Investigation of Purification of Oligosaccharides Produced Using Twin-Screw Extrusion

A THESIS

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Dedication

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To my three brothers, Adel, Danial and Fazel who practiced teamwork with me since I was born, for their support and putting a smile on my face even on the toughest of days.

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Abstract

Lactose, a low value dairy product, was polymerized to a soluble dietary fiber called polylactose using twin-screw extrusion. Non- enzymatic browning reactions occurring during extrusion produce colored compounds and hydroxymethylfurfural. This research focuses on development and optimization of a purification method for polylactose using activated carbon and resins. Polylactose was ground, solubilized in water at three different concentrations (50 mg/mL, 100 mg/mL and 150 mg/mL). Forty mL of polylactose solution was flowed through the filtration system utilizing gravity. The filtration system was rinsed with 20 mL double distilled water. The rinse water plus the filtrate collected were freeze dried to achieve a powder form suitable for different analysis.

42.4% Purolite A874 resin followed by 42.4% Activated carbon and 3% diatomaceous earth was proven to have the best purification results increasing dietary fiber content from 54.48% to 92.24% while reducing the hydroxymethylfurfural content by 90.91% to a level that was lower than the maximum level determined by the Joint FAO/WHO Expert Committee on Food Additives HMF limit in polydextrose (0.1%). Furthermore, the effect of polylactose solution concentration for filtering 40 mL polylactose solution was determined. Using 50 mg/mL concentration achieved the highest dietary fiber content. This could be since less polylactose had to interact with the same amount of filtration material resulting in removing more impurities and caramelization products. However, the hydroxymethylfurfural content reduction was the same for 50 mg/mL and 100 mg/mL concentrations.

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1.Introduction

Lactose is a component of whey and a by-product of cheese production. The demand for cheese continues to increase, causing a proportional increase in the production of whey and lactose (Chambers and Ferretti, 1979). Utilizing the produced lactose in order to avoid economic and environmental issues is crucial. Previous work performed by Tremaine, et al. showed that lactose can be polymerized into an indigestible ingredient called polylactose using reactive extrusion (Tremaine et al., 2014).

Public awareness of nutrition and reviewing the nutritional labeling of food products has increased significantly (Cowburn and Stockley, 2005). This awareness is followed by more demand for foods with demonstrable health benefits (Borra and Bouchoux, 2009).

Prebiotic non-digestible carbohydrates are recognized as functional foods. Functional foods refer to those similar in appearance to conventional foods but have demonstrated physiological benefits (Loo et al., 1999). Polylactose could fit the definition of dietary fiber and be classified as prebiotic (FDA 2020). Abernathy et al. determined polylactose fermentability and the effect on the microbiome (Abernathy et al., 2019). They confirmed that polylactose is highly fermentable and provided a beneficial change in the gut microbiome.

To ensure maximum nutritional benefits, polylactose composition was analyzed for an artifact of the heating process hydroxymethylfurfural (HMF). High levels of HMF in food products could impose health risks on the consumer although it naturally exists in some products like honey (Monien et al., 2009). This highlights the importance of filtration processes used in the food industry to purify products and limit detrimental compounds.

Adsorption of undesirable compounds using activated charcoal and resins is utilized for purification processes in the food industry (Carabasa et al., 1998). The potential to produce low-cost activated carbon from agriculture by-products such as cane trash, bagasse, corn cobs and seed shells makes activated carbon a proper option for filtration material (Ahmedna et al., 1997). Furthermore, exhausted activated carbon and

resins can be regenerated and used again in the process which can eliminate waste (Aljohani et al., 2018).

There were three objectives for this project. The first was to produce and purify polylactose for an animal trial using the method developed by Kuechel (Kuechel, 2017). The second objective was to develop and optimize a filtration method to purify polylactose and reduce the HMF content while maintaining dietary fiber content. The third objective was to evaluate polylactose dietary fiber content, and changes to the DP2 peak (which was assumed to be lactose) during lactase treatment. The goal of the third objective was to understand the potential effect of human digestion on the fiber quantity and to determine if the DP2 peak contains only lactose or non-digestible disaccharides.

2. Literature Review

2.1 Dietary fiber

2.1.1 Definition and labeling requirements

The United States federal government has emphasized the education of the public about the potential health benefits of diets high in dietary fiber, and many organizations have developed programs to educate consumers (Guthrie et al., 1992).

In 2016, the FDA changed the definition of dietary fiber and announced seven isolated or synthetic non-digestible carbohydrates as meeting the definition. FDA defines dietary fiber that can be declared on the Nutrition and Supplement facts label as certain naturally occurring fibers that are "intrinsic and intact" in plants and added isolated or synthetic non-digestible soluble and insoluble carbohydrates that FDA has determined have beneficial physiological effects to human health (FDA, 2020). These effects include lowering blood glucose and cholesterol levels, reduced calorie intake, increasing the frequency of bowel movements, increased mineral absorption in the intestinal tract, and reduced energy intake (for example, due to the fiber promoting a feeling of fullness). The Nutrition Facts Label final rule defines "dietary fiber," as "non-digestible soluble and insoluble carbohydrates (with 3 or more monomeric units), and lignin that are intrinsic and intact in plants; isolated or synthetic non-digestible carbohydrates (with 3 or more monomeric units) determined by FDA to have physiological effects that are beneficial to human health."(FDA, 2020). In addition to intact and intrinsic fibers, FDA identified the following isolated or synthetic non-digestible carbohydrates as meeting the dietary fiber definition: Beta-glucan soluble fiber, Psyllium husk, Cellulose, Guar gum, Pectin, Locust bean gum, Hydroxypropylmethylcellulose (FDA, 2020).

In 2018, FDA approved eight additional non-digestible carbohydrates to meet the definition of dietary fiber. The eight additional dietary fibers include mixed plant cell wall fibers (a broad category that includes fibers like sugar cane fiber and apple fiber, among many others), arabinoxylan, alginate, inulin, and inulin-type fructans, high amylose starch (resistant starch 2), galactooligosaccharide, polydextrose, and resistant maltodextrin/dextrin (FDA, 2018a). Petitions can be submitted to FDA under 21 CFR

10.30 requesting an amendment to the list of isolated or synthetic non-digestible carbohydrates that meet the definition of dietary fiber (FDA, 2018b). This petition will include requested action, statement of grounds, environmental and economic impact, and certification of truth and legitimacy of the information provided (21CFR10.30, 2019). FDA evaluates the scientific evidence based on data and information provided by citizen petition, the publicly available scientific literature, comments received in response to the notice, and comments received in response to the 2016 science review (FDA, 2016). When physiological effects of proposed dietary fiber to human health are determined FDA will recognize it as dietary fiber and therefore it could be mentioned on the nutritional labeling.

2.2 Soluble dietary fibers

2.2.1 Galactooligosaccharides

2.2.1.1 GOS synthesis

GOS are non-digestible carbohydrates with the potential to modulate intestinal microbiota. GOS are mainly constituted by galactose units and obtained from lactose by the action of ß-Galactosidase. Normally β-glycosidases hydrolyze substrates formed by a monosaccharide attached to another polyol by a β bond. This β bond is more commonly in form of β1’4 and β1’6 rather than β1’3 and β1’2 (Otieno, 2010). For example, β-glycosidases could hydrolyze lactose to galactose and glucose. However, β-glycosidases could also catalyze transgalactosylation reactions (Petzelbauer et al., 2000). Two main reactions catalyzed by ß-galactosidase activity on lactose are hydrolysis and transgalactosylation (Gonawan, 2019). Transgalactosylation occurs when instead of just hydrolyzing lactose, β-galactosidase transfers the galactose unit to another carbohydrate to result in oligosaccharides with a higher degree of polymerization (DP) (Kim et al., 2004). Transgalactosylation is affected by temperature, pH, enzyme origin, and substrate concentration (Vera et al., 2011).

The primary studies with ß-Galactosidase suggest at least three steps are involved:

1) enzyme + lactose -> enzyme-lactose
2) enzyme-lactose-> galactosyl-enzyme + glucose
3) galactosyl-enzyme + acceptor -> galactosyl- acceptor + enzyme

If the acceptor is water, free galactose is formed by hydrolysis. On the other hand, if the acceptor is a sugar, the result is galactooligosaccharides (Mahoney, 1998).

According to the Food and Agriculture Organization (FAO) and World Health Organization (WHO), probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Nutrition Division, 2009). Lactic bacteria (LAB), bifidobacteria, certain yeasts, and bacilli are the most common types of microbes used as probiotics. Probiotics are consumed as part of fermented foods or dietary supplements. When live microorganisms are used as enzyme sources for GOS synthesis, they could also provide the double advantage of providing probiotics as well as prebiotics (Otieno, 2010). ß -Galactosidase, also known as lactase, is isolated from different sources and the activity of the enzyme varies with the source. See Table (1) for examples of how GOS varies with source and production process.

2.6 β- Galactosidase

β-galactosidase has two main applications in the food industry: hydrolysis of lactose to glucose and galactose for lactose-intolerant people, and the production of GOS (Husain, 2010). GOS produced using β-galactosidase through transgalactosylation pathway contains oligomers ranging from the degree of polymerization (DP) 2 to 10 (Coulier et al. 2009). β-Galactosidase catalyzes the hydrolysis of β(1-4) glycosidic linkage of β- galactosides which are carbohydrates containing galactose β linked components like lactose and lactulose (Gonawan, 2019).

β-galactosidase is found in microorganisms including bacteria, fungi, and yeast or plants (Nagy et al., 2001). ß-galactosidase, also known as lactase, has been isolated from different sources. *Kluyveromyces lactis, Bacillus circulans, Aspeergillus oryzae, Bifidobacterium bifidum,* and *Sporobolomyces singularis* are among enzyme sources utilized to produce β-Galactosidase enzyme used in industrial production of GOS (Table

1). Using different enzyme sources by manufacturers results in GOS with different degrees of polymerization ranging from DP2 to DP10, branching, and degree of purity that could be used in various products (Coulier et al., 2009). In some cases, more than one type of ß-Galactosidase could be used. See table (1) for a summary of GRAS notices approved and the enzyme source used.

Transgalactosylation is affected by temperature, pH, enzyme origin, and substrate concentration (Vera et al., 2011). Vera et al. report that galactose has a stronger competitive inhibitor role in transgalactosylation than in hydrolysis. They also report that glucose at low concentrations has an activating effect on the reaction of transgalactosylation. At high concentrations (above 200 mmole kg^{-1}) this will change to an inhibitory effect. As described by Vera et al. (2011) the transgalactosylation activity increases with temperatures of 40 to 55C. The temperature has the same effect on hydrolytic activity for this enzyme. Furthermore, pH has a strong effect on ß-Galactosidase kinetics as catalysis with this enzyme is mainly acid catalysis (Prenosil et al., 1987). Vera et al. also reports pH between 2.5-5.5 as the optimum range for transgalactosylation activity of ß-Galactosidase

Table (1) – Summary of GRAS notices for different galactooligosaccharides produced using ß-Galactosidase enzymes

Company Name and Country	GRAS Notice Number	Product Commercial Name	ß-Galactosidase Source	GOS Purity
Neo Cremer, South Korea	729	'Nature's GOS; Mother's/Beauty oligo; Goat Oligo-CL	B. circulans (but further ferments with Saccharomyces cerevisiae to remove sugar)	30- 75%
Vitalus, Canada	671, 721	Vitagos	Aspergillus oryzae and Kluyveromyces lactis	>62%
Nestle Nutrition, USA	620	Nestle GOS	Aspergillus oryzae	46%
New Francisco Biotech Corp, China	518, 569	King-Prebiotics GOS	B. circulans (but further ferments with Kluyveromyces lactis to remove sugar)	27- 99%
Clasado, USA	484, 495	Clasado, Bimuno	Bifidobacterium bifidum	46% (syrup) -80% (powder)
Friesland Domo, The Netherlands	236	Vivinal	B. circulans	>57%
GTC Nutrition, USA	285, 286	Purimune	B. circulans	>94.5%
Yakult, Japan	334	Oligomate	Sporobolomyces singularis & Kluyveromyces lactis	>56%
International Dairy Ingredients, Canada	489	Floraid	Aspergillus oryzae	>28% (syrup), 39% (powder)

2.2.2 Polydextrose

2.2.2.1 Polydextrose synthesis

Polydextrose (PD) is a soluble dietary fiber produced chemically by polymerizing glucose and sorbitol under vacuum in presence of an acid. PD is considered a dietary fiber. PD was first invented as a polysaccharide by Hans H. Rennhard Lyme at Pfizer Inc., New York. Melt polymerization is used as the synthesis method with maltose and glucose as starting materials. The patent mentions citric acid as a preferred catalyst and preferred pressure of less than 300 mm. They mention the average molecular weight for their polymer is usually from 1000 to 2400 (Rennhard Lyme 1973).

According to polydextrose product and process patent (Patent number 5,728,397), PD is commercially manufactured by melt polymerization of glucose and maltose using edible acids as catalysts (Fuisz, 1997). Citric acid and phosphoric acid are commonly used. The acid used could be any non-volatile, edible, organic polycarboxylic acid. PD produced following this patent has an average molecular weight of 1500 to 3600 Da (Fuisz, 1997).

PD is prepared by conduction heating under vacuum and is reported to contain a (1→2), (1→3), (1→4), or (1→6)-linked glucopyranose backbone branched at the O-3, O-4, or O-6 position (Wang et al., 2018, Stumm and Baltes, 1997). PD can be prepared using microwave irradiation using glucose and phosphoric acid. Wang et al. prepared PD by microwave irradiation of glucose in the presence of phosphoric acid (Wang et al., 2018). They report purified PD with an average molecular weight of 2.131 kDa was obtained. They used FT-IR to analyze the structure of PD. They identified both α- and β-glycosidic bonds configurations in the product. They reported their synthetic PD exhibited a strong hydroxyl radical scavenging activity and proposed it could be utilized as an antioxidant in the food industry (Wang et al., 2018).

Wang et al. also studied the feasibility of a rapid and efficient microwave mediated method to synthesize PD (Wang et al., 2014). They mixed glucose and sorbitol (8.9:1, w/w) followed by the addition of 10% (v/w) water and 1.2%(v/w) phosphoric acid. The mixture was subjected to microwave irradiation at 120°C for 2 min. They report

that temperature plays a pivotal role in PD synthesis rate and yield ratio with 120°C being the optimum temperature. Also, the reaction time was an important factor. They reported that their PD is had an average Mw of 2.131 kDa and a degree of polymerization of 13 (Wang et al., 2014). The degree of polymerization for commercially produced polydextrose is variable. More than 90% of molecules have a DP between 3 and 30. The average DP for polydextrose is 12 with an average molecular mass of 2000 Da (Lahtinen et al., 2010)

The production of a fructoglucan similar to polydextrose was reported by Manley-Harris and Richards and is produced by the heating of anhydrous, amorphous, acidified sucrose (Manley-Harris and Richards, 1993). They suggest that higher yields could be achieved by heating for a longer period of time or at higher temperatures. However, increased color may appear which can be reduced under vacuum. The polymer they produced is highly branched and of low molecular weight with an average DP of 25. It contains both fructose and glucose.

2.2.3Polylactose

2.2.3.1 Production of polylactose

Tremaine et al. used twin-screw extrusion to polymerize dried lactose, or mixtures of lactose and glucose, with citric acid as the acid catalyst (Tremaine et al., 2014). Different levels of glucose did not affect the yield however 2% of citric acid had a higher yield than 1% citric acid. They also report the generated oligomers, polylactose, had a degree of polymerization that predominantly ranged from 3-5. Added glucose in the raw mix resulted in lower extruder torque and specific mechanical energy (SME), and extrusion products of a lighter color. This would have beneficial effects in reducing energy consumption and possibly increasing throughput (Tremaine et al., 2014). However, Tremaine reports that the concentration of citric acid did not affect any of the process responses (Tremaine, 2012). She also observed that with an increase in SME, the extrudates brightness decreased since the caramelization rate is dependent on heat transfer and SME.

Reid has evaluated the effect of citric acid concentration on the yield of indigestible oligomers after the extrusion of polylactose (Reid, 2015). Additionally, she evaluated the effect of feed rate on yield and efficiency by using two different feed rates (Reid, 2015). Citric acid concentrations of 2%, 4%, and 6% with 20% glucose, and the remaining weight percentage of lactose were used. These three formulas were extruded at two feed rates, 15kg/hr, and 30 kg/hr. She observed that feed rate greatly affected both SME and torque as by increasing the feed rate, the motor torque increased while the SME decreased (Reid, 2015). Furthermore, significant differences were observed in color measurements of the two feed rates showing that in lower feed rate samples, more browning occurred (Reid, 2015). Reid also reports that the sample prepared at the lower feed rate for the 2% and 4% citric acid formula had the greatest concentration of low molecular weight soluble fiber levels. However, for the 6% citric acid formula, no difference in low molecular weight soluble fiber (LMWSF) between the two feed rates were observed. 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 (Reid, 2015). Kuechel and Schoenfuss have developed a microwave reactor method for the polymerization of lactose and glucose. Citric acid was used to catalyze the reaction (Kuechel and Schoenfuss, 2018). They report that the quantity of oligosaccharides produced using the minimum amount of water necessary to achieve consistent heating was much lower than that achieved by reactive extrusion. They also studied the effect of formula variation on the polymerization process. Kuechel tried polymerizing lactose from deproteinized permeate powder using extrusion (Kuechel, 2017). She reported the darker brown color in the final product could be from the elevated citric acid level that was necessary to produce fiber (90% permeate powder, 10% citric acid) coupled with the composition of the ingredients. In this experiment, she was able to achieve about 4.25% LMWSDF (Kuechel, 2017).

Polylactose prepared by extrusion contains HMF and some residual citric acid, which reduces its application in the food industry if not removed. Thus, it is important to evaluate a purification process that produces a product rich in dietary fiber that could be

used by the functional food and nutraceutical industries. Polylactose purity is important to place a competitive product in the market.

2.3 Extrusion

Extrusion is often used in the food industry. Extrusion can be used for conveying, mixing, shearing, separation, heating or cooling, encapsulation, flavor generation, and sterilization (Guy, 2001). At the beginning of the process, the variables are set in order to achieve the proper physical and chemical changes within the barrel of the machine. After that, while processing, these conditions must be maintained to ensure that the extrudate variables are kept at the required levels. Extrusion is relatively low in processing costs with high productivity and is applicable for producing a wide range of products (Guy, 2001). Reactive extrusion combines chemical processes of polymer synthesis or modification with extrusion (Covas et al., 1995). This means melting, blending, structuring that happens in an extruder forms polymer or modifies the present polymers. The extruder serves as a reaction vessel during the reactive extrusion process. Using materials with a low polymerization heat in reactive extrusion makes high throughput possible (Fink Johannes, 2013). Reactive extrusion is widely used to produce different industrial polymers. For example, as a result of continuous polymerization, amines can be grafted on resins that increase the toughness. This reaction is utilized in the production of bullet-proof glass (Covas et al., 1995). Grafting reactions modify the original polymer chemically and physically. Multiple polymers are obtained by reactive extrusion including polystyrene, polyamide 12, and polylactide (Shishuang et al., 2004, Wollny et al., 2003, Jacobsen et al., 2000). Polylactides (PLA) are biodegradable composites that can be used for plastic films and containers (Raquez et al., 2008). PLA is produced by reactive extrusion of lactic acid or lactide via ring-opening polymerization reactions (Jacobsen et al., 2000). Polyamides are thermoplastic polymers that are widely used as engineering materials and they show good mechanical properties at higher temperatures, in comparison to commodity plastics (Covas et al., 1995). Compounds like acetylated, n-octenylsuccinylated, and phosphorylated waxy maize starches can be prepared by

extrusion and can be used for flavor encapsulation to retain flavors in foods (Murúa-Pagola et al., 2009).

2.3.2 Extruders

Extruders are available in single or twin-screw configurations, with various internal configurations. The appropriate type of extruder is selected based on physical and sensory properties of the end product, ingredients, production rate, source of energy, and cost. The four most commonly used types of cooking extruders currently are: single-screw 'wet' extruders, single-screw 'dry' extruders, single-screw interrupted-flight extruders, and twin-screw extruders (Guy, 2001). All extruders consist of a screw which is suspended only from the drive end of the barrel and rest on the product on the exit end. The screw consists of a shaft with a keyway or hexagonal shape on which different elements are slipped on before being put in place. In twin-screw extruders, each screw has a modular component that gives it the advantages of arranging elements in different configurations and replacing and moving the shaft to change the clearance in the barrel (Heldman and Hartel, 1997). Each screw wipes the walls of barrels and pushes the product forward, backward, or kneads the product to develop the desired characteristics (Heldman and Hartel, 1997, Guy, 2001). Reactive extrusion is utilized to form polymers. This involves polymerizing a liquid or solid monomer during the residence time in the extruder barrel to form high molecular weight melt materials (Fink Johannes, 2013). This method combines the chemical process of polymerization and extrusion into one process occurring in an extruder (Covas et al., 1995). Both single and twin-screw extruders could be utilized for reactive extrusion. Continuous polymerization or polycondensation techniques have been developed industrially on twin-screw extruders (Covas et al., 1995). The extruder barrel acts as a chemical reactor that holds the reactant oligomer or monomer in a solid, liquid, or molten state (Beyer and Hopmann, 2018).

2.3.2.1 Twin-screw extruder

The direction of rotation of screws in this type of extruder categorizes them into two main groups: a counter-rotating twin-screw extruder and a co-rotating twin-screw

extruder (Heldman and Hartel, 1997). This can be further subdivided into intermeshing and non-intermeshing extruders, depending on how the elements on both screws interact with each other. The non-intermeshed twin-screw extruder is like two single-screw extruders sitting side by side with a small portion of the barrel in common. In intermeshing twin-screw extruders, the screws partially overlap in the barrel track forming an “8” shape. (Guy, 2001). One advantage of using a twin-screw extruder for reactive extrusion is that melts with different viscosities can be processed without a need for solvents as a medium (Beyer and Hopmann, 2018). Moreover, using a twin extruder for the reactive extrusion process increases the surface/ volume ratio as a new thin surface layer is continuously created on the screws (Covas et al., 1995). This enhances mixing, reaction and, heat transfer. Also, since multiple barrel sections and injection ports could be included in the design of a twin-screw extruder, back mixing if a product with its reactants is prevented (Covas et al., 1995). Extrusion motor torque provides a direct indication of the energy absorbed by the material due to the shear pressure of the elements and/or die. Fluctuation in torque is an indication of process instability during the extrusion process. The effect of different moisture contents, die temperatures, screw speed, shaft-elements, and their order and mass flow rate on extruder operation need to be evaluated when designing extrusion processes.

2.3.2.1.1 Effect of die temperature

If a die is included in the extruder design, as the viscosity of molten ingredients decreases with temperature increase, the die pressure decreases (Akdogan, 1996).

2.3.2.1.2 Effect of mass flow rate

An increase in the mass flow rate can increase the degree of fill in the barrel. Specific feed loading (SFL) is the measure of the degree of fill in the barrel defined as:

$$SFL = \frac{mass\ flow\ rate\ (\frac{kg}{h})}{screw\ speed\ (rpm)}$$

As the mass flow rate increases, SFL will increase at constant screw speed. Moreover, the extruder throughput and die pressure drop will (Akdogan, 1996).

2.3.2.1.3 Effect of screw speed

SFL will decrease as screw speed increases causing the pressure drop to decrease. Pressure drop along the die can be used to determine the flow properties of the sample. An increase in the screw speed results in a reduction in the melt viscosity. Higher screw speed causes a higher shear rate in the extruder which causes a reduction in the melt viscosity reducing the pressure drop (Akdogan, 1996). The higher screw speed causes lower torque, more work into the polymer matrix, more mixing and, more degradation of the polymer due to higher temperature (Giles Jr et al., 2004).

2.3.2.1.4 Effect of extrusion barrel temperature

The temperature in the barrel is another factor affecting extruded food products. High barrel temperatures and protein content decrease the expansion index in functional blends with different proportions of corn starch and whey protein concentrate. This could be due to increased fragmentation and loss of protein solubility (Amaya-Llano et al., 2007). Protein content influences expansion because of protein's macromolecular structure, conformation, and formation of widespread networks through covalent and nonbonding interactions that happen during extrusion (Amaya-Llano et al., 2007). Higher temperature leads to a greater vapor pressure and increased driving force for expansion; however, it could lead to a decrease in melt viscosity and greater tendency for extrudate to collapse in puffed products. Barrel temperature decreases the nutritional quality of the product including levels of vitamins like ß- carotene and tocopherol (Guy, 2001). High temperatures promote the Maillard reaction (Guy, 2001). During reactive extrusion, the barrel temperature changes the material viscosity which subsequently changes the reaction speed and progress within the extruder. Temperature buildup or initial high temperature of the barrel can cause material degradation or change the degree of polymerization (Beyer and Hopmann, 2018). Hwang et al report that at 160°C of

extrusion temperature, 43.9% GOS and PD were produced that was mostly consisted of GOS with lower molecular size. As they increased the temperature, the degree of polymerization increased. At 200°C most of the mixture was polydextrose (Hwang et al., 1998).

2.3.2.1.5 Effect of moisture content

Wet extrusion is known as extrusion at higher than 40% moisture content. Extruders could be used as continuous bioreactors for starch-based foods since enzymatic action can be coupled with thermal and mechanical shear (Akdogan, 1999).

Moisture is one of the main factors controlling the efficiency of enzymatic starch hydrolysis in extruders. Excess moisture in food results in complete starch gelatinization which will normally result in a higher degree of starch hydrolysis (Akdogan, 1996, Akdogan, 1999). The optimum moisture content in three feed streams was determined to be about 55-60% for complete conversion (Akdogan, 1999). Moreover, samples extruded at higher moisture content were more saccharified. High moisture also influences rheological properties and distribution of shear, mixing, and mechanical heat (Akdogan, 1996). Higher moisture content prevents viscous dissipation of energy in the extruder barrel due to low melt viscosity and reduces the pressure build-up in the barrel(Heldman and Hartel, 1997). The lower the starch content of the sample, the lower the degree of gelatinization would be and hence extrusion expansion would be lower. During the extrusion of high moisture protein-based food, protein structures are disrupted. For these samples (moisture content above 60%) plastification of the protein mix can happen at high temperatures (>150°C) but the lower the moisture content is, the higher temperature is required for plastification (Akdogan, 1999).

Other studies have shown that increased feed moisture and reducing barrel temperature reduces non-protein nitrogen in high protein products however these conditions did not have any significant influence on cysteine and methionine content (Chaiyakul et al., 2009). The NPN involves a wide group of components, including urea, ammonium salts, single amino acids, small peptides, amines, amides, and nucleotides, and all of them end in ammonium as the final chemical form. Loss of these two amino

acids, cysteine, and methionine, are not influenced by temperature (140–180 °C) and moisture (30–40 g/100 g) levels. Lysine content of extrudate was significantly reduced with increasing barrel temperature or decreasing feed moisture content due to the Maillard reaction. Lysine is lost because of a heat-induced reaction between the reducing groups of sugars and amino groups of protein polypeptide chains. Both terminal α-NH2 groups and the ε-NH2 groups of lysine side chains could be involved. Lysine may also form cross-linkages with other amino acid residues forming lysinoalanine and lanthionine which have reduced digestibility. Moreover, with the loss of amino acids, protein nutritional values decrease (Chaiyakul et al., 2009). In this research, they also have reported that increasing raw material moisture could improve the final extrudate moisture. In the case of polylactose production, condensation polymerization happens within the extruder barrel. These reactions are prone to chain scission if moisture is present during extrusion (Morris, 2016). Chain scission causes a decrease in molecular weight while changing mechanical properties. The moisture also causes bubble formation in the extrudate while exiting the extruder since water expands as pressure is reduced. Preferably, water is avoided for the sugar polymerization process and sugars are used in an anhydrous state since the presence of water causes depolymerization of polymers (Leuk, 1942). Water, if available while the polymerization occurs, should not be in a state capable of dissolving monomers. For example, for dextrose polymerization if superheated steam is used, polymerization still happens since it does not dissolve dextrose (Leuk, 1942).

2.4 Purification of carbohydrate preparations

Carbohydrates that are produced or purified using processes involving heat are commonly purified to obtain the final product. The main goal of this purification is to remove undesirable chemicals and colors and concentrate the desirable fractions. Different strategies used for carbohydrate purification are adsorption, diafiltration, nanofiltration, ion exchange, moving bed chromatography, and selective fermentation. However only adsorption and ion exchange are commercially used (Scott and Vera, 2016).

Adsorption is commonly used to purify contaminated fluids that are unacceptable in color, smell, and taste. Adsorption involves the separation of a substance of one phase by concentration on the surface of the adsorbing phase. This transferring process continues until a dynamic equilibrium conditions are reached. Activated carbon and ion exchange resins are some of the typical adsorbents (Carabasa et al., 1998). Adsorption separates one phase of a mixture by forming bonds between the rest of the mixture and the adsorbing phase until dynamic equilibrium is reached. An adsorption isotherm demonstrates the relationships between equilibrium concentrations of the adsorbate in the adsorbing phase and fluid phase at a fixed temperature (Carabasa et al., 1998).

2.4.1 Activated carbon

Carbon is commonly used in food processing for sugar refining and water filtration. Color and protein can be removed using activated carbon (Scott and Vera, 2016). Diatomaceous earth can be coupled with carbon to remove the carbon fines from eluent. In order to make sure the decolorization process is safe and hygienic, the decolorization material should have a large surface area, good porosity, and pH sensitivity. Carbon is very ordered, regular, and has multi- porous-like layers. The large specific surface area of activated charcoal allows the disaccharide molecules to take up these spaces and be separated from the mixture.

2.4.1.1 Production of activated carbon

In the United States, activated charcoal is usually derived from coal and to a lesser extent from coconut shells, wood, or peat. Used coal is ground to a powder then mixed with a binder. The mixture is pyrolyzed and activated to produce granular activated carbon (Pendyal et al., 1999). Most activated charcoals sold commercially are from non-renewable sources like coal. However, agriculture by-products such as cane trash, bagasse, corn cobs, and seed shells are used to produce activated charcoal. These sources have the advantage of offering low-cost sources. Depending on carbon's porosity, surface area, pore size distribution, bulk density, particle size, amount of water-

soluble minerals and its total ash content, the efficiency of activated carbon to absorb the color, ash, or other components changes. In the case of sugar decolorization, the carbon used should have a suitable pore size to adsorb a mixture of polydispersed components from highly concentrated sugar solutions. Moreover, the ash content of this carbon should be low since ash is not desirable in refined sugar. Ahmedna, et al (1997) compared granulated activated carbon made from rice hulls, rice straw, soybean hulls, and pecan shells mixed with molasses or H_3PO_4 against a reference carbon. Their findings showed that activated carbon made from rice hulls and rice straw had the best decolorizing power with a relative efficiency of about 85% of that achieved by the reference carbon. However, for dilute sugar solutions of 10 Brix, the difference between decolorization by reference carbon and rice hulls was not significant. (Ahmedna et al., 1997).

In another experiment, Ahmedna compared activated carbons made from sugarcane bagasse and pecan shells. Results showed carbon made from sugarcane bagasse, steam activated pecan shell and chemically activated pecan shell were as effective as commercial carbons in sugar decolorization. This could be due to their highly developed surface area (Ahmedna et al., 1997).

The exhausted carbon can be regenerated using chemical and thermal methods (Aljohani et al., 2018). Thermal regeneration involves heating the charcoal to 800°C and it is commercially widely used (LENNTECH, 2020). However, this requires investment in a furnace and causes high carbon losses. Some equipment designed for thermal regeneration of activated carbon use the rotary kiln to perform drying, heating, regeneration, and cooling phases in one machine (Metso, 2012, Carlson and Ebben, 2020)

2.4.1.2 Activated carbon characteristics affecting efficiency

Ahmedna et al. concluded that in sugar decolorization, the total surface area of carbon, pore-size distribution, and chemical reactivity are the main characteristics affecting efficiency (Ahmedna et al., 2000). Large surface area may increase the absorption rate, but its adsorptive capacity could be low because it lacks the proper pore size to contain the material. Macropores could help rapid diffusion of color while smaller

pores are necessary to maintain the adsorbed particles. Pore structure and chemistry are directly dependent on pyrolysis temperature, composition, and structure of raw material, and method of activation. Using a curvilinear relationship, they showed the optimal decolorization by activated carbon would occur around a macro- plus mesopore area/total surface area (MMSA/TSA) ratio of 0.25 and a total surface area between 1000 m2/g and 1220 m2/g. However, the negatively charged surface formed by dissociation of specific typed of surface oxides may reduce color removal. The highest percent color removal was achieved by carbons having no carboxylic groups or no negative charge at pH 7. They also report carbon's efficiency in removing sugar colorants decreased with an increasing amounts of negative charges on the surface (Ahmedna et al., 2000). Kuhn and Filho produced fructooligosaccharides in stirred reactors containing 50% sucrose and enzyme. They used activated charcoal with particle diameters between 0.25 and 0.40 mm at 40C until equilibrium was attained. Their results showed good adsorption affinity for fructooligosaccharides, but less for glucose, fructose, and sucrose. They report the activated charcoal fixed-bed column was effective in separating the mixture of sugars, especially fructooligosaccharides and glucose and fructooligosaccharides and fructose, and the best separations were obtained using 15% ethanol solution (v/v) as eluent at 50 °C. This method helped develop a FOS with higher market value, lower production costs, and a robust process (Kuhn and Filho, 2010).

Mudoga and co-workers (Mudoga et al., 2008) have compared the decolorization capabilities of beet pulp activated carbons with commercial sugar decolorizing activated carbons. The beet carbons were prepared using direct carbonization or impregnating with phosphoric acid at a lower temperature. Samples with the best performances were those with neutral pH and low conductivity leading to a more nonpolar surface. They observed the activated carbon obtained from sugar beet pulp by direct carbonization for 5 h at 750 °C under a stream of carbon dioxide has a decolorization performance that can compete with commercially used activated carbons.

Mixing activated charcoal with water and heating has been used to reduce the fine particles of activated charcoal. This also helps to eliminate the air from pores (Campos et al., 2017). Campos and co-workers report activated charcoal reduces the color of chicory

extract drastically. However, it did not affect the content of FOS. Cleared extract treatment with 1% powdered activated charcoal at 55 °C for 30 min was sufficient to remove most of the pigments and most of the residual turbidity without affecting the FOS and sugar contents.

Carabasa and co-workers evaluated the HMF reduction in peach juice after treating it with activated carbon. They reported the content of HMF decrease with carbon-juice contact time. They also observed at a given contact time and given temperature, the HMF content linearly decreased with increasing concentration of carbon in the juice (Carabasa et al., 1998). They observed that with an increase of temperature the adsorption rate on activated carbon increases.

Carabasa et al. report the most reduction in the percentage of HMF for 0.06 g carbon per g juice at 50°C compared to 10°C, 20°C, 30°C and 40°C. Results also show that the content of HMF decreases with the contact time. According to Carabasa et al., carbon-juice contact time of l0-15 min with a temperature from 30 to 50°C is enough to improve the color of juice, and drive it to 'normal' color. Longer contact times unnecessarily prolonged the process and obtained similar results (Carabasa et al., 1998).

Morales et al. (2006) tested activated charcoal to remove mono and disaccharides from honey supplemented with maltodextrins. They also evaluated different stirring conditions of the sample with activated charcoal and water/ethanol solutions. They observed that by increasing the ethanol ratio, the recovery rate of mono and disaccharides increased.

Satyawali and co-worker's acid-activated charcoal using sulfuric acid, phosphoric acid, nitric acid, and hydrochloric acid. They also used thermal and steam activation for different agro-residues such as bagasse, sawdust, bagasse fly ash, wood ash, and rice husk ash. Also, select commercial activated carbons were also examined. The activated carbon samples were evaluated for color removal efficiencies with biomethanated distillery spent wash. Samples were centrifuged and vacuum filtered after mixing with charcoal. They report that among the various samples tested, the phosphoric acid-treated bagasse displayed the highest color removal (50%). They reach the conclusion that activated carbon is capable of color removal as well as removing phenols from molasses

spent wash. Also, melanoidins, the primary cause of color in distillery wastewater, underwent monolayer adsorption, following the Langmuir equation (Satyawali and Balakrishnan, 2007).

Activated carbon has been reported to remove mono and disaccharides in GOS purification due to carbon's hydrophobic nature (Scott and Vera, 2016). GOS is absorbed to activated carbon since it has a higher hydrophobicity compare to mono and disaccharides. This allows mono and disaccharides to elute.

2.4.2 Application of resins and adsorbents

Interaction between atoms and molecules of the adsorptive and adsorbent could be utilized for the separation of different components. The adsorbent is a site with certain electronic and sterical properties. Most adsorbents are not categorized by their exterior surface but by their inner porous surface (Shih et al., 2019). Adsorption processes can be grouped into three categories:

a) Physisorption: Commonly reversible, mainly based on van der Waals forces, dipole forces, dipole-dipole forces, and dispersion forces as well as induction forces which are usually below 50 kJ/mol (Condon, 2020).
b) Chemisorption: Chemical bonding between the adsorbent and adsorptive, in the range of 60- 450 kJ/mol, caused by covalent or ionic bonding (Condon, 2020).
c) Ionosorption: Ion transfer happens. An ion from a solution can be exchanged with a similarly charged ion present on a stationary solid particle called ion exchange resin. This chemical reaction can be reversible. IE reactions are stoichiometric. Resins can be periodically regenerated to restore their original ionic form. Cation and anion exchange resins have been widely used for water softening and water deionization (Bhatnagar & Kumar, 2012).

As described by Kammerer et al., adsorption happens in four consecutive steps. First, the compound that we want to separate is transformed from one phase to the subsurface and builds up around adsorbent. Next, film diffusion happens in which there is the transport of the chemical through the subsurface. Thirdly, surface diffusion or pore diffusion happens. Surface diffusion is diffusion along the inner surface upon adsorption and pore

diffusion is transportation into the pores of the adsorbent through diffusion by the pore fluid. Fourthly, interaction with the active sites of the adsorbent happens (Kammerer et al., 2011).

The major difference between ion exchange and adsorption is that in ion exchange, ions are not removed from the solution, but they are replaced by ions bound by the solid phase via electrostatic interactions to achieve electroneutrality. Due to the fact that it would be less complex if resins are devoid of functional groups, most theories and models used for adsorption could be used for ion exchange (Dutcher et al., 2013). Adsorption and ion exchange processes using resins have led to the development of well-designed filtration processes. Adsorbent and ion exchange materials are categorized based on their matrix composition, polarity, chemical and physical resistance, particle size, inner and specific surface area, density, porosity, and pore radius distribution (Gusain et al., 2020). Ion and salts can be removed using weak acid and basic resins (Scott and Vera, 2016). Saccharides are weak electrolytes therefore they have little interaction in their natural forms with ion-exchange resins and resins can be used for ion removal without affecting sugar yield. Ion exchange resins are used for cationization of aqueous sugar solutions by passing the solution through ion exchange resin containing strong acid cationic exchange resin in hydrogen form (Rousseau and Lamotte 1982). Additionally, the anion exchange resin can remove color bodies in the sugar solution.

Synthetic resins are polymeric adsorbents with large internal surface areas manufactured by polycondensation or polymerization (Kammerer et al., 2011). For the production of gel resins, styrene and divinylbenzene (2-12%) are blended with the same amount of water in a chemical reactor. The mixture is dispersed by stirring which produces small globules with a size of about 1 mm. Then benzoyl peroxide is added and initiates the radical chain reaction and polymerization that leads to the formation of small beads of polystyrene/ divinylbenzene molecules (Kammerer et al., 2011). Ion exchange resins have been used to produce colorless sucrose crystals. The microporous styrene-divinylbenzene resin with quarternary amine functional group was used to remove Maillard reaction products from sugar solution (Serpen et al., 2007). Furthermore, a microporous copolymeric resin made up of a monovinyl aromatic monomer

functionalized with hydrophilic groups has been reported to be used for the decolorization of sugar solutions (Stringfield, 1990). Ion exchangers are also used for decaffeination of liquid extracts like coffee, tea, or cocoa (Dawson-Ekeland, 1991). Campos and co-workers also used a mixture of anionic/cationic resins to demineralize the yacon extract that is rich in FOS and simple sugar. They reported color (ΔE) dropped from 31.6 to 0.5. Additionally, this step removed salts and helped eliminate the other ionic substances such as polymers and pigments. According to Nobre et al. (Nobre et al., 2014), resins have more affinity for mono and disaccharides than oligosaccharides.

2.4.2.1 Purolite resins

Purolite® A860S is a macroporous polyacrylic polymer crosslinked with divinylbenzene in the form of spherical beads with a functional group of quaternary ammonium with Cl^- anion form. A860S is primarily used for the decolorization of sugar solutions and as an organic scavenger (Purolite®). Most quaternary ammonium compounds have the general formula of $R_4N^+X^-$ and are the type of cationic organic nitrogen compound. The symbol R marks the support structure of the resin called the matrix. Functional groups are bound to that matrix. Matrices are formed from styrene, acrylic acid, or methacrylic acid, which can undergo polymerization with crosslinking agents, such as divinylbenzene or other divinyl monomers (Henke et al., 2019). N represents the nitrogen and X marks the negatively charged ion that allows an exchange of anions. Quaternary ammonium compounds exist in many different structures; however, they are all made of a positively charged species while bearing a hydrophobic moiety nearby. Low molecular weight quaternary ammonium compounds, polymeric quaternary ammonium compounds and immobilized quaternary ammonium compounds are some types of quaternary ammonium compounds (Loontjens, 2013). The N atom is covalently bonded to four organic groups that are positively charged, balanced by the X negative ion. For the A860S formula, the ion is Cl^- and it's a strong-base anion exchanger (De Mancilha and Karim, 2003). Carvalho et al. report A860 resin removed furfural compound formed during xylitol production from hemicellulosic hydrolysate from 0.056 to 0.01 (g dm^{-3}) rather than HMF. A860 reduced HMF from 0.176 to 0.076(g

dm $^{-3}$) (De Carvalho et al., 2004). This resin is reported to have resistance to organic fouling and is efficiently regenerated. It also has a highly reversible adsorption (Purolite®).

Purolite® C150SH is a macroporous polystyrene crosslinked with divinylbenzene in the form of spherical beads with sulfonic acid as the functional group and ionic form of H^+. The sulfonic acid functional group consists of R SO_2OH, which consists of a sulfur atom, S, bonded to a carbon atom that may be part of a large aliphatic or aromatic hydrocarbon, R (Senning, 1992). This resin is primarily used for demineralization of sugar solutions, citric acid, gelatin, and collagen and deashing of sweetener. This resin is reported to have excellent resistance to osmotic and thermal shock (Purolite®).

Purolite® A847S is a gel polyacrylic resin crosslinked with divinylbenzene in the form of spherical beads with tertiary amine functional groups and the deprotonated form of an amine, base. It is primarily used for sugar applications, lactic acid, and citric acid. This resin has resistance to organic fouling and against osmotic shock attrition. Moreover, A847S is reported to have high regeneration efficiency with low rinse volumes and high purity. (Purolite®).

All Purolite® resins (A860S, C150SH and A847S) are food grade and kosher certified.

2.4.2.2 AMBERLITE™ FPA53 OH and AMBERSEP® 200H

AMBERLITE™ FPA53 OH and AMBERSEP® 200H are two resins initially used for polylactose purification (Kuechel, 2017). Since as a part of Megazyme integrated total dietary fiber kit, Ambersep and Amberlite resins have demonstrated their ability to remove color compounds, organic acids, and minerals including calcium, magnesium, and sodium, they were chosen to be used for polylactose purification study.

AMBERLITE is an anion exchange containing tertiary amine functionality on a gel-type acrylic matrix. It is mostly used for decolorization in conjunction with AMBERLITE. Because of AMBERLITE's high basicity, it can be an excellent choice for

the removal of weak organic acids. This resin can withstand a maximum temperature of 50°C (Megazyme, 2012a).

AMBERSEP® 200H is a strong acid, cation exchange resin based on sulphonic acid exchange groups on a polystyrene matrix. This resin can be regenerated with HCl or H_2SO_4 and can stand a maximum temperature of 135°C (Megazyme, 2012b). This resin is not food grade.

2.4.3 Techniques of adsorption and ion exchange

Currently, adsorbent and ion exchange materials are used in different processes. Bed processes with fixed bed reactors, batch processes in agitated reactors, and moving bed processes the in reactor with moving solid phase are some of the most commonly used processes.

Bed processes are the most commonly used for fluid adsorption and ion exchange processes. In this process, resin loading is continued until the concentration of the compound targeted to be removed leaving the bed exceeds a certain level which indicated the exhaustion of the resin capacity. This capacity is referred to as breakthrough. For the purpose of efficiency, columns filled with ion-exchange material or adsorbents in different loading states or one absorber with several adsorption layers are connected in series or parallel (Kammerer et al., 2011). In batch processes, the adsorbent is agitated in a defined volume of fluid. After reaching equilibrium, by filtration or sedimentation, the adsorbent is removed (Kammerer et al., 2011). Moving bed processes performed in reactors with a moving solid phase are a continuous sorption process. The sorbent material crosses the system from top to bottom without being kept in suspension. The solution containing the adsorptive is added in countercurrent flow. The flow rate of the adsorbent can be controlled however, some of the major disadvantages of this process are high investment costs, and the complexity of resin dosage and suspension and the control of the fluid and solid phase transport (Kammerer et al., 2011).

2.5 Non-enzymatic browning reactions effect on sugar extrusion products

Browning reaction can occur during processing and storage of products. Below we will further review these reactions and their products.

2.5.1 HMF

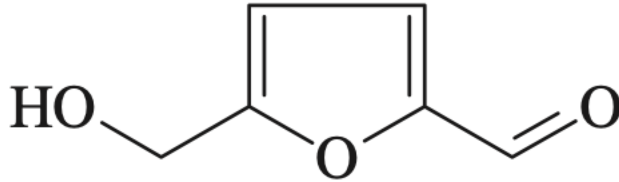

Figure 1. Chemical structure of Hydroxymethylfurfural

Hydroxymethylfurfural (HMF) is naturally present in honey which is produced by the action of normal honey acidity on reducing sugars and sucrose, usually at room temperature. However, when honey undergoes thermal processing to delay crystallization and reduce microbial load, HMF content increases (Shapla et al., 2018). Thermal processing such as extrusion can cause the formation of compounds called neo-formed contaminants (NFCs) such as acrylamide and HMF (Capuano and Fogliano, 2011). Masatcioglu et. al investigated effect of reducing sugars (D- glucose, and D- ribose) on furfural and HMF formation. (Masatcioglu et al., 2015). Their preliminary experiment indicated that HMF content was at trace levels in products using D- ribose. They also reported the addition of sodium bicarbonate into the formulation slightly decreased HMF formation levels of the extrudate which might be due to increased pH. However, when ammonium bicarbonate was used in the product, HMF content was significantly higher due to increased rates of Maillard reaction using free ammonia released from ammonium bicarbonate (Masatcioglu et al., 2015).

Table (2) - Range of HMF content (mg/kg) in different food commodities (Capuano and Fogliano, 2011)

Food Commodities	HMF Content (mg/L)	Method
Coffee	100-1900	LC-UV
Instant Coffee	400-4100	LC-UV
Decaffeinated Coffee	430-494	LC-UV
Malt	100-6300	LC-UV
Barley	100-1200	LC-UV
Honey	10.4-58.8	DNPH-LC-UV
Beer	3-9.2	DNPH-LC-UV
Jam	5.5-37.7	LC-UV
Red Wine	1-1.3	LC-UV
Balsamic Vinegar	316.4-35251.3	LC-UV
Cookies	0.5-74.5	LC-UV
White Bread	3.4-68.8	LC-UV
Whiskey (Straight)	2-8.2	LC-UV
Roasted Almond	9	LC-UV
Caramel Products	110-9500	LC-UV

2.5.1.1 Occurrence in Food

HMF is not present in fresh or untreated foods and it accumulates during the heat treatment and storage of carbohydrate-rich products (Capuano and Fogliano, 2011). The main foods with high levels of HMF include honey, coffee, fruit juices, candies, cakes, tomato paste, processed cereal products, heat-treated milk, and alcoholic beverages. The other source of HMF in food is related to the ingredients used. The addition of caramel solution, flavoring, honey or heat-processed dairy product such as dried whey can increase the levels of HMF in a product. HMF has been used as a marker of thermal

processing, storage, or abuse practices. HMF was first used as an indicator of adulteration with invert syrups (Stadler et al., 2009).

2.5.1.2 Regulatory information/ industry standards on HMF limits

Codex Alimentarius Commission has established a limit of 80 mg/kg for honey. However, Codex notes that many countries have been requesting to change the limit to 40 mg/kg. There are also other countries who are opposed to any reduction in the HMF level arguing that this could create a technical barrier to world trade for honey produced in countries with high ambient temperatures. The level of 80 mg/kg has therefore been retained in the standard but within square brackets (FAO/WHO, 2000). Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established the limit of not more than 0.1% for 5-Hydroxymethylfurfural in polydextrose(JECFA, 1995). This translates to not more than 1000 mg HMF per 1 kg polydextrose.

2.5.1.3 HMF main formation pathways

Thermal processing applied to food products can cause desirable or undesirable reactions between food components. From a food safety and nutrition perspective, the measurement of undesirable reaction products is very important. This is related to the health concerns related to undesirable reactions. Therefore, Maillard reaction products have been widely researched in a variety of heat-treated foods. Upon heating at high temperatures, sugars decompose into furfural compounds by Maillard reactions (MR) or caramelization. Caramelization requires higher temperatures than Maillard reactions (Kroh, 1994). Hydroxymethylfurfural (HMF) can be produced as a common intermediate product of MR or by heating hexose directly in fruit juice, jam, milk powder, and infant formula, especially when pH is low (Surh and Tannenbaum, 1994).

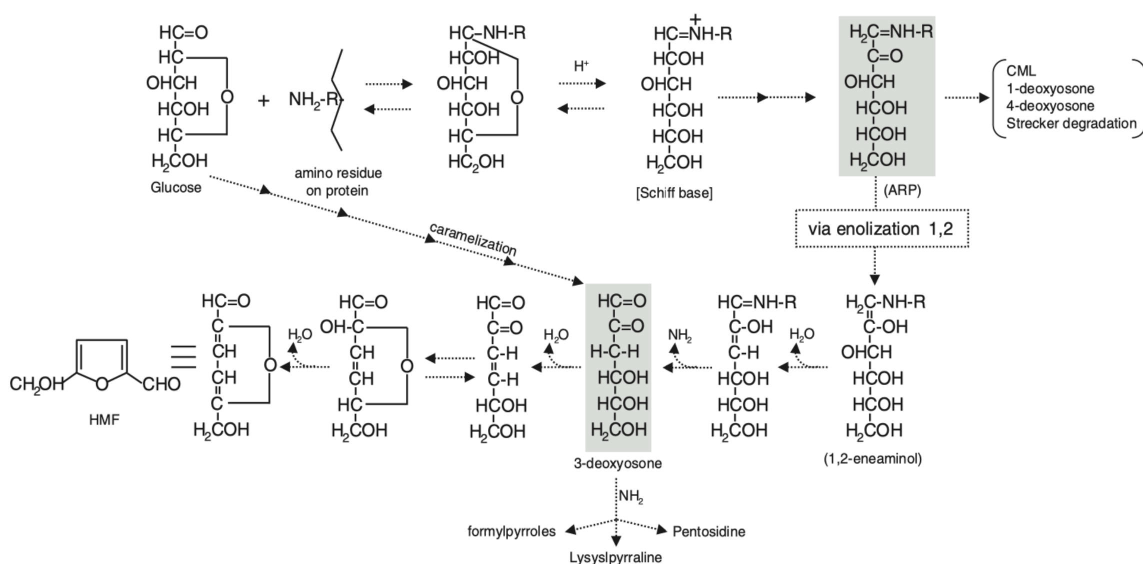

Figure 2. Scheme of the main routes for HMF formation during heat processing of foods (Stadler et al., 2009)

Ketoses generate more HMF than aldoses. The presence of a catalyst increases the HMF formation yield. Kuechel reported a 0.09% HMF concentration in presence of 2.02% citric acid in polylactose (Kuechel, 2017). Studies show fructose is the most reactive sugar compare to sucrose and glucose under acidic conditions. Moreover, the presence of amino acids slightly enhances the formation of HMF from glucose and sucrose while it does not enhance the formation of HMF from fructose. However, acidic conditions improved the rate of HMF formation from fructose relative to glucose (Perez Locas and Yaylayan, 2008). At 1 mM H2SO4, 42% of fructose was converted into HMF versus 31% for glucose (Perez Locas and Yaylayan, 2008).

Perez and co-workers investigated the mechanism of HMF formation using C-labeled precursors (Perez Locas and Yaylayan, 2008). For this purpose, they refluxed sucrose in methanol in the presence of p-toluensulfonic acid monohydrate and analyzed the sample using GC/MS. Under acidic conditions, when methanol is used as the solvent, fructofuranosyl cation can form which will react with the solvent and produce methyl fructofuranoside. This will prevent the formation of HMF from fructose through the 3-deoxyglucosone pathway (3- DG). The low temperature of refluxing methanol (65 °C) will prevent formation of HMF through the fructofuranosyl cation pathway.

Alternatively, if sucrose was being hydrolyzed without the formation of fructofuranosyl cation, both fructose and glucose moieties can generate HMF through a less efficient 3-DG pathway (Perez Locas and Yaylayan, 2008). Their results show sucrose generated more HMF per mol compare to both fructose and glucose at different temperatures. sucrose and fructose generate HMF through fructopyranosyl cation pathway and glucose generates HMF through 3-deoxyglucosone (3-DG) pathway. They also proposed that the major pathway of sucrose decomposition is the direct formation of fructofuranosyl cation in addition to glucose and 1,6-anhydro- glucose (levoglucosan), a known degradation product of glucose and cellulose. This is proposed based on the comparison of HMF generation relative to free fructose in different oligosaccharides.

2.5.1.4 Health risks

It has been shown that HMF is rapidly absorbed from gastrointestinal tracts in rats and mice (Germond et al., 1987). However, it has been reported that absorption and transport of HMF are more when cells are exposed to higher HMF concentrations (Capuano and Fogliano, 2011, Delgado-Andrade et al., 2008). In addition, the food composition, i.e. fiber content, might affect HMF uptake (Capuano and Fogliano, 2011). The major pathway of HMF in the body comprises early oxidation of HMF to 5-hydroxymethyl-2-furanoic acid (HMFA) followed by conjugation to glycine to yield N-(5-hydroxymethyl-2-furoyl) glycine (HMFG, the glycine conjugate of HMFA) as main metabolites, which are quickly discharged in the urine (Cajnko et al., 2020). HMF at high concentrations is cytotoxic, and irritating to the eyes, upper respiratory tract, skin, and mucous membranes (Monien et al., 2009). HMF has been shown to induce and promote aberrant crypt foci (ACP, preneoplastic lesions) in the rat colon (Archer et al., 1992). HMF mutagenic and genotoxic potential have been tested in vitro in bacterial test systems and results suggest low or no mutagenic effects (Aeschbacher et al., 1981). In contrast, it has been shown to be mutagenic in *S. typhimurium* (Sommer et al., 2003). Data on dietary exposures are very limited. Additional studies are therefore needed to

assess average, medium, and maximum intake for different populations and segments of the population.

2.5.1.5 HMF mitigation in food

Selection of raw materials and ingredients for the product can reduce the levels of HMF in a final product. Selecting materials less reactive for MR, or simply removing the active substrate of MR through blanching, soaking with inhibitors or physical removal of the substrate can reduce the rate of MR reactions (Capuano and Fogliano, 2011). The formulation could reduce MR rate by the addition of inhibitors such as salts, sulfites, organic acids, and hydrocolloids (Rannou et al., 2016). Moreover, controlling the thermal conditions of processing can decrease the formation of HMF. Appropriate storage conditions to avoiding excess temperature over product shelf life can also help to control HMF levels (Capuano and Fogliano, 2011). Furthermore, HMF and MR products can be removed after processing using fermentation or resin adsorption.

2.5.1.6 HMF analysis

Colorimetric methods were developed in the 1950s to determine HMF levels in heated dairy products (Capuano and Fogliano, 2011). Spectrophotometric methods can be carried out either by direct measurement of the absorption or by preparing a derivative (indirect methods) (Capuano and Fogliano, 2011). Colorimetric and spectrophotometric methods have several drawbacks including being tedious, prone to interference, and employ toxic chemicals.

Oral and co-workers report that heat treatment and acid digestion used as part of sample preparation for HMF quantification may increase the formation of HMF since it is composed of hexoses when heated with acid. Therefore, the results can be misleading in analyses to determine HMF binding when using the acid-heat treatment in some foods (Oral et al., 2014).

Truzzi et al. compared two official methods for the determination of HMF in honey, the spectrophotometric White method and the HPLC method (International Honey

Commission) for the determination of HMF in unifloral honey and honeydew samples with a very low HMF content (<4 mg/kg). They compared quality parameters like the limit of detection (LOD), the limit of quantification (LOQ), and the linearity range. With samples containing >1 mg/kg HMF (34 samples), the t-test revealed generally no statistically significant differences, except for one acacia honey sample. For samples with an HMF content of <1 mg/kg (9 samples), generally significant differences in HMF concentration ($P < 0.05$) were observed. In general, the spectrophotometric method gave higher values than the HPLC method (20–30% higher) for samples with HMF contents in the range 0.5–1 mg/kg and about 70% higher for samples with HMF content <0.5 mg/kg) (Truzzi et al., 2012).

Truzzi's results agree with Zappala et al results who compared the spectrophotometric method (White) with the HPLC method (Zappala et a. 2005). They also achieved higher HMF values through spectrophotometric methods compare to HPLC. They confirmed suggestions given by the International Commission of Honey to not use the Winkler method for determining HMF in honey, because of carcinogenic of p-toluidine and of the low precision of this method (Zappalà et al., 2005).

Capillary electrophoresis can also be utilized for HMF analysis (Teixidó et al., 2010). Rizielio et al developed a fast micellar electrokinetic capillary chromatography (MEKC) mode employed to capillary electrophoresis. They report that this rapid MEKC method for HMF quantification provides accurate and precise results suitable for routine quality control analysis of honey (Rizelio et al., 2012).

2.5.2 Caramelization

Caramelization is a nonenzymatic browning reaction. Sugars darken to form brown colored polymers under alkaline or acidic conditions when heated above their melting point. HMF and furfural are precursors for color development (Kroh, 1994). HMF is made from hexoses through acid-catalyzed dehydration and cyclization mechanism (Capuano and Fogliano, 2011). Furfural and methylfurfural are produced mainly from pentoses. HMF formation is dependent on temperature, pH, water activity, acidity, presence of bivalent metals, organic or inorganic acids, or salts in the reaction

media. The formation of caramel colors increase as temperature and pH increases (Srinivasan and Kirk 2017). During caramelization, the reducing carbohydrates, directly undergo 1, 2 enolization, dehydration, and cyclization reactions (Capuano and Fogliano, 2011). In other words, sucrose is hydrolyzed to glucose and fructose. Further degradation of these compounds forms HMF (Antal et al., 1990).

Caramelization requires higher temperatures than the Maillard reaction to develop but does not require the presence of any amino acids or proteins (Srinivasan and Kirk 2017). Similarly, different sugars have a different impact on the formation of HMF; for example, fructose is apparently twice as reactive as glucose (Capuano and Fogliano, 2011). Colors produced by caramelization could affect the quality and consumer perception. Color can be used as a measurement of reaction extension in the product since the appearance of color could be due to the formation or degradation of compounds undergoing reactions (Francis, 1995).

2.5.2.1 Caramel colors

Caramel colors are a group of similar polymeric compounds formed from five and six membered rings with different properties (Srinivasan and Kirk 2017). These colors are dark brown to black liquids or solids having an odor of burnt sugar and a pleasantly bitter taste (Sengar and Sharma, 2014) and are produced by heating food-grade carbohydrates including dextrose, lactose, malt syrup, molasses, starch hydrolysates and fractions and sucrose (FDA, 21CFR73.85, 2019). Food grade acids, alkalis, and salts are used to assist the caramelization such as acetic acid, citric acid, ammonium hydroxide, sodium, and phosphate. Catalysts can increase the reaction rate and affect the specific type of color produced (Srinivasan and Kirk 2017). According to 21CFR73.85 caramel shall not have more than 10 ppm lead, 3 ppm arsenic and 0.1 ppm mercury.
According to JECFA 1992; Codex 1996 there is four types of caramel colors:

- Caramel Color I (also known as plain or spirit caramel)
- Caramel Color II (caustic sulfite caramel)
- Caramel Color III (ammonia caramel, baker's and confectioner's caramel)

- Caramel Color IV (known as sulfite-ammonia, soft drink caramel, or acid-proof caramel).

Class I caramel colors are made by heating a carbohydrate without a source of an ammonium or sulfite ion. Acids or bases are used to generate the color. Class II caramel colors are formed in the presence of a sulfite source and the absence of ammonium ions. Caramel added to beers and other alcoholic beverages are examples of this group. Class III caramel color is made in the presence of a source of an ammonium ions and absence of sulfite ions source. This caramel is reddish-brown and is used in bakery products and syrups. Class IV caramel is formed in presence of both ammonium and sulfite ion sources. This produces a very brown color is used in soft drinks, baked goods, and syrups (Srinivasan and Kirk 2017). Isoelectric points (pIs) of caramel helps determine the possibility of application of caramel in different products. Caramels may be positive (pI 5.0–7.0), negative (pI 4.0–6.0), and spirit (pI < 3.0) types (Sengar and Sharma, 2014). Table (3) lists the analytical requirements for the classification of caramel colors. The complete analytical methodology for each of the above specifications can be found in the Compendium for Caramel Color (JECFA, 2000)and the Food Chemicals Codex (Codex 1996).

Table (3) - JECFA 200 classification of caramel

Parameters	Class I- E150 a	Class II- E 150 b	Class III- E150 c	Class IV- E150 d
Color intensity	0.01-0.12	0.06-0.10	0.08-0.36	0.10-0.60
Total nitrogen (%)	<0.10	<0.2	1.3-6.8	0.5-7.5
Total Sulphur (%)	<0.3	1.0-3.5	<0.3	1.4-10.0

2.5.3 Maillard reaction

Maillard reaction (MR) products contribute to the flavor and color of food products. MR involves three stages of reaction: early, advanced and final. They happen concurrently in food during processing (Nursten, 2005). The early stage involves the condensation of a carbonyl group followed by Amadori rearrangement. In food, it translates to the reaction of a reducing sugar with a free amino group. Since the concentration of primary amines in food is higher than secondary amines, primary amines are more important. The Amadori rearrangement is not reversible and takes place even at 25°C. The products made in the first step are colorless without absorption in UV light (280 nm) (Hodge, 1953).

The second step also known as the intermediate stage, includes sugar dehydration and amino acid degradation as Amadori products degrade into different compounds depending on pH (Purlis, 2010). Low pH favors the 1,2- enolisation pathway which results in the formation of HMF or furfural (Nursten, 2005). The products of this stage are colorless or yellow with strong absorption in the UV range (Nursten, 2005).

The third step or final stage includes aldol condensation, aldehyde amine condensation, and heterocyclic nitrogen compounds formation (Hodge, 1953, Nursten, 2005). The compounds formed in this step are colored with high molecular mass and are called melanoidins (Rannou et al., 2016)). HMF is considered a final product of the MR during food processing (Capuano and Fogliano, 2011).

3. Purification of polylactose using activated carbon, AMBERLITE™ FPA53 OH and AMBERSEP® 200H ion-exchange resins

3.1 Introduction

The extrusion process, as a continuous high-temperature short-time process, can change the functional and nutritional properties of food components (Masatcioglu et al., 2015). One concern due to heat treatment of food is the formation of harmful compounds such as HMF and acrylamide (Capuano and Fogliano, 2011). HMF in high concentrations is cytotoxic causing eye irritation and harming mucous membranes (Shapla et al., 2018). However, the main concern for HMF is due to its conversion to SMF (5-sulfoxymethylfurfural) which has genotoxic properties (Monien et al., 2009). Fresh honey has a low or minimal HMF concentration. The presence of simple sugars and acid in products like honey enhances HMF production (Shapla et al., 2018). Higher HMF concentration is an indication of poor storage conditions or excess heating of food products including honey(Zappalà et al., 2005). Since HMF is present in a wide range of food products, it is considered one of the main quality indexes of products like molasses and sugar (Shapla et al., 2018). HMF in honey could be formed during acid-catalyzed degradation of hexose (Fallico et al., 2008). The reaction starts with enolization. Glucose converts to 1,2-enediol then water elimination happens at C-3 and C-4 which leads to the production of 1,2- diulose (3,4-dideoxyosone), which after cyclization to a hemiacetal, a dihydrofuran, releases another molecule of water, producing HMF (Belitz, 2004).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established the limit of not more than 0.1% for 5-hydroxymethylfurfural in polydextrose (JECFA, 1995). The Codex Alimentarius Standard commission has set the maximum limit for HMF in honey at 40 mg/kg (with a higher limit of 80 mg/kg for honey originating from tropical regions) (The Codex Alimentarius, 2009). Gökmen et al. reported that lowering pH by adding citric acid to a dough containing glucose, reduced the acrylamide content of cookies by 67% while significantly increasing HMF levels (Gökmen et al., 2007). Thus, alternative mitigation strategies are needed to reduce HMF

levels after processing. HMF can be removed after processing using activated charcoal and resin adsorption.

Carbon in form of activated charcoal is used in the food industry for decolorization of different products including juice or in sugar refining. The large specific surface area of activated charcoal allows the disaccharide molecules to take up these spaces and be separated from the sugar mixture (Aljohani et al., 2018). Oligosaccharides from corn starch hydrolysates have been separated using size exclusion chromatography, ultrafiltration, and charcoal with diatomaceous earth (Waniska and Kinsella, 1980).

Furthermore, resins can be used to exchange anion or cations with sample solutions as they are used in water softening and water deionization (Bhatnagar and Kumar, 2012).

Polylactose is produced using twin-screw extrusion using lactose, glucose, and citric acid Depending on the process conditions, it is possible to have an HMF content above the Codex specification limit for an analogous ingredient, polydextrose. The purpose of this study was to investigate the contributions of activated charcoal and adsorptive resins to decolorization and HMF reduction in polylactose. The main objective was to reduce the HMF concentration to comply with the limits presented in the JEFCA polydextrose specification (JECFA, 1995) as well as to reduce citric acid concentration in the final product.

3.2 Materials and methods

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

3.2.1 Materials

3.2.2 Large scale benchtop carbon filtration

A glass column (~2463 cm^3) was filled with 10 g of diatomaceous earth (Fisher Scientific) as the lowest layer. Then a mixed-bed ion exchange resins was added to the column on top of diatomaceous earth. This consisted of 50 g of Amberlite FPA 53 OH-

and 50 g of Ambersep 200 H+ (Megazyme International). 400 g of Cabot NORIT GAC 1240 PLUS granular activated carbon (Cabon Norit Americas Inc.) was added on top as the last layer. A 20% w/v solution of polylactose in water was prepared with the ground polylactose produced using twin-screw extruder in University of Minnesota pilot plant following appendix A1 procedure. The prepared column was rinsed with 3000 mL of DI water and then 800 mL of the 20% w/v solution of polylactose was added to the column. 1000 mL of DI water was used to rinse the column after the polylactose solution eluted. All samples eluted at a rate of approximately 3 mL/min. Filtration materials were discarded and column was rinsed with double distilled water before packing the column for next filtration batch. All filtrates were mixed before spray drying.

3.2.3 Spray drying of polylactose

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

3.2.4 Chemical analysis

Dietary fiber

The integrated total dietary fiber assay procedure item number K-INTDF lot 9020101 (Megazyme International, Bray, Ireland) was used to measure the low molecular weight soluble dietary fiber (LMWSDF) that formed during extrusion process based on AOAC Method 2009.01 with some alterations. D-ribose (Sigma Aldrich) was used as the internal standard for high performance liquid chromatography (HPLC) analysis. The column used for separation of the carbohydrates by HPLC analysis was a Transgenomics CHO-411 column (Omaha, NE, USA). A Sedex 85 LT low temperature evaporative light scattering detector (ELSD –LT) (Shimadzu Corporation) was used to detect the compounds from the HPLC. The HPLC conditions used were a column temperature of

80°C, flow rate of 0.3 mL/min and a double distilled water mobile phase. The ELSD nebulizer temperature was set at 40°C and the nitrogen pressure was 250 kPa.

Lactose/ glucose

Lactose and glucose left in the extrusion product were measured using the lactose/sucrose/D-glucose enzymatic assay procedure item number K-LACSU lot 190220-1 (Megazyme International). A Shimadzu UV-1800 spectrophotometer was used for measuring absorbance (Shimadzu Corporation, Kyoto, Japan).

Citric acid

Citric acid left in the extrusion product was measured using the Megazyme citric acid assay kit item number K-CITR Lot 180802 (Megazyme International). A Shimadzu UV-1800 spectrophotometer was used for measuring absorbance (Shimadzu Corporation, Kyoto, Japan).

HMF

HMF was quantified using a method adapted from Truzzi et al., 2012. Polylactose samples were diluted in double distilled water to a concentration of 100 mg/mL and filtered through a 0.45 µm syringe filter. The sample was injected into a Shimadzu LC-2010 HT system with a UV-Vis detector (Shimadzu Corporation). All HPLC analyses used an YMC Pack ODS-AM column (YMC CO. Ltd., Kyoto, Japan). The conditions used were an isocratic mobile phase, water: methanol 95: 5v/v; flow rate, 0.4 mL/min; injection volume, 20 µL; column temperature 30°C; detection, 285 nm. The standard curve for polylactose HMF quantification (R^2= 0.999) used 5 standard solutions of HMF in double distilled water ((9.38×10^{-5} mg/mL, 1.88×10^{-4} mg/mL, 3.75×10^{-4} mg/mL, 7.50×10^{-4} mg/mL and 1.50×10^{-3} mg/mL).).

Absorption at 420 nm

The absorbance at 420 nm was used to measure the amount of brown pigments in polylactose sample. A 100 mg/mL polylactose in reverse osmosis water solution was

prepared and absorbance was measured using the Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation).

Hunter L, a, b

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

3.2.5 Statistical analysis

The values presented are the means of triplicate determinations. The data obtained for polylactose before and after mixed bed carbon filtration was analyzed using a t-test (R Studio Version 1.2.1335 © 2009-2019 RStudio, Inc.).

3.3 Results and discussion

The filtration set-up used successfully reduced lactose, glucose, and citric acid content in the filtered product (Table 4). Activated carbon can remove mono and disaccharides due to their hydrophobic nature (Scott and Vera, 2016). The addition of ion exchange resin to activated carbon was reported to remove more low molecular weight impurities than carbon alone, increasing the soluble fiber content of polylactose (Kuechel, 2017). Hernandez et al. has used different concentrations of ethanol solutions to desorb mono and di-saccharides from the carbon in the separation of oligosaccharides (Hernández et al., 2009). However, the present work demonstrates that mono and di-saccharides removal from polylactose is possible using an aqueous solution without ethanol. The mixed bed carbon filtration system reduced lactose content from 20.56% in the unfiltered sample to 9.22% in the filtered sample (Table 4). Glucose content was reduced from 7.78% to 2.83% and citric acid content was reduced from 2.01% to 0.04 %

in the filtered product (Table 4). Kuechel also reported a reduction in citric acid content while using the same filtration method (Kuechel, 2017). However, she reports lactose content increased and glucose content was unchanged after filtration.

Table (4) - Compositional analysis of polylactose and polylactose filtered using three different materials

Sample	LMWSDF (%)[1,2]	Lactose (%)[1]	Glucose (%)[1]	Citric acid (%)[1]
Polylactose	54.48±0.32[a]	20.56±0.41[a]	7.78±0.12[a]	2.01± 0.02[a]
Mixed Bed Carbon Filtered Polylactose	72.45 ± 0.35[3,b]	9.22±1.19[b]	2.83±0.22[b]	0.04 ± 0.04[b]

[1] Values are means ± one standard deviation

[2] Low molecular weight soluble fiber

[3] Measured by Medallion lab using Fiber method (AOAC 2011.25)

[a-b] Means without a common superscript letter within the same column are significantly different ($p<0.05$)

The filtration set-up used increased the dietary fiber content by almost 18%. One of the proposed methods for polydextrose purification suggests to contact the polydextrose solution in methanol, ethanol, or isopropanol with a bleaching agent like hydrogen peroxide and then filtering and drying of the final product(Torres 1987). Another method reports contacting aqueous polydextrose solution with a bleaching agent at 25-90ºC and pH of 2.5-9 and then filtering and drying of the final product (Rennhard and Hans, 1973). The second method can be carried out with a solvent pH adjustment or addition of carbonates of potassium, sodium, calcium, or magnesium (Rennhard and Hans, 1973).

Ion exchange resins used in the filtration set up eliminated the chromatographic peak proposed by Kuechel to be due to caramelization products (Kuechel, 2017). Caramelization products include caramel colors, HMF, and many other compounds (Capuano and Fogliano, 2011).

Our filtration was also able to significantly remove 98.26% of HMF content and reduced the HMF content from 0.05% in the unfiltered sample to 0.0009% in the filtered

sample (Figure 3 and Table 5). These results are similar to those reported by Kuechel (2017) as we achieved both HMF content reduction and color removal after filtration of polylactose.

Carabasa et al reported a reduction in the HMF content of fruit juice using activated carbon. They also report that increasing contact time of the fruit juice and activated charcoal as well as increasing the temperature, increases the adsorption rate of HMF to activated carbon (Carabasa et al., 1998). These researchers reported that after 50 minutes of contact with the activated carbon at a temperature of 20°C, HMF was reduced by 40%. Our results show with the 3 mL/min elution rate which results in more than 4 hours of contact with activated carbon and ion- exchange resins at room temperature (20-22°C) HMF content was reduced by 98.26%. Contact time could be adjusted to achieve a faster elution.

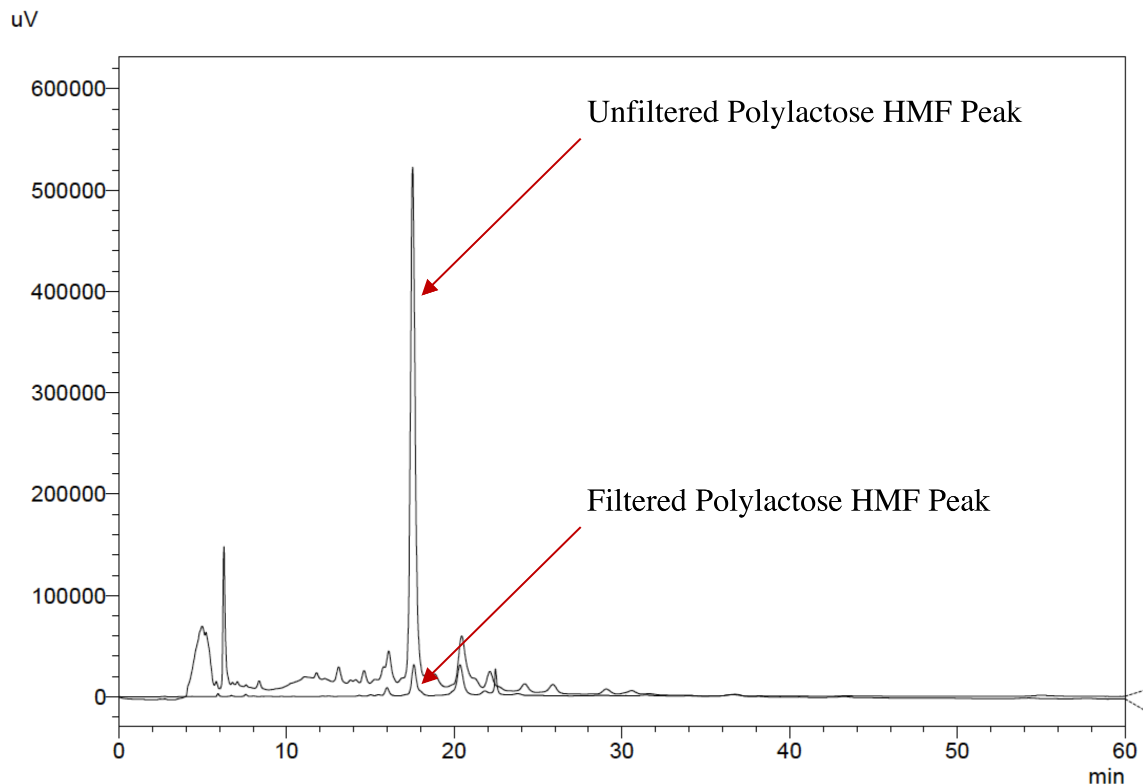

Figure 3. HPLC-UV chromatogram showing the HMF peak before and after filtration through a mixed bed carbon: ion exchange: diatomaceous earth column after spray drying.

Table (5) –HMF content for polylactose and mixed bed carbon filtered polylactose

Sample	**mg HMF per 100 mg polylactose[1]**
Polylactose	0.05±0.15[a]
Mixed Bed Carbon Filtered Polylactose	0.0009±1.23[b]

[1] Values are means ± one standard deviation

[a-b] Means without a common superscript letter within the same column are significantly different ($p<0.05$)

The Hunterlab "L" scale is a measure of dark or lightness and a low L value (0-50) indicates a darker color, and a high number (51-100) indicating a lighter color (HunterLab, 2016).

The "a" scale is the indication of red vs green with a positive number means red and a negative number means green (HunterLab, 2016).

The "b" scale is the indicator of yellow vs blue with a positive number indicating yellow and a negative number indicating blue (HunterLab, 2016).

Table (6) - Polylactose color before and after filtration treatment

Sample	*L*[1]	*a* [1]	*b* [1]
Polylactose	93.94±0.001[a]	-0.144±0.001[a]	1.114±0.001[a]
Mixed Bed Carbon Filtered Polylactose	100.11±0.01[b]	-0.093±0.001[b]	-0.173±0.001[b]

[1] Values are means ± one standard deviation (n=3)

[a-b] Means without a common superscript letter within the same column are significantly different ($p<0.05$)

Filtration using Ambersep, Amberlite, and carbon increased the "L" and "a" values while decreasing the "b" value (Table 6). This work demonstrates the filtration procedure used changed polylactose color to a lighter, less green with more blue color. Colored compounds are formed during the thermal decomposition of sugar. In

polylactose production, the presence of citric acid can assist with the formation of caramel colors (21CFR73.85, 2019). Caramels are polar compounds (Henke et al., 2019). Due to the lack of presence of amino acids and nitrogen in polylactose, Maillard reactions are not likely to produce melanoidins. In the sugar industry, a strong- base anion resin is used to remove colorants from sugar solution (Broadhurst and Rein, 2003). Most colorants in sugar solutions are in anionic form at usual pH conditions (Henke et al., 2019). That is why Amberlite, a commercial strong-base anion (SBA) resin containing quaternary amino groups could be used for the decolorization of sugars. However caramels are relatively uncharged (Guimarães et al., 1996). Therefore, the presence of activated charcoal is necessary to remove color. The polystyrenic matrix of Ambersep with the aromatic group in the structure has a high affinity to aromatic groups which are found in colorant molecules but styrenic resins do not retain caramels(Henke et al., 2019). This also emphasizes the application of activated charcoal to solve this issue by removing caramel colors. Cruz-Tirado et al. reported that the adsorption of colored compounds onto resin do not depend on the temperature of filtration, but did increase as the concentration of resins increased (Cruz-Tirado et al., 2018). This is due to the added surface area by introducing more resin material. They report observing a clearer sugar cane sample as L value increase and a decrease in a and b value (Cruz-Tirado et al., 2018).

Figure 4. Unfiltered polylactose (right) mixed bed carbon filtered polylactose (left)

Our designed filtration method significantly increased the pH from 2.66 ± 0.03 to 3.95 ± 0.03 ($P<0.05$). This could be due to significant reduction of citric acid content after filtration (Table 4).

3.4 Conclusion

Ambersep and Amberlite resins were chosen since their performance as a part of Megazyme integrated total dietary fiber kit have been tested and their ability to remove color compounds and organic acids have been demonstrated. Filtration of polylactose using activated carbon, Ambersep, and Amberlite resulted in an increase in LMWSDF. This filtration reduced lactose, glucose, and HMF content in the final product. The HMF content was decreased to lower than the limit set by JEFCA/FAO for polydextrose (0.1%). The color was successfully removed resulting in a white powder convenient to be used in the different dietary supplement or food formulations. Overall, it is clear that this filtration set up is an efficient purification method for polylactose. However, the application of the food-grade purification system explained in the next chapter addresses food safety and regulatory concerns associated with Ambersep resin.

4. Purification of polylactose using activated carbon and ion-exchange resins

4.1 Introduction

Adsorption is widely used for purifying contaminated fluids with undesirable color, smell, or taste. Adsorbents and ion exchange material are used in different processes including bed processes with fixed bed reactor, column chromatography processes, and moving bed processes (Kammerer et al., 2011). Carbon and resins are both used in the purification of high fructose corn syrup. Activated carbon is used for color removal and the removal of color precursors and sugar degradation products. Ion exchange resins are used to eliminate ionic materials from fructose syrup (Atiyeh and Duvnjak, 2005)

Carbon is commonly used to refine carbohydrate-containing food products and for water filtration. The exhausted carbon can be regenerated using chemical and thermal methods (Aljohani et al., 2018). Campos and co-workers report activated charcoal reduces the color of chicory extract while maintaining the FOS (soluble fiber) content (Campos et al., 2017). Carabasa and co-workers reported activated carbon decreased the HMF content of peach juice (Carabasa et al., 1998). Morales et al reported that charcoal removed mono and disaccharides from honey samples supplemented with maltodextrin (Morales et al., 2006).

Ion-exchange resins are synthetic resins that are polymeric adsorbents and have a large surface area for selective adsorption and desorption of molecules (Kammerer et al., 2011). A large surface area, multiple active sites, and mechanical stability are among the factors that differentiate an efficient adsorbent (Gusain et al., 2020). Moreover, the size of the material being adsorbed affects the efficiency as the smaller size compounds are adsorbed more quickly and easily(Li et al., 2015). pH affects the surface charges of resins and can regulate the adsorption process (Gusain et al., 2020). The ability of resins to adsorb unwanted compounds is responsible for their application in purification processes. Some resins including resins designed and made by Purolite are used for the removal of heavy metals from water (Sofińska-Chmiel and Kołodyńska, 2016). Ion exchange resins

are widely used for decaffeination of liquid extracts and for sugar decolorization (Stringfield, 1990, Serpen et al., 2007, Dawson-Ekeland, 1991).

Purolite® A860S, C150SH, and A847S resins are microporous spherical beads primarily used for sugar decolorization. These resins are regeneratable using a backwash method by running water backward through the bottom of the bed. The backwash will free particulate matter, clear the bed of bubbles, and rearrange the resin particles ensuring minimum resistance to flow (Sofińska-Chmiel and Kołodyńska, 2016). Carvalho et al. report A860 resin removed furfural compound formed during xylitol production from hemicellulosic hydrolysate from 0.056 to 0.01 (g dm -3). The resin also reduced HMF from 0.176 to 0.076(g dm $^{-3}$) (De Carvalho et al., 2004)

Some lactose loss may be observed while using resin filtration. It is recommended that to reduce lactose losses during ion exchange processing by 43%, gel structured anion resin (A847S) should be coupled in series with a gel structured cation resin (Smith, 1999). Jamil et al. report that the granulated activated carbon adsorbs more hydrophobic compounds than Purolite A860S (Jamil et al., 2019).

Polylactose is produced using a twin-screw extruder using lactose, glucose, and citric acid. This process can result in production of HMF through caramelization reactions. Depending on the processing conditions, higher concentrations than desired may be present. We hypothesized that activated charcoal and resin adsorption could be used as a means for decolorization, reducing HMF content, and reducing the citric acid content while maintaining high dietary fiber levels. The objective of this study was to develop a filtration method using food-grade resins and carbon to decolor, reduce HMF concentration of polylactose to comply with the limit presented in the JEFCA polydextrose specification (JECFA, 1995), and reduce the citric acid content.

4.2 Materials and methods

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

4.2.1 Materials

4.2.2 Small scale benchtop filtration using Purolite resins and activated carbon

A Bio-Rad 20 mL disposable polypropylene column (Hercules, CA, USA) was filled with 0.5 g of diatomaceous earth (Fisher Scientific Education, S25298) and 7 g of NORIT GAC 1240 PLUS granular activated carbon (Cabot Norit Americas Inc.) and 7 g of Purolite resin (Purolite A860 resin (polyacrylic macroporous, chloride form) or Purolite C150 resin (polystyrenic microporous, strong acid cation resin, hydrogen form) or Purolite A847 resin (polyacrylic Gel, Weak Base Anion Resin, Free Base form)). It was then rinsed with 20 mL of reverse osmosis water which was disposed. Forty mL of polylactose solution in reverse osmosis water (50 mg/mL or 100mg/mL or 150 mg/mL) was added to the column. Twenty mL of reverse osmosis water was used to rinse the column. All samples eluted at a rate of approximately 1 mL/min. The collected filtered sample and rinse water were freeze dried and used for further analysis.

4.2.3 Chemical analysis

Dietary fiber

The integrated total dietary fiber assay procedure item number K-INTDF lot 9020101 (Megazyme International, Bray, Ireland) was used to measure the low molecular weight soluble dietary fiber (LMWSDF) that formed during the extrusion process based on AOAC Method 2009.01 with some alterations. D-ribose (Sigma Aldrich) was used as the internal standard for high performance liquid chromatography (HPLC) analysis. The column used for HPLC analysis was a Transgenomics CHO-411 column (Omaha, NE, USA) . A Sedex 85 LT low temperature evaporative light scattering detector (ELSD) (Shimadzu Corporation) was used instead of a refractive index detector. The HPLC conditions used were a column temperature of 80°C, flow rate of 0.3 mL/min and a double distilled water mobile phase. The ELSD nebulizer temperature was set at 40°C and the nitrogen pressure was 250 kPa.

Lactose/ glucose

Lactose and glucose left in the extrusion product before and after filtration were measured using the lactose/sucrose/D-glucose enzymatic assay procedure item number K-LACSU lot 190220-1 (Megazyme International). A Shimadzu UV-1800 spectrophotometer was used for measuring absorbance (Shimadzu Corporation, Kyoto, Japan).

Citric acid

Citric acid left in the extrusion product was measured using the Megazyme citric acid assay kit item number K-CITR Lot 180802 (Megazyme International). A Shimadzu UV-1800 spectrophotometer was used for measuring absorbance (Shimadzu Corporation, Kyoto, Japan).

HMF

HMF was quantified using a method adapted from Truzzi et al., 2012. Polylactose samples were diluted in double distilled water to a concentration of 100 mg/mL and filtered through a 0.45 µm syringe filter. The sample was injected into a Shimadzu LC-2010 HT system with a UV-Vis detector (Shimadzu Corporation). All HPLC analyses used an YMC Pack ODS-AM column (YMC CO. Ltd., Kyoto, Japan). The conditions used were an isocratic mobile phase, water: methanol 95: 5v/v; flow rate, 0.4 mL/min; injection volume, 20 µL; column temperature 30°C; peak detection at 285 nm. The standard curve for polylactose HMF quantification (R^2= 0.999) used 5 standard solutions of HMF in double distilled water ((9.38×10^{-5} mg/mL, 1.88×10^{-4} mg/mL, 3.75×10^{-4} mg/mL, 7.50×10^{-4} mg/mL and 1.50×10^{-3} mg/mL).).

Absorption at 420 nm

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

Hunter L, a, b

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

4.2.4 Statistical analysis

The values presented are the means of duplicate determinations. All data were analyzed with univariate analysis of variance (ANOVA) coupled with a Tukey Honest Significant Difference (HSD) test using R Studio (R Studio Version 1.2.1335 © 2009-2019 RStudio, Inc.).

4.3 Results and discussion

4.3.1 Impact of different resin types used in carbon filtration system

Three different Purolite ion-exchange resins along activated carbon were compared to identify the most efficient filtration system. The resins provided by Purolite included A860, C150, and A847.

All filtration systems showed an increase in soluble dietary fiber content while reducing lactose, glucose and citric acid content as the previously designed filtration using Ambersep, Amberlite with activated carbon also did (Table 4). Activated carbon can remove mono and disaccharides due to their smaller size during the size exclusion process. This will increase the LMWSDF content as they are larger molecules. This also explains the reduction in glucose and lactose content after filtration.

Table (7) - Compositional analysis of unfiltered polylactose and polylactose filtered using three different methods[1]

Sample	LMWSDF (%)[2]	Lactose (%)	Glucose (%)	Total[3] (%)	Citric acid (%)	pH
Polylactose	54.48±0.32 [c]	20.56±0.41 [b]	7.78±0.12 [b]	82.82	2.01± 0.02 [c]	2.66±0.01 [a]
A860-Carbon	93.77±3.89 [a]	17.90±1.27 [b]	1.41±0.00 [a]	113.08	0.31±0.09 [a]	3.19±0.02 [b]
C150-Carbon	72.25±0.70 [b]	8.15±0.89 [a]	3.96±1.87 [c]	84.36	1.55±0.12 [b]	3.72±0.02 [b]
A847-Carbon	92.24±2.05 [a]	8.55±0.81 [a]	3.90±1.27 [c]	104.69	0.02±0.05 [a]	5.30±0.14 [c]

[1] Values are means ± one standard deviation (N = 2),

[a-c] Means without a common superscript letter within the same column are significantly different ($p<0.05$)

[2] Low molecular weight soluble fiber

[3] Total of LMWSDF, lactose and glucose

The total of LMWSDF, lactose, and glucose exceeds 100% for A860-Carbon and A847-Carbon systems. This is due to the drastic increase in the LMWSDF content of samples treated with these systems.

Using anion exchange resin paired with cation exchange resin is a common practice to ensure complete deionization of a product. But our initial results showed pairing anion exchange resins with cation exchange resins interfered with HMF removal. This could be since they neutralize each other reducing absorbance of HMF as a hydrophilic compound.

The polylactose sample had 0.046±0.00 mg HMF per 100 mg sample before filtration treatment. All filtration systems resulted in a significant reduction in HMF content. The A860-Carbon system reduced the HMF content by 91.73% to 0.0038±0.00 mg HMF per 100 mg sample (Table 8). The C150-Carbon system reduced the HMF content by 91.08% to 0.0041±0.00 mg HMF per 100 mg sample and the A847-Carbon

system reduced the HMF content by 91.95% to less than 0.0037±0.00 mg HMF per 100 mg sample.

Table (8)- Polylactose HMF content comparison for samples treated with different filtration systems

Filtration system	**mg HMF per 100 mg unfiltered polylactose**	**mg HMF per 100 mg filtered polylactose**	**HMF reduction %**
A860-Carbon	0.046±0.00	0.0038±0.00 [a]	91.73 [a]
C150-Carbon	0.046±0.00	0.0041±0.00 [a]	91.08 [a]
A847-Carbon	0.046±0.00	0.0037±0.00 [a]	91.95 [a]
Resin Mix- Carbon	0.046±0.00	0.027±0.00 [b]	41.30 [b]

[1] Values are means ± one standard deviation (N = 2),

[a-b] Means without a common superscript letter within the same column are significantly different ($p<0.05$)

The 860 resin was reported to remove furfural compounds formed during xylitol production from hemicellulosic hydrolysate rather than HMF (De Carvalho et al., 2004). HMF ($C_6H_6O_3$) formation is interconnected to that of furfural (C_4H_3OCHO). HMF could form from furfural under conditions in which dehydration of monosaccharides proceeds (Karinen et al., 2011). HMF is the product of hydroxymethylation of furfural (Lecomte et al., 1999). A860S resin has a quarternary ammonium functional group which has a hydrophobic moiety (Loontjens, 2013). This could explain why this resin had the least HMF reduction as HMF is a hydrophilic compound. Furfural has a topical polar surface area of 13.1 Å^2 and HMF has a TPSA of 50.4 Å^2 (PubChem, 2020). TPSA can affect the absorption as the higher TPSA is, the more the surface sum over all polar atoms or molecules is. More surface area provides more available sites for adsorption.

Mixing anion exchange and cation exchange resins for demineralization is a common practice (Atiyeh and Duvnjak, 2005). However, our initial results showed mixing resins reduces HMF removal efficiency since by mixing cation and anion exchange resins only a 41.30% reduction in HMF content was observed.

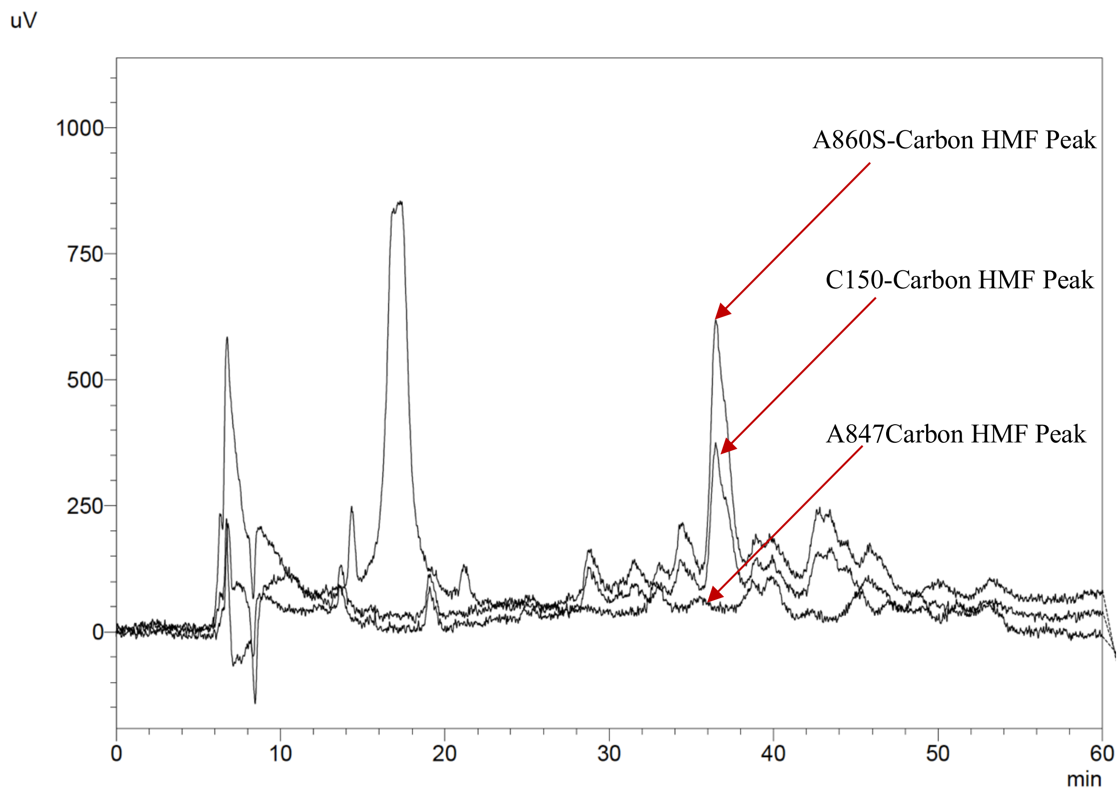

Figure 5. Comparison of HMF peak of polylactose filtered using three different purification systems using Shimadzu LC with UV-Vis detector

All filtration systems increased the pH from the initial 2.66±0.01. The A847-Carbon system had the largest pH increase (5.30±0.14) as it also reduced the citric acid content to the lowest amount in the conditions we evaluated (Table 7). The A860-Carbon system showed the least pH increase (3.19±0.02) although it did decrease citric acid content significantly, and more than the C150-Carbon system. The C150-Carbon system increased the pH to 3.72±0.02. The C150-Carbon system had the least reduction in citric acid content.

All filtration systems increased "L" value and "a" value while decreasing "b" value (Table 9). This work demonstrates that the filtration procedures used changed solutions of ground polylactose color to lighter, less green with more blue color as was also observed in the filtration system reported by Kuechel (Kuechel, 2017). Hunter L, a, and b values are color scales based on Opponent-Color Theory which assumes that the

receptors in the human eye perceive color as pairs of opposites. “L” is light vs dark, “a” is red vs green, and “b” is yellow vs blue (Hunter, 1975). A860-Carbon, C150-Carbon, and A847-Carbon showing the same values for L, a and b show human eye perceives all three samples the same color (Table 9).

Absorbance at 420 nm can be used to measure the amount of brown pigments from Maillard and caramelization reactions in a product (Kroh, 1994). All filtration systems reduced absorbance at 420 nm. Carabasa et al. also reported a decrease in the absorbance at 420nm as color compounds are removed (Carabasa et al., 1998). The presence of citric acid in polylactose production increases caramel color production (21CFR73.85, 2019, Reid, 2015). The A860S resin, like Amberlite, is a strong- base anion resin with quaternary ammonium functional group (Purolite®). These types of resin are frequently used in sugar refining (Davis, 2001). The C150 and A847 resins have aromatic groups within their styrenic matrices which increase their affinity to absorb aromatic groups present in colorant molecules but these resins do not retain caramels (Henke et al., 2019). This explains the need for activated carbon in the filtration design to remove colors.

Table (9) – Hunterlab values and absorbance at 420 nm for polylactose and polylactose filtered using three different systems after resolubilizing dried filtrate

Sample	**L**	**a**	**b**	**absorbance at 420 nm**
Polylactose	78.40±0.001 [a]	-5.39±0.001 [a]	23.71±0.001 [a]	0.143±0.00 [a]
A860-Carbon	85.32±0.001 [b]	-1.93±0.001 [b]	9.32±0.001 [b]	0.051 ±0.001 [b]
C150-Carbon	85.32±0.001 [b]	-1.93±0.001 [b]	9.32±0.001 [b]	0.023±0.001 [c]
A847-Carbon	85.32±0.001 [b]	-1.93±0.001 [b]	9.32±0.001 [b]	0.076±0.00 [d]

[1] Values are means ± one standard deviation (N = 2),

[a-d] Means without a common superscript letter within the same column are significantly different ($p<0.05$)

4.3.2 Impact of order of resin and carbon in carbon filtration system

In order to further evaluate the purification, the order of resin and carbon was studied. Jamil et al. report granulated activated carbon followed by Purolite resin treatment removed more hydrophobics and Purolite followed by granulated activated carbon removed more hydrophilic (Jamil et al., 2019). Our results showed treatments using activated carbon followed by Purolite resins absorbed more of the low molecular weight dietary fiber content compared to treatments using Purolite resins followed by activated carbon (Table 10). We propose this is a result of activated charcoals pores adsorbing mono and disaccharides since they are smaller in size. This leaves resins unsaturated to remove undigestible carbohydrates with degree of polymerization greater than 2 (LMWSDF). The common order for purification systems used for high fructose corn syrup is activated carbon for color removal followed by ion-exchange for demineralization (Atiyeh and Duvnjak, 2005). However, our study showed Purolite resins followed by activated carbon had better retention of LMWSDF in the filtrate. As a part of the integrated dietary fiber analysis, ash (total minerals) were measured to correct the dietary fiber content. The insignificant ash content could suggest low total mineral content of polylactose after filtration. The low mineral content of polylactose could explain why de-mineralization is not as important for polylactose as it is for high fructose corn syrup.

Passing polylactose solution through Purolite resin first reduced citric acid to a greater extent. The exception was for Purolite A874 resin where the citric acid reduction was the same for both orders. Purolite resin- activated carbon order for treatments using Purolite A860 and C150 did not make a difference in lactose content. However, treatment using A847 resin followed by carbon resulted in significantly lower lactose content compared to the reverse order.

Purolite resin- activated carbon order for treatments using Purolite A847 did not make a difference in glucose content however for treatments using A860 or C150 resins followed by carbon had significantly lower glucose content compare to the reverse order.

Table (10) - Compositional analysis of polylactose filtered using different methods and changing resin-carbon order[1]

Sample	LMWSDF (%)[2]	Lactose (%)	Glucose (%)	Citric acid (%)	HMF (%)
A860-Carbon	93.77±3.89[c]	17.90±1.27[b]	1.41±0.00[a]	0.31±0.09[b]	0.0038±0.00[a]
Carbon-A860	56.91±1.58[a]	15.46±0.59[b]	11.43±1.24[b]	0.01±0.02[a]	0.0130±0.00[c]
C150-Carbon	72.25±0.70[b]	8.15±0.89[a]	3.96±1.87[c]	1.55±0.12[c]	0.0041±0.00[a]
Carbon-C150	52.21±0.38[a]	11.75±1.11[a]	7.21±0.29[d]	0.25±0.01[d]	0.0059±0.00[b]
A847-Carbon	92.24±2.05[c]	8.55±0.81[a]	3.90±1.27[c]	0.02±0.05[d]	0.0037±0.00[a]
Carbon-A847	54.58±0.32[a]	30.50±0.59[c]	3.30±0.93[c]	0.02±0.02[d]	0.0042±0.00[a]

Values are means ± one standard deviation (N = 2),

[a-d] Means without a common superscript letter within the same column are significantly different ($p<0.05$)

[2] Low molecular weight soluble fiber

All filtration systems had a significant reduction in HMF content compare to unfiltered polylactose. The A860-Carbon system reduced the HMF content by 91.73% while the Carbon- A860 treatment had a 71.73% reduction. The C150-Carbon system reduced the HMF content by 91.08% while the Carbon-C150 had an 87.17% reduction, and the A847-Carbon system reduced the HMF content by 91.95% while the Carbon-A847 system had a 90.86% reduction. HMF is hydrophilic and highly soluble in water (Capuano and Fogliano, 2011). HMF has topological polar surface area of 50.4 $Å^2$. These results show filtrate passed through resin first had less HMF content which supports Jamil et al. observations reporting Purolite A860 resins followed by granulated activated

carbon removed more hydrophilic compounds (Jamil et al., 2019). The filtration systems that consisted of resin, followed by activated carbon had higher LMWSDF content. This could be due to resins being saturated by HMF and other soluble color compounds. Mono and disaccharides are also smaller molecules compare to LMWSDF that could saturate the resins pores and allow LMWSDF to pass. Moreover, activated carbon placed after resins can further remove mono and disaccharides due to their higher hydrophilic tendency compare to LMWSDF and let LMWSDF remain in the eluent. LMWSDF are carbohydrates with the degree of polymerization more than 2 which their elution results in an increase in the dietary fiber content.

Furthermore, filtrate passed through resin first had higher pH compare to the filtrate passed through carbon first.

4.3.3 Impact of different polylactose concentrations on filtration

The A847-Carbon system significantly increased soluble dietary fiber content for all concentrations. However, as the polylactose solution concentration increased, the LMWSDF content decreased. An increase in the polylactose solution concentration did not significantly change glucose and citric acid content. Lactose content was the highest for 100mg/mL polylactose solution followed by 15 mg/mL polylactose solution. The A847-Carbon system reduced the HMF content of both 50 mg/mL and 100mg/mL polylactose solution by 91.85% to less than 0.0037±0.00 mg HMF per 100 mg sample. It reduced the HMF content of 150 mg/mL polylactose solution by 84.85% to less than 0.0069±0.00 mg HMF per 100 mg sample (Table 11). These results demonstrate that the concentration of the solution did affect the efficiency of HMF removal.

The A847-Carbon system significantly increased the pH from 2.66 for unfiltered polylactose. However, this increase was the same for 50mg/mL solution and 100 mg/mL solution. After filtration, the pH increased for the 50 mg/mL solution to 5.30±0.14 as citric acid content decreased to the lowest, for 100 mg/mL pH increased to 5.45±0.49 as citric acid content decreased and for 150 mg/mL pH increased to 3.91±0.01. 150 mg/mL had the least pH increase and citric acid content decrease.

Table (11) - Compositional analysis of different concentrations of polylactose solution filtered using Purolite A847-Carbon [1]

Polylactose Concentration (mg/mL)	LMWSDF (%)[2]	Lactose (%)	Glucose (%)	Citric acid (%)	HMF (%)
50	92.24±2.05[b]	8.55±0.81[a]	3.90±1.27[a]	0.02±0.05[a]	0.0037±0.00 [a]
100	81.78±3.86[c]	33.29±2.77[c]	5.36±001[a]	0.05±0.01[a]	0.0037±0.00 [a]
150	76.78±3.63[a]	24.54±0.74[b]	1.92±0.39[a]	0.06±0.09[a]	0.0069±0.00[b]

1 Values are means ± one standard deviation

2 Low molecular weight soluble fiber

a-d Means without a common superscript letter within the same column are significantly different ($p<0.05$)

4.4 Conclusion

Filtration of polylactose using activated carbon and Purolite resins resulted in an increase in LMWSDF. This filtration reduced lactose, glucose, citric acid, and HMF content in the final product. The HMF content was decreased to lower than the limit set by JEFCA/FAO for the polydextrose specification (JECFA, 1995). The color was removed which resulted in a white powder similar to what was achieved by Ambersep, Amberlite, and carbon filtration in previous experiments. Furthermore, replacing Ambersep with food-grade resins addresses the regulatory concerns of polylactose production for food, supplement, or feed formulations. The order of resin and activated carbon experiments demonstrated that systems using resin followed by activated carbon had better dietary fiber retention in the filtrate and higher pH while systems using activated carbon first provided filtrate with lower pH. Resin followed by activated carbon systems reduced HMF content more than activated carbon followed by resin systems. Considering all the factors analyzed, we suggest the A874-Carbon system as an efficient purification system for polylactose clean-up.

For evaluating the efficiency of the purification process, three different polylactose solution concentrations were filtered using the A874-Carbon system. As the

concentration of the polylactose solution increased, the LMWSDF content decreased. However, the HMF content reduction was the same for 50 mg/mL and 100 mg/mL of polylactose solution.

For scale-up purposes, through-put is a major factor. Filtering 100 mg/mL polylactose solution will provide more dried filtered polylactose product compare to filtering the same volume of 50 mg/mL polylactose solution. Although both products will have the same HMF content, the product from 50 mg/mL polylactose solution filtration will have slightly higher LMWSDF content.

5. Effects of lactase on polylactose

5.1 Introduction

β-Galactosidase (lactase) is one of the most useful enzymes in the dairy industry and is used to hydrolyze lactose for the production of lactose-free products or it is utilized for producing galactooligosaccharides from lactose by a transgalactosylation reaction. β-Galactosidase is located in the brush border of the small bowel mucosa of humans and other mammals and is used to digest milk (Azcarate-Peril et al., 2017). A deficit in the lactase enzyme causes lactose malabsorption since the disaccharide cannot be absorbed and is instead fermented by gut microbiota, leading to the development of clinical symptoms characteristic of lactose intolerance (Leis et al., 2020)

The β-Galactosidase enzyme can be prepared from fungi like *Aspergillus niger* or yeast like *Kluyveromyces lactis* to be used in industry (Belitz, 2004). *Kluyveromyces lactis, Bacillus circulans, Aspeergillus oryzae, Bifidobacterium bifidum* and *Sporobolomyces singularis* are among enzyme sources utilized to produce β-Galactosidase enzyme used in industrial production of GOS (Table 1). Ha-Lactase^{TM} is a liquid ß-galactosidase which is produced by fermentation of *Kluyveromyces lactis* on a vegetable substrate (HANSEN, 2020, CHR-HANSEN, 2020). Application of β-Galactosidase enzyme produced by different sources makes the production of different GOS purities possible. β-Galactosidase obtained from *Kluyveromyces lactis* has high lactose hydrolysis activity (Kim et al., 2004). β-Galactosidases from *A. oryzae* and *B. circulans* exhibited high transgalactosylation activity (Guerrero et al., 2015). β-galactosidases from fungi sources optimally work between pH 2.5-5.4 while yeast and bacterial produced enzymes are active between pH 6.0- 7.0 (Husain, 2010). Vera et al. also report pH between 2.5-5.5 as the optimum range for ß-galactosidase (Vera et al., 2011). Fungal β-galactosidases are more sensitive to galactose inhibition but are thermostable (Boon et al., 2000). Bacterial β-galactosidases have good stability and high activity (Picard et al., 2005).

β-Galactosidase catalyzes the hydrolysis of β(1-4) glycosidic linkage of β-galactosides which are carbohydrates containing galactose β linked components like

lactose and lactulose (Gonawan, 2019). The selectivity of this enzyme depends on the presence of tertiary structure and amino acids in the active site and concentrations of lactose as donor and acceptor of the transgalactosylated galactose (Guerrero et al., 2015).

Monosaccharides could act as inhibitors to β-galactosidase (Gonawan, 2019). This explains why the catalytic activity of this enzyme is reduced the longer it is present in the reaction system. Vera et al. report that galactose has a stronger competitive inhibition role in transgalactosylation than in hydrolysis. Yang and Okos suggested a competitive product inhibition for lactase by galactose assuming that the glucose molecule leaves the active site of enzyme first, leaving a covalent galactosyl- enzyme complex (Yang and Okos, 1989). However, the enzymatic hydrolysis of lactose occurs at low lactose concentration, and as lactose concentration increases the transgalactosylation reaction rate increases which results in the production of oligosaccharides (Iwasaki et al., 1996). Different models have been developed to explain β-galactosidase performance. Some models describe galactooligosaccharide synthesis as well as simultaneous lactose hydrolysis while still including product inhibition (Boon et al., 1999). Kim et al. investigated the effect of galactose and glucose on lactose conversion showing lactose hydrolysis and transgalactosylation happens concomitantly at high substrate concentrations. They developed a model in which products such as galactose and glucose did not inhibit the reaction of lactose conversion at high concentrations of lactose, and both the lactose and glucose produced were used as acceptors. Their results showed that galactose acts as an inhibitor for β-galactosidase at a lactose concentration of below 50mM (Kim et al., 2004). Galactose is not a typical competitive product inhibitor however it competes with the substrate at the binding site for the hydrolysis reaction at a low concentration of lactose. Kim also suggests glucose acts as a better acceptor for transgalactosylation reaction, but it is not an inhibitor for hydrolysis. GOS produced using β-galactosidase contains oligomers ranging from DP2 to DP10 (Coulier et al., 2009). Coulier et al report DP2 compounds include lactose and other oligomers. This could suggest the peak observed in dietary fiber analysis of polylactose for DP2 could also include other compounds than lactose possibly causing fiber like physiological effects (Figure 6).

Polylactoseproduced in 2016 and 2018 by reactive extrusion of lactose, glucose, and citric acid dry blend contained different lactose contents (21.92 vs. 10.25%) and different soluble dietary fiber contents (45.19 vs. 85.65%). Both lots were used in separate animal trials, and the lot produced in 2016 had much greater prebiotic effects than the lot produced in 2018 (Abernathy, 2020).Abernathy reports that 2016 polylactose significantly increased cecum weight which is an indicator of fermentation. It also altered species abundance, increased cecal acetate and propionate, and reduced liver lipids and fat pad weight. However, 2018 polylactose did not change liver lipids, fat pad weight, and body composition as much as 2016 polylactose did (Abernathy, 2020).

A potential reason for the different effects could be the DP2 peak containing compounds with fiber characteristics. In order to make the diet containing 6% fiber, more amount of the 2016 polylactose was fed to the rats to achieve the same fiber content as polylactose 2018 since polylactose 2016 had lower LMWSDF content. This means more DP2 fiber could have been was present in the polylactose 2016 diet. This chapter evaluates the effect of lactase (Ha-Lactase^{TM}) enzyme on dietary fiber levels of polylactose to see if β-galactosidase hydrolyzes dietary fiber in the polylactose sample This matter is of interest since this enzyme is not applied to the sample as a part of integrated dietary fiber analysis, but is present in the human body, if polylactose is digested, it will not survive to the large intestine for fermentation by gut microbiota. Moreover, the possibility of the presence of fiber with the degree of polymerization of 2 needs to be evaluated to help understand differences observed during feeding trials. The presence of this fiber could change physiological effects of polylactose in the human body, and could be the reason for the incredible results observed in the first trial when compared with other prebiotic fibers used in that study (Abernathy, 2020).

5.2 Materials and methods

The materials and methods are described in this section. The extended methods can be found in Section A.4 of the Appendix.

5.2.1 Materials

Ha-Lactase^{TM} (Chr-Hansen Holding A/S, Denmark) enzyme was used to treat polylactose lots produced in 2016 by Kuechel and in 2018 by Monsefi which were used in animal trials. Both batches were filtered using activated carbon, Ambersep and, Amberlite, then spray dried.

5.2.2 Lactase treatment

Five gr of polylactose was dissolved in double distilled water to a final volume of 50 mL and filtered using activated charcoal, Ambersep, and Amberlite. Lactase^{TM} 5200 (Chr-Hansen Holding A/S, Denmark) (0.15 mL) was added to the flask, and samples were incubated for 24 hours at 4ºC. After the incubation period samples were boiled for 5 minutes to deactivate the enzyme. Samples were then freeze dried and used for further analysis.

5.2.3 Chemical analysis

Dietary fiber

The integrated total dietary fiber assay procedure item number K-INTDF lot 9020101 (Megazyme International, Bray, Ireland) was used to measure the low molecular weight soluble dietary fiber (LMWSDF) that formed during the extrusion process and possibly after lactase treatment based on AOAC Method 2009.01 with some alterations. D-ribose (Sigma Aldrich) was used as the internal standard for high-performance liquid chromatography (HPLC) analysis. The Column used for HPLC analysis was a Transgenomics CHO-411 column (Omaha, NE, USA). Sedex 85 LT low-temperature evaporative light scattering detector (ELSD) (Shimadzu Corporation) was used. The HPLC conditions used were a column temperature of 80°C, a flow rate of 0.3 mL/min, and a double distilled water mobile phase. The ELSD nebulizer temperature was set at 40°C and the nitrogen pressure was 250 kPa.

Lactose/ glucose

Lactose and glucose left in the extrusion product were measured before and after lactase treatment using the lactose/sucrose/D-glucose enzymatic assay procedure item number K-LACSU lot 190220-1 (Megazyme International). A Shimadzu UV-1800 spectrophotometer was used for measuring absorbance (Shimadzu Corporation, Kyoto, Japan).

5.2.4 Statistical analysis

The values presented are the means of triplicate determinations. The data obtained for polylactose before and after mixed bed carbon filtration was analyzed using a t-test (R Studio Version 1.2.1335 © 2009-2019 RStudio, Inc.).

5.3 Results and discussion

Two lots of polylactose (2016 and 2018) were treated with β-galactosidase to evaluate the effect of lactase enzyme on dietary fiber levels of polylactose and obtain more information about the composition of peak observed for DP2 compounds in the dietary fiber analysis. Lactase treatment significantly reduced the lactose content of polylactose 2018 by 45.83% and by 43.52% for polylactose 2016 (Table 12). The Dietary fiber content of polylactose 2018 filtered using Ambersep, Amberlite, and activated carbon was 85.69% and for polylactose 2016 was 45.19% (Table 12). After treatment with the lactase enzyme, the dietary fiber was reduced to 79.77% for polylactose 2018 and slightly increased to 45.22% for polylactose 2016 (Table 12). Lactase treatment reduced dietary fiber content of polylactose 2018 only by 6.9% and enzyme treatment slightly increased dietary fiber content by less than 1% for polylactose 2016. The results from this study showed Ha-Lactase treatment used did not significantly change the dietary fiber content. Although it did significantly reduce the lactose content measured using Megazyme lactose kit, it did not eliminate it. The chromatograph obtained from dietary fiber analysis was also used to quantify the DP2 peak and it does not show about 50% reduction for the DP2 peak (Figure 6). The DP2 peak decreased by 1.62% for

polylactose 2016 and decreased by 13.24% for polylactose 2018. This could suggest the DP2 contains other compounds besides lactose that were not hydrolyzed by lactase. In the case of animal trials conducted using polylactose 2016 and 2018, considering a 6% fiber diet made with polylactose 2016 as the fiber source, 13.27g polylactose would be required to prepare 100 g feed. This amount of polylactose 2016 would provide 1.96g DP2 material. In comparison, using polylactose 2018 to make up the same 6% diet would require 7g polylactose for 100g feed which would contain 1.3g DP2 material. This could suggest that the difference observed between the two animal studies is led by the difference between the DP2 amount provided in the diets.

β-galactosidase catalyzes the hydrolysis of β 1,4 galactosidic linkages (Anisha, 2017). As a part of dietary fiber analysis, polylactose samples were digested using pancreatic α-amylase (PAA) and amyloglucosidase (AMG) (Megazyme, 2020). pancreatic α-amylase hydrolyses α-1,4-glycosideic internal bonds of starch, yielding glucose and maltose (Gurung et al., 2013). Amyloglucosidase cleaves α(1-6) glycosidic linkages, as well as the last α(1-4) glycosidic linkages at the nonreducing end of amylose and amylopectin, producing glucose (Gurung et al., 2013). We could suggest that besides lactose there are other compounds present in polylactose with DP2 but a different type of bond than β 1,4 galactosidic or bonds affected by PAA and AMG. This explains why β-galactosidase treatment did not completely take DP2 peak away and why PAA and AMG did not affect these compounds. Moreover, β-galactosidase catalyzes the release of terminal β-D-galactosyl residues(Eda et al., 2014). We can suggest this causes the release of some of terminal β-D-galactosyl residues that are connected to the oligomers with DP3 or more by β 1,4 galactosidic linkages, resulting in a decrease in the DP3 and above peaks which directly will decrease the LMWSDF content of polylactose as observed in polylactose 2018 after lactase treatment.

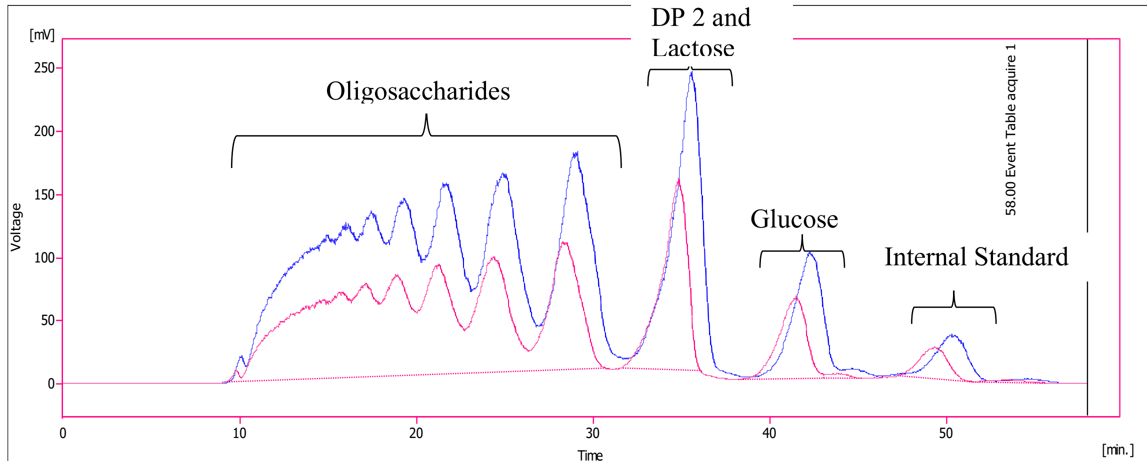

Figure 6. HPLC chromatogram comparing polylactose 2018(blue) and polylactose 2018 that was treated with Ha-Lactase (pink).

Kim et al. demonstrated galactose acts as an inhibitor for lactose hydrolysis only at low lactose concentration (Kim et al., 2004). Since lactose concentration of treated and untreated polylactose are higher than what Kim et al. suggest, we can conclude galactose did not have an inhibitory effect on lactose hydrolysis. Moreover, this galactose could have been used in the transgalactosylation reaction for the formation of trisaccharides (Kim et al., 2004).

Table (12) - Compositional analysis of polylactose and polylactose treated with Ha-Lactase1

Sample	Treatment	LMWSDF (%)[2]	Lactose (%)	DP2 %
Mixed Bed Carbon Filtered Polylactose 2018	Ha- Lactase 5200	79.77±1.87	5.55±0.0.37	22.72±0.86
Mixed Bed Carbon Filtered Polylactose 2016	Ha- Lactase 5200	45.22±0.15	12.37±0.11	15.16±0.0
Mixed Bed Carbon Filtered Polylactose 2018	None	85.69±1.53	10.25±0.86	26.19±0.13
Mixed Bed Carbon Filtered Polylactose 2016	None	45.19±1.65	21.92±0.66	15.41±0.04

[1] Values are means ± one standard deviation

[2] Low molecular weight soluble fiber

5.4 Conclusion

Using β-galactosidase commercialized by Chr-Hansen Holding as Ha-Lactase enzyme, the lactose content of the polylactose powder was significantly reduced, but the soluble dietary fiber content was not. This is beneficial for the production of polylactose as a soluble dietary fiber that survives digestion to the large intestine and can then be fermented. These results suggest polylactose as dietary fiber is not severely affected by lactase in the human body. The DP2 peak evident on the chromatogram, which had been

assumed to be lactose, did not change significantly with lactose treatment. This suggests that the DP2 peak is comprised of other components besides lactose. Isolation of the DP2 peak and further evaluation of chemical bond types and polylactose chemical structure could lead to a better understanding of polylactose effects during consumption.

6. Concluding remarks and next steps

Lactose, a component of whey and a by-product of cheese production was efficiently polymerized to produce oligosaccharides by a continuous process using twin-screw extruder. Different purification methods were developed at bench-top and scale-up size.

This research demonstrated that different materials could be used for the purification of polylactose. Polylactose was purified using activated carbon, ion exchange resins, and diatomaceous earth. Purification increases the dietary fiber content of polylactose while reducing HMF and citric acid content. Moreover, color compounds were removed to provide a white powder convenient to be used in different product formulations. Evaluations showed that lactase does not affect the dietary fiber content of polylactose, but it does reduce the lactose content. This could suggest that there are other compounds besides lactose present in polylactose that alter its prebiotic performance.

The developed purification method could be scaled up to prepare filtered polylactose in greater quantities. In addition, further characterization of polylactose is necessary to understand polylactose behavior in the human body. This could be useful to prove the physiological benefits of polylactose in order to be identified as a dietary fiber. More extensive analysis of different components of polylactose could help specify their prebiotic role. For example, separation of DP2 peak and analyzing its components could identify compounds similar to GOS products with physiological effects in the human body. Furthermore, using the same filtration materials, different filtration designs including column filtration and stir bed filtration systems could be evaluated.

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8. Appendix

A.1 Extended methods for scale-up polymerization of sugars to polylactose

A.1.1 Production of polylactose using twin screw extruder

Objective: The objective of this procedure is to develop a method to produce polylactose at a scaled-up quantity using twin screw extruder.

Material:

α-Lactose Monohydrate
Glucose
Citric Acid

Equipment:

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

Procedure:

1. Weigh ingredients for a batch of 76% lactose, 20% glucose and, 4% citric acid in a plastic bucket and pour all ingredients in the ribbon blender.
2. Close both the grated cover and the top cover of the ribbon blender. Mix for 2 minutes in both forward and reverse directions. Press stop and let the blades come to a complete stop before changing directions.
3. Open the top cover of the ribbon blender and continue mixing in either forward or reverse direction to get the powder out of the ribbon blender.
4. Slowly open the trap door at the bottom of the ribbon blender and pour the mix into a plastic bag. To make sure the powder gets into the bag, take the top of the bag and wrap it around the exit of the ribbon blender. Make sure to carefully control the flow through the trap door.
5. Use the spatula to scrape off the remaining of the mix into the bag.
   The pilot plant staff will put the extruder dies together and will start the extruder by pouring the feed into K-Tron feeder.

   Extrusion conditions:

   i. Barrel zones #2, #3, #4, #5, #6 - 238°C
   ii. Barrel zone #7 - no heating. Temperature maintained by heat transfer control system model H47212DT.
   iii. Feed rate - 15 kg/hr Feed rate controlled by K-Tron Soder K-ML-KT20 loss-in-weight feeder. Screw speed: 250 rpm.
   iv. Die plate – none

6. Start collecting the sample after the conditions are met. As the product comes off extruder allow it to drop on the metal tray in thin layers.

7. Change trays as it is full of sample. Allow the full trays to cool down on cooling rack.

Extruder set-up

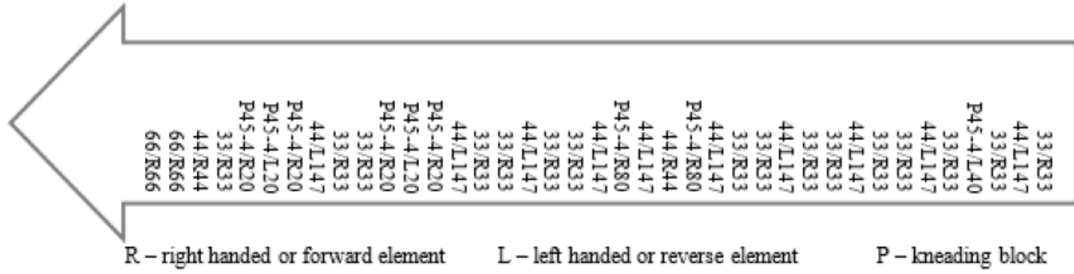

Figure A7. Screw design for Buhler 44mm twin-screw extruder used to polymerize lactose.

A.2 Extended methods for filtration of polylactose

A.2.1 Filtration of polylactose using activated carbon, Ambersep and Amberlite

Objective: The objective of this procedure is to clean up the extrusion product to remove color, citric acid and HMF while maintaining dietary fiber level.

Material:

Megazyme Amberlite FPAS53 (OH^-)

Megazyme Ambersep 200 (H^+)

NORIT GAC 1240 Plus granular activated carbon (Cabot Norit Americas, Inc.)

Diamaceous earth

Equipment:

Analytical balance

Beaker

Stir Bar

Stir plate

Funnel

Glass column

Glass bottle (1 L)

Erlenmeyer flask (2 L)

Graduated cylinder (500 mL)

Procedure:

1. Prepare the glass column filter pack by pouring 10 g diatomaceouss earth, 50 g of each resin and, 400 g of activated carbon.
2. Rinse the filter pack with 3 L of DI water and toss the collected water.

3. Add 800 mL of 20% W/V polylactose solution to the column and allow it to elute. Collect the eluent.
4. Rinse the filter with 1 L DI water and collect the eluent. Spray dry the eluent to get the dry filtered polylactose.

A.2.2 Filtration of polylactose using activated carbon and Purolite resins

Objective: The purpose of this experiment is to develop a filtration method that with bee used for scaled up polylactose purification prior to a human study.

Material:

Purolite®-A860S,

Purolite®-C150SH

Purolite®-A847S

NORIT GAC 1240 Plus granular activated carbon (Cabot Norit Americas, Inc.)

Diatomaceous earth

Equipment:

Analytical balance

Beaker

Stir Bar

Stir plate

Funnel

Test tube

Disposable column (Bio-Rad) 87

Glass vacuum manifold (Supelco)

Procedure:

1. Prepare a 50mg/mL solution of polylactose by dissolving the product in RO water.
2. Prepare column by adding 0.5g diatomaceous earth followed by 7 gr activated carbon and 7 gr resin.
3. Place a test tube under the column, and rinse the column using 20 mL DD water.
4. Change the test tube and add 40 mL polylactose solution to the column followed by 20 mL DD water.
5. Collect the filtrate for freeze drying or spray drying.

A.2.3 Spray drying of filtered polylactose

Objective: The purpose of this experiment is to dry the filtration eluent from liquid to solid dry powder form.

Materials:

Filtration eluent

Equipment:

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

Rubber mallet

Procedure:

1. The pilot plant staff will set-up the spray dryer. Spray drier needs to warm up. The conditions used are:
    - Inlet temperature: 185°C
    - Outlet temperature: 90°C
    - Flow rate: 220 mL/min
    - Atomizer : 24,000 rpm

2. Place the inlet hose in the sample bucket and start running the product into spray drier.

3. Occasionally tap the spray dryer with the rubber mallet to loosen the powder from the sides of the dryer.

4. open the trap door and collect the dried samples

A.3 Extended methods for analysis of polylactose and filtered polylactose

A.3.1 Dietary fiber quantification

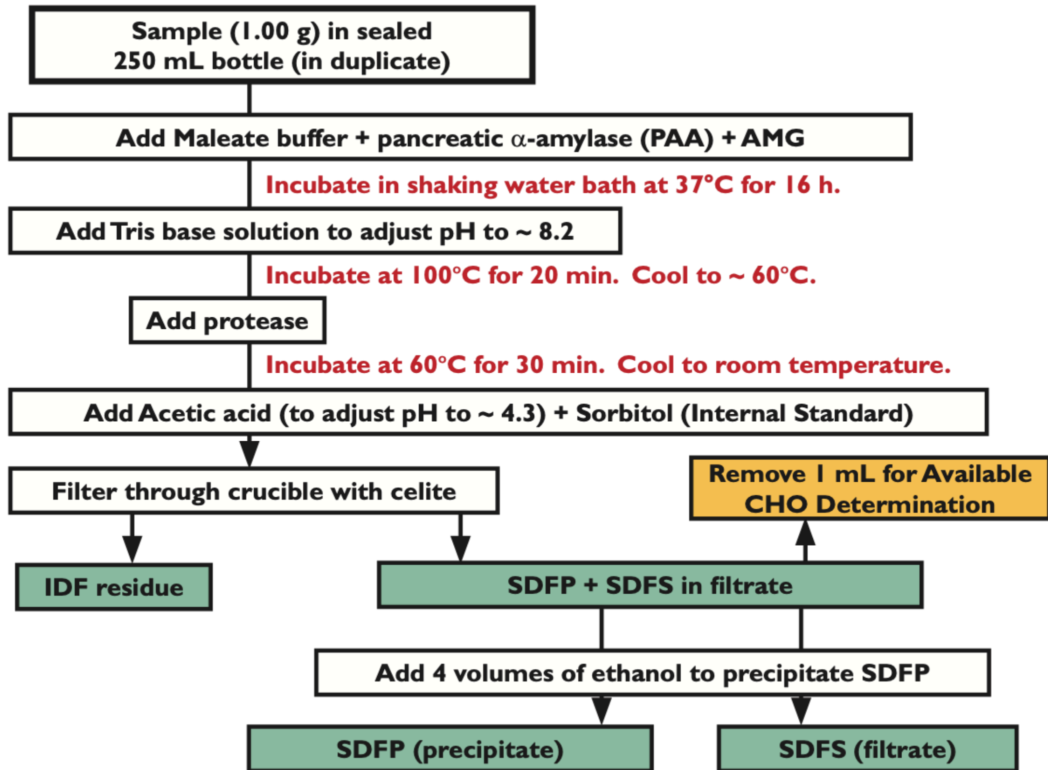

Figure A8. Principle of the Integrated (Codex compliant) Total Dietary Fiber assay procedure showing separate measurement of IDF, SDFP and SDFS.

Objective: The purpose of this experiment is to measure the content of total dietary fiber in the polylactose product filtered.

Material:

Megazyme Integrated Total Dietary (K-INTDF)

Maleic acid

Distilled water

Calcium chloride dihydrate

Tris buffer salt

Glacial acetic acid

Sodium hydroxide

Ground polymerization product

Ribose

Ethanol, 95% v/v

Distilled water

Ethanol, 78% v/v

Celite, acid washed

Acetone

Crucibles with residue

Weigh paper

Soy protein isolate (positive control)

Kjeldahl tablets

Sulfuric acid, 95-98%

Distilled water

32% NaOH

4% Boric acid

0.1 N HCl

0.2 Methyl red

Equipment:

Beaker (2000 mL)

Volumetric flask (1L, 2L)

Stir bar

Stir plate

pH meter with calibration standards, 4.0, 7.0, 10.0 (Accumet AB15/15+)

250 mL plastic bottle with a cap

Shaking water bath for 37°C incubation

Non-shaking water bath for 60°C and 95-100°C incubation

Pipettor

Volumetric flask (1 L)

Fritted crucibles: 50 mL, pore size coarse: 40-60 µm

Drying oven

Dessicator

Analytical balance

Transfer pipette

Graduated cylinder (10 mL, 25 mL)

Vacuum pump

Rubber fitting for vacuum filter flask

Vacuum filter flask (250 mL, 500 mL)

Muffle oven

Analytical balance

Volumetric flask (1L)

Metal spatula

Weigh boat

Kjeldahl digestion block (Büchi)

Kjeldahl distillation unit (Büchi)

250 mL Erlenmayer flask

10 mL buret

Ring stand

Buret clamp

Round bottom flask (250 mL)

Rotovap (Büchi)

Beaker (600mL)

Weigh boat

Disposable column (Bio-Rad) 87

Glass vacuum manifold (Supelco)

Glass test tube

Graduated cylinder (25 mL)

Vacuum pump

Pipettor

Centrifuge tube (50 mL)

Parafilm

Freeze dryer

Freeze dryer flask

Vortex mixer

5 mL disposable syringe

0.45 µm syringe filter

HPLC vial with cap and septum

Volumetric flask (100mL)

5 mL disposable syringe

0.45 µm syringe filter

HPLC vial with cap and septum

High Performance Liquid Chromatography (HPLC) system (Beckman Coulter) with Evaporative Light Scanning Detector (ELSD) (Shimadzu SEDEX Model 85-LT LT-ELSD)

Reagent preparation:

1. 4 M NaOH: Prepare the solution by dissolving NaOH in distilled water to achieve a concentration of 160 g/L.

2. Sodium maleate buffer (50mM, pH 6.0 plus 2 mM $CaCl_2$) :
   a) Calibrate the pH meter.
   b) Dissolve 11.6g of maleic acid in 1600 mL of distilled water.
   c) Adjust the pH to 6.0 with 4M NaOH.
   d) Add 0.6 g of calcium chloride dihydrate and adjust the volume to 2 L.

3. Pancreatic α-amylase (50 Units/mL)/AMG (3.4 Units/mL):
    a) Dissolve 0.10 g of purified porcine pancreatic α-amylase in 290 mL of sodium maleate buffer immediately before use.
    b) Stir for 5 minutes and add 0.3 mL of AMG.

4. 0.75 Tris base solution
    a) Add 90.8g of Tris buffer salt to approximately 800 mL of distilled water and dissolve.
    b) Adjust the volume to 2L using a volumetric flask.

5. 2M Acetic acid solution
    a) Add 115 mL of glacial acetic acid to a 1L volumetric flask.
    b) Dilute to 1L with distilled water.

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

7. 32% Sodium Hydroxide
    a) Add 320g of sodium hydroxide tablets to a 1L volumetric flask. Bring to volume with distilled water.
    b) Dissolve the sodium hydroxide. Add more water to bring to volume if needed.

Procedure:

1. Weigh duplicate samples: 1.000 ± 0.005 g samples accurately into 250 mL Fisherbrand® soda glass, wide mouth-bottles.
2. Addition of Enzymes: Wet the sample with 1.0 mL of ethanol and add 40 mL of pancreatic α-amylase/AMG mixture to each bottle. Cap the bottles. Transfer the

bottles to a shaking incubation bath and secure the bottles in place with the springs or in a rack in the shaker frame.

3. Incubation with pancreatic α-amylase/AMG: Incubate the reaction solutions at 37°C and 150 rpm in orbital motion in a shaking water bath for exactly 16 h (e.g. 5.00 pm to 9.00 am).
4. Adjustment of pH to approx. 8.2 (pH 7.9-8.4), Inactivation of α-amylase and AMG: After 16 h, remove all sample bottles from the shaking water bath and immediately add 3.0 mL of 0.75 M Tris buffer solution to terminate the reaction. Slightly loosen the caps of the sample bottles and immediately place the bottles in a water bath (non-shaking) at 95-100°C, and incubate for 20 min with occasional shaking (by hand). Using a thermometer, ensure that the final temperature of the bottle contents is > 90°C (checking of just one bottle is adequate).
5. Cool: Remove all sample bottles from the hot water bath (use appropriate gloves) and cool to approx. 60°C.
6. Protease treatment: Add 0.1 mL of protease solution with a positive displacement dispenser (the solution is viscous). Incubate at 60°C for 30 min.
7. pH adjustment: Add 4.0 mL of 2 M acetic acid to each bottle and mix. This gives a final pH of approx. 4.3.
8. Internal standard: Add 100 mg of ribose as an internal standard to each bottle and mix well.
9. Precipitation SDFP: Pre-heat the sample to 60°C and add 192 mL (measured at room temperature) of 95% (v/v) EtOH pre-heated to 60°C. Mix thoroughly and allow the precipitate to form at room temperature for 60 min.
10. Filtration setup: Tare crucible containing Celite® { to the nearest 0.1 mg. Wet and redistribute the bed of Celite® in the crucible, using 15 mL of 78% (v/v) EtOH from the wash bottle. Apply suction to crucible to draw Celite® onto the fritted glass as an even mat.
11. Filtration: Using the vacuum, filter precipitated enzyme digest through the crucible. Using a wash bottle with 78% (v/v) EtOH, quantitatively transfer all remaining particles to crucible. Retain filtrate.

12. Wash: Using a vacuum, wash residue sequentially with two 15 mL portions of the following: 78% (v/v) EtOH, 95% (v/v) EtOH and, acetone.
13. Dry crucibles containing residue overnight in 105°C oven. If a forced air oven is used, loosely cover the crucibles with aluminum foil to prevent loss of dried sample.
14. Cool crucible in a desiccator for approx. 1 h. Weigh crucible containing dietary fiber residue and Celite® to the nearest 0.1 mg. To obtain residue mass, subtract tare weight, i.e., weight of dried crucible and Celite ®.
15. Protein and ash determination: The residue from one crucible is analyzed for protein and the second residue of the duplicate is analyzed for ash. Perform protein analysis on residue using Kjeldahl or combustion methods (Caution should be exercised when using a combustion analyzer for protein in the residue. Celite® volatilized from the sample can clog the transfer lines of the unit). Use 6.25 factor for all cases to calculate mg of protein. For ash analysis, incinerate the second residue for 5 h at 525°C. Cool in the desiccator and weigh to the nearest 0.1 mg. Subtract crucible and Celite® weight to determine ash.
16. Determination of HMWDF: Subtract ash and protein from average residue weight for calculation of HMWDF.
17. Filtrate recovery and concentration: save the filtrate from one of the sample duplicates to use in case of spills or if duplicate SDFS data is desired. Transfer one half of the filtrate of the other sample duplicate to a 500 mL evaporator flask and evaporate to dryness under vacuum at 60C. Alternatively, evaporate as much as ethanol you can using the rotovap and freeze dry the rest of the sample.
18. Deionization of sample: Add 5 mL of deionized water to the evaporator flask and swirl the flask for approx. 2 min to dissolve the sample. Transfer the solution to a sealable polypropylene 20 mL container. Fill a Bio-Rad disposable column with 4 g each of freshly prepared and thoroughly mixed, Amberlite FPA 53 (OH-), and Ambersep 200 (H+). Hydrate the resins with 20 ml DDW and toss the eluent. Add 2 mL of sample solution to the top of the column. Elute the column at a rate of 1.0 mL/min. When the sample has entered the resin, add 2 mL of distilled water to the

resin and allow this to percolate in. Then add approx. 20 mL of deionized water to the top of the column and continue to elute at a rate of 1.0 mL/min. Transfer the eluate to a 250 mL round bottom rotary evaporator flask and evaporate to dryness under vacuum at 60C or freeze-dry.

19. Add 2 mL of deionized water to the flask and redissolve the sugars by swirling the flask for approx. 2 min. Using a Pasteur pipette, transfer the solution to a polypropylene storage container.
20. Preparation of samples for LC analyses: Transfer the solution to a 10 mL disposable syringe and filter through a 0.45 µm filter. HPLC set up condition:

    - Reversed-phase
    - Mobile phase: DDW
    - Sample injection volume: 20 µL
    - Column: Transgenomics CARBO-Sep CHO-411
    - Column temperature: 80°C
    - Flow rate: 0.3 mL/min
    - ELSD Detection conditions: 40C and 350 kPa

21. Determine the response factor for D-glucose: Since D-glucose provides an LC refractive index (RI) response equivalent to the response factor for the non-digestible oligosaccharides that make up SDFS the LC is calibrated using D-glucose, and the response factor is used for determining the mass of SDFS.
22. Obtain the values for the peak areas of D-glucose and internal standard from the 3 chromatograms. The reciprocal of the slope obtained by comparing the ratio of peak area of D-glucose/peak area of internal standard (y-axis) to the ratio of the mass of D-glucose/mass of internal standard (x-axis) is the "response factor". Determine the average response factor (typically 0.97 for D-sorbitol).

Calculations:

$$\%Ash =$$

$$\frac{(Weightofashedcruciblewithceliteandfilterate) - (Weight\ of\ crucible\ and\ celite)}{Weight\ of\ residue} \times 100$$

$$Ash(mg) = Residue \times \left(\frac{\%Ash}{100}\right) \times 1000$$

$$\%N = \frac{1.4007 \times (mL\ HCl\ sample - mL\ HCl\ Blank) \times N\ of\ HCl}{Sample\ Weight\ (g)}$$

$$\%Protein = \%N \times Conversion\ Factor$$

$$Response\ factor = \frac{Peak\ area\ of\ interenal\ standard}{Peak\ area\ of\ Glucose} \times \frac{mass\ of\ Glucose\ in\ standard}{Mass\ of\ internal\ standard\ in\ standard}$$

$$HMWSDF\ \left(\frac{mg}{100g}\right) = \frac{(Weight\ of\ residue\ (mg) - ash(mg) - protein(mg))}{sample\ weight\ (g)}$$

$$\%HMWSDF = \frac{HMWSDF\left(\frac{mg}{100g}\right)}{1000}$$

$$LMWSDF\left(\frac{mg}{100g}\right) = \frac{\left(RF \times Internal\ Standard\left(\frac{mg}{ml}\right) \times \left(\frac{Peak\ area\ LMWSDF}{Peak\ area\ internal\ standard}\right) \times 100\right.}{Sample\ weight\ (g)}$$

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

A.3.2 Hydroxymethylfurfural quantification

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

Material:

HPLC grade methanol

Hydroxymethylfurfural standard

Double distilled water

Ground polylactose

Equipment:

Volumetric flask

Analytical balance

Shimadzu LC-2010C HT

YMC Pack ODS-AM C18 Column

HPLC vials and caps

Procedure:

1. Prepare 5% methanol solution to be used as mobile phase: Add 50 mL HPLC grade methanol to a 1L volumetric flask and bring to volume with double distilled water.
2. Prepare 5 concentration of HMF standard in DDW for HMF standard curve used for unfiltered polylactose. (0.05 mg/mL, 0.075 mg/mL, 0.1 mg/mL, 0.15 mg/mL and 0.2 mg/mL).
3. Prepare 5 concentration of HMF standard in DDW for HMF standard curve used for unfiltered polylactose. (9.38×10^{-5} mg/mL, 1.88×10^{-4} mg/mL, 3.75×10^{-4} mg/mL, 7.50×10^{-4} mg/mL and 1.50×10^{-3} mg/mL).
4. Prepare a 100mg/mL solution of polylactose in DDW.
5. Filter each solution using a 0.45 µm syringe filter into a HPLC vial.
6. Set up the Shimadzu HPLC with the following conditions:
   - Reversed-phase
   - Mobile phase: 5%methanol

- Sample injection volume: 20 µm
- Column – YMC Pack ODS-AM C18 Column, 250 x 4.6 mm I.D.
- Column temperature - 30°C
- Flow rate: 0.4 mL/min
- Absorbance: 285 nm

7. Run each standard and plot the peak area vs. the HMF concentration to make the standard curve.

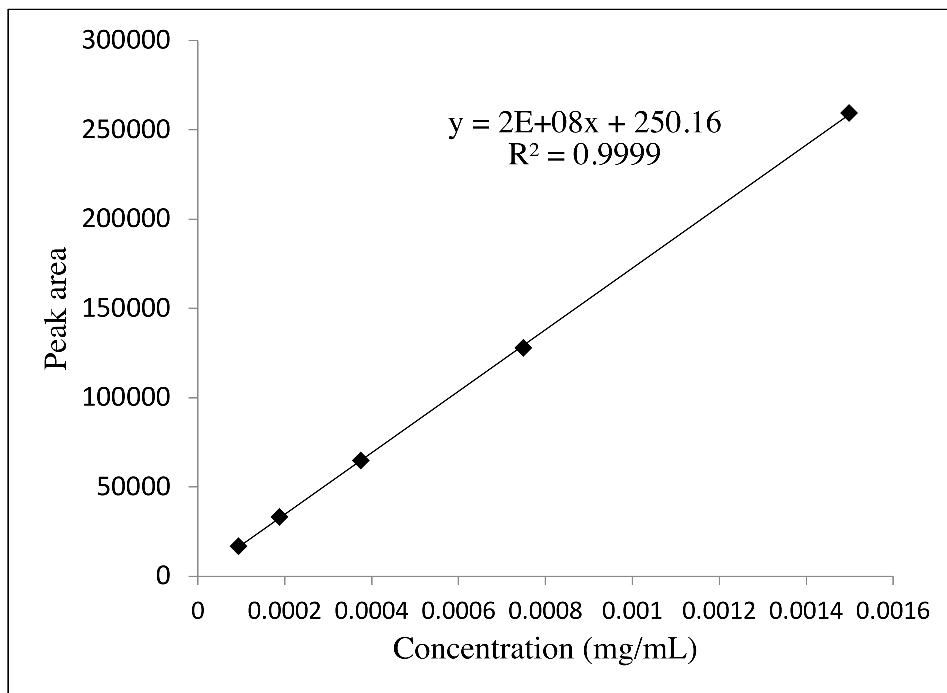

Figure A9. Calibration curve used for HMF quantification of carbon-resin filtered polylactose used for animal trial 2019

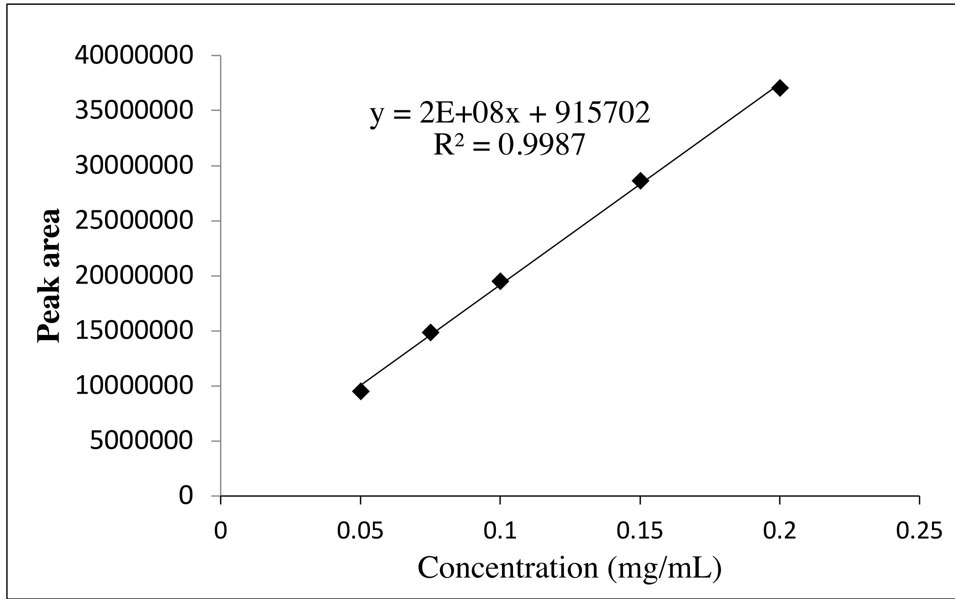

Figure A10. Calibration curve used for HMF quantification of unfiltered polylactose used for animal trial 2019

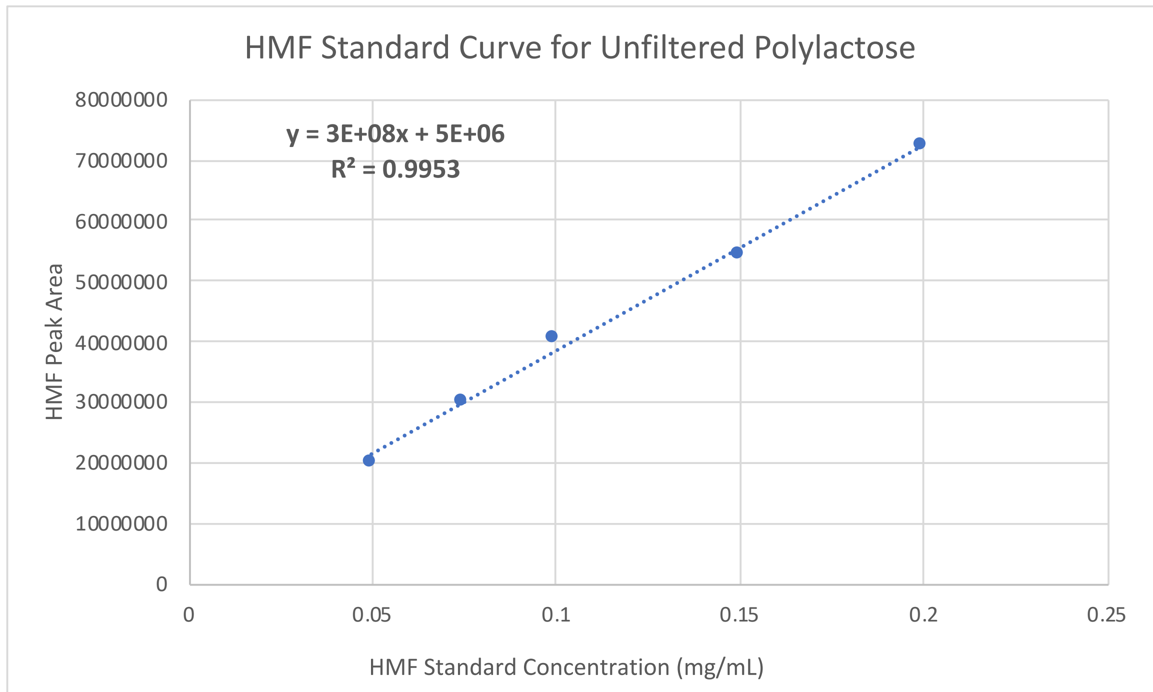

Figure A11. Standard curve used for unfiltered polylactose HMF quantification

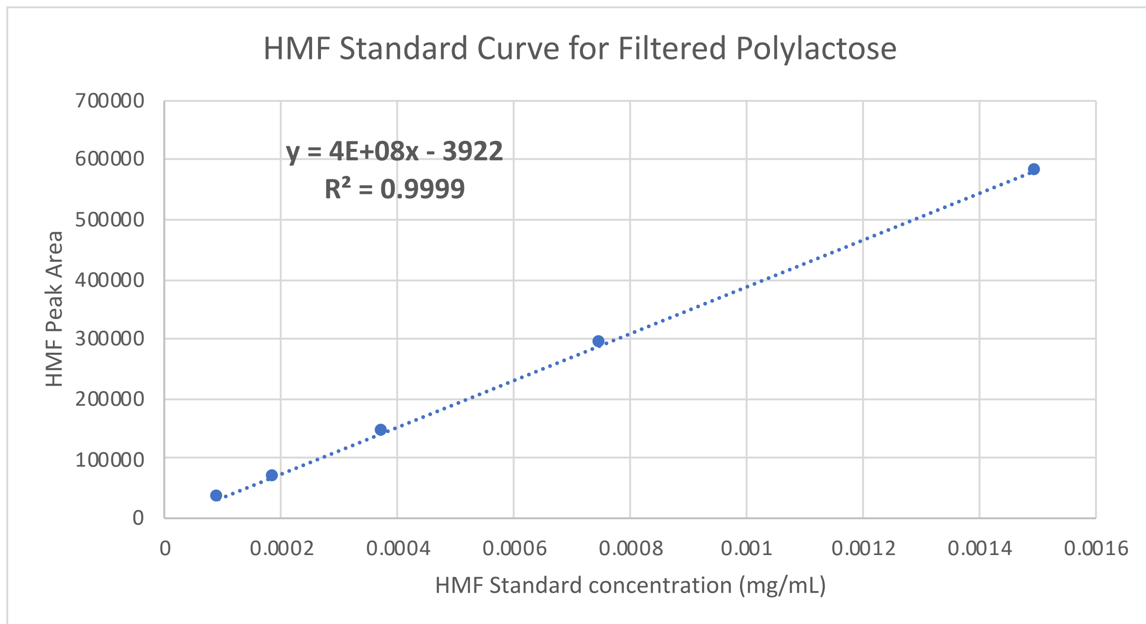

Figure A12. Standard curve used for filtered polylactose HMF quantification

Calculations:

$$HMF\left(\frac{mg}{mL}\right) = \frac{HMF\ peak\ area - Line\ intercept}{Line\ slope}$$

A.3.3 Lactose quantification

Objective: The objective of this experiment is to quantify lactose and D-glucose content of polylactose sample.

Material:

Megazyme Lactose Sucrose/D-Glucose Kit (K-LACSU)

Glacial acetic acid

1M sodium hydroxide

Distilled water

Ground polylactose

Equipment:

pH probe with calibration standards, 4.0, 7.0, 10.0 (Accumet AB 15/15+)

50 and 1000 mL volumetric flasks with glass stoppers

15 mL Centrifuge tubes

Aluminum foil

125 mL plastic bottle

Weigh boats

Analytical balance

Small glass test tubes with screw caps

Test tube rack

Positive displacement pipettor

Water bath

Vortex mixer

Cuvettes

Shimadzu UV-1800 spectrophotometer set at 510nm

Procedure:

1. Prepare GOPOD: Dilute the contents of bottle 3 (GOPOD Reagent Buffer) to 1 L with distilled water (this is solution 3). Use immediately. Dissolve the contents of bottle 4 in 20 mL of solution 3 and quantitatively transfer this back into the bottle containing the remainder of solution 3. Cover this bottle with aluminum foil to protect the enclosed reagent from light. Stable for ~ 3 months at 2-5°C or > 12 months below -10°C.
2. Prepare a blank: 0.4 mL of distilled water in a test tube.
3. Prepare glucose control: add 0.1 of D- glucose standard solution (Bottle 5) +0.3 mL distilled water in two test tubes.

4. Prepare samples: Sample should contain: D-glucose + sucrose + lactose at a concentration of 0.02-0.5 mg/mL. The starting formula was 10.7 Lb lactose and 6 Lb glucose out of a 22.2 Lb batch. Prepared 2 mg/mL solution of the sample which according to starting formula the concentration in assay would be:

$$\frac{10.7Lb\ Lactose}{22.2Lb\ batch} = \frac{x}{100} \rightarrow x = 48.19\%\ Lactose$$

2mg/ml: $\frac{x}{2mg} = \frac{48.19}{100} \rightarrow x = 0.96mg\ Lactose$

0.2 mL of 2mg/mL: $\frac{x}{0.2mL} = \frac{0.96\ mg}{2mg} \rightarrow x = 0.09mg/mL\ Lactose$

$$\frac{6Lb\ Glucose}{22.2Lb\ batch} = \frac{x}{100} \rightarrow x = 27.03\%\ Glucose$$

2mg/ml: $\frac{x}{2mg} = \frac{27.03}{100} \rightarrow x = 0.54\ mg\ Glucose$

0.2 mL of 2mg/mL: $\frac{x}{0.2mL} = \frac{0.54\ mg}{2mg} \rightarrow x = 0.05\ mg/mL\ Glucose$

Pipette 0.2mL of 2mg/mL sample (Just dissolved in DDW , Did not to do enzyme inactivation and sugar extraction step) to 4 test tubes, add 0.2 mL sodium acetate buffer to two of them and 0.2 mL of previously prepared frozen solution 2 (ß- galactosidase: Bottle 2 of kit dissolved in 19 mL of sodium acetate buffer) to two of the test tubes.

5. Incubate blank, glucose and, samples in a water bath at 50 °C for 20 minutes.
6. Add 3 mL GOPOD reagent (Previously made and frozen in aluminum wrapped bottles).
7. Incubate all tubes again at 50 °C for 20 min.
8. Transfer to cuvettes and measure absorbance against blank at 510 nm.

Calculations:

Absorbance of A: Absorbance of tubes with Sodium acetate

Absorbance of C: Absorbance of tubes with Solution 2

$$F = \frac{100\ \mu g\ Glucose}{average\ absorbance\ of\ glucose\ standards}$$

$$D - Glucose\% \left(\frac{W}{W}\right) =$$
$$\left(\frac{absorbance\ of\ A}{0.2mL}\right) \times (F) \times (\frac{1}{1000}) \times (\frac{mL\ of\ volume}{mg\ of\ sample}) \times 100$$

$$Lactose\% \left(\frac{W}{W}\right) =$$
$$\left(\frac{absorbance\ of\ C - absorbance\ of\ A}{0.2mL}\right) \times (F) \times (\frac{1}{1000}) \times (\frac{mL\ of\ volume}{mg\ of\ sample}) \times \frac{342}{180} \times 100$$

A.3.4 Citric acid quantification

Objective: The objective of this experiment is to measure the citric acid content of polymerization products.

Material:

Megazyme citric acid test kit (K-CITR)
DI water
Polylactose

Equipment:

Graduated cylinder (25 mL)
Beaker (100 mL)
Polypropylene tubes (25 mL)
Volumetric flask (100 mL)
Small glass test tubes with screw caps

Test tube rack

Positive displacement pipettor

Vortex Mixer

Cuvettes

Parafilm

Shimadzu UV-1800spectrophotometer set at 510nm with UV Probe2.43software

Reagent Preparation:

1. Solution 1: Use the contents of bottle 1 as supplied. Stable for > 2 years at 4C.
2. Solution 2: Dissolve the contents of bottle 2 in 16 mL of distilled water. Stable for > 1 year at 4¬∞C or stable for > 2 years below -10C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes).
3. Solution 3: Usethecontentsofbottle3assupplied. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottle in an upright position. Swirl the bottle to mix contents before use. Stable for > 2 years at 4C.
4. Solution 4: Carefully dissolve the contents of one of bottle 4 in 0.55 mL of distilled water. Stable for 4 weeks at 4 C or > 6 months below -10C.
5. Solution 5: Use the contents of bottle 5 as supplied. Stable for >2 years at 4C.

Procedure:

1. Dissolve 0.125 g of polylactose in DI water and bring it to volume using a 100 mL volumetric flask. The concentration of citric acid in each cuvette needs to be 1.0-100 µg.
2. Prepare blank by pipetting 2 mL DI water (at 25 C), 0.50 mL solution 1, 0.20 mL solution 2 and 0.02mL suspension 3 into test tube.

3. Prepare sample by pipetting 1.80 mL DI water (at 25 C), 0.20 mL sample, 0.50 mL solution 1, 0.20 mL solution 2 and 0.02mL suspension 3 into test tube.
4. Mix, transfer to cuvettes and, read absorbances at 340 nm against the water. after approx. 4 minutes (A_1).
5. Add 0.02 mL solution 4 to blank and samples cuvettes, mix and read absorbances at 340 nm against the water after 5 minutes. (A_2)

Calculations:

$$\Delta A = \Delta A\ Sample - \Delta A\ Blank$$

$$Citric\ acid\ concentration = 0.4177 \times \Delta A$$

A.4 Extended methods for treatment of polylactose with lactase enzyme

A.4.1 Lactase treatment of polylactose

Objective

The purpose of this method is to treat polylactose filtered used for the animal study with lactase and evaluate the effect of lactase on dietary fiber content.

Material

Polylactose

Ha-Lactase^{TM} 5200

Equipment

Analytical balance

Micropipette

Volumetric flask

Procedure

1. Dissolve 5 gr polylactose in double distilled water in a 50 mL volumetric flask, and add water to reach final volume of 50 mL.
2. Pipette 0.15 mL Ha-Lactase 5200 into each flask.
3. Incubate samples at 4ºC for 24 hours.
4. After the incubation period, boil samples for 5 minutes in order to deactivate the enzyme.