Manipulation of Starch Digestibility in Particle Form

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Abstract

This work investigates ways to prevent and manage hyperglycemia using preventive nutrition. Uncontrolled and chronic hyperglycemia is a global health issue leading to many health problems including diabetes. This thesis details the manipulation of highly retrograded starch particles in order to produce particles that are digested slowly to release glucose at a prolonged and moderate rate to prevent this. The first section of this study utilized acid hydrolysis to alter starch structure and change digestibility. The hydrolysis treatment showed that hydrolysis of native starch prior to particle formation changed the structure in a way that increased digestibility. The second section of this work introduced polyphenols into the particles which only a marginal effect on digestion. Overall the actual process of retrograding and making the particles themselves appeared to create particles that were more resistant to digestion. These could be used in a product to deliver a moderate glycemic response.

Resumé

Ce travail examine les moyens de prévenir et de gérer l'hyperglycémie à l'aide d'une nutrition préventive. L'hyperglycémie chronique et non contrôlée est un problème de santé mondial qui entraîne de nombreux problèmes de santé, notamment le diabète. Cette thèse détaille la manipulation de particules d'amidon fortement rétrogradées afin de produire des particules qui sont digérées lentement pour libérer le glucose à une vitesse prolongée et modérée afin d'éviter cela. La première partie de cette étude utilisait l'hydrolyse acide pour modifier la structure de l'amidon et modifier la digestibilité. Le traitement d'hydrolyse a montré que l'hydrolyse de l'amidon natif avant la formation de particules modifiait la structure de manière à augmenter la digestibilité. La deuxième partie de ce travail a introduit dans les particules des polyphénols n'ayant qu'un effet marginal sur la digestion. Globalement, le processus de rétrogradation et de fabrication des particules elles-mêmes semblait créer des particules plus résistantes à la digestion. Ceux-ci pourraient être utilisés pour créer un produit qui donne une réponse glycémique modérée.

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1 Introduction

1.1 General introduction

Chronically uncontrolled glycemia can have many negative health outcomes for an individual. Habitual consumption of foods eliciting elevated glycemic and insulinemic responses is associated with chronic conditions such as obesity, diabetes, cardiovascular disease, and chronic inflammation, the last of which is thought to be a precursor to the occurrence of certain cancers[1]. Hyperglycemia has also been directly linked to a higher incidence of deaths related to breast cancer[2]. In 2012, the World Health Organization reported an estimate of 2.2 million deaths that year were related to high glycaemia[3]. These conditions represent a substantial economic burden as well. In 2012, the health care costs of diabetes alone were estimated to be over $322 billion in the United States[4]. A 2016 study reported a 2.5% increase in global prevalence of diabetes and estimated global costs of diabetes care to be $825 billion[5].

Consumption of carbohydrate-rich foods can have a great impact on blood glucose levels and the overall health of the individual[6]. A 2006 study on hyperglycemia found often it impaired fasting glucose levels and impaired glucose tolerance which led to both inflammation and type 2 diabetes, with these conditions thought to be reciprocal[7]. Additionally, this work highlighted the effect hyperglycemia has on glycosylated end products in inflammation. This research also highlighted that poor glycemic control and increased presence of cytokines and other inflammatory markers were associated with progressed type 2 diabetes symptoms, with further work remaining to be done about the predictive value of these markers for the onset of pre-diabetes. Supporting this, there have been studies showing that a lowered glycemic response has been able to reduce obesity, aid in management of diabetes, and improve the HDL to LDL cholesterol ratio[8]. Hyperglycemia has been linked to peripheral vascular disease(PVD) with a higher risk for not only PVD but a

general increase in systolic blood pressure as well[9]. This epidemic is multifaceted and will not decrease without intervention, so solutions that incorporate prevention as well as treatment must be implemented through different channels including public health policy and adaptive forms of applied health care. Innovative food forms eliciting a moderate and prolonged glycemic response may help prevent negative health effects both in the short and long term. It is important to look at existing diets to identify solutions and areas to improve on. However, currently there is a lack of practical and easily accessible means for consumers to manage glycemia. Glucose is the primary contributor to stimulate an insulinemic response and digestible carbohydrates will have the greatest influence on blood glucose, with the glycemic index (GI) representing the increase in blood glucose in the 2 hour period following ingestion (Compared to a reference of glucose)[10]. Potatoes for example, when baked boiled or mashed, will have a high GI and increased intake will increase the risk of type 2 diabetes[11]. With carbohydrate-rich foods contributing heavily to postprandial glycemia, functionalizing dietary starches would be a good starting point to minimize chronic hyperglycemia.

Starch is the main glycemic carbohydrate in most diets and therefore consumption has a major impact on the postprandial glycemic response in the body. It is found in cereals, tubers, legumes, and other plant-based foods which are essential parts of the average diet and are consumed in many different forms and often over the course of a day. Processing of starch, such as extrusion, can increase glycemic response, which is common for breakfast carbohydrates like muffins or cereals[12]. Constantly consuming foods with a high glycemic load will cause problems with insulin secretion and glucose tolerance. Preventive action could be provided via starch tailored to deliver a prolonged and moderate glycemic response that could relieve the glycemic load and not overwhelm the insulinemic response. Incorporating other food sources such as fruits to

functionalize starch could also provide appealing ways to make a synergistic product[13]. The goal would be a product that favours starch that is hydrolyzed at a slower rate in the intestinal tract and produces a subsequent reduction of the length and magnitude of the glycemic response, contributing to relief on the insulinemic response[14].

Optimization of starch-bases products for a better glycemic response can be achieved through various means, including changing starch structure or introducing polyphenols, which have been shown to interact with starch digestive enzymes. This could be applied by incorporating starch into a fruit matrix rich in polyphenols, such as a berry smoothie, a product known to have desirable organoleptic properties[15]. This could aid in further control of digestibility of starch.

Using starch in the novel form of cooked particles gives the potential for a product that is easy to manipulate and that can be delivered in a product that is appealing to consumers. This work will investigate how manipulation of starch in particle form can be used to control *in vitro* digestion kinetics of starch.

1.2 Scope

This work aims to identify means for producing nutritional foods for consumers that would elicit a prolonged and moderate glycemic response. In order to prevent and manage Type 2 diabetes or other conditions where hyperglycaemia is problematic. We propose a starch product in a particle form that can be easily incorporated into a fruit matrix, which can be an ideal vehicle, as the fruit itself contains active compounds which may contribute to the slowed release of glucose. This could be turned into a commercial product that would offer consumers a convenient and acceptable food product to provide a mediated postprandial glycemic response This product could exist in the form of a smoothie or fruit beverage.

1.3 Hypotheses

1. It is hypothesized that changes in starch molecular and supramolecular structure will directly impact the digestion rate of starch in the form of particles and can be used to influence digestion kinetics *in vitro.*
2. It is hypothesized that the type of starch used to make the particles will affect the digestion rate based on composition and amylose: amylopectin content unique to that species of starch.
3. It is hypothesized that the presence of polyphenols from berries will impact starch digestion.

1.4 Objectives

1. To identify a relationship between the organization of starch structure and rate of digestion and identify ways to manipulate starch digestion to favour a slowed and controlled digestion of starch.
2. To select the optimal type of starch and conditions in which to produce starch particles that are slowly digestible or resistant to digestion *in vitro.*
3. To asses the ability of polyphenols from fruit and other sources to slow the rate of starch digestion *in vitro.*

2 *Literature Review*

2.1 Functional foods as applied to regulating the glycemic response

In targeting diet related health issues, functional foods have become an increasingly common option to incorporate as part of lifestyle adjustment. Functional foods are defined by health Canada as the following: "A *functional food* is similar in appearance to, or may be, a conventional food, is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions"[16]. Agriculture and Agri-food Canada defined functional foods as "foods enhanced with bioactive ingredients and which have demonstrated health benefits, such as probiotic yogurt, or breads and pasta with added pea fibre"[17]. A chapter in the book *Nutraceuticals and Natural Product Derivatives* outlined the role of functional foods for targeting glycemia and managing diabetes which highlighted foods including plants and berries that contain fibers and polyphenols shown to lower glycemic and insulinemic peaks [18]. Nutraceuticals are products derived from food sources that are used for medicinal purposes. This paper also emphasized the importance of standardization of dose for nutraceuticals that would be used towards management of glycemia and diabetes treatment. In a 2017 review, Alkhatib *et al.* detailed the mechanisms in which various foods imparted physiological benefits towards managing diabetes[19]. What is of particular interest to this current study is the creation of a starch-based product that would contain polyphenols. Foods containing starch have a role in targeting hyperglycemia and can be applied diabetes treatment. Resistant starch (RS) specifically acting as dietary fiber has been able to interact with the microorganisms in the digestive tract and produced effects such as improved glucose tolerance and insulin sensitivity and lower postprandial blood glucose levels in individuals with Type 2 Diabetes as well as healthy individuals[20]. This study showed that consumption of 60g of RS was able to significantly lower postprandial glycemia

and the work suggested that incorporating foods that naturally contain or have added RS into diets is beneficial to diabetes management and prevention. These functional foods could be whole grains or legumes or starch particles high in resistant starches that promote the growth and activity of gut microbiota. Specifically microbiota including Lactobacillus and Bifidobacteria, which secrete the hormones like Peptide YY which reduces appetite and modulate other hormones such as ghrelin hormones that is involved in hunger and satiety[21].

2.1.1 Starch as a functional ingredient

When using a functional food and preventive nutrition approach towards managing glycemia, it is important to consider what food to manipulate. Preventive nutrition is the application of nutrition science to help prolong life expectancy, promote healthy lives, and delay or prevent the onset of disease[22]. Foods that already have a large role in determining the blood glucose levels in the body like starch can be used to provide a tailored glycemic response. Starch is a glucose polymer that will have a direct influence on post prandial blood glucose levels[23] and is a major constituent of the Western human diet[14]. For foods high in starch content, texture is a variable factor depending on cooking time, heating temperature, or other forms of modulation. A functional food must have consumer acceptance in order to have effective commercial value, therefore it must be made acceptable to consumers in order to incentivize consumptions and impart the benefits[24]. This is the reasoning behind developing an appealing and easily marketable product like a smoothie containing starch particles.

2.2 Starch

2.2.1 Starch composition

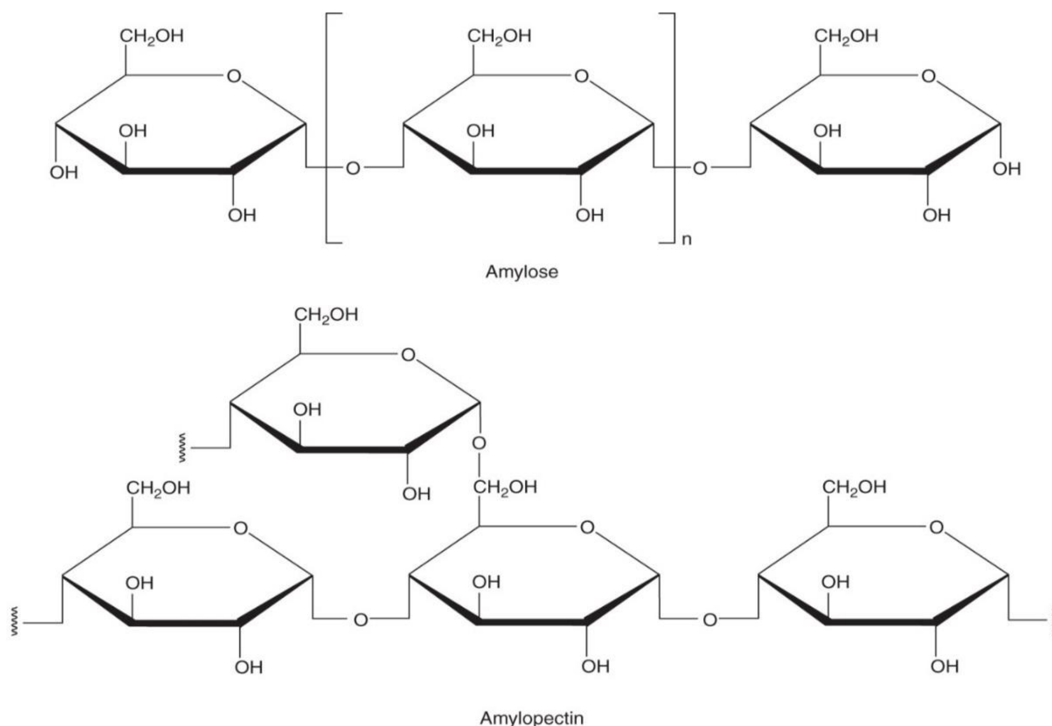

Figure 1. Chemical structure of Amylose and Amylopectin.[25]

In order to apply starch as a functional ingredient, the structure must be understood sufficiently to select for what is desirable. Here what is desired is to form a starch that is more readily digested.

Native starch is found in the form of semi-crystalline granules[26]. Starch is a homopolymer of glucose, consisting of two macromolecules; amylose and amylopectin, the structures of which are shown in Figure 1. Amylose has a molecular weight of approximately 10^5-10^6 g/mol while amylopectin is a much larger molecular with a molecular weight of 10^7-10^9 g/mol [27]. The amylose molecule is a linear chain of α-D-glucopyranosyl units joined by (1→4)linkages[28]. Amylopectin is a highly branched molecule of D-glucopyranosyl units joined by (1→4)linkages with branching via (1→6)linkages approximately every 40-50 units [28]. Amylose molecules can have minor

branching as well, but are generally essentially linear, as these branches will have no influence on the supramolecular structure[29].

The ratio of amylose: amylopectin drives the crystallinity of native starch and differs depending on plant source[27]. The amorphous region of native starch is made up of amylose molecules while the crystalline region is made up of branched amylopectin. Potato starch has approximately 26.9% amylose content while corn starch has approximately 20.9%[30]. Waxy starch for example has very little amylose content while genetically modified starches can have up to 80% amylose content and therefore lower crystallinity in their native form[29].

2.2.2 Gelatinisation and retrogradation

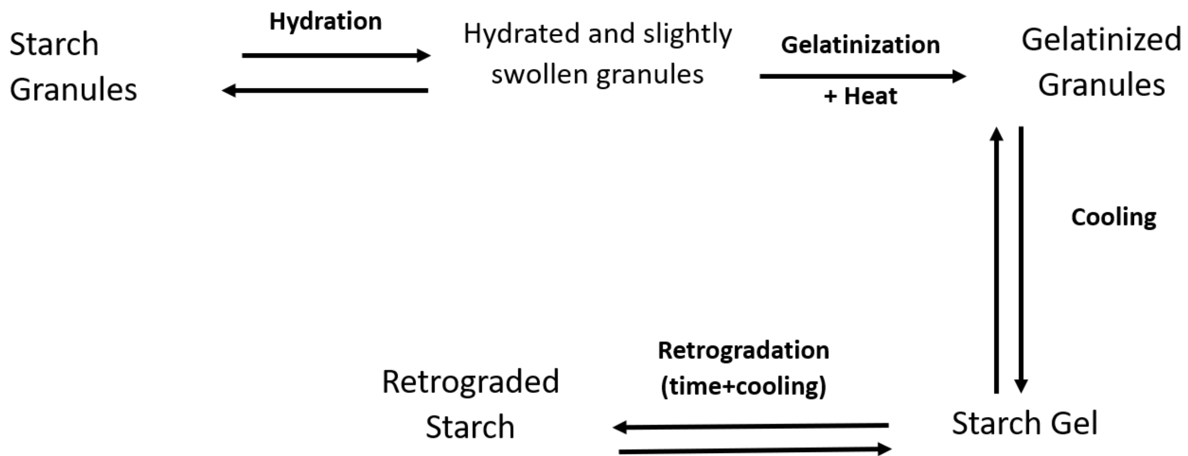

Figure 2. Schematic outlining starch granule undergoing gelatinisation and retrogradation and subsequent arrangement of starch structure.

Important transformations of starch structure are gelatinisation and retrogradation, as shown in Figure 2. Gelatinization is a loss of order of starch structure that occurs when starch is heated in excess water, which disrupts the structure of starch and causes swelling of the granules upon

shearing[31]. This creates a hot starch paste. The temperature at which this occurs will depend on the starch source and amylopectin and amylose content. Hydrogen bonds in the amorphous region are disrupted and water is absorbed (hydration) which pulls apart the double helical crystallites and results in an irreversible loss of order to the granule[32].

Following heating and gelatinisation, after these bonds break down there will be a reformation or reorganization in the starch gel. This process is called retrogradation, where the chains reaggregate and form a viscoelastic gel upon cooling[33]. This will cause the amylopectin and amylose chains to rearrange. When starch is retrograded, the amylose molecules will reform into crystallites. After cooling, the retrograded structure will be more ordered due to these crystallites that have formed from aggregated amylose helices. Specifically, the crystallites of amylose will reform making a more rigid structure which can subsequently make the starch less accessible to degradation. Retrograded starch structure has been shown to possess some enzyme resistance[34]. This was attributed to their being a highly aggregated portion of amylose which limits enzyme accessibility to the starch structure. Retrogradation can be distinguishable between species of starch based on amylose: amylopectin content of the native starch.

Mild hydrolysis can aid in retrogradation. Acid can hydrolyze amylose and amylopectin into smaller molecules. For example, corn starch that has undergone hydrolysis for a short period prior to gelatinisation and retrogradation will have lower molecular weight amylose that can reaggregate readily[35]. Additionally since corn starch is high in amylose content, it will undergo more retrogradation than starch with a lower amylose content[35].

2.2.3 Crystallinity

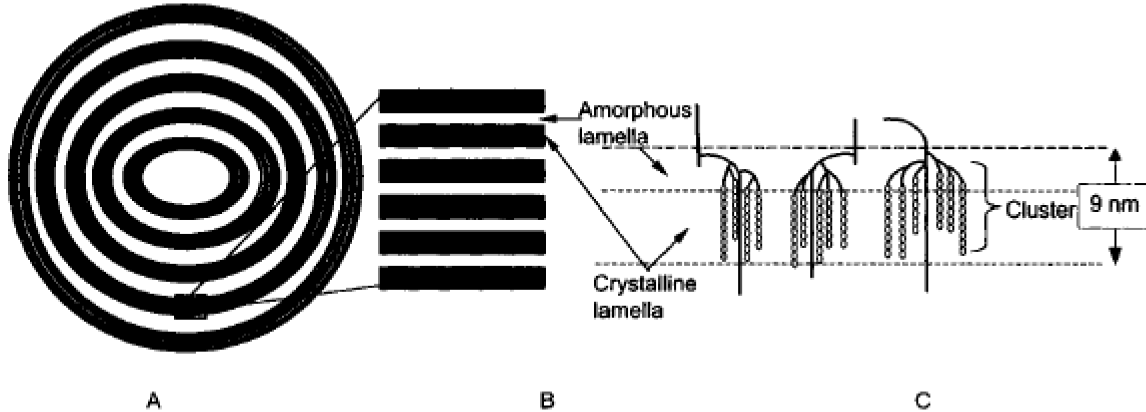

Figure 3. Diagram of starch crystalline and amorphous structure. A shows a starch granule. B shows amorphous and crystalline regions of starch. C shows amylopectin double helix clusters which make up the crystalline region of native starch[27].

The supramolecular structure of native starch can be described as a helical structure, with amylose and amylopectin being packed into round granules in the amyloplasts of the plant[30]. This is illustrated in Figure 3. The helical structure of the molecules is made up of six anhydrous glucose residues per turn[31]. Early characterizations of native starch structure identified starch granules as having a outer lamellar amorphous region that is loosely packed and non-resistant surrounding an inner crystalline and tightly packed resistant core of 90% branched molecules (amylopectin)[36]. This research also stated that potato starch in particular is clearly separated into these layers. Different types of starches have slightly different arrangements, and this will dictate how they are gelatinised and subsequently retrograded.

Retrograded starch also contains crystalline regions. Crystallites are composed of highly ordered arrangements of doubles helices of amylose or amylopectin. Native starch granules have

crystallinity due to tightly packed amylopectin and retrograded starch has crystallinity due to tightly packed amylose double helices that have aggregated during the cooling period.

Granule structural characteristics are what influence starch granule hydrolysis, (rather than enzyme properties) and this is what determines how the gelatinised and retrograded structures will appear and how enzymes or other forms of hydrolysis will interact with these structures. For example, a more highly crystalline starch granule will retrograde more readily[37]. Different kinds of starch will have varying granule compositions, with potato starch granules being progressively eroded via exo-corrosion from the α-amylose enzyme hydrolysis with somewhat strong enzyme resistance[38]. More resistant starch granules usually contain longer amylopectin side chains and as a result will have more amylopectin structures that remain after heating and cooling[38].

Work done examining the molecular order of enzyme-resistant retrograded starch showed that these starches are high in double helical formations (60-70% of the structure) and lower in crystallinity (25-30% of the structure)[34]. This suggested that crystallites in retrograded starches are smaller and less perfectly packed than in granular starch and that the general structure is mainly aggregated double helical linear amylose chain segments with small regions of B-type crystalline packing. Retrograded starch is usually accompanied by an increased degree of crystallinity with some appearance of those B-type crystallites[33].

2.3.4 Probing starch structure using thermal characteristics

DSC (Differential Scanning Calorimetry) has been used to measure thermal changes related to starch structure since the 1970s. Stevens and Elton published a paper describing how DSC was applied towards defining heat endotherms and allowing calculation of heat gelatinisation enthalpy for each sample[39]. DSC progressively heats a sample across a temperature range and then can

record the exothermic or endothermic release of heat associated to whatever phase transition is occurring. This allowed them to compare the properties of different starches to each other. DSC is now a common method of measuring starch molecular order. Since starch crystallinity is a major contributor to starch molecular order, DSC can provide a relative estimate of crystallinity and molecular order. The gelatinisation of starch and loss of order of starch structure can be measured as an endothermic or (exothermic) event. Starch crystalline regions have a greater amount of inter- and intra- molecular non-covalent bonds compared to the amorphous region therefore a more crystalline structure requires a greater heat enthalpy to break the structure, and can be used to indicate the molecular order of starch samples[40]. DSC records the temperatures at which this phase transition or melting occur.

Thermal studies on starch have established a correlation between amylopectin content and gelatinization enthalpy (ΔH) and other thermal properties as well as a negative correlation between amylose content and peak gelatinization temperature (T_p)[41].

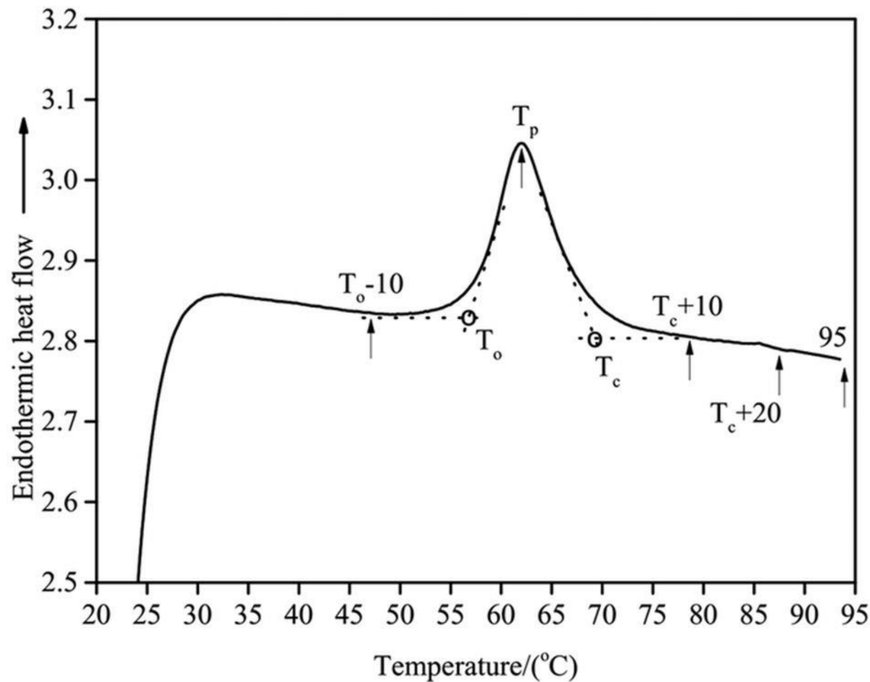

Figure 4. Example of a DSC thermogram showing onset (T_{o}), peak (T_p) and conclusion (T_c) temperatures. [42]

Measuring a thermal transitional event can provide insight on the composition of a starch sample. Usually within the temperature range of 0-130°C, the phase transition event being recorded is the melting point where there is a loss of order in starch structure. The temperature at which a phase transition event commences is called the onset temperature (T_o). The temperature at which a phase transition event peaks is called the peak temperature (T_p). The temperature at which a phase transition event concludes is called the conclusion temperature (T_c). These temperatures change depending on how readily the starch structure is degraded and loses order. For example, if the phase transition occurs at a higher temperature, then more energy is required to disrupt molecular order which would indicate that the sample is more crystalline or contains more densely packed crystallite bodies.

Differential Scanning Calorimetry (DSC) technology can be used to measure the phase transition that accompanies the breakdown of starch structure. In retrograded starch the phase transition that is being measured is melting of amylose crystallites and can provide an indirect measure of crystallinity through estimation of starch molecular order. Lower transition enthalpy (ΔH) is mostly reflective of loss of double helical amylopectin and crystallite content in native starch[43].

Decreases in melting temperatures suggest loss of order, which could be attributed to defective or imperfect or smaller crystalline regions formed[44].The onset temperature for corn starch is related to amylose content. A higher temperature is needed to induce melting in the absence of amylose-rich amorphous regions and the thermal transition events of high-amylose corn starch are less sharp and defined and broader than a more amylopectin heavy starch samples, meaning that the difference between onset and conclusion temperatures are also wider and the peak temperatures are slightly lower[44]. It has also been noted that addition of external factors, polyphenols for example, can cause a shift in onset and peak temperatures from loss of order of starch structure[13].

2.4 Starch digestibility

2.4.1 Digestion of starch

Structure of starch will directly influence its functionality, including digestibility. Starch supramolecular structure is important in determining how it is digested since it will dictate hydrolysis interactions. Tester *et al.* discussed starch structure as it relates to digestibility. They highlighted that the extent of ordered structure is a major driver of starch digestibility. They also emphasize the importance of starch granule size and how damage, change, or reconfiguration of starch structure through events like gelatinisation or retrogradation can also influence starch digestibility in a significant way[45]. Amylose and amylopectin content will dictate the characteristics of starches and these will change through processes such as cooking or retrograding. Without gelatinisation native starch would be poorly digested in the human system.

To apply this knowledge to the current project, how the starch is digested and absorbed in the intestinal tract must be understood. Englyst *et al.* classified starch into different categories, in terms of their nutritional relevance, based on hydrolysis by starch digestive enzymes. Starch that is hydrolyzed and releases glucose within the first 20 minutes of digestion is classified as Rapidly Digestible Starch (RDS), while starch that is hydrolyzed within the 20 to 120 minute period is classified as Slowly Digestible Starch (SDS). If starch is not hydrolyzed within 120 minutes, it is classified as Resistant Starch (RS) as it would reach the colon and escape digestion[46]. When starch is digested slowly, glucose is also released slowly and at a steady rate into the bloodstream, resulting in a moderate and prolonged glycemic response as well as a moderate insulinemic response[47]. Favouring starch that is digested at a slower digestion rate (or k-value) in food would therefore allow for management of glycaemia and prevention of hyperglycaemia and associated conditions[48]. Work done on SDS consumption examined the physiological response which resulted

in lower insulin levels during digestion, prolonged exogenous glucose oxidation, and lower circulating levels of non-esterified fatty acids[23]. These benefits were in addition to the slow and prolonged release of glucose, and especially beneficial to diabetic patients, who also saw decrease in hormones such as glucagon-like peptide which regulates gastric emptying and can be helpful in maintaining homeostasis.

2.4.2 Starch hydrolysis

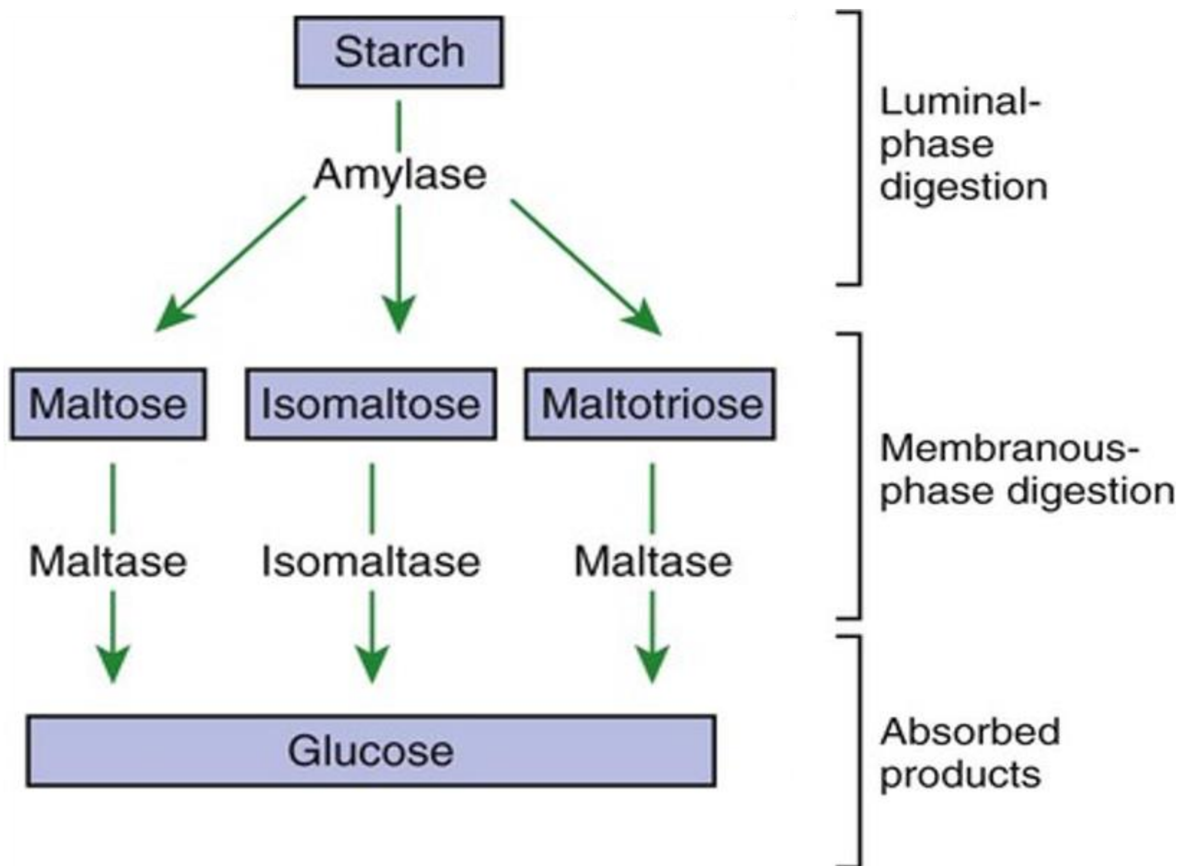

Figure 5. Diagram outlining the role of enzymes in cleaving starch molecules into glucose ready for absorption in the intestinal tract.[49]

Starch structure influences the accessibility of digestive enzymes, including α-amylase and glucosidase, to starch and this is one factor that establishes the rate of digestion[50]. Salivary α-

amylase and pancreatic α-amylase both participate in the hydrolysis of starch into glucose residues in the form of α-dextrins, with the pancreatic form of amylase responsible for the majority of the hydrolysis owing to there being longer contact time with the starch bolus as compared to the salivary form, since salivary α-amylase is rapidly degraded by acid in the stomach as it moves through the digestive tract [51]. However, some α-amylase may remain to interact with the starch since contact with the gastric fluid, with the lower pH, may be protected by the bolus itself having a large surface area. The enzyme α-glucosidase participates in the next step of starch degradation, hydrolyzing the α-dextrins into glucose which are absorbed in the small intestine. Brush border enzymes sucrase-isomaltase, lactase, maltase-glucoamylase, and trehalase finish starch digestion by cleaving the disaccharide substrate releasing glucose[52]. Enzymes do not perform preferential hydrolysis onto the amorphous regions of the starch structure. Retrogradation will open starch structure to make it more digestible by starch digestive enzymes[27]. The rate at which these events occur establishes how readily available glucose is and determines the final RDS, SDS, and RS fractions[53].

It has been shown that larger starch granules undergo slower digestion, due to larger granules having smaller relative surface area to volume ratio[53]. Creating a starch particle with a larger surface area could produce the same response. Specific surface area refers to the actual surface area of starch available for interaction with external factors and takes into account pores in the starch structure[54]. For example, potato starches are usually more crystalline and compact whereas corn starch granules contain more pores or channels. Digestion by the enzyme α-amylase can also increase the number of pores and the specific surface area.

The interaction of the starch digestive enzyme, α-amylase, with starch is primarily what drives starch digestion. Limiting activity of α-amylase with starch by physical or biochemical means may reduce digestion kinetics, which may effectively slow the rate of glucose release.

2.4.3 Resistant starch and digestibility

Resistant starch is starch that escapes digestion in the small intestine, reaches the colon and can interact with the microflora present there. While it is not hydrolyzed by starch digestive enzymes and therefor not used as an energy source for the body, the recent focus on the colon and gut microbiome may provide new value to resistant starch fractions. A study on how resistant potato starch impacted both glycemia and satiety showed that the RS sample elicited a significantly decreased glycemic response where the different treatments consisted of glucose, RS+water, or RS+glucose which suggested that RS may have a prebiotic effect[55]. RS has shown promise both in reducing the postprandial glycemic and insulinemic response in the body and also interacted with the microbiome for various outcomes. These included anti-inflammatory effects potentially through proliferation of the Bacillus and Bifidobacterium in the colon which can change short-chain fatty acid concentrations and can influence satiety and appetite regulation pathways in the body[56]. Another area of interest for RS interacting with the microbiome is potential effects on satiety after consumption, compared to consuming more rapidly digested starch since a smaller fraction will reach the colon[57] A 2014 review from Dupuis *et al*. listed different methodologies used to increase RS content of food starches, some of which included physical treatments such as hydrothermal or enzymatic treatments[58].

A test where a resistant starch meal of raw potato starch (R) was compared to a meal containing gelatinised starch (S), which is more rapidly digested, showed a 9-fold increase in the postprandial

glucose concentration of the S meal compared to the R meal. Increasing RS content may be useful towards creating a product that will deliver a reduced glycemic response.

2.4.4 *In vitro methods of quantifying starch digestion*

As starch is hydrolyzed by digestive enzymes, this produces free glucose molecules. By measuring the glucose content over the course of digestion period, this can estimate the rate at which starch is digested. Englyst *et al.* first introduced the classifications of RDS, SDS, and RS and the specialized method for starch digestion. Edwards *et al.* published a paper in 2014 that offered an alternative to the Englyst method of measuring starch digestion using first order kinetics of amylolysis to fit the data[59]. In 2014 Minekus *et al.* published a standardized digestion method that could be applied to all different types of food and allowed better comparison between papers.[60] Rate of starch digestion in the small intestine can be modelled *in vitro* by measuring glucose release then using first order kinetics to describe the rate and amplitude at which digestion occur. Amplitude describes how much starch is digested. Using enzyme kinetics can provide a good model of starch amylolysis since this process typically follows a first-order kinetic reaction, with the amount of substrate, in this case starch particles, diminishing as the hydrolysis reaction with the enzymes proceeds[59]. The following integrated first order kinetic equation $[glucose] = C + A(1-e^{-kt})$ can be used to illustrate the release of glucose. Using this model allows the presentation of curves describing glucose formation or percent of total starch (%TS) digested to be generated. The k (digestion rate), C (intercept), and A (Amplitude) values derived from the modified kinetic equation, with t being the time of digestion, can provide meaningful and more accurate quantitative comparisons between samples that better reflect digestion kinetics than quantifying RDS/SDS/RS.

2.4.5 Starch structure and digestibility

How enzymes interact with starch will be determined by the structure. The degree of retrogradation of starch will dictate these interactions. Digestibility will be influenced by the molecular order of native starch and the final molecular order of retrograded starch. Retrograded structure with fewer crystallites is more open and allows enzymes to hydrolyze starch with easy access to the substrate. The amorphous region of starch is most readily digestible and provides the most accessibility to digestive enzymes[45]. Higher crystallite content makes starch more resistant to digestion since enzymes can not easily access these tightly packed region and any amylopectin content is more difficult to hydrolyze due to the presence of hindering branches[61]. Previous work has shown that high-amylose starch gels that have retrograded have the most compact structure allowing only minor amylose hydrolysis to occur[62]. This is because, after gelatinisation, the high amylose content will rapidly reform into tightly packed crystallite structures. For example, highly crystalline starch structures have been shown to reduce accessibility of α-amylase to starch since the enzyme is not able to access the substrate[63]. Previous studies have been able to manipulate starch digestion by altering branch length of the macrostructures amylopectin and amylose that make up the structure of starch, again where the increase in branch density was able to slow the rate at which starch was digested[47]. Additionally treatments that have been shown to favour highly branched amylopectin molecules in starch have been shown to have poor digestibility[64]. Therefore, this shows that alteration to the crystalline structure of starch is an extremely important aspect of controlling starch digestibility.

Since native starch is poorly hydrolyzed by starch digestive enzymes, gelatinization results in increased absorption of glucose in the duodenum and small intestine[26]. Excessive absorption early in the digestion process is what leads to the elevated levels of blood glucose and the potential for

hyperglycemic postprandial periods. Favouring amylose content in the native starch used to form the starch particles would provide the more crystalline structure that is desired to lower digestion rates, once they have been gelatinised and retrograded. Highly retrograded starch is quite resistant to digestion, as previously mentioned due to the high crystallite content. Retrogradation of starch will decrease the total starch that can undergo hydrolysis by amylose enzymes, which is attributed to structure limiting enzyme binding[65].

2.5 Modifying digestibility

2.5.1 Heat moisture treatment (HMT)

Heat moisture treatment (HMT) is the physical modification typically applied to starch to alter the amorphous and crystalline regions of starch. It involves incubating low moisture content native starch at a temperature above the glass transition temperature but below the gelatinisation temperature, which can result in crystalline disruption[66]. Studies using heat-moisture treatment to increase crystallinity of waxy potato starch granules showed that HMT was responsible for increased SDS content. This was explained by HMT forming shorter double helix crystallites allowing partial enzyme accesibility[67]. Investigation into the relationship between temperature-cycled crystallinity of native rice starch and digestibility also produced results that used HMT to favour SDS. This research demonstrated that longer periods of temperature cycling showed a decrease in RDS content that was attributed to changes in crystallinity of the starch, causing more crystalline structures to be formed that decreased enzyme susceptibility to starch[68]. This suggested that longer exposure times can produce more enzyme resistant starches. Another paper used HMT on cooked starch to manipulate brown rice flour in rice cakes and showed that the treatment increased the hardness and cohesion of the cakes, suggesting that the HMT was creating a protective shell around the starch granules, increasing thermal stability through strengthening

amylose-amylopectin interactions[69]. As a result, the cooked rice cakes showed decreased digestibility. Processes like HMT and annealing of gelatinized starch have been shown to decrease RDS while increasing SDS and RS, with the amylopectin structures dictating the final digestibility measurements[70]. These treatments change the susceptibility of starch to enzyme degradation by enhancing interactions in the starch chains and causing already existing crystallite structures to strengthen through aggregation. Some work has been done looking into the relationship between HMT and retrogradation, which showed that the retrogradation process will be directly influenced by the rearrangement of the starch chains within both the amorphous and crystalline regions of the granule brought on by HMT[71]. This means that treatments which disrupt the arrangement of amylose and amylopectin within the granule prior to retrogradation can be applied towards manipulating the final structure of starch after retrogradation has occurred. This current research plans to build on this and investigate if increasing crystallinity to decrease digestibility, through similar processes, could be applied towards cooked starch particles in novel ways.

2.5.2 Acid treatment

In addition to enzymes, acid can also hydrolyse starch. Acid hydrolysis of native starch consists of two distinct phases over time; the first being fast acid hydrolysis of the amorphous starch structure followed by a second stage where the densely packed crystalline regions of the starch granules are hydrolysed [72]. This is because the hydrolysis of the former allows access to the latter. It has long been established that the amorphous region of starch is more susceptible to these kinds of treatments compared to the crystalline region[36]. Crystalline regions of starch are more resistant to acid hydrolysis and therefore are hydrolyzed slower. Unlike with enzyme hydrolysis, acid hydrolysis does appear to preferentially hydrolyze different regions of starch. Past work has confirmed the concept of preferential hydrolysis of the amorphous region of starch which consists

of amylose[53]. This is because the loosely packed amylose is more accessible than tightly packed crystallites or amylopectin which is branched with glucosyl- or maltosyl- units. Hydrochloric acid (HCl) treatment has been explored as a method for changing the crystalline structure of starch, with exposure times of over 4 days producing a higher degree of crystallinity with increasing hydrolysis time[73]. This can be applied as a treatment before starch is introduced to enzymes that would hydrolyze starch, changing the rate at which it would normally be digested. This current work aims to apply acid treatment when making retrograded starch particles to change the accessibility of enzymes during simulated digestion.

2.5.3 Polyphenols

Targeting starch digestive enzymes directly can also lead to reduced release of glucose into the digestive system. Past research has attempted to utilize phenolic compounds as a means of inhibiting the enzymes involved in starch digestion. This was investigated in a study with luteolin, a flavonoid, administered in doses of 100 and 200mg per kg doses to rats, but the results showed that it was unsuccessful as an inhibitor of α-glucosidase in order to achieve and anti-glycemic effect, compared to a control[74]. Later work on phenolics has shown more promise. Research done with phenolics from finger millet seed coat that contained phenolic compounds (10% w/w) was able to bind to and non-competitively inhibit α-amylase, α-glucosidase, and pancreatic amylase, thereby slowing the rate of the starch hydrolysis by these enzymes[75].

This action can also be observed in fruits containing phenolics. Plant extracts such as berries that are rich in polyphenols have been shown to inhibit the starch digestive enzymes α-amylase and α-glucosidase[76]. Research on berries has shown that regular consumption has been able to reduce the risk of Type II diabetes with anthocyanins thought to be playing a role in this, inhibiting postprandial glycemia and insulinemia after carbohydrate consumption[77]. This has been theorized

to be due to anthocyanins interacting with pancreatic starch digestive enzymes and regulating digestion of carbohydrates into glucose. This can be used as an advantage when designing a product where limiting hydrolysis of starch and release of glucose is the goal. Incorporating elements from berries could provide a two-fold benefit of introducing polyphenols that would limit enzyme activity while also improving the consumer appeal of a product by using healthy and popular fruits.

Among fruit, berries have been found to have high polyphenol content. Flavonoids found in berries have been shown to slow the digestion of starch. Work done using extracts from berries showed inhibitory potential with ellagitannins thought to be the phenolic responsible, but this remained unconfirmed[50]. The role of the individual polyphenols in the inhibition of starch digestive enzymes compared to the berry extracts which contain mixes of polyphenols including flavonoids as well as ellagitannins remains to be explored. A recent review on raspberries and the polyphenolics found in raspberries addressed the ability of the berry extracts ability to inhibit both α-amylase and α-glucosidase and noted the potential for its application towards influencing starch digestion[78]. The inhibitory effect on starch digestion enzymes from ellagitannins, proanthocyanins, and catechins found in berries has been theorized to be additive [79]. For this reason, it would be interesting to investigate how different sources of polyphenols impact starch digestion.

Investigation into the presence of natural levels of flavonoids in starch digestion showed that they have the ability to interfere with both α-amylase and α-glucosidase, shifting the amount of RDS to alter digestion and shift to a higher proportion of SDS and RS[80]. Raspberries and strawberries in particular have been found to have an inhibitory effect on enzymes involved in starch digestion[50]. Raspberry extracts containing phenolic compounds (ellagic acid, cyanidin-diglucoside, pelargonidin-3-rutinoside, and catechins) were shown to directly inhibit the activity of α-

glucosidase with uncertainty regarding the exact mechanism[81]. One consideration when working with starch and phenolics is the potential for interactions that could have an effect on the overall functionality and quality of the product. A 2015 review on starch-phenolic interaction listed how phenolic-rich plant extracts were able to alter such properties of starch including gelatinisation and retrogradation through the presence of non-covalent interactions which would directly influence digestibility[13]. What these interaction create is a V-type amylose inclusion complex, which entraps the compounds and protects them from degradation[13]. Looking to novel sources for the kinds of flavonoids in food sources like berries would be beneficial for developing a product that would lower starch digestion rates.

2.6 Creating a product

This work aims to create particles that are digested at a slower rate through application of different techniques and concepts. Acid hydrolysis can be used to change the molecular order of retrograded starch particles. Exposing starch to acid hydrolysis for different durations and at different concentrations could create particles with different structures. The molecular order of these particles can be estimated using DSC and their digestibility can be measured *in vitro* using a simulated digested method. A structure function relationship can be estimated by comparing these results. The size and relative surface area of the particles as well as the introduction of polyphenols could also impact the starch particle digestibility. The retrogradation process itself may contribute to digestibility of the particles. Combining these different conditions and treatments could create a particle with optimal digestibility.

3 Chapter 1: Starch structure and digestibility

3.1 Introduction

Starch crystalline structure is the main characteristic to consider when designing a food product such as this that aims to slow digestion. Previous research has shown that the ratio of crystalline to amorphous regions in starch granules will greatly impact the digestibility of starch. A past study on starch digestibility showed that an approximate 100 fold increase in rigidity of starch in gel form showed a 5% decrease in hydrolysis by α-amylase due to reduced accessibility of the amorphous region of starch[62]. Translating this into constructing a starch particle would mean focusing on controlling the structure of the particles to favour a more compact and crystalline structure as to limit enzyme accessibility. The particles with more crystalline content would be desirable as they would be more resistant to enzyme hydrolysis and therefore less digestible.

It has been established that acid hydrolysis of starch is capable of influencing the in vitro digestibility of starch, with there generally being an increase in RDS and SDS at the expense of RS which may result in an overall slower digestion rate due to the increase in SDS[82]. This is due to the structure being disrupted and there being room for enzyme access created. Acid hydrolysis of native starch consists of two distinct phases over time; the first being fast hydrolysis of the amorphous starch structure followed by a second stage where the densely packed crystalline regions of starch granules are hydrolysed [72]. If different extents of exposure can produce different degrees of crystalline structure, this could be used to create a series of particles with different susceptibilities to enzyme hydrolysis. Therefore, changing the starch structure via hydrolysis could change the digestibility of the particles.

In terms of starch structure measurement using DSC, acid hydrolysis of starch generally increases the onset temperature of phase transitions of retrograded starches, with the reasoning behind this

thought to be that acid hydrolysis accelerates starch retrogradation by generating smaller molecules that could cause disorder of structure upon the recrystallization of the starch gels[82].

The present study used a hydrochloric acid (HCl) treatment of corn and potato starch, either in the native or the particle form. The goal was to change the crystalline content of starch in in a way that would create a range of crystalline structures through different exposure times. Treatment was done before or after heating to determine if gelatinisation and retrogradation were able to amplify or minimize the effects of the hydrolysis in any way. This allowed a range of crystallinities to be created and DSC technology was employed to estimate relative molecular order of these particles. The interest lay in the relationship between crystalline structure and starch digestibility and how these two factors would then be measured and compared.

The objective of this section was to determine if the relationship between increased crystallinity was correlated to decreased starch digestibility. The additional goal was to determine which acid treatments and which molarities of HCl showed optimal digestion kinetics and how these factors affected the rate of digestion (k). Digestibility was measured through measuring release of glucose over time to create a digestion curve or digestogram and supply digestion kinetics. Faster digestion kinetics where there was an increase in extent of acid hydrolysis were to be expected, as increased hydrolysis result has been previously shown to increase crystallinity.

3.2 Materials and methods

3.2.1 Source materials

The following enzymes: α-Amylase (type VI-B, from porcine pancreas), pancreatin (8x USP from porcine pancreas), pepsin (from porcine gastric mucosa), amyloglucosidase (from *Aspergillus niger*) were obtained from Sigma-Aldrich Corporation (St. Louis, Missouri, United States). All enzymes were stored at -20ºC. Potato starch and corn starch were obtained from Sigma-Aldrich Corporation (St. Louis, Missouri, United States).

3.2.2 Starch particle formation

Starch particles were designed according to Figure 6.

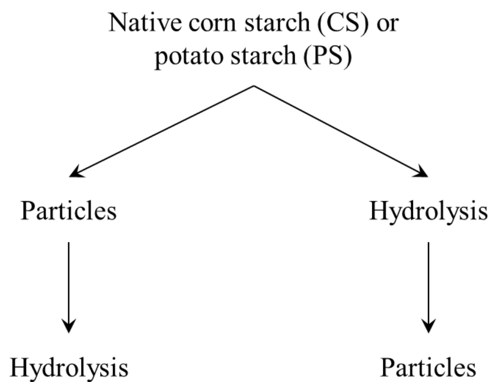

Figure 6. Formation of starch particles based on at which point acid hydrolysis was incorporated.

Starch was suspended into a slurry (20% w/w starch in water dH_2O with 200 ppm NaN_3 to inhibit microbial growth). This mixture was stirred constantly and heated to approximately 75-80°C to ensure complete gelatinisation of starch[83]. The resulting paste was deposited onto aluminum foil as particles (5mm in diameter) using a syringe shown in Figure 7. The particles were then sealed in plastic so as to retain consistent moisture content, then stored at 4°C for at minimum 24 hours prior to *in vitro* digestion to allow for complete retrogradation.

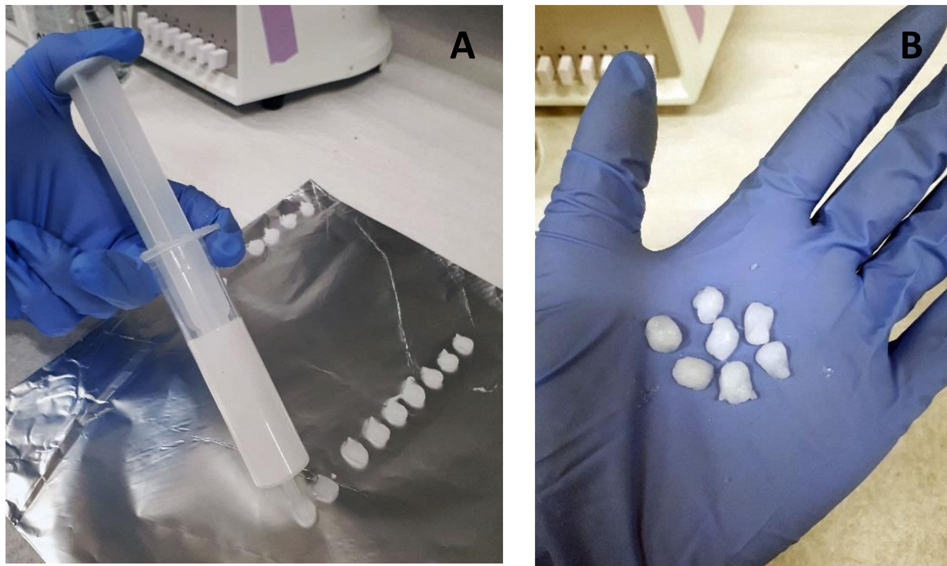

Figure 7. (A) Deposition method for formation of starch particles. (B)Fully retrograded potato starch particles after 24 hours of storage at 4°C.

3.2.3 Hydrolysis of native starch and starch particles

Corn starch (CS) or Potato Starch (PS) were subjected to acid hydrolysis treatment pre- or post-formation of particles as described in Figure 6. Native starch or starch particles was stirred in dilute HCl (2M or 3M) at a 1:1 (mass: volume) ratio, at room temperature for 24, 48, 72, 96, or 120 hours. These were referred to as 1, 2, 3, 4, or 5 Day hydrolysis periods. Neutralization was performed with an equivalent amount of NaOH (2M or 3M) and pH manually was adjusted to 7. Native starch was recovered by centrifugation (15000 RPM, k-Factor 455, 15 min) whereas starch particles were simply filtered, and all hydrolyzed materials were washed three times with dH_2O[84]. Hydrolyzed starches were then dried overnight at room temperature before use in particle formation. Both native starch and starch particles were stored at 4°C until use.

3.2.4 *Moisture content*

Moisture content of the starch samples was measured using a Moisture Analyzer (Mettler Toledo, Columbus, Ohio, USA). Samples of approximated 1.5-2.0g were measured in the tray and the percent moisture content was subtracted from the initial digestion sample weights and used to calculate Total Starch (TS) content. This measurement allowed the progression of starch digestion to be tracked and allowed for digestion kinetics to be calculated. The resistant starch fraction was calculated by subtracting the amounts of SDS and RDS from the amount of Total Starch (TS). RS= TS-RDS-SDS. The amount of total starch in a sample can be calculated using moisture content and starch content of a given sample.

$$Total\ Starch = Moisture\ Content\ x\ Mass\ of\ starch\ Sample$$

3.2.5 *In vitro digestion (IVD)*

Glucose release from starch digestion was measured with an IVD method described by Minekus *et al.* (2014) with modifications[60]. Samples consisted of 3g of starch particles suspended in 2mL of water. Digestion was conducted in a shaking water bath at 37 °C and consisted of: 1. an oral phase, 2 minutes at pH 7 with α-amylase (1500 U/mL from porcine pancreas, Sigma Aldrich St. Louis, Missouri, United States).); 2. a gastric phase, 2 hours at pH 3 with pepsin (25 000 U/mL from porcine gastric mucosa, Sigma Aldrich St. Louis, Missouri, United States).), and 3. an intestinal phase for 2 hours at pH 7 with pancreatin (2mg/mL from porcine pancreas, Sigma Aldrich St. Louis, Missouri, United States)[60] and amyloglucosidase (AMG) (280 U/mL from Aspergillus niger, Sigma-Aldrich St. Louis, Missouri, United States). A glucometer (Accu-Chek®

Perfoma Glucometer, Roche Pharmaceuticals, Mississauga, Ontario, Canada) was used to measure concentration of glucose concentration (mmol/L) upon commencement of the intestinal phase and every 20 minutes for the 2 hour duration of the intestinal phase of IVD. The glucometer reading was used to calculate amount of starch digested using methods from Sopade and Gidley, 2009[85].

Advancement of starch digestion monitored by the appearance of free glucose in solution was calculated in percent of available starch digested as: $\frac{G \times V / 180}{m \times S / 161}$, where V = volume of digesta (L), 180 = molecular weight of glucose (g/mol), m = mass of test sample digested (g), S = mass fraction of starch in test sample as is (%), 161 = molecular weight of starch anhydroglucose monomeric units (g/mol), and G = glucose concentration in the digesta (g/L).

3.2.6 Differential Scanning Calorimetry (DSC)

DSC was used to estimate the degree of molecular order of starch in the particles[86]. This was performed using a Nano-DSC (TA Instruments, New Castle, Delaware, USA). Samples consisted of between 5 and 15mg/ml of finely crushed particles dispersed in dH_2O. Samples were heated from 5°C to 130°C at 1°C/min and a pressure of 3ATM. Heat flow was measured in µW against a reference cell filled with dH_2O[87]. Peak (T_p), onset (T_o), and conclusion (T_c) temperatures were recorded in °C and area under the curve (AUC, in µW/°C) was estimated using NanoAnalyze software (TA Instruments, New Castle, Delaware, USA). AUC was used to calculate ΔH.

3.2.7 Statistical analysis

All samples were prepared and analyzed in triplicate. Digestograms showing rate of glucose release over digestion period were fitted with a first order kinetic equation

$$[\%glucose] = C + A(1 - e^{-k_D t})$$

where [% glucose] is defined as $[free\ glucose] \times 100 / [total\ glucose\ in\ sample]$.

A 2-way ANOVA followed by a Tukey's test (post-hoc pair-wise comparison) was used to identify significance in digestion rate (k_D) and the Amplitude (A) values of the first order kinetic equation and also the onset, peak, and conclusion melting temperatures and melting enthalpies between different acid treatments. These tests were run for 4 groups (particles from potato starch hydrolyzed before formation of the particles, particles from potato starch hydrolyzed after formation of the particles, particles from corn starch hydrolyzed before formation of the particles, particles from corn starch hydrolyzed after formation of the particles) and aimed at comparing the different acid treatments (differing by time of treatment and concentration of acid) within the four groups, Significance was set to $p<0.05$.. Systat 13 statistical analysis software was used.

3.3 Results and Discussion

3.3.1 Overall impact of factors

Acid hydrolysis appeared to significantly impact the order of starch structure as well as the rate at which the particles were digested. Acid treatment of native starch or retrograded starch particles did have significant effects on starch molecular order as evidenced by the DSC results. These different treatments changed the starch structure but did not change the way it was digested in any major capacity. The choice of starch also did not appear to impact digestion. This is consistent with corn and potato starch having slightly different structures and characteristics. For this reason, starch source is a more important consideration for starch particle formation. Things such as ease of particle formation and organoleptic properties such as mouthfeel and not the actual glycemic response will be impacted more by starch source.

3.3.2 Manipulation of starch structure

3.3.2.1 Structure of particles formed from acid treated native starch

Table 1. Thermal properties of corn starch (CS) particles formed from native starch exposed to HCl hydrolysis (2M or 3M) for 1-5 days. Melting enthalpy (ΔH), onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) of melting measured by DSC. Mean (n=2)± SEM. Different letters in one column of table indicate significant differences between all individual samples for that value. Samples sharing letters are not significantly different ($p \leq 0.05$).

Sample	T_o (°C)	T_p(°C)	T_c(°C)	ΔH (kJ/g)
Particles from 1 Day 2M CS	69.2±0.2^{a}	73.5±0.2^{ac}	78.4±0.6 ^{acd}	5.2±0.9^{acd}
Particles from 2 Day 2M CS	68.1±0.8^{a}	71.8±1.7^{ad}	76.1±2.0 ^{a}	5.0±1.0^{acd}
Particles from 3 Day 2M CS	71.9±0.9^{a}	76.2±0.1^{bcde}	80.5±0.5 ^{adf}	5.1±0.7^{acd}
Particles from 4 Day 2M CS	70.0±1.0^{a}	77.5±1.1^{bcf}	80.9±0.1 ^{adg}	6.5±0.1^{ad}
Particles from 5 Day 2M CS	71.5±0.5^{a}	77.9±0.3^{bcg}	83.8±0.3 ^{ae}	5.7±0.0^{acd}
Particles from 1 Day 3M CS	68.3±0.3^{a}	73.8±0.1^{aefgh}	79.2±0.2 ^{adh}	6.6±0.1^{ad}
Particles from 2 Day 3M CS	71.8±0.3^{a}	77.2±0.2^{bchi}	84.1±0.1 ^{bcefgh}	3.1±0.0^{bc}
Particles from 3 Day 3M CS	72.5±0.5^{a}	78.3±0.3^{bchj}	84.6±0.5 ^{befghi}	4.6±0.4^{acd}
Particles from 4 Day 3M CS	72.1±2.5^{a}	75.0±2.2^{aefgijk}	81.0±3.0 ^{acdi}	5.9±0.3^{acd}
Particles from 5 Day 3M CS	68.8±1.8^{a}	76.7±0.2^{bcdhk}	84.1±0.8 ^{bcefgh}	3.8±0.2^{acd}
Normal Corn Starch Particle	65.0^{a}	69.5^{a}	74.7 ^{ad}	6.6^{acd}

Table 2. Thermal properties of potato starch (PS) particles formed from native starch exposed to HCl hydrolysis (2M or 3M) for 1-5 days. Melting enthalpy (ΔH), onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) of melting measured by DSC. Mean (n=2)± SEM. Different letters in one column of table indicate significant differences between all individual samples for that value. Samples sharing letters are not significantly different ($p \leq 0.05$).

Sample	T_o (°C)	T_p(°C)	T_c(°C)	ΔH (kJ/g)
Particles from 1 Day 2M PS	63.3±0.8^{a}	68.4±0.2^{c}	74.0±1.0^{ac}	5.4±0.4^{a}
Particles from 2 Day 2M PS	65.9±0.4^{ac}	70.7±0.1^{d}	75.7±1.2^{adgh}	5.2±0.5^{a}
Particles from 3 Day 2M PS	69.4±0.5^{bcd}	74.2±0.1^{efg}	79.9±0.1^{efgh}	5.5±0.1^{a}
Particles from 4 Day 2M PS	69.6±0.5^{bc}	74.9±0.2^{f}	78.4±0.4^{dfgh}	6.7±0.4^{a}
Particles from 5 Day 2M PS	68.0±0.0^{c}	73.2±0.1^{g}	77.7±0.1^{cg}	6.7±0.2^{a}
Particles from 1 Day 3M PS	71.7±0.2^{bd}	74.8±0.1^{ef}	79.3±0.3^{h}	5.8±0.4^{a}
Particles from 2 Day 3M PS	75.3±1.6^{bd}	79.1±0.5^{b}	83.4±0.4^{be}	4.5±1.1^{a}
Particles from 3 Day 3M PS	74.1±0.1^{b}	79.4±0.2^{b}	85.4±1.1^{b}	4.3±0.8^{a}
Particles from 4 Day 3M PS	74.4±0.2^{b}	80.3±0.0^{b}	84.9±0.7^{b}	6.7±0.1^{a}
Particles from 5 Day 3M PS	72.9±0.6^{bd}	79.0±0.2^{b}	83.8±0.3^{b}	6.2±0.3^{a}
Normal Potato Starch Particle	62.4^{a}	66.5^{a}	72.0^{a}	5.4^{a}

The melting temperatures and enthalpies for particles formed from native corn starch exposed to HCl hydrolysis are shown in Table 1. There were no significant differences within the onset temperatures for these samples. The peak and conclusion melting temperatures were almost all significantly higher than the values for the control of untreated corn starch particle with longer duration and higher molarity of HCl treatment showing higher temperatures. Significant peak temperatures ranged from 76.2±0.1°C to 78.3±0.3°C, compared to the control (65.0°C) and the conclusion temperatures ranged from 81.0±3.0°C to 84.6.3±0.3°C compared to the control (69.5°C). For ΔH, only the particles made from corn starch exposed to 3M HCl for 2 days showed a significantly lower melting enthalpy of 3.1±0.0 kJ/g compared to the control (6.6 kJ/g) while the rest were not significantly different.

Much like the particles formed from the acid treated native corn starch, Table 2. shows significance in the melting temperatures of particles formed from potato starch exposed to HCl hydrolysis. Here nearly all the particles in this category showed significantly higher melting temperatures compared to the control of normal potato starch particles. Significant onset temperatures (T_o) ranged from 68.0±0.0 to 74.4±0.2 °C compared to a control (62.4°C), peak temperatures (T_p) from 68.4±0.2 to 80.3±0.0°C and conclusion temperatures (T_c) from 77.7±0.1 to 85.4±1.1°C . There were no significantly different melting enthalpy values.

3.3.2.2 Structure of Acid Treated Particles

Table 3. Thermal properties of corn starch (CS) particles exposed to HCl hydrolysis (2M or 3M) for 1-5 days. Melting enthalpy (ΔH), onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) of melting measured by DSC. Mean (n=2)± SEM. Different letters in one column of table indicate significant differences between all individual samples for that value. Samples sharing letters are not significantly different ($p \leq 0.05$).

Sample	T_o (°C)	T_p(°C)	T_c(°C)	ΔH (kJ/g)
1 Day 2M CS Particles	66.3±2.6[a]	70.5±0.1[a]	75.0±2.0[a]	4.8±1.0[a]
2 Day 2M CS Particles	64.4±0.7[a]	70.8±0.0[a]	76.4±0.4[a]	4.8±0.1[a]
3 Day 2M CS Particles	64.8±0.3	71.3±0.5[a]	77.6±1.5[a]	3.7±0.8[a]
4 Day 2M CS Particles	66.3±2.8[a]	71.8±1.0[a]	76.6±0.6[a]	5.3±0.3[a]
5 Day 2M CS Particles	64.0±1.5[a]	71.1±0.6[a]	76.4±0.9[a]	4.2±0.7[a]
1 Day 3M CS Particles	65.8±0.3[a]	71.2±0.8[a]	76.8±1.3[a]	4.9±0.9[a]
2 Day 3M CS Particles	64.1±0.6[a]	70.9±0.2[a]	75.9±0.3[a]	4.9±0.1[a]
3 Day 3M CS Particles	63.8±0.3[a]	71.0±0.2[a]	76.8±0.3[a]	4.2±0.0[a]
4 Day 3M CS Particles	63.5±0.5[a]	70.7±0.6[a]	77.4±1.3[a]	3.3±0.5[a]
5 Day 3M CS Particles	62.6±0.6[a]	71.0±0.2[a]	76.6±0.4[a]	3.5±0.2[a]
Normal Corn Starch Particle	65.0[a]	69.5[a]	74.7 [a]	6.6[a]

Table 4. Thermal properties of potato starch (PS) particles exposed to HCl hydrolysis (2M or 3M) for 1-5 days. Melting enthalpy (ΔH), onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) of melting measured by DSC. Mean (n=2)± SEM. Significance set to $p<0.05$. Letters indicates significance. Values sharing letters are not significant.

Sample	T_o (°C)	T_p(°C)	T_c(°C)	ΔH (kJ/g)
1 Day 2M PS Particles	61.0±1.0[a]	66.6±0.3[c]	71.5±0.6[c]	5.7±0.3[a]
2 Day 2M PS Particles	62.2±1.6[a]	68.0±0.2[acd]	73.9±0.1[acd]	5.4±0.3[a]
3 Day 2M PS Particles	62.2±0.8[a]	68.4±0.2[ace]	74.1±0.7[ace]	5.8±0.1[a]
4 Day 2M PS Particles	63.5±0.1[a]	69.1±0.1[acf]	73.8±0.4[acf]	5.3±0.3[a]
5 Day 2M PS Particles	63.2±1.0[a]	69.9±0.5[ag]	74.9±0.1[acg]	4.9±0.1[a]
1 Day 3M PS Particles	62.2±0.8[a]	67.8±0.2[ac]	73.0±0.2[ac]	5.5±0.1[a]
2 Day 3M PS Particles	61.7±2.2[a]	67.8±1.4[ac]	72.2±1.6[ac]	5.4±0.2[a]
3 Day 3M PS Particles	64.5±1.0[a]	71.8±0.1[bdfg]	77.7±0.4[bgh]	4.5±0.1[b]
4 Day 3M PS Particles	64.2±0.7[a]	71.2±0.0[befg]	76.5±0.1[bdefgh]	4.6±0.1[b]
5 Day 3M PS Particles	64.5±0.6[a]	70.9±0.4[bdefg]	75.4±0.0[ah]	5.2±0.0[a]
Normal Potato Starch Particle	62.4[a]	66.5[ac]	72.0[ac]	5.4[a]

The melting temperatures and enthalpies of the acid treated corn starch particles are shown in Table 3. There were no significant differences shown in any of the onset, peak or conclusion temperatures. There were also no significant differences shown in the ΔH values.

The melting temperatures and enthalpies of the acid treated potato starch particles are shown in Table 4. There were no significant differences between the onset temperatures. For the peak temperatures, the 3, 4, and 5 day 3M treatments (71.8±0.1°C, 71.2±0.0°C, and 70.9±0.4°C) were all significantly higher compared to the control (66.5°C). Similar results were seen for the conclusion temperature where only the 3 and 4 day 3M treated particles (76.5±0.1°C, and 75.4±0.0°C) were significantly higher than the control (72.0 °C). Again the 3 and 4 day 3M treated particles were the only treatments with ΔH values (4.5±0.1 kJ/g and 4.5±0.1 kJ/g) significantly lower than the control (5.4 kJ/g).

3.3.3 Starch particle digestibility

3.3.3.1 *Acid hydrolysis of native starch and digestibility*

Digestion Rate (K) of Particles from Acid Treated Native Corn Starch

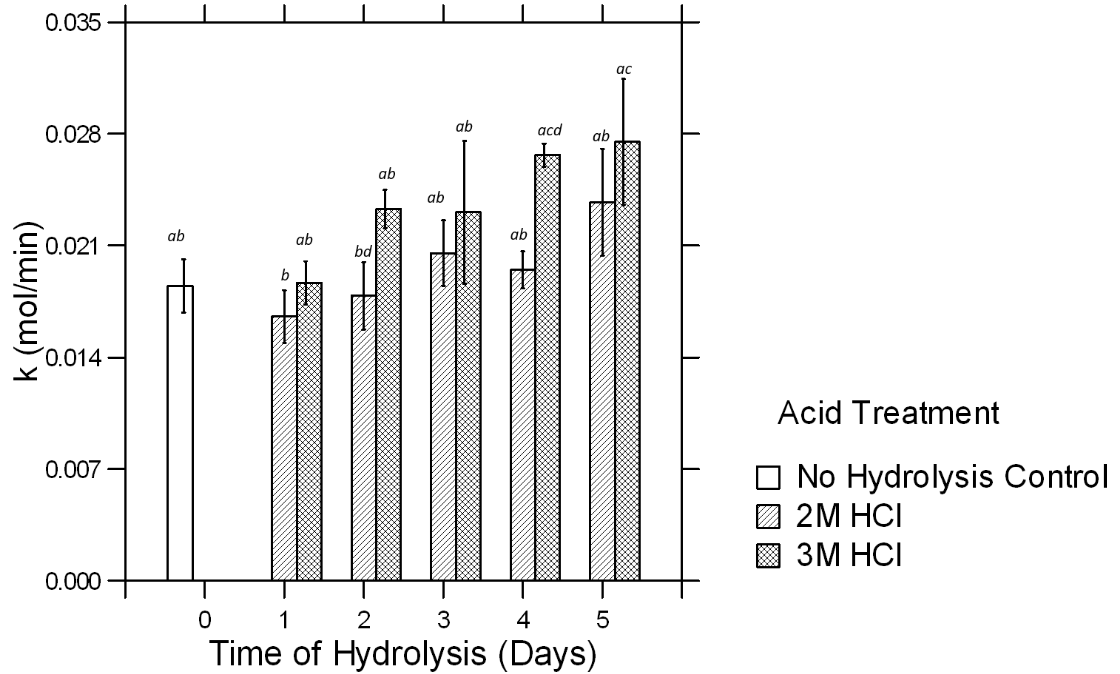

Figure 8. Digestion rate (k) of particles formed from native corn starch exposed to HCl hydrolysis for between 1-5 Days. Calculated from release of glucose being fitted to integrated kinetic equation. Mean (n=3)±SEM. Different letters above bar indicate significant differences between all individual samples for that value. Samples sharing letters are not significantly different ($p \leq 0.05$).

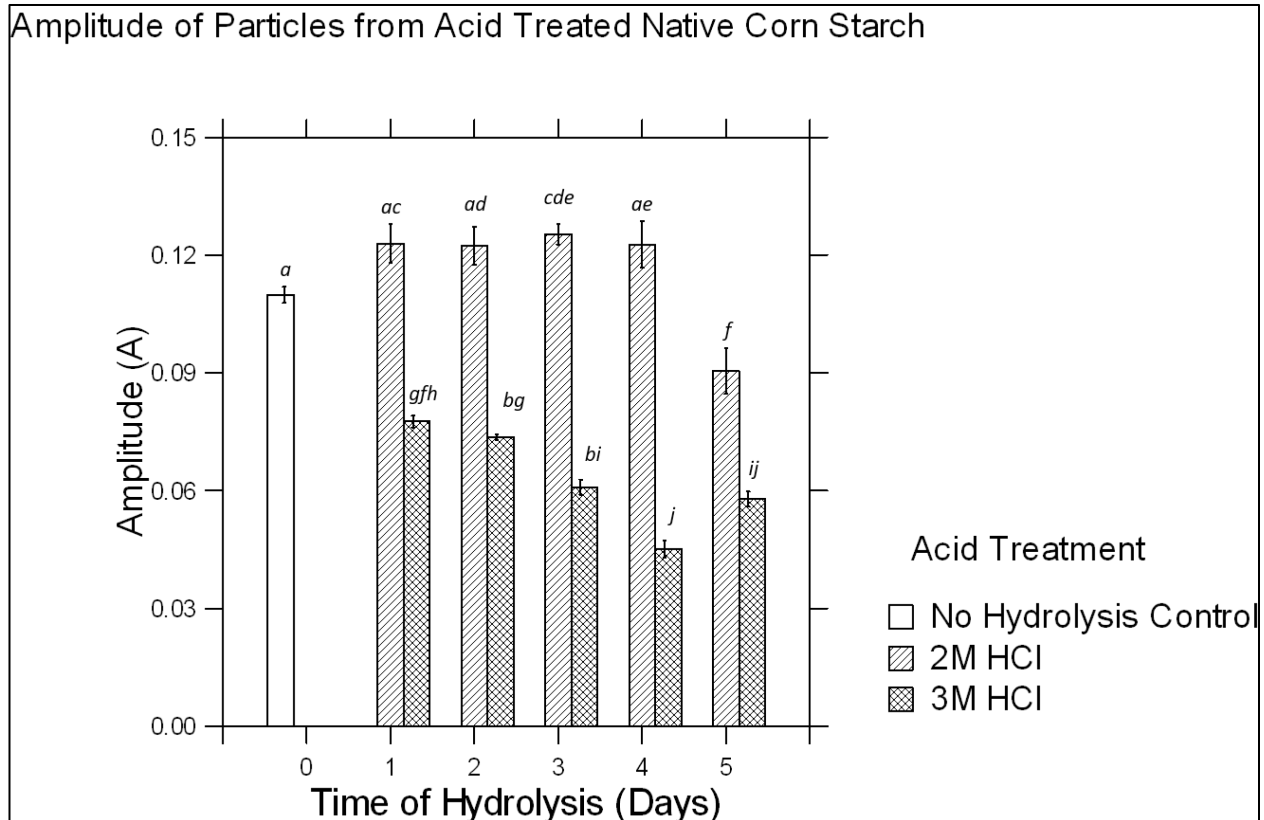

Figure 9. Amplitude (A) of particles formed from native corn starch exposed to HCl hydrolysis for between 1-5 Days. Calculated from release of glucose being fitted to integrated kinetic equation. Mean (n=3)±SEM. Different letters above bar indicate significant differences between all individual samples for that value. Samples sharing letters are not significantly different ($p \leq 0.05$).

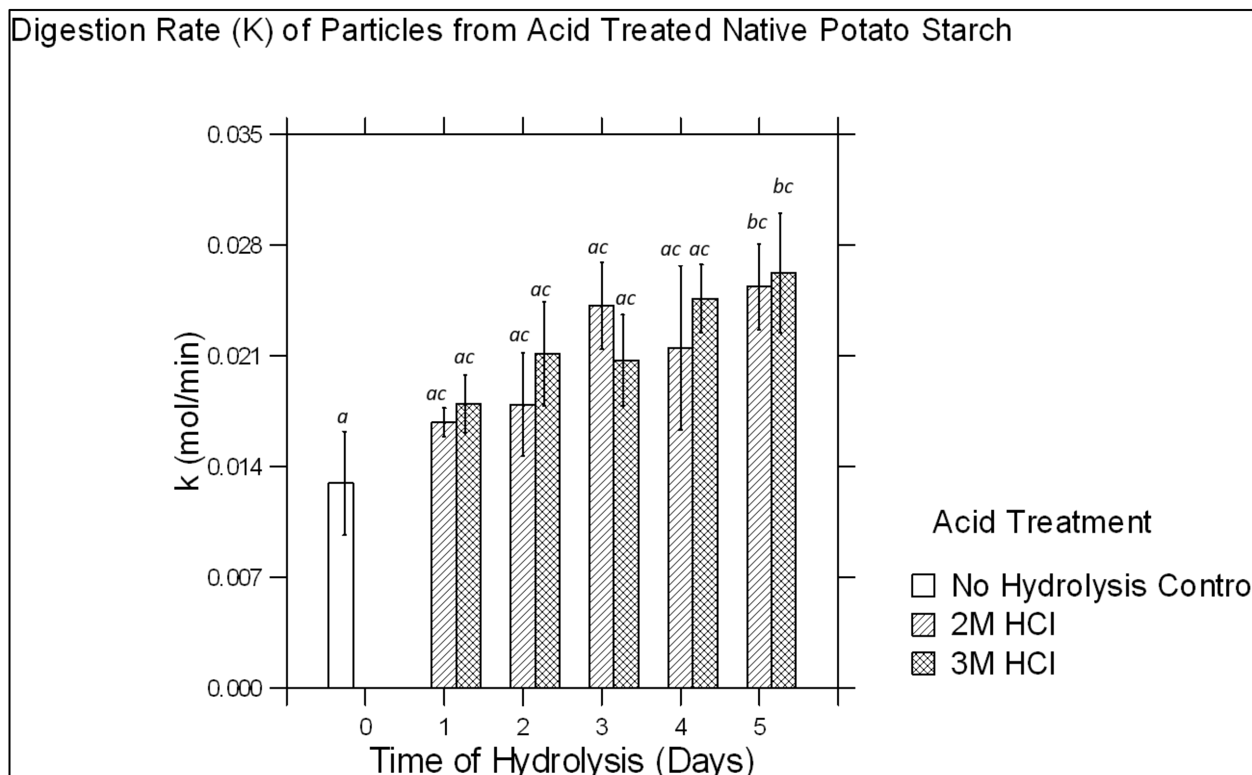

Figure 10. Digestion rate (k) of particles formed from native potato starch exposed to HCl hydrolysis for between 1-5 Days. Calculated from release of glucose being fitted to integrated kinetic equation. Mean (n=3)±SEM. Different letters above bar indicate significant differences between all individual samples for that value. Samples sharing letters are not significantly different ($p \leq 0.05$).

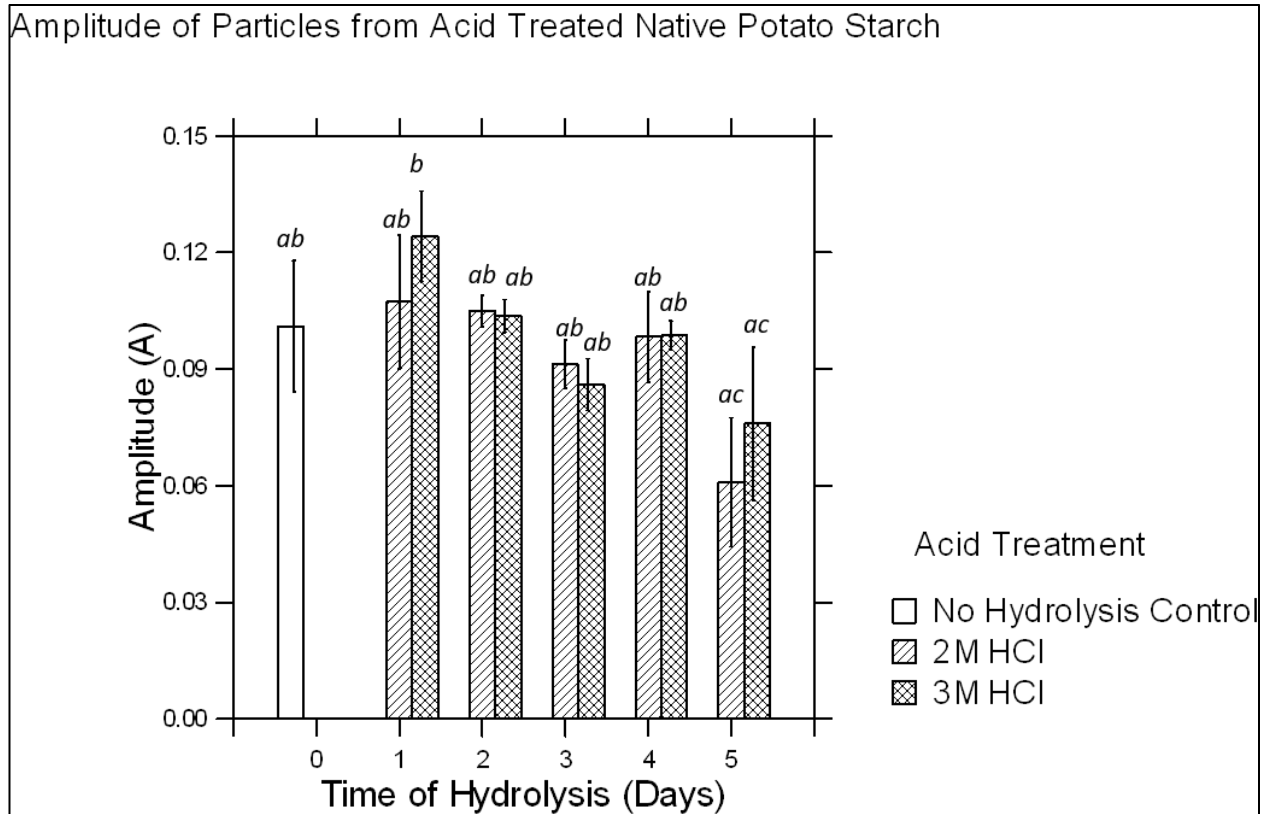

Figure 11. Amplitude (A) of particles formed from native corn starch exposed to HCl hydrolysis for between 1-5 Days. Calculated from release of glucose being fitted to integrated kinetic equation. Mean (n=3)±SEM. Different letters above bar indicate significant differences between all individual samples for that value. Samples sharing letters are not significantly different ($p \leq 0.05$).

The digestion rates (k value) of particles formed from acid treated native cornstarch are shown in Figure 8. This value is reflective of the speed of digestion. All acid treated particles had increasing digestion rates but only the particles formed from the starch exposed to the 4 and 5 day 3M HCl treatment had k values (0.027±001 mol/min and 0.027±003 mol/min) that were significantly higher than the control (0.018±0.002 mol/min).

The amplitudes (A value) of particles formed from acid treated native cornstarch are shown in Figure 9. This value is reflective of the total amount of starch digested by the end of the simulated digestion and represents the extent of digestion. Acid treatment appeared to decrease the A value with the longer duration and higher molarity treatments having significantly lower A values compared to the control (0.110±0.002). These were the particles from the 5 Day 2 M treatment (0.060±0.004) and the particles from the 1,2,3,4 and 5 day 3M treatments (0.078±0.001, 0.074±0.00, 0.061±0.001, 0.045±0.002, and 0.058±0.001).

The digestion rates (k value) of particles formed from acid treated native potato starch are shown in Figure 10. Again, all acid treated particles had increasing digestion rates but only the particles exposed to the acid treatment for longer durations had significantly higher digestion rates. There were the particles formed from the starch exposed to the 5 day 2 M and 3M HCl treatments which had k values (0.025±002 mol/min and 0.026±002 mol/min) that were significantly higher than the control (0.013±0.002 mol/min).

The amplitudes (A value) of particles formed from acid treated native potato starch are shown in Figure 11. Acid treatment appeared to decrease the A value with the longer duration and higher molarity treatments but none were significantly lower than the control (0.101±0.013).

3.3.4 Digestibility of acid treated particles

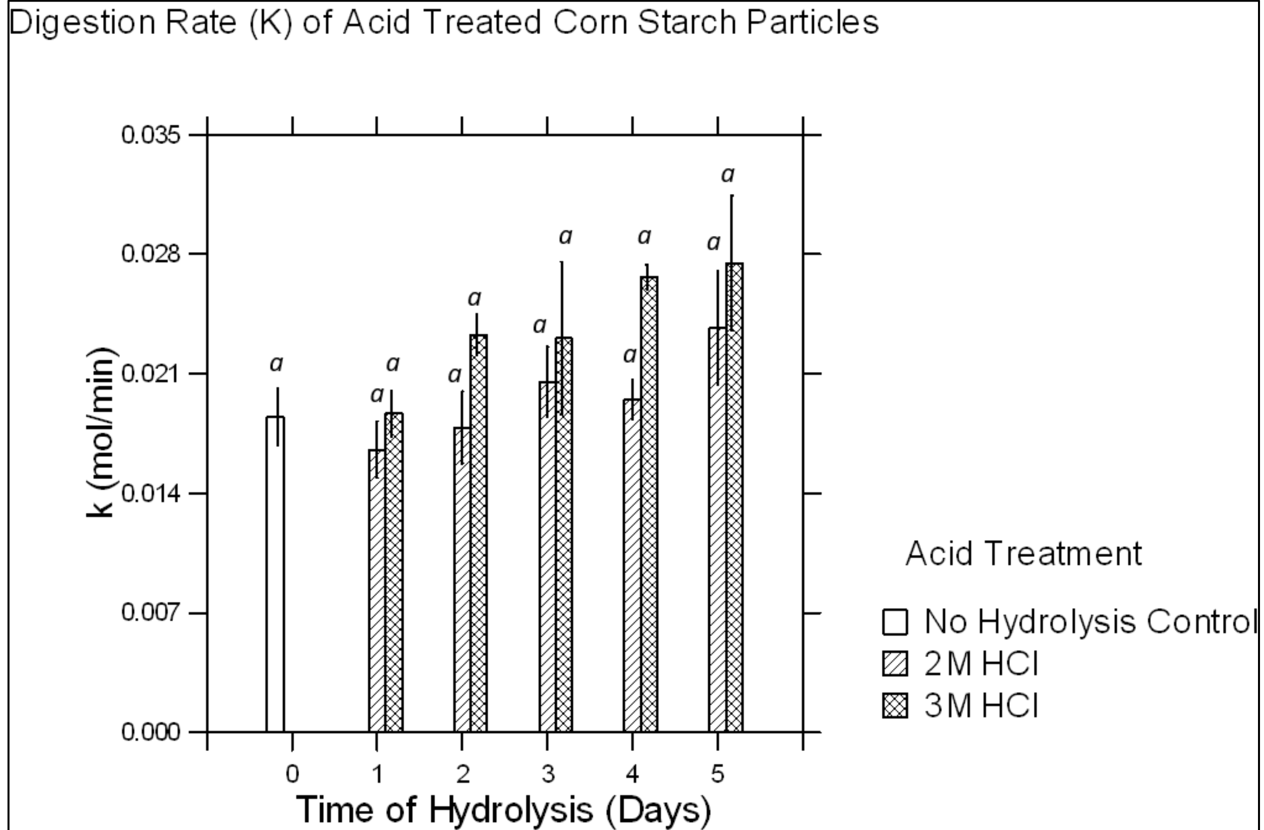

Figure 12. Digestion rate (k) of starch particles exposed to HCl hydrolysis for between 1-5 Days. Calculated from release of glucose being fitted to integrated kinetic equation. Mean (n=3)±SEM. Different letters above bar indicate significant differences between all individual samples for that value. Samples sharing letters are not significantly different ($p \leq 0.05$).

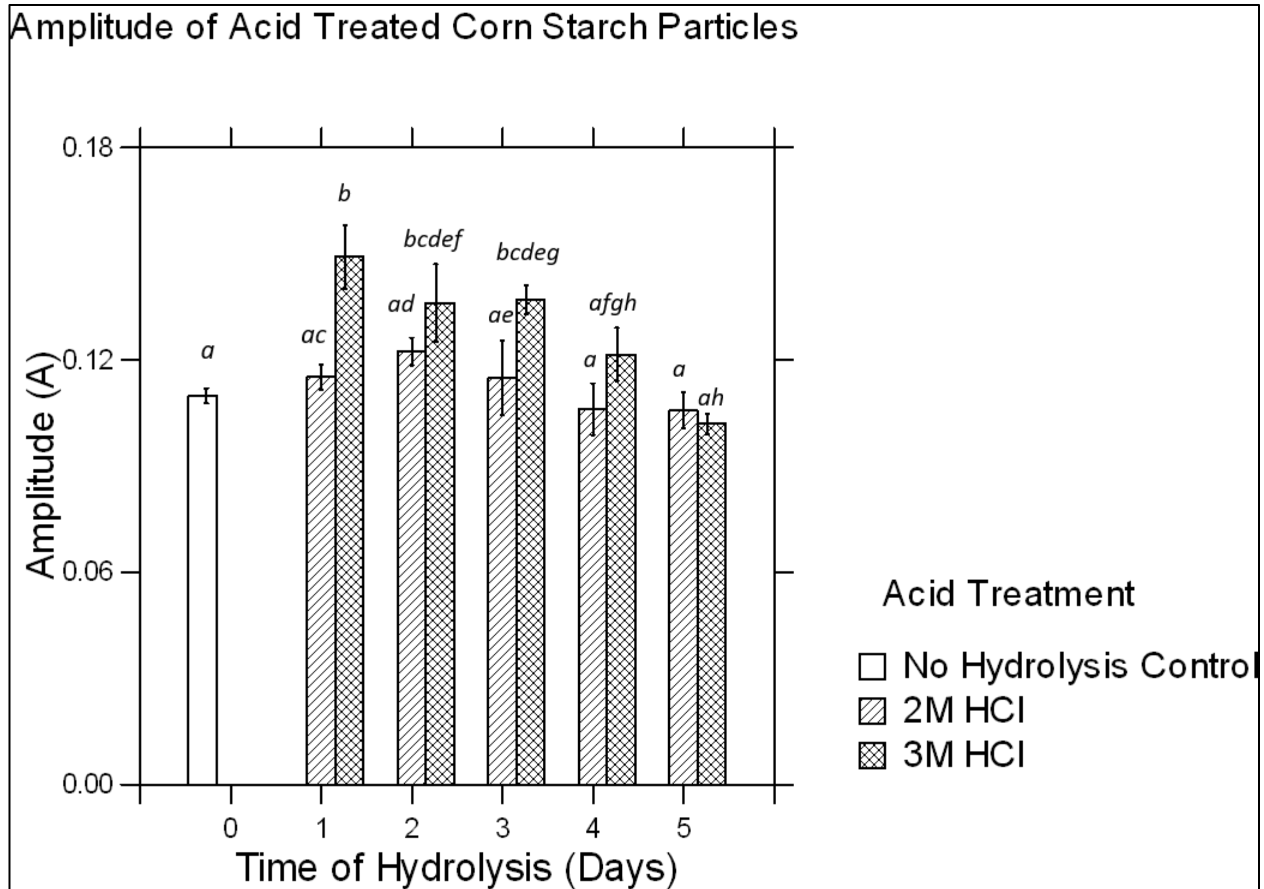

Figure 13. Amplitude (A) of starch particles exposed to HCl hydrolysis for between 1-5 Days. Calculated from release of glucose fitted to integrated kinetic equation. Mean (n=3)±SEM. Different letters above bar indicate significant differences between all individual samples for that value. Samples sharing letters are not significantly different ($p \leq 0.05$).

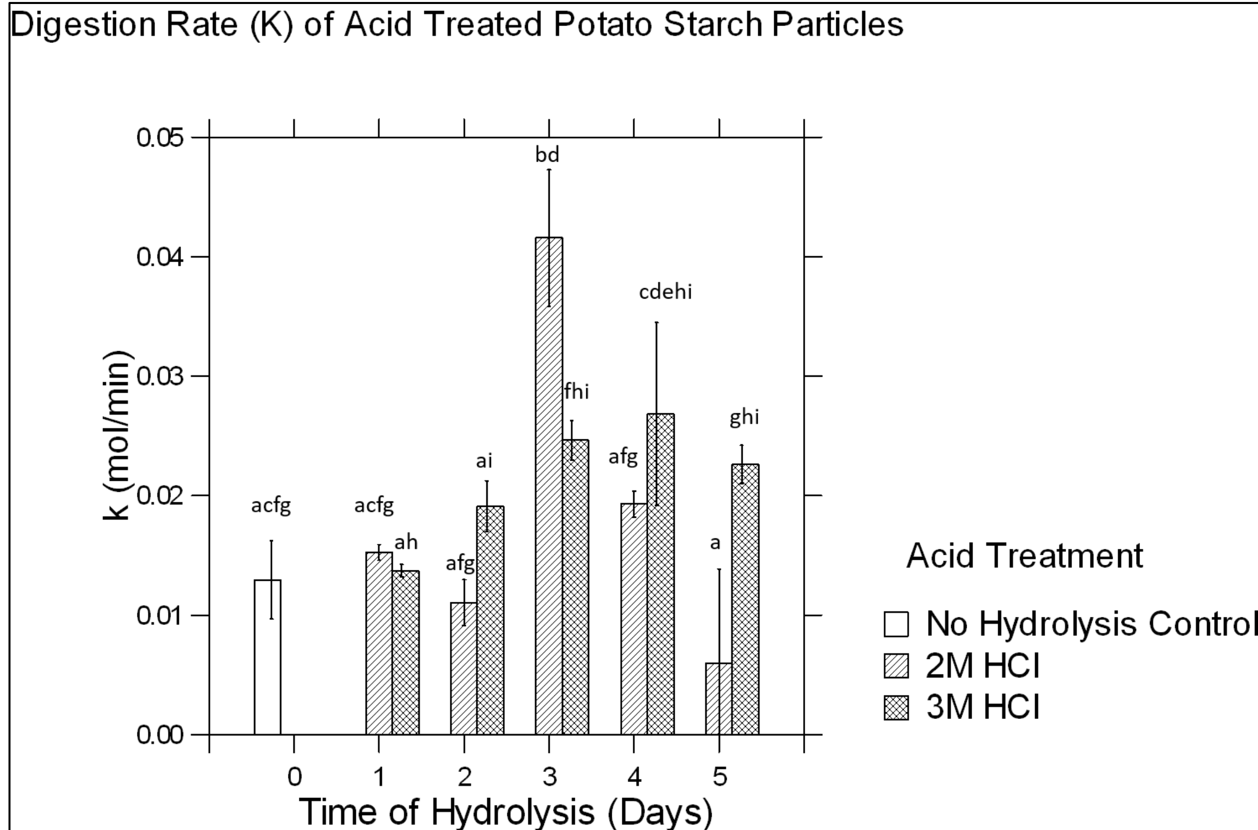

Figure 14. Digestion rate (k) of starch particles exposed to HCl hydrolysis for between 1-5 Days. Calculated from release of glucose being fitted to integrated kinetic equation. Mean (n=3)±SEM. Different letters above bar indicate significant differences between all individual samples for that value. Samples sharing letters are not significantly different ($p \leq 0.05$).

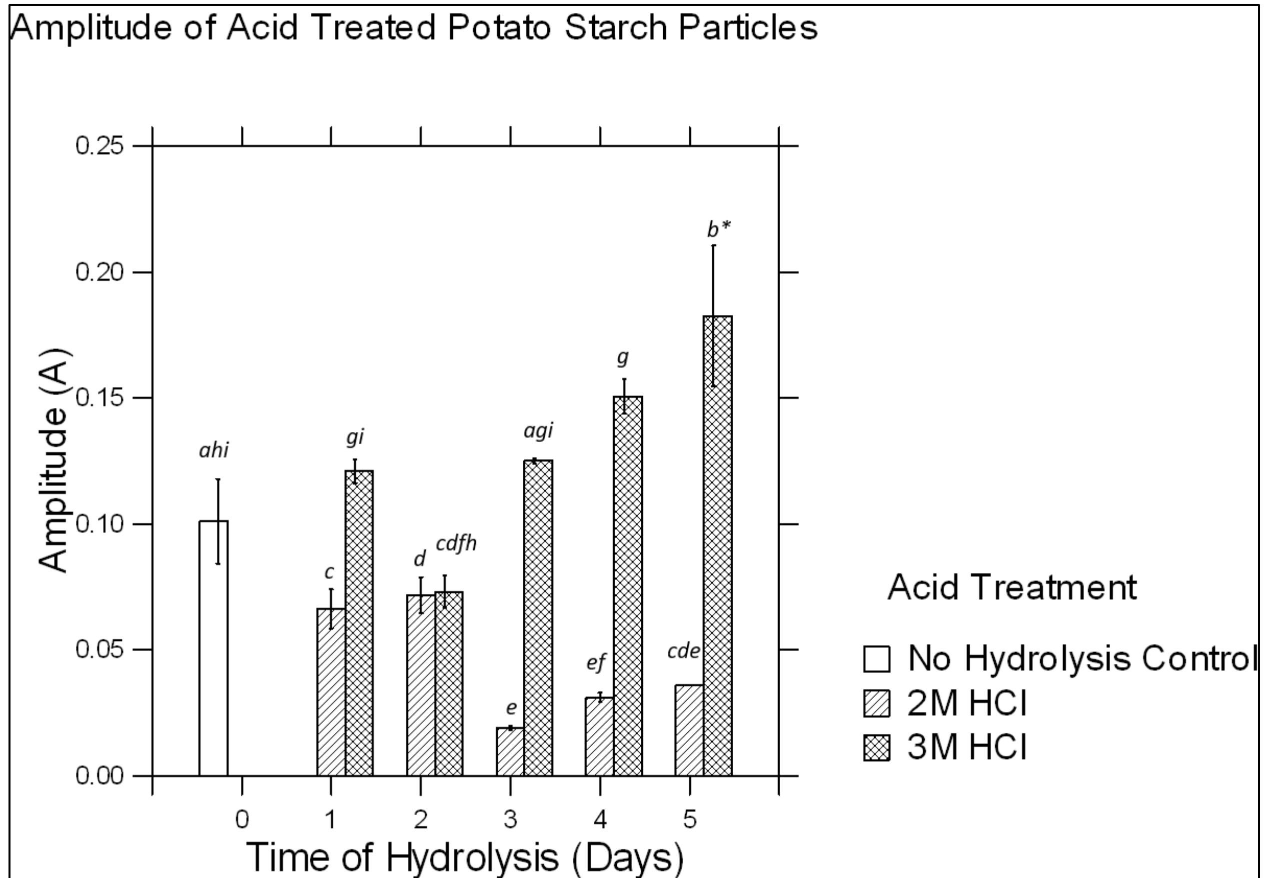

Figure 15. Amplitude (A) of starch particles exposed to HCl hydrolysis for between 1-5 Days. Calculated from release of glucose being fitted to integrated kinetic equation. *indicates n=1, outliers removed. Mean (n=3)±SEM. Different letters above bar indicate significant differences between all individual samples for that value. Samples sharing letters are not significantly different ($p \leq 0.05$).

The digestion rates (k value) of acid treated corn starch particles are shown in Figure 12. All acid treated particles had slightly increasing digestion rates with increasing duration and molarity of acid treatment, but none were significantly different than each other or the control.

The amplitudes (A value) of acid treated corn starch particles are shown in Figure 13. The only significant differences observed between the control A-value (0.110±002) were that the 1, 2 and 3

day 2M treated particles A values (0.115±003, 0.122±003, and 0.115±008) were significantly higher.

The digestion rates (k value) of acid treated potato starch particles are shown in Figure 14. There was no distinct pattern in these digestion rates. The 3 Day 2M acid treated particles (0.42±004 mol/min) and the 3,4, and 5 day 3M treated particles had K values (0.025±002 mol/min, 0.027±006 mol/min, and 0.023and ±001 mol/min) that were all significantly higher than the control (0.013±013 mol/min)

The amplitudes (A value) of acid treated potato starch particles are shown in Figure 15. Again, no clear pattern was shown. The control had an A value of 0.101±0.013 and different treatments produced amplitudes that were significantly higher and lower than the control.

3.4 Discussion

Overall acid hydrolysis of native starch prior to particle formation appeared to have an impact on both the structure and digestibility of these starch particles. This was seen to a lesser extent in retrograded particles exposed to acid hydrolysis after particle formation. Significant differences were usually only seen for longer durations of acid hydrolysis or higher molarity treatments, suggesting that a more prolonged treatment is necessary for structural differences to occur in a way that impacted digestion rates. Differences were also noted depending on whether the acid treatment was performed before or after particle formation. Hydrolysis on native corn and potato starch showed similar effects in melting properties and digestion kinetics compared to each other. Acid treated retrograded corn starch and potato starch particles however showed different digestion kinetics.

Previous literature values for melting parameters of potato starch with approximately 66-87% moisture content (similar to current particles) showed T_o to be 57°C, T_p at 62-64°C, and T_c at 70-81°C with the ΔH value at 16 J/g of starch or 0.016kJ/g[88]. Melting temperatures for waxy maize ranged from approximately 38-72, with a peak at 52 and potato ranging from 38-80 with a peak at 60[41].

The melting temperatures for the particles studied in this work were all slightly higher than these literature values. This suggests that acid treatment and retrograding process could favour crystallization in the particles. Decreases in melting temperatures suggest that defective or less than perfect or smaller or fewer crystalline regions are being formed[44]. Therefore, increased crystalline regions may account for increased melting temperatures. The increased melting temperatures could be suggesting that after the acid treatments and retrogradation processes, these particles contained higher amylopectin content combined with more crystallites formed from the amorphous region of the native starch granule. The acid hydrolysis prior to retrogradation may have be more effective in influencing the crystalline regions of the final particles.

Acid hydrolysis, due to preferential hydrolysis of amylose or amorphous regions of starch initially increases the double helix content of native starch[82]. This can be estimated by the change in enthalpy required to cause the starch structure to lose order, with a higher enthalpy being indicative of a higher molecular weight of the particles. The onset temperature for corn starch is related to amylose content with a higher temperature needed to induce melting in the absence of amylose-rich amorphous regions and the thermal transition events of corn starch high in amylose being less sharp and defined and more broad as more amylopectin heavy starch[44]. Potato starch amylopectin has longer chain length which at higher temperatures and for this reason melts at a higher temperature.

Previous work has identified highly waxy rice starch that was retrograded at 4°C to have a k value constant (calculated from % of total starch hydrolysis) in the range of 0.0476±0.040 for starch retrograded for 2 days and 0.454±0.018 for starch retrograded for 4 days[89]. This study on highly waxy rice starch also showed melting temperatures between 40 and 60 °C with enthalpy values of 36.7±3.7% and 67.2±3.1% (measured as percent relative to native starch)[89]. It was observed that as melting enthalpy increased, digestion rate was reduced. A study on melting enthalpies of various retrograded starches showed that waxy maize that was retrograded for 2 and 4 days had ΔH of 12.3 J/g and 13 J/g respectively and normal amylose potato starch had 13.6 J/g to 14.4 J/g while a high amylopectin sample had a lower value of 13.6 J/g and 13.5 J/g [41]. These values are much lower than any enthalpies recorded in the current data. This could be explained by the high levels of retrogradation in the starch particles compared to previous work.

This current work observed that acid treatment to native starch decreased melting enthalpies. Subsequently digestion rates were increased. The DSC results also indicated that there were structural changes occurring, and the changes seen in the digestion rates supported this. The ΔH required to melt the structure was increasingly low with increased acid treatment, meaning that the crystallite structures in the particles had a reduced molecular weight (MW) which requires a lower enthalpy for melting to occur. What could have happened was that the hydrolysis treatment was preferentially attacking the amorphous region of the native starch granules, producing lower MW amylose in the granule. Once gelatinisation and retrogradation took place during the particle formation process, the low MW amylose may have reformed into imperfect crystallite structures. Increased amylose content is associated with lower transitional temperatures since unordered amylose chains in the amorphous lamellae are increased along with amylose tie-chains in the crystalline lamellae, which acts as defects or kinks in the double-helical crystal wall, weakening

the structure and making it easier to melt[43]. The presence of the crystallite structures could be what caused the increased melting temperatures. It has also been theorized that the increase in crystallinity seen after acid hydrolysis could be owing to the decoupling of individual double helices to realign into a more crystalline structure[82].The shorter amylose molecule crystallites could be less densely packed and allow more accessibility for starch digestive enzymes as well as require a lower enthalpy to break down, explaining the lower ΔH and higher k values. Total gelatinization enthalpy increases with increased amylopectin and water content and the same can be applied for a melting temperature[44].

In terms of amplitude or the amount of starch digested, this value was typically lower than the control with increased molarity or duration of acid treatment. What may have occurred was a pre-hydrolysis of the starch before the enzymes are introduced. The enzymes would have had less substrate available to hydrolyse readily so less glucose was released. This could be due to the amorphous region being reaggregated into a denser crystalline region.

Acid hydrolysis treatment of the already retrograded corn starch particles did not significantly change the rate of digestion. This is confirmed by the DSC results showing that no significant structural changes were occurring as a result of HCl hydrolysis. So, the hydrolysis here was not hydrolyzing the chains in a way that changed how enzymes could access the starch structure in any significant way. Or at least not in any significant way compared to any retrogradation that has already taken place due to particle formation. The acid treatment neither increased nor decreased the digestion rate significantly, suggesting that acid hydrolysis has more of an impact on starch structure before retrogradation.

Overall, there is not much impact brought from the hydrolysis treatment of these particles, potentially due the retrogradation process already dictating digestion to the point where the hydrolysis treatment brings no additional influence.

Previous work shows that the ΔH of retrograded starch is usually higher than that of native starch which represents the melting of starch crystallites formed from retrogradation and of residual crystallites remaining after gelatinization[90]. In this work, the untreated controls of potato and corn starch particles were particles that have been left to retrograde without any additional treatments. The particles involving acid treatments consistently showed ΔH values that were lower than the controls while the melting temperatures were generally higher. This suggests that the lower ΔH values were mostly reflective of poorer quality crystallite structures that required less enthalpy to melt[43]. This may suggest that the untreated particles were already high in crystallite content and able to hinder enzyme hydrolysis without any additional treatment.

Treating the native starch may have lowered the MW of the structure and as a result made them more easily hydrolyzed by enzymes since there was a larger specific surface area present allowing the starch digestive enzymes to have the necessary accessibility to the glycosidic linkages. The rate of digestion was changed but not in a way that was favourable to a prolonged and moderate glycemic response. This could be due to the fact that the pre-treatment was on native starch that has not undergone any retrogradation and the structure is more susceptible to hydrolysis compared to the retrograded particles which had already been retrograded at the time of acid hydrolysis. There may have been already densely packed crystallites formed that were resistant to hydrolysis. Post treatment of the particles may provide more ideal digestion kinetics for a moderate glycemic response.

3.5 Summary

Overall what these results showed was that in order to create more rapidly digested starch particles, treating the native starch was the better option. In order to try and create a more slowly digested particle, with a lower digestion rate, acid-treating particles post particle formation is the route to explore more closely. Studies on the digestion rate of native starch by enzymes have shown that digestion rate is proportional the specific surface area of the substrate[91]. A larger particle has a smaller specific surface area and a lower digestibility. What may be the biggest driver of starch digestion is whether the particles have been retrograded more so than the acid treatments.

In terms of hydrolysis treatment, going forwards with future work on longer hydrolysis period or more hydrolysis after retrogradation would provide more information on optimal conditions for slowly digested particles. The next step would be to extend hydrolysis time to longer than 5 days to observe the extended effects of hydrolysis. After this point it could be observed when the "amorphous region" is fully hydrolyzed, which is then shearing the branches of the amylopectin, leaving behind the more rigid and crystalline amylose structure which is what would make the particles more resistant to further hydrolysis from starch digestive enzymes. This combined with the retrogradation process could achieve the slowest digestion rates possible.

4 *Chapter 2: Polyphenols and starch digestibility*

4.1 Introduction

This portion of the work examines the interaction between polyphenols and starch in, specifically polyphenols from berries. Berries are of interest as a functional food due to their high anti-oxidant capacity and polyphenol content. They also have the benefit of being a popular food can appeal to consumers if incorporated into a product. Past work has identified berries as major contributors for polyphenols with the potential to inhibit starch digestive enzymes[92].

A 2011 study by Clegg *et al.* that looked at blueberry and raspberry polyphenols showed that the addition of these components to starch based foods did not result in a significant reduction to the glycemic response however this was an isolated study and contrary to other works so more work is required on the subject[93]. McDougall *et al.* have investigated the role of polyphenols from fruits and berries[92,94]. Their work has provided much insight towards how polyphenols can inhibit starch degradation. They observed that anthocyanin-rich extracts from blueberries and blackcurrant were able to significantly inhibit glucosidase activity. They theorized that the anthocyanins were competitively binding to the active site of the enzymes since they are structurally similar to the normal enzyme substrate of maltose. They also suggested that the combination of ellagitannins and anthocyanins from fruits like raspberries could synergistically increase the overall effect by inhibiting both of α-amylase and glucosidase.

This current work selected cranberry and blueberry as polyphenols sources. This choice was to study the effects of less researched berries as well as to incorporate native Canadian berries into the research.

Cranberries (*vaccinium oxycoccus)* are a rich source of polyphenols, predominantly flavonoids like anthocyanins[95]. For cranberry, the most abundant phenolic compounds are *p*-coumaric, sinapic, caffeic and ferulic acids, as identified by gas chromatography in combination with mass specrtrometry[96] Cranberries contain a high proportion of tannins as well as smaller amount of catechins and epicatechins. *In vitro* studies on cranberry extracts have shown that tannins have been able to inhibit α-amylase activity, and are more effective than an equivalent amount of grape, pomegranate, or cocoa at inhibiting glucoamylase activity suggesting that the polyphenols interacted to varying degrees with the two enzymes[97]. A 2013 study on various fruits showed that cranberry tannins were able to have a high inhibition efficacy for α-amylase and a moderate effect on glucoamylase (amyloglucosidase) with potential to slow starch digestion[98].

Blueberries also reside in the *Ericaceae* family and *Vaccinium* genus and have shown anti-diabetic properties such as improving insulin sensitivity[99]. Blueberries have also been shown *in vivo* to elicit a positive insulinemic response by increasing insulin sensitivity in obese, nondiabetic, and insulin-resistant men and women compared to a control after consumption for 6 weeks, particularly to compensate for high-fat diet-induced hyperglycemia[100]. The structure of anthocyanidins consists of 2 benzene rings joined by a heterocyclic ring, with many different forms existing owing to glycosylation or acylation[77]. Blueberries have been identified to contain mainly anthocyanins, with the most abundant of these being malvidin-3-galactoside, cynidin-2-O(2^G-xylosylrutinoside) and cyanidin-3-O-rutinoside as identified by HPLC coupled with diode array detection (DAD)[101].

This shows the promise for further investigation towards using blueberries to reduce postprandial hyperglycemia.

To have contrasting types of structures, green tea was selected as the third source of polyphenols. Catechins, the primary flavonoid polyphenol found in green tea extracts are of interest in starch hydrolysis inhibition[102]. It has been shown that polyphenols present in green tea (epicatechin, epigallocatechin, epicatechin gallate, epigallocathecin gallate) present at a concentration of 0.5-120 µg/mL were able to inhibit α-amylase activity[103]. Notably green tea has been shown to also have effects on inhibiting α-glucosidase which is not consistently seen in all berries[104]. A study on how hydrolysis was impacted by polyphenols from tea was done in 2012 which showed that the interaction between starch and phenolics reduced hydrolysis through interactions with starch digestive enzymes, dependent on the structure of the starch.[105]. Noteworthy was that green tea extracts had more effect and interaction during starch cooling and setting of the gel network of cooked starch while the black tea interactions occurred during the heating phase. This difference may have been due to green teas containing many low-molecular weight catechins while black teas contained more complex polyphenols. Experiments performed where extracts were precooked with the starch showed that both were effective as inhibitors. This work also confirmed previous work that noted that hydrolysis was lower in starches with higher gelatinisation temperatures, contrasting corn to potato starch. This work showed that these polyphenols had an effect on starch hydrolysis through the interactions but also impact on the enzymes involved in starch hydrolysis.

Further work on tea extracts has highlighted their ability to inhibit starch hydrolysis by α-amylase. The interaction was solidified through a 2013 study establishing amylose as the main component of starch that the tea polyphenols are interacting since tests done with waxy or high amylopectin content starch showed little change to starch hydrolysis[106]. This work suggested the formation of complexes between amylose and the polyphenols present in tea, with the presence of the polyphenols showing disruption of the normal formation of double helices and ordered structure

in starch as well as non-covalent interactions with other components such as free fatty acids. Ismail *et al.* compared extracts from green tea, oolong, and black tea[107]. What this study showed was that polyphenol composition is more important than quality, though the concentration of 2mg/mL was necessary to show significant effects.

Anthocyanins are one polyphenol that is proposed to inhibit salivary and pancreatic α-amylase, as well as α-glucosidase and slow the release of free glucose and the subsequent effect on hyperglycemia, suggesting that if berries were consumed habitually with meals in the form of drink products for example, that this could provide management of the postprandial glycemic respone[77]. Various plant foods have a range of polyphenol contents and bioavailability depending on the source and can vary depending on processing or ripeness. Apples alone can range from 0.1-10g/kg of PE content depending on the variety[108]. It can be estimated that the normal daily intake of polyphenols is 1 g/day for a balanced diet that would include several servings of fruit or vegetables[108]. This can be used to estimate the polyphenol content for starch particles containing crude extracts from berries. Dark pigmented berries have an estimated range of 130-160mg of anthocyanin/100g of fresh fruit[77]. Blueberries and cranberries, both of which are native to Canada, are examples of such berries. There is also some thought around starch-phenolic compounds controlling release and bioavailability of phenolic compounds but further work remains to be done on this subject[13].

The objective of this portion of the work was to determine if incorporating polyphenols from berries into the starch particles already exposed to an acid hydrolysis treatment further inhibited starch particle digestion. We hypothesized that the addition of flavonoids in crude polyphenol extracts from berries would inhibit the digestive enzymes responsible for hydrolyzing starch, and in turn further slow the rate of digestion of starch more than particles alone without any

polyphenols added. The objective of this section was to observe how the polyphenols present in this fruit impacted digestion of starch in the form of particles and to determine which were most effective.

4.2 Materials and methods

4.2.1 Materials and reagents

The digestive enzymes α-Amylase (type VI-B, from porcine pancreas), pancreatin (8x USP from porcine pancreas), pepsin (from porcine gastric mucosa), amyloglucosidase (from *Aspergillus niger*) were purchased from Sigma-Aldrich Corporation (St. Louis, Missouri, United States). Potato Starch and Corn starch were purchased from Sigma-Aldrich Corporation (St. Louis, Missouri, United States). Cranberry powder was purchased from Mountain Rose Herbs (Eugene, Oregon, United States), green tea powder was purchased from Teaki Hut (San Antonia, Texas, United States), and blueberry powder was purchased from Z Natural Foods (Palm Beach, Florida, United States).

4.2.2 Isolation of polyphenol compounds

Polyphenolic crude extracts were obtained from freeze dried powders of cranberry (CRAN), blueberry (BLUE), and green tea (GT). 40g of freeze-dried powder was extracted three times with a total 120 mL of 70% acidified methanol (70:30:1 v/v, methanol: water: HCl). Each extraction step consisted in 15 min sonication[109] followed by 16 h methanolic extraction period and collection of the solvent by filtration through a 0.2µm PDFE filter paper. The combined extracts were then evaporated with a Thermo Scientific Savant® SPD121P SpeedVac® Concentrator at 60°C and a vacuum pressure of 0.1 torrs. Once the solvent was completely evaporated, the samples were resuspended in water and collected and concentrated, then dried at 60°C in a convection oven to evaporate the water and concentrate the extracts again.

4.2.3 Measurement of Polyphenol Content

Total phenolic content of the samples was estimated using the Folin-Ciolcateu (F-C) method as described by Ainsworth and Gillespie (2007)[110]. Samples of 20 mg of each PE were dissolved in

95% (vol/vol) methanol and gallic acid was used as a standard. Samples were loaded into a 96-well microplate and read at an absorbance of 765nm. From these values a standard curve was calculated and the mole equivalent of phenolic per g of starch particle was calculated.

4.2.4 Formation of particles

Starch particles were formed as previously outline in Section 3.2.3. Only four of the previous acid treatments were selected from the previous work to include the addition of crude polyphenol extracts. Four of the previous samples were selected from the previous work to include the addition of crude polyphenol extracts.: Corn Starch Particles exposed to 3M HCl treatment for 24 Hours (1 Day 3M Acid Treated CS Particles), Potato Starch Particles exposed to 3M HCl treatment for 120 Hours (5 Day 3M Acid Treated PS Particles), Native Corn Starch exposed to 2M HCl treatment for 24 hours used to form particles (Particles from 1 Day 2M Acid 1019 Treated Native CS), and Native Potato Starch exposed to 2M HCl treatment for 120 hours used to form particles (Particles from 5 Day 2M Acid Treated Native PS). To each of these conditions, one of three PEs was added, either CRAN, BLUE, or GT at a ration of 0.0125g of PE per 1 g of Native PS or 0.0123g of PE per 1g of Native CS. This was done to establish a relative reaction of 3g of PE/1kg of particles. This ratio was determined based of the weight of cooked particles prior to IVD and after retrogradation and storage at 4°C.

4.2.5 Moisture content and weight identification

A Mettler Toledo HE53 Moisture Analyzer was used to identify the starch content as previously outlined in section 3.2.4.

4.2.6 In vitro digestion

The prepared samples were digested using *In vitro* Digestion (IVD) procedures as previously outlined in section 2.2.5 to measure starch digestion kinetics[60]. The portion of the research added

the element of crude PEs. For each of the four acid treatments, there was one of three crude PEs added (CRAN, BLUE, or GT) as well as a control in the form of acid treated particles with no polyphenol added. Each of the conditions was performed in triplicate. Across the 2 hour IVD period, a glucometer was used to measure the glucose concentration which was in turn was used to quantify rate of release of glucose as an indicator of rate of starch digestion[85].

4.2.7 Differential scanning calorimetry

DSC was performed as previously outlined in section 2.2.6.

4.2.8 Statistical Analysis.

All samples were prepared and analyzed in triplicate. Digestograms showing rate of glucose release over digestion period were fitted with a first order kinetic equation ($[\%glucose] = C + A(1-e^{-kt})$). A 1-way ANOVA was used to identify significance in digestion rate (k_D) and the Amplitude (A) values of the first order kinetic equation and also the onset, peak, and conclusion melting temperatures and melting enthalpies between different PE contents within an acid treatment. This was followed by a Tukey's test to identify significant differences using a pairwise comparison between all means within an acid treatment. Significance was set to $p<0.05$. Systat 13 statistical analysis software was used.

4.3 Results and discussion

4.3.1 Estimation of Polyphenol Content

Table 5. Results of Total Polyphenol Assay. Estimated polyphenol equivalents calculated from standard curve using gallic acid as a standard. Displayed are the amount of polyphenols present per gram of starch particle sample, either corn starch (CS) or potato starch (PS). Mean (n=3)± SEM *Mean (n=2)± SEM For CRAN (outlier removed).

PE Sample	Equivalent mmol of polyphenols	Mol of Polyphenol /g of powdered PE	mol eq. of PE per gram of kg of CS particle	mol eq. of PE per gram of kg of PS particle
GT	0.0840±0.0008	0.0404±0.0004	0.4967±0.0048	0.5048±0.0049
CRAN*	0.0128±0.0009*	0.0062±0.0004*	0.0765±0.0053*	0.0777±0.0054*
BLUE	0.0175±0.0012	0.0084±0.0006	0.1027±0.0069	0.1044±0.0070

Table 5. shows the equivalent values of polyphenols estimated for each PE extract and the amount of polyphenols in each particle sample, based on the amount of PE that was added to the starch slurry. From this assay, it was determined that the green tea PE had the highest polyphenol content as compared to cranberry and blueberry PEs, and subsequently these particles contained polyphenols at a higher concentration per g of starch particle.

4.3.2 Polyphenols and structure results

Table 6. Thermal properties of particles from 1 day 2M acid treated native CS with PE. Melting enthalpy (ΔH), onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) of melting measured by DSC. Mean (n=2)± SEM. Different letters in a column indicate significant differences between all individual samples for that value. Samples sharing letters are not significantly different ($p \leq 0.05$).

Sample	T_o (°C)	T_{max}(°C)	T_c(°C)	ΔH (kJ/g)
Particles from 1 Day 2M Acid Treated Native CS (Control)	63.8±0.1[a]	70.5±0.1[a]	75.0±2.0[a]	5.2±0.9[a]
Particles from 1 Day 2M Acid Treated Native CS with Green Tea PE	67.4±0.6[a]	81.5±1.6[bc]	96.5±0.5[b]	1.6±0.0[b]
Particles from 1 Day 2M Acid Treated Native CS with Cranberry PE	69.3±2.0[a]	85.7±1.3[c]	97.0±1.9[b]	1.6±0.1[b]
Particles from 1 Day 2M Acid Treated Native CS with Blueberry PE	72.5±6.5[a]	78.9±0.1[b]	92.4±0.6[b]	1.8±0.3[b]

Table 7. Thermal properties of particles from 5 day 2M acid treated native PS with PE. Melting enthalpy (ΔH), onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) of melting measured by DSC. Mean (n=2)± SEM. Different letters above bar indicate significant differences between all individual samples for that value. Samples sharing letters are not significantly different ($p \leq 0.05$).

Sample	T_o (°C)	T_p(°C)	T_c(°C)	ΔH (kJ/g)
Particles from 5 Day 2M Acid Treated Native PS (Control)	65.7±3.5[a]	69.5±53.[a]	72.6±2.4[a]	6.7±0.2[a]
Particles from 5 Day 2M Acid Treated Native PS with Green Tea PE	78.5±0.5[ac]	83.7±0.9[a]	97.1±1.6[bc]	3.6±1.0[bc]
Particles from 5 Day 2M Acid Treated Native PS with Cranberry PE	72.2±0.2[ac]	79.7±0.0[a]	85.4±0.5[ac]	6.2±0.1[ac]
Particles from 5 Day 2M Acid Treated Native PS with Blueberry PE	81.8±3.1[bc]	89.6±5.4[a]	96.5±3.5[ac]	2.5±0.3[b]

Table 8. Thermal properties of 1 day 3M acid treated CS particles with PE. Melting enthalpy (ΔH), onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) of melting measured by DSC. Mean (n=2)± SEM. Different letters within a column indicate significant differences between all individual samples for that value. Samples sharing letters are not significantly different ($p \leq 0.05$).

Sample	T_o (°C)	T_p(°C)	T_c(°C)	ΔH (kJ/g)
1 Day 3M Acid Treated CS Particles (Control)	65.8 ± 0.3^{a}	71.2 ± 0.8^{a}	76.8 ± 1.3^{a}	4.9 ± 0.9^{a}
1 Day 3M Acid Treated CS Particle with Green Tea PE	63.5 ± 0.1^{bc}	69.6 ± 0.1^{ac}	77.4 ± 0.7^{a}	4.7 ± 0.0^{a}
1 Day 3M Acid Treated CS Particle with Cranberry PE	62.4 ± 0.2^{b}	68.9 ± 0.0^{bcd}	74.7 ± 0.2^{a}	4.9 ± 0.2^{a}
1 Day 3M Acid Treated CS Particle with Blueberry PE	64.6 ± 0.6^{ac}	70.2 ± 0.0^{ad}	77.2 ± 1.0^{a}	4.5 ± 0.2^{a}

Table 9. Thermal properties of 5 day 3M acid treated PS particles with PE. Melting enthalpy (ΔH), onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) of melting measured by DSC. Mean (n=2)± SEM. Different letters within a column indicate significant differences between all individual samples for that value. Samples sharing letters are not significantly different ($p \leq 0.05$).

Sample	T_o (°C)	T_p(°C)	T_c(°C)	ΔH (kJ/g)
5 Day 3M Acid Treated PS Particles (Control)	64.5 ± 0.6^{a}	70.9 ± 0.4^{a}	75.4 ± 0.0^{a}	5.2 ± 0.0^{a}
5 Day 3M Acid Treated PS Particle with Green Tea PE	63.5 ± 0.1^{a}	72.5 ± 0.1^{b}	78.8 ± 0.1^{b}	4.0 ± 0.1^{b}
5 Day 3M Acid Treated PS Particle with Cranberry PE	64.8 ± 0.2^{a}	72.9 ± 0.1^{b}	79.7 ± 0.4^{b}	4.1 ± 0.2^{b}
5 Day 3M Acid Treated PS Particle with Blueberry PE	63.3 ± 0.4^{a}	72.6 ± 0.3^{ab}	78.8 ± 0.3^{b}	3.8 ± 0.2^{b}

The DSC results for the melting temperatures and enthalpies of particles made from 1 day 2M acid treated native CS with PE are shown in Table 6. The T_o is not significantly different than the control but the GT, CRAN, and BLUE samples T_p (81.5±1.6, 85.7±1.3 and 78.9±0.1) and T_c (96.5±0.5, 97.0±1.9, and 92.4±0.6) are all significantly higher than the respective control values. The CRAN T_p is also significantly higher than the BLUE T_p while none of the T_c values for GT, CRAN or BLUE are significantly different than each other. The melting enthalpy values for the GT, CRAN,

and BLUE samples (1.6±0.0, 1.6±0.1, and 1.8±0.3) are all significantly lower than the melting enthalpy of the control (5.2±0.9), but not significantly different among themselves.

The DSC results for the melting temperatures and enthalpies of particles made from 5 day 2M acid treated native PS with PE are shown in Table 7. Only the BLUE T_o (81.8±3.1) is significantly higher than the control (65.7±3.5), there are no significant differences within the T_p values and only the GT T_c value is significantly higher than the control (72.6±2.4). None of the PE samples show significant differences from each other for the melting temperatures. The melting enthalpy values for the GT and BLUE samples (3.6±1.0, and 2.5±0.3) are both significantly lower than the melting enthalpy of the control (6.7±0.2), with the BLUE melting enthalpy being lower than any of the other samples in this condition.

The DSC results for the melting temperatures and enthalpies of 1 day 3M acid treated CS particles with PE are shown in Table 8. The T_o for GT and CRAN (63.5±0.1 and 62.4±0.2) are significantly lower than the control (65.8±0.3). CRAN T_p (68.9±0.0) is lower than the control (71.2±0.8). The CRAN onset temperature is also significantly lower than that of the BLUE particles. There are no significant differences within the T_c or ΔH values.

The DSC results for the melting temperatures and enthalpies of particles made from 5 day 3M acid treated native PS with PE are shown in Table 9. There are no significant differences within the T_o values . The GT and CRAN T_p (72.5±0.1 and 72.9±0.1) and T_c (78.±0.1, 79.7±0.40, and 78.8±0.3) are all significantly higher than the respective control values for T_p (70.9±0.4) and T_c (75.4±0.0). None of the PE values are significantly different among themselves. The melting enthalpy values for the GT, CRAN, and BLUE samples (4.0±0.1, 4.1±0.2, and 3.8±0.2) are all significantly lower

than the melting enthalpy of the control (5.2±0.0), but not significantly different among themselves.

4.3.3 *Polyphenols and digestibility*

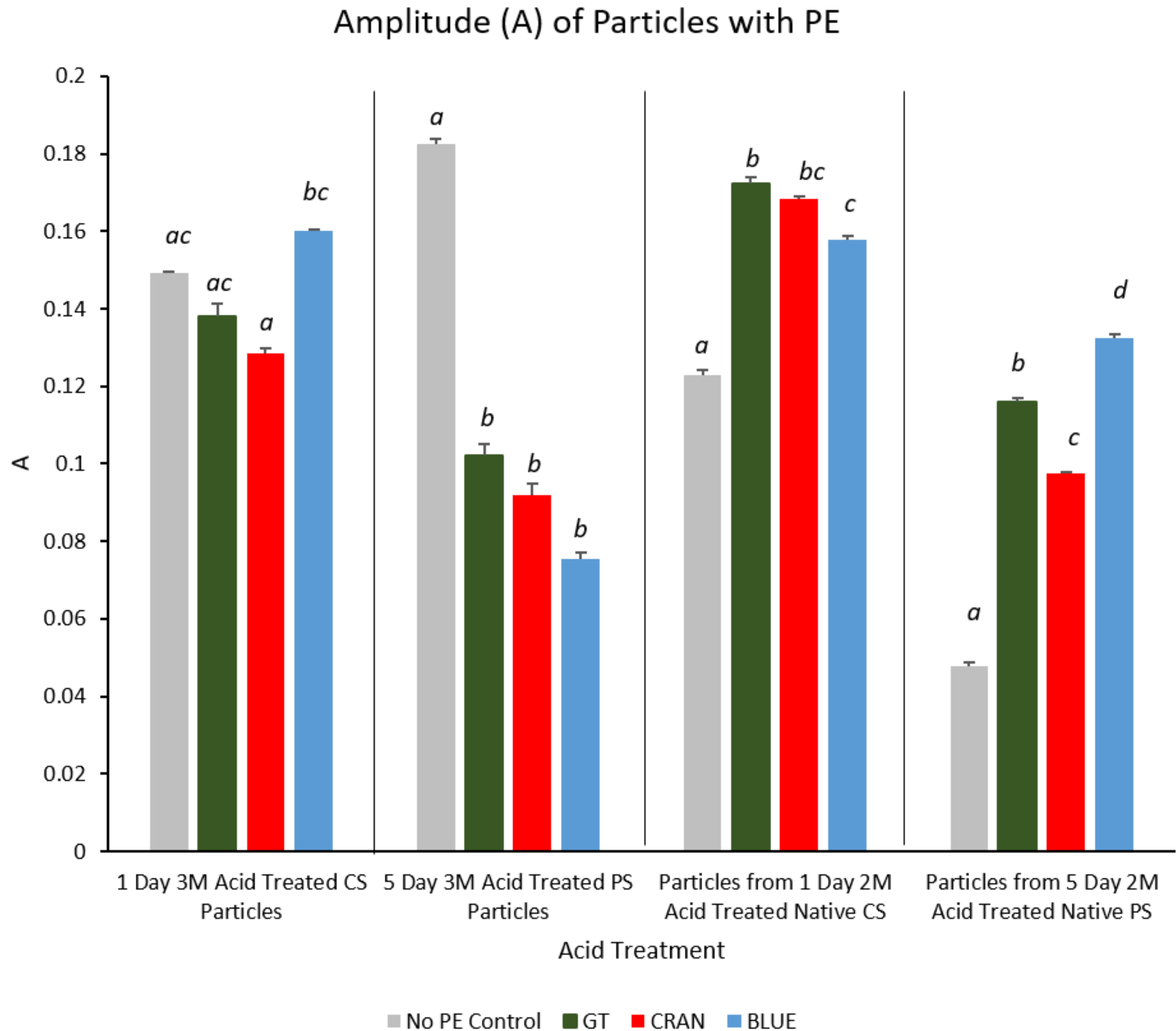

Figure 16. Amplitude (A) of acid treated starch particles containing crude polyphenol extracts (PE) either Green Tea (GT), Cranberry (CRAN) or Blueberry (BLUE). Mean (n=3)±SEM. Significance set to $p<0.05$. Letters indicates significance. Different letters above bar indicate significant differences between all individual samples for that value. Means compared within an acid treatment only. Samples sharing letters are not significantly different ($p \leq 0.05$).

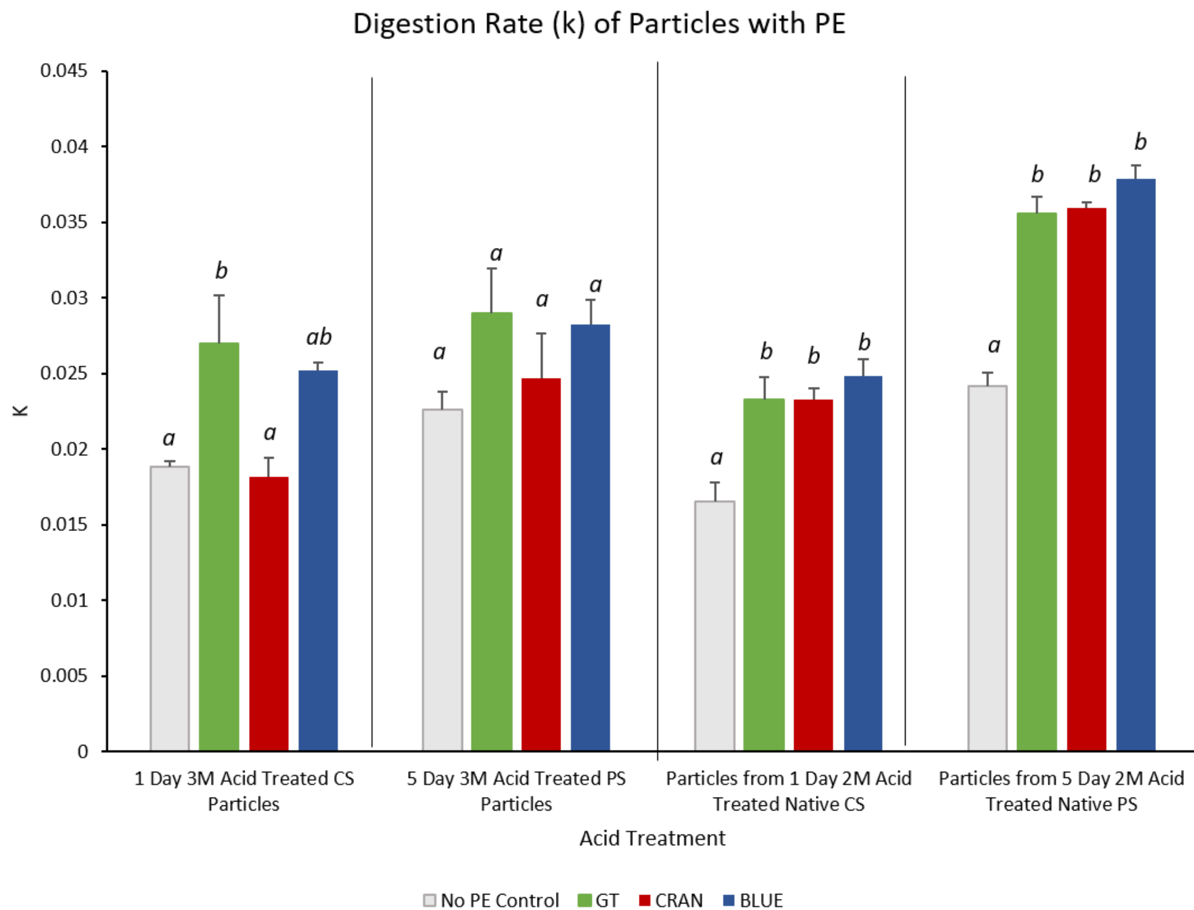

Figure 17. Digestion rate (k) in mol/min of acid treated starch particles containing crude polyphenol extracts (PE) either Green Tea (GT), Cranberry (CRAN) or Blueberry (BLUE). Mean (n=3)± SEM. Significance set to $p<0.05$. Different letters above bar indicate significant differences between all individual samples for that value. Means compared within an acid treatment only. Samples sharing letters are not significantly different ($p \leq 0.05$).

The amplitudes (A value) of each of the kinetic equations for the digestion rates for each particle samples are shown in Figure 16. The only A values for the particles formed from 1 Day 2M HCl treated native corn starch, the GT, CRAN, and BLUE A values (0.172±0.004, 0.168±0.002 and 0.158±0.001) are all significantly higher than the control rate (0.123±0.004) but only GT and

BLUE are significantly different than each other. The k values for the particles formed from 5 Day 2M HCl treated native potato starch with GT, CRAN, and BLUE (0.116±0.003, 0.098±0.002, and 0.132±0.003) are all significantly higher than the control rate (0.048±0.001) and all A values for this set of conditions are significantly different than each other.

For the 1 Day 3M HCl treated CS particles, the A values of the PE samples are not significantly different compared to the control but the BLUE A value (0.160±0.006) is significantly higher than the CRAN A value (0.128±0.004). For the 5 day 3M HCl treated PS particles the GT, CRAN, and BLUE A values (0.102±0.006, 0.092±0.004 and 0.075±0.002) are all significantly lower than the control rate (0.183±0.021) but not significantly different than each other.

The digestion rates (k values) are shown in Figure 17. The k values for the particles formed from 1 Day 2M HCl treated native corn starch with GT, CRAN, and BLUE (0.023±0.001,0.023±0.001, and 0.025±0.001) are all significantly higher than the control rate (0.017±0.001) but not significantly different than each other. Similar results are seen for the k values for the particles formed from 5 Day 2M HCl treated native potato starch with GT, CRAN, and BLUE (0.036±0.001,0.036±0.001, and 0.038±0.001) are all significantly higher than the control rate (0.024) but not significantly different than each other.

For the acid treated particles fewer instances of significance were observed. For the 1 Day 3M HCl treated CS particles, only the GT particles k value (0.027±0.003) was significantly higher than the control (0.019±0.001). There was also significance in the GT sample being higher than the CRAN sample but not the BLUE sample. For the 5 day 3M HCl treated PS particles there were no differences observed in the k values.

4.3.4 Discussion

The presence of the polyphenols in samples conditions that did show significance compared to the control further increased the melting temperatures as well as consistently lowered the ΔH compared to the controls with no PE added.

Previous work has shown the presence of lipids changed melting temperatures, with the removal of lipids leading to a more densely packed and crystalline structure with increased melting point and temperature[43]. This concept could be applied to the presence of polyphenol molecules. Presence of other food ingredients can increase the gelatinisation temperatures due to alteration of crystallite formation. Sugars for example increase the gelatinization temperature and enthalpy of PS due to the sugar-sugar interactions stabilizing the amorphous regions of the starch samples[44].

The presence of the polyphenol structures appeared to be acting in an opposite way, potentially enabling a more extensive hydrolysis by the acid treatment. Overall the addition of polyphenols increased the rate of digestion slightly which means the starch was being digested slightly faster. The k values of the samples containing PEs were significantly higher than the controls but not from each other, meaning that the presence of any crude polyphenol extracts were making the particles more digestible compared to the control alone but there were not significant differences depending on the types of polyphenols used. In the acid treated pre formed particles, the presence of the polyphenols lowered the amplitude slightly, meaning less starch was being hydrolysed. The opposite was happening with the particles made from the acid treated native starch. The amplitude was higher when there were polyphenols present meaning that the polyphenols may have enabled more starch to be hydrolyzed.

Instead of inhibiting enzyme activity as hypothesized, the presence of these extracts could be creating a more open starch structure, disrupting the crystalline regions of starch, making the bonds more accessible to starch digestive enzymes, hence a more rapid digestion rate. As well the molecular order appears to be decreased as evidenced by the lower ΔH values.

4.3.5 Summary

Compared to each of the conditions respective controls, the acid treated particles without any polyphenols added, there was usually an increase in digestion rates and amount of starch digested with a PE added. This addition created particles that were digested at a slightly faster rate, suggesting enzyme interactions were favoured. In terms of changing structure and altering digestibility in a way that favoured slower digestion of starch, adding polyphenols had only a marginal effect. The main effect appeared to be from the previous section with the acid treatments, and with the particle making process acting as the main influences on digestion. The high degree of order produced from retrogradation appeared to slow digestion sufficiently.

The polyphenols did not have the expected inhibitory effect on the starch digestive enzymes, at least at this concentration. Their role can be considered negligible in comparison to the role that retrogradation and acid hydrolysis treatments are playing. The higher melting temperatures from the DSC analysis are not corresponding with lower digestion rates. What could have occurred was that the presence of polyphenols themselves required a higher transitional temperature to degrade, rather than them contributing to the starch structure being more crystalline. What this data did show was that GT was more effective at changing the digestion rate, which could be attributed to the increased polyphenol content in the GT extracts. Cranberry and blueberry showed some promise as well. More investigation would be needed into polyphenol contents and different concentrations in future work. Further work into isolating specific polyphenols or using higher

concentrations of these crude PEs to observe if they produce beneficial effect in order to create a more prolonged and moderate glycemic response would be the next steps. In this work, however, it was the acid treatment that provided the most significant effect at minimizing digestion, as well as the formation process of making and retrograding the particles themselves. The smaller surface area and compact structure of the particles was most likely what slowed starch enzyme hydrolysis.

5 Conclusion and future work

What this work showed was highly retrograded acid treated particles that are digested at a lower rate can be produced. The main aspect of this appeared to be the gelatinisation and retrogradation process involved in the initial formation of the particles. What is being created is mostly likely particles high in crystallites that are resistant to digestion by enzymes. Additional longer acid treatments on the native starch could create particles that have an imperfect crystalline structure which can increase digestibility. This work emphasized the importance of crystallinity as a driver of starch digestion. Polyphenol content showed minimal effects to starch particle digestion and further work may be needed to determine if their role is necessary in creating optimally digested particles. Isolation of specific compounds or higher concentration of crude polyphenols extracts could be investigated as future directions.

The large size of the particles themselves created a smaller relative surface area for which enzymes could interact with the substrate. This most likely limited digestion compared to previous studies on starch digestion. With this knowledge, production of a product containing highly retrograded starch particles can move forward. The next steps would be applying this towards human trials to measure the glycemic response *in vivo.*

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