose to each beaker, record actual weight, and swirl to mix.
7. Refrigerate if not continuing with the next step right away.
8. Continue with filtration step.

Filtration

Materials and Equipment

- Digested samples in 600 mL beakers from enzyme digestion step
- Non-shaking water bath for 60°C incubation
- Ethanol, 95% v/v
- Muffle oven
- Fritted crucibles: 50 mL, pore size coarse: 40-60 μm
- Celite, acid washed (Sigma)
- 500 mL vacuum filter flasks for each sample plus one for washings
- Acetone
- Drying oven
- Analytical balance
- Vacuum system (pump, rubber tubing)
- Vacuum filter flasks
- Rubber fitting to connect crucibles to the filter flask

Reagent Preparation

1. Crucibles
    a. Ash the crucibles overnight in a muffle oven at 525 °C.
    b. Remove any residual Celite and ash
    c. Soak in a soapy cleaning solution at room temperature for 1 hour.
2. Rinse crucibles with water and reverse osmosis water.
3. Rinse with 15 mL acetone and air dry.
4. Ethanol, 78% v/v
    a. Place 180 mL RO water into a 1-L volumetric flask.
    b. Dilute to volume with 95% v/v ethanol.
    c. Mix.

Procedure

1. Prepare crucibles.
    a. Add approximately 1.0 g of Celite to each dried crucible and dry at 130 °C for one hour (to constant weight).
    b. Cool crucibles in a desiccator for 1 hour and record the mass of the crucible plus Celite.
2. Precipitation of higher molecular weight soluble dietary fiber.
    a. Preheat the sample to 60 °C using a waterbath.
    b. Add 192 mL of 95% (v/v) EtOH which has been preheated to 60 °C.
    c. Mix thoroughly by swirling and allow precipitate to form at room temperature for 60 minutes.
3. Filtration.
    a. Use 15 mL of 78% EtOH, wet and redistribute the bed of Celite in the crucible.
    b. Apply suction to the crucible to draw the Celite onto the fritted glass in an even layer.

c. Using a vacuum, filter the precipitated enzyme digest through the prepared crucible and Celite.
d. Quantitatively transfer all remaining particles with 15 mL of 78% (v/v) EtOH.
e. Retain this filtrate for further analysis.

4. Using a vacuum, wash the residue with the following solutions, in order.
    a. Two quantities of 15 mL of 78% (v/v) EtOH
    b. Two quantities of 15 mL of 95% (v/v) EtOH
    c. One quantity of 15 mL of acetone
    d. This is waste and does not need to be retained.
5. Dry the crucibles.
    a. Dry the crucibles overnight in a 105 °C oven.
    b. Cool the crucibles in a desiccator for approximately 2 hour.
6. Weigh the residue.
    a. Weigh the dried crucible containing the dietary fiber residue and Celite.
    b. Subtract the crucible and Celite weight to determine the weight of the residue.
7. Continue on to the LMWSDF step with the filtrate. To determine HMWDF, continue on with the residue to the protein and ash determination steps. Note that one crucible with residue will be used for ash determination and the other crucible with residue will be used for protein determination.

Preparation for HPLC Analysis

Materials and Equipment

- Filtrate obtained from previous filtration step
- Rotary evaporator
- Evaporator flasks (500 mL and 250 mL)

- Pipettes
- Double distilled water
- Amberlite FPA 53 (OH^-) ion exchange resin (Megazyme)
- Ambersep 200 (H^+) ion exchange resin (Megazyme)
- Polypropylene disposable column with one-way stopcock (Bio-Rad)
- Vacuum manifold block with pump
- Test tubes
- Analytical balance
- 5 mL disposable syringe
- 0.45 μm syringe filter
- HPLC vial

Procedure

1. Filtrate evaporation.
    a. Transfer about half of the filtrate to a 500 mL evaporator flask.
    b. Evaporate to dryness under a vacuum at 60 °C.
    c. Add 5 mL of double distilled water to the evaporator flask and swirl the flask for about 2 minutes to fully dissolve it.
2. Preparation of the deionization system.
    a. Mix together approximately 4 g of Amberlite FPA 53 (OH^-) and 4 g of Ambersep 200 (H^+).
    b. Place the Amberlite/Ambersep mixture into a Bio-Rad disposable column and place into a glass vacuum manifold block. Place a test tube under each column to catch the washings. Adjust the valves of the vacuum manifold so that they are partially open.
    c. Wash each column with 20 mL of double distilled water and use a vacuum to ensure that all water has eluted. After washing, replace test tubes with clean test tubes to catch filtrate.
3. Deionization of the sample.

a. Adjust the valves of the vacuum manifold so that they are closed.
b. Transfer 2 mL of the reconstituted filtrate to the top of the prepared Bio-Rad disposable column.
c. Add 2 mL of double distilled water to the column and allow to permeate.
d. Adjust the valves of the vacuum manifold so that a slow drip (approximately 1.0 mL per minute) is achieved.
e. Add 20 mL of double distilled water to the top of the column and elute (approximately 1.0 mL per minute).
f. Transfer the filtrate to a 250 mL evaporator flask.

4. Evaporation of deionized sample.
    a. Evaporate to dryness under a vacuum at 60 °C.
    b. Add 2 mL of double distilled water to the evaporator flask and swirl the flask for about 2 minutes to fully dissolve it.
5. Microfiltration of the deionized sample.
    a. Transfer the sample to a 5 mL disposable syringe.
    b. Filter through a 0.45 μm filter into an HPLC vial.

HPLC Analysis

Materials and Equipment

- Microfiltered sample in HPLC vial from previous filtration step
- Glucose
- Ribose
- 100 mL volumetric flasks
- Double distilled (DD) water
- Disposable plastic pipettes
- HPLC vials and caps
- High performance liquid chromatography (HPLC) system with evaporative light scattering detector (ELSD) with 100 μL sample loop

- Sodium form ligand-exchange column with 4% cross linked styrene divinylbenzene Polymer (Transgenomics CHO-411) (7.8 x 300 mm stainless steel)
- Column performance check standard oligosaccharide (Phenomonex ALO-3038)

Reagent Preparation

1. Glucose/Ribose Response Factor Solutions
    a. Weigh 0.1, 0.2 and 0.3 grams of glucose into three separate 100 mL volumetric flasks.
    b. Weigh 0.2 grams of ribose into each flask.
    c. Dilute to volume with DD water.
    d. Transfer ~1.5 mL of each solution to separate labeled HPLC vials using plastic pipettes.

Procedure

1. Prepare the HPLC and ELSD detector
    a. Set the following conditions
        - Elution type: isocratic
        - Mobile phase: double distilled water
        - Flow rate: 0.3 mL/min
        - Column temperature: 80 °C
        - Run time: 60 minutes
        - Collection time: 55 minutes
        - Sample injection volume: 10 µL
        - Detection: ELSD (40 °C and 250 kPa)
    b. Allow to equilibrate for 40-60 minutes.
2. Run the performance check standard to determine the demarcation point between a degree of polymerization of two and three.

3. Run samples.
4. Run each extrudate sample and record the chromatogram.
    a. Determine peak areas of all peaks with a degree of polymerization of greater than three.
5. Run each response factor solution and record the chromatogram.
    a. Determine peak areas.

Calculation of LMWSDF

1. Determine response factor for glucose and ribose.
    a. Plot the ratio of peak area of D-Glucose/peak area of D-ribose (y-axis) vs. the ratio of the mass of D-glucose/mass of D-ribose (x-axis).
    b. Determine a best fit line using linear regression.
    c. The reciprocal of the slope of that line is the response factor.
2. Calculate LMWSDF (mg/100g)

$$LMWSDF\ \left(\frac{mg}{100g}\right) = Response\ Factor \times \frac{Peak\ Area\ of\ LMWSDF}{Peak\ Area\ of\ Ribose} \times \frac{weight\ of\ ribose\ (mg)}{weight\ of\ original\ sample\ (g)} \times 100\ g$$

3. Calculate LMWSDF %

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

Ash Determination

Materials and Equipment

1. Crucible containing residue from filtration step
2. Muffle oven
3. Analytical balance

Procedure

1. Ash the crucible with Celite and residue.
    - Ash the crucibles at 525 °C in a muffle oven for 6 hours. Allow to cool before removing from the oven.
2. Cool the crucibles to room temperature in a desiccator.
3. Weigh the crucible plus residue.
4. Subtract the weight of the crucible and Celite to determine ash content.

Protein Determination

Materials and Equipment

- Crucibles with residue from previous filtration step
- Weigh paper
- Kjeldahl digestion block and flasks (Buchi)
- Kjeldahl tablets
- 95-98% sulfuric acid
- Reverse osmosis (RO) water
- Sodium hydroxide
- 4% boric acid
- 250 mL Erlenmeyer flasks
    - N HCl
- Methyl red
- Buret

Reagent Preparation

Prepare a 32% sodium hydroxide solution using a volumetric flask, RO water, and sodium hydroxide pellets.

Procedure

1. Transfer the residue.

    a. Transfer the residue as quantitatively as possible with a metal spatula to weigh paper.
    b. Fold the weight paper around the sample and put into a labeled weigh boat until the protein analysis is performed.
2. Prepare flasks.
    a. Add samples (and weigh paper) to Kjeldahl flasks.
    b. Along with the samples, run 3 blanks with weigh paper only, and 3 positive controls with something like whey protein to ensure the method works.
    c. There will be at least one blank from the dietary fiber procedure.
3. Perform the digestion.
    a. Place one Kjeldahl tablet and 10 mL of 95-98% sulfuric acid into each flask.
    b. Digest at 420 °C for 90 minutes.
    c. Allow to cool overnight.
    d. Add 50 mL of RO water.
4. Perform the distillation.
    a. Prepare a 250 mL Erlenmeyer flask for each sample with 50 mL of boric acid and two drops of methyl red.
    b. Using the Buchi distillation unit, transfer 150 mL of sodium hydroxide to the flask with the digested sample.
    c. Distill the sample into the receiving flask using steam and the Buchi distillation unit.

       Note: Make sure that you have enough sodium hydroxide and water for your sample before you start.
5. Titrate the solution.
    a. Titrate the blank with HCl to a pink endpoint.
    b. Titrate all samples with HCl to the same pink endpoint.
6. Calculate protein content.

a. Convert amount of HCl to amount of nitrogen, and then convert to protein using a 6.25 factor.

Calculation of HMWDF

1. Determine the correction for the blank.

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

2. Calculate HWMDF (mg/100g)

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

3. Calculate HMWDF %

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

6.3 Extended Methods for Acid Whey

6.3.1 *Yogurt Making*

Objective

The purpose of this experiment is to produce acid whey which can be used for further analysis and experimentation. Plain yogurt is produced, and then centrifuged to separate out excess acid whey to create Greek yogurt. The resulting acid whey is treated and condensed. Further experiments, such as washing and drum drying are performed with the concentrated acid whey.

Materials and Equipment

34 kg of Grade A whole milk

16 grams of YFL702 culture from Danisco

pH meter

Milk cans

Can pasteurizer

Desludging centrifuge, Westfalia SB7

Glass evaporator

6” twin drum dryer, Buflovak

Beckman Coulter Avanti J-E centrifuge with JA-14 rotor

250 mL centrifuge tubes

Reverse osmosis (RO) water

Procedure

Yogurt Make

1. Heat 34 kg of milk in a milk can to 82°C in a can pasteurizer.
    a. Hold the milk at 82°C for 30 minutes, and then immediately cool to 43°C.
2. Add 16 grams of YFL702 Danisco culture.
3. Monitor the pH of the yogurt until it drops to 4.60, and then refrigerate overnight.

Acid Whey

1. Allow the yogurt to warm up to room temperature.
2. Run the yogurt through a desludging centrifuge
    a. Retain the supernatant (acid whey).
3. Evaporate water out of the acid whey using a glass evaporator. Follow step 3a for one batch of whey and step 3b for a second batch of whey.
    a. Concentrate the whey 10-12x or until approximately 1 liter is obtained.

OR

b. Pasteurize the acid whey using the can pasteurizer to heat the whey to 82°C for 30 minutes.
    i. Evaporate water out of the acid whey using a glass evaporator. Concentrate the whey 10-12x or until approximately 1 liter is obtained.

4. Store the whey either frozen or refrigerated until further analysis is conducted.

Acid Removal

1. Divide the condensed acid whey into three portions.
    a. The first portion will be left untreated and should be the smallest portion, use approximately one fifth of the total.
    b. The second portion will be washed once, and so you will lose some product during washing. Use approximately two fifths of the total.
    c. The third portion will be washed twice and should be the largest portion since you will lose a lot during the washing product. Use approximately two fifths of your condensed whey.
2. Leave the first portion untreated.
3. Wash the second portion once.
    a. Use a 2:1 ratio (by weight) of product to RO water and mix thoroughly in a large glass beaker.
    b. Divide the mixture evenly into 6 250 mL centrifuge tubes.
    c. Centrifuge at 1000xg for 3 minutes at 23°C.
    d. Decant off the supernatant and retain the precipitate.
4. Wash the third portion twice.
    a. Use a 2:1 ratio (by weight) of product to RO water and mix thoroughly in a large glass beaker.
    b. Divide the mixture evenly into 6 250 mL centrifuge tubes.

c. Centrifuge at 1000xg for 3 minutes at 23°C.
d. Decant off the supernatant and retain the precipitate.
e. Repeat steps 4a to 4d.

Drum Drying

1. Assemble and warm up the 6" twin drum dryer.
    a. Scrape off any oil or residual product.
    b. The drums should be at approximately 250°C
2. Run a slurry of maltodextrin and water through the drum dryer for approximately 5 minutes to clean off any previous residue.
    a. Allow all maltodextrin to run through, wipe off drums, and empty the sample collection plate.
3. Run the washed twice product through the drum dryer first.
    a. Collect sample.
4. Run the washed once product through the drum dryer second.
    a. Collect sample.
5. Run the not washed produce through the drum dryer last, as it will be much stickier and harder to clean.
    a. Periodically scrape the blades to clean off the product and prevent overheating and uneven heating.

6.3.2 *Protein by Dumas*

Objective

The purpose of this experiment was to measure the amount of protein in the various fractions of acid whey. These results will allow us to determine the protein content after production, condensing, and various clean up processes.

Materials and Equipment

- Tin foil weigh paper (Elementar, Mt. Laurel, NJ, USA)

- Dumas protein analysis system (Buchi DuMaster D-480, Buchi, Flawil, Switzerland)
- Drum dried acid whey product, ground by mortar and pestle
- Concentrated acid whey
- L-Aspartic Acid, reagent grade (Sigma, St. Louis, MO, USA)

Procedure

1. Start up and calibrate the Buchi Dumas system by running two blanks and two standards of 200 mg acid.
2. Weight out approximately 250 mg of sample, in duplicate, into the tin weigh paper.
3. Run the samples on the Dumas system and record the nitrogen percentage.
4. Use a 6.25 protein factor to convert nitrogen to protein.

6.3.3 *Protein by BCA*

Objective

The purpose of this experiment was to measure the amount of protein in the acid whey.

Materials and Equipment

- Pierce Bicinchoninic Acid (BCA) Protein Assay Kit containing the following:
    - BCA Reagent A: sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide
    - BCA Reagent B: 4% cupric sulfate
    - Albumin standard ampules: 1 mL ampules containing bovine serum albumin (BSA) at 2mg/mL in 0.9% saline and 0.05% sodium azide
- Acid whey samples
- 2 mL vials

- 5 mL test tubes with caps
- 2 mL micro-cuvettes
- Vortexer
- Graduated cylinder
- Large glass bottle with cap
- Water bath at 60°C
- Pipetter and tips

Sample and Reagent Preparation

1. Sample preparation.
    a. Prepare sample solutions of each sample using reverse osmosis water so that the concentration is between 5 and 250 μg/mL. (Absorbance readings after incubation should be between 0.1 and 1.5, adjust concentration as necessary. The condensed whey before drying use 0.10 g/250 mL and condensed whey after drying use 0.05 g/250 mL.)

2. Standard curve preparation.
    a. Prepare the standard solutions in small 2 mL vials using the dilution scheme below.

Vial	Volume of Water (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	1400	200 of stock	250
B	800	800 of vial A dilution	125
C	900	600 of Vial B dilution	50
D	800	800 of Vial C dilution	25
E	800	200 of Vial D dilution	5
F	800	None	0

3. Prepare working reagent.
    a. Mix 50 parts BCA Reagent A with 1 part BCA Reagent B, ex: 50 mL Reagent A with 1 mL Reagent B.
    Each sample and standard will require 2.0 mL of working reagent per replicate.
    b. Prepare enough buffer for all samples and standards to be tested in each run of the assay.

Procedure

1. Pipette 0.1 mL of each standard and unknown sample replicate into a labeled test tube. Measure the samples and standards in triplicate.
2. Add 2.0 mL of the working reagent to each tube and vortex.
3. Cover and incubate tubes at 60°C for 30 minutes.
4. Allow all tubes to cool to room temperature.
5. Transfer the solution to micro-cuvettes and read on a spectrophotometer at 562 nm using water as the blank to zero the spectrophotometer. Read all samples within 10 minutes to avoid error from excess color change.

Calculation of Protein Content

1. Subtract the average 562 nm absorbance measurement of the blank standard replicates from the 562 absorbance measurement of each standard and unknown sample readings.
2. Plot the average blank corrected 562 nm measurements for each BSA standard vs. its concentration in μg/mL and obtain a standard curve by linear regression. Use the standard curve to calculate the concentration of protein in each unknown sample.

6.3.4 *Moisture by Gravimetric*

Objective

The purpose of this experiment was to measure the amount of moisture in the drum dried acid whey, and starting materials.

Materials and Equipment

- Drum dried acid whey product, ground by mortar and pestle
- Concentrated acid whey
- Atmospheric oven (100°C)
- 65 mm diameter fluted aluminum pans
- Vacuum oven (65°C)
- Desiccator with absorbent material such as Dri-Rite
- Analytical balance

Procedure

1. Preheat atmospheric oven to 100°C.
    a. Label aluminum pans with sample identification
    b. Dry aluminum weighing dishes in the oven for one hour.
    c. Place pans into a desiccator to cool to room temperature.

2. Weigh 1.0-1.5 grams of sample into each pan, analyzing in triplicate.
    a. Record the weight of the empty pan and the weight of the sample.
3. Pre-dry the concentrated acid whey samples
    a. Pre-heat a water bath to 95°C
    b. Cover a cookie sheet with tinfoil
    c. Place the pans with sample onto the cookie sheet and place on top of the water bath.
    d. Let dry for approximately 30 minutes.
4. Pre-heat vacuum oven to 65°C
    a. Place aluminum dishes with sample into vacuum oven.
    b. Dry to constant weight at a pressure of -86 kPa to -96 kPa.
    c. Admit dried air into the oven during drying at about 120 mL/min dried by passing through a solid desiccant.
    d. Allow samples to dry for 16 hours.
5. Stop the vacuum pump and turn off the oven.
    a. Slowly allow dried air into the oven to release the vacuum.
6. Remove the pans with sample from the oven and place into a desiccator.
    a. Allow to cool to room temperature.
    b. Record final weight of pan plus sample.

Calculation of Moisture Content

$$\%\ Moisture\ (w/w) = \frac{initial\ weight\ of\ sample - weight\ of\ dried\ sample}{initial\ weight\ of\ sample} \times 100$$

6.3.5 *Ash*

Objective

The purpose of this experiment was to measure the amount of ash, or mineral content, in the drum dried acid whey.

Materials and Equipment

- Drum dried acid whey product, ground by mortar and pestle
- Muffle furnace (550°C)
- Ashing crucibles
- Desiccator with absorbent material such as Dri-Rite
- Analytical balance

Procedure

1. Pre-ash crucibles.
    a. Ash in a muffle furnace at 550°C for 12 hours.
    b. Allow muffle furnace to cool to below 100°C before removing crucibles.
    c. Place crucibles into a desiccator to cool to room temperature.
2. Weigh 1.0-1.5 grams of sample into each crucible, analyzing in triplicate.
    a. Record the weight of the empty crucible and the weight of the sample.
3. Ash crucibles with sample.
    a. Ash in a muffle furnace at 550°C for 12 hours.
    b. Allow muffle furnace to cool to below 100°C before removing crucibles.
    c. Place crucibles into a desiccator to cool to room temperature.
4. Record final weight of crucible plus sample ash.

Calculation of Ash Content

$$\%\ Ash(w/w) = \frac{weight\ of\ ash}{initial\ weight\ of\ sample} \times 100$$

6.3.6 *Fat by Mojonnier*

Objective

The purpose of this experiment was to measure the amount of fat in the drum dried acid whey.

Materials and Equipment

- Drum dried acid whey product, ground by mortar and pestle
- 65 mm diameter fluted aluminum pans
- Desiccator with absorbent material such as Dri-Rite
- Mojonnier-type fat extraction flasks
- Corks stoppers for Mojonnier flasks
- Reverse osmosis (RO) water
- Ammonium hydroxide, concentrated
- Phenolphthalein indicator
- Ethanol, 95%
- Diethyl ether, certified ACS, BHT stabilized
- Petroleum ether
- Fume hood

Procedure

1. Preheat atmospheric oven to 100°C.
    a. Label aluminum pans with sample identification
    b. Dry aluminum weighing dishes in the oven for one hour.
    c. Place pans into a desiccator to cool to room temperature.
    d. Record the weight of each dried pan.
2. Weigh 1.00 gram of solid sample into the lower bulb of a Mojonnier flask or 10.00 grams of liquid sample, analyzing in triplicate.
    a. Add 9-mL of RO water into each flask.

b. Mix the sample with the water and make sure there are no dry spots in the bottom of the bulb.
c. If the sample is liquid, weigh 10 grams of sample into the lower bulb of a Mojonnier flask, and do not add any additional water.

3. Prepare two blanks.
    a. Add 9-mL of RO water into each flask, and no sample.
4. Add 1.5 mL ammonium hydroxide to each prepared Mojonnier flask and mix thoroughly by rocking the flask.
5. Add 3 drops of phenolphthalein indicator to help to indicate the interface between aqueous and ether layers.
6. Perform the first extraction.
    a. Add 10 mL of ethanol. Stopper the flasks and mix by rocking the flask for 15 seconds. Periodically release pressure by loosening the cork as necessary.
    b. Add 25 mL of ethyl ether. Stopper the flasks and mix by rocking the flask for 1 minute. Periodically release pressure by loosening the cork as necessary.
    c. Add 25 mL of petroleum ether. Stopper the flasks and mix by rocking the flask for 1 minute.
    d. Place the flasks into the Mojonnier centrifuge. Turn the crank 15 times and then let go of the handle and allow the machine to stop on its own. There should be a clean separation of aqueous and ether phases. If not, centrifuge again.
    e. Decant the supernatant (ether layer) into the pre-dried weighing pan.
        i. Be careful not to pour any solids or any of the aqueous phase into the pan.

ii. The pan will only fit about half of the ether layer at a time. Pour in half of the ether layer, let it evaporate, and then pour in the second half.

iii. Evaporate the ether from the pans on the hot plate of the instrument while conducting the second extraction.

7. Perform the second extraction.
    a. Add 5 mL of ethanol. Stopper the flasks and mix by rocking the flask for 15 seconds. Periodically release pressure by loosening the cork as necessary.
    b. Add 15 mL of ethyl ether. Make sure to rinse the neck of the flask when adding the ether. Stopper the flasks and mix by rocking the flask for 1 minute. Periodically release pressure by loosening the cork as necessary.
    c. Add 15 mL of petroleum ether. Make sure to rinse the neck of the flask when adding the ether. Stopper the flasks and mix by rocking the flask for 1 minute.
    d. Place the flasks into the Mojonnier centrifuge. Turn the crank 15 times and then let go of the handle and allow the machine to stop on its own. There should be a clean separation of aqueous and ether phases. If not, centrifuge again.
        i. If the interface of the aqueous and ether layers is below the neck of the flask after resting, add water to bring the level approximately halfway up the neck, then centrifuge again.
    e. Decant the supernatant (ether layer) into the pre-dried weighing pan.
        i. Be careful not to pour any solids or any of the aqueous phase into the pan.

ii. The pan will only fit about half of the ether layer at a time. Pour in half of the ether layer, let it evaporate, and then pour in the second half.

f. Evaporate the ether from the pans on the hot plate of the instrument while conducting the third extraction.

8. Perform the third extraction.
    a. Add 15 mL of ethyl ether. Make sure to rinse the neck of the flask when adding the ether. Stopper the flasks and mix by rocking the flask for 1 minute. Periodically release pressure by loosening the cork as necessary.
    b. Add 15 mL of petroleum ether. Make sure to rinse the neck of the flask when adding the ether. Stopper the flasks and mix by rocking the flask for 1 minute.
    c. Place the flasks into the Mojonnier centrifuge. Turn the crank 15 times and then let go of the handle and allow the machine to stop on its own. There should be a clean separation of aqueous and ether phases. If not, centrifuge again.
        i. If the interface of the aqueous and ether layers is below the neck of the flask after resting, add water to bring the level approximately halfway up the neck, then centrifuge again.
        ii. If there is not clear separation between the phases, let the sample sit for 20-30 minutes and then centrifuge again.
    d. Decant the supernatant (ether layer) into the pre-dried weighing pan.
        i. Be careful not to pour any solids or any of the aqueous phase into the pan.
        ii. The pan will only fit about half of the ether layer at a time. Pour in half of the ether layer, let it evaporate, and then pour in the second half.

e. Evaporate the ether from the pans on the hot plate of the instrument or under a hood.

9. Dry the pans with extracted fat in an atmospheric oven at 100°C for 2 hours.
    a. Place pans into a desiccator to cool to room temperature.
    b. Record the weight of each dried pan with fat, and the blank pans with residue.

Calculation of Fat Content

$$\%\ Fat(w/w) = \frac{M_1 - M_2 - B}{M_0} \times 100$$

M_1 = initial weight of sample plus dish

M_2 = final weight of extracted fat plus dish

M_0 = initial weight of sample

B = average mass of residue in blanks