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Impact of Particle Size and Water Content on Particle Breakdown and Starch Digestibility of
Chickpea-based Snacks during in Vitro Digestion

By

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THESIS

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ABSTRACT

Chickpea (*Cicer arietinum L.*) is an agriculturally-important legume crop that is an excellent source of proteins, fiber, and minerals. Therefore, developing chickpea-based snacks could add value to chickpeas, as well as provide consumers with snack products rich in protein and fiber. As two main components, there are around 24% protein and 54% carbohydrate in chickpea that could be influenced by processing methods. Many studies have focused on the effect of processing on in vitro digestibility of starch and nutrient availability in legumes. Previous studies have found that the water uptake during digestion plays an important role in the breakdown of starch-based food matrices. However, the interaction between particle size, water content, and modifications in nutrient release and digestibility is not clear. The objective of this project was to investigate the effect of particle size and water content on particle breakdown and starch digestibility of chickpea-based snacks during dynamic in vitro digestion.

To achieve this goal, chickpeas puree and cracker were produced with different particle size: fine puree, fine cracker, coarse puree, coarse cracker. For all treatments, chickpea samples were mixed with simulated saliva for 30 s and were incubated in the Human Gastric Simulator (HGS) for up to 180 min. Gastric digesta were removed from the bottom of HGS every 30 min to be analyzed and sample aliquots were also incubated in a shaking water bath (37℃ at 100 rpm) with simulated intestinal juice for up to 180 min. Intestinal digesta were removed from simulated intestinal juice every 30 min up to 180 min to be analyzed. For whole gastric digesta, pH, moisture content, and gastric emptying were measured. Gastric digesta were then separated into 3 phases: liquid, suspended solid, and solid phase. For the suspended solid phase, particle size was measured using laser diffraction, while image analysis was used to measure the particle size and number of particles in the solid phase. The starch hydrolysis and protein hydrolysis were

measured in all three phases through analysis of reducing sugars and free amino groups, respectively.

It was found that both the initial particle size and drying influenced the breakdown rate and the digestibility of starch and protein in chickpea snacks. Across the four treatments, the pH decreased from 3.38-4.00 after 30 min and to 1.61-1.14 after 180 min gastric digestion. The pH of the fine puree was the highest from 60-150 min compared with the other treatments, as it had the highest buffering capacity of the four treatments. For the gastric emptying of dry matter, the emptying rate (k) was significantly ($p < 0.05$) higher for fine puree compared to the other treatments, which may be due to the higher pH and lower water holding capacity observed in the fine puree. The particle size of the solid phase was quantified through the x50, or median particle area. The x50 of fine puree was constant during gastric digestion due to the smallest particle size, while fine cracker and coarse cracker rapidly decreased in size from 0-30 min due to changes in the product matrix that occur during drying. The coarse puree had the largest particle size at all digestion times, due to the remaining large pieces of skin that were not broken down during milling. The hydrolysis of starch and protein in the liquid phase during gastric digestion had a similar trend: fine cracker>coarse cracker>fine puree>coarse puree, which was impacted by both initial particle size and drying. The reducing sugars and free amino groups released from fine puree were higher than coarse puree, and fine cracker was higher than coarse cracker due to the influence of initial particle size. Similarly, the water content also impacted starch and protein hydrolysis in the chickpea snacks, crackers showed higher protein and starch hydrolysis than puree.

Due to their high protein content and potentially low glycemic index, developing chickpea-based snacks could result in nutritious, slowly-digestible high protein snacks.

Therefore, this study is essential to understand how the properties and processing of chickpeas, such as particle size and moisture content, impact their breakdown and macronutrient digestibility. This information will help to develop chickpea-based snacks with specific functional properties to control starch and protein digestion.

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1 INTRODUCTION

1.1 Background

Chickpea (*Cicer arietinum L.*) ranks third in world legume production and first in the Mediterranean basin, and is the only pulse crop with an anticipated rise in planted area in the United States (Gupta, Liu, and Sathe 2019). In California, about 10,000 acres of chickpea were grown per year between 2011 to 2017, and in 2019 chickpea production increased to 13,400 acres (USDA/NASS 2019 State Agriculture Overview for California n.d.). Increased interest in chickpea production could be due to chickpeas being a good source of protein, carbohydrate, fiber, and minerals (Long et al. 2019, Gupta, Liu, and Sathe 2019). Protein content in chickpea ranges from 20.9 to 25.27% and the total lipid content is low, ranging from 4.5 to 6.0 g oil/100 g of chickpea seed (Rachwa-Rosiak, Nebesny, and Budryn 2015). Besides nutritional value, chickpea is also considered to be a low glycemic index (GI) food. Previous studies have suggested that pasta prepared with chickpea flour had a lower GI compared to pasta prepared with wheat flour (I. Goñi and Valentín-Gamazo 2003). Due to their high protein content and potentially low glycemic index, developing chickpea-based snacks could result in nutritious, slowly-digestible snack products that are also rich in protein. However, it is essential to understand how the properties and processing of chickpeas, such as particle size and moisture content, impact their breakdown and macronutrient digestibility. This information will help to develop chickpea-based snacks with specific functional properties to control starch and protein digestion.

Previous studies have attempted to describe the effect of processing methods on physicochemical property changes of chickpeas such as water content and texture. It has been indicated that certain processing methods can significantly influence the moisture and nutrient

content as well as texture. For example, after drying and roasting, moisture is removed, which can help preservation (Moreira, Vazquez, and Chenlo 2002). Processing such as soaking and boiling increases the moisture content by water absorption (Gupta, Liu, and Sathe 2019). Nutrient content can either increase or decrease after processing. The decrease or elimination of antinutrients, such as trypsin inhibitors, tannins, or chymotrypsin inhibitors after thermal processing (e.g. boiling or microwave cooking) have been found due to the heat labile nature of these antinutrients (Alajaji and El-Adawy 2006; Gupta, Liu, and Sathe 2019). In some studies, fat and ash content has been found to decrease after processing such as boiling because of leaching during the boiling process. Crude fiber has been found to increase significantly in chickpeas after processing such as boiling, pressure cooking, and roasting due to the formation of protein-fiber complexes (R. Mittal et al. 2012). In addition, the texture is another property that can be influenced by processing. For example, it has been observed that the hardness of chickpeas decreased after soaking due to hydration of the dried seed (Shafaei, Masoumi, and Roshan 2016; Yildirim, Öner, and Bayram 2013). In contrast, the hardness of chickpea-based snacks increased quickly when the moisture content was reduced during frying (Debnath, Bhat, and Rastogi 2003), and many studies found similar results that drying could increase the hardness of foods such as eggplant, potato, and ginkgo seeds (Boateng et al. 2021; Ferrão et al. n.d.; Moon, Pan, and Yoon 2015). However, the effect of moisture content of chickpeas on texture and breakdown during digestion has not been studied.

Besides focusing on the effect of processing on physicochemical property changes of chickpea products, previous studies have also investigated the effect of processing on nutrient digestibility, such as starch and protein. It has been indicated in many studies that compared with raw chickpea, thermal processing decreases resistant starch content due to starch gelatinization

and decreases protein solubility because of protein denaturation, and thus increases the

digestibility of starch and protein (Aguilera et al. 2009; Frias et al. 2000; Ma et al. 2011).

Additionally, some studies found that the combination of high temperature and moisture could

lead to a greater increase in available starch (reducing sugars) which was caused by greater

disruption of the cellular structure (Eyaru, Shrestha, and Arcot 2009). For example, Wang et al.

(1997) investigated the combined effects of soaking, water, and steam blanching on the nutrients

of cowpea, and found that only soaking did not influence starch gelatinization, but when it was

combined with steam-blanching, the influence of soaking increased starch gelatinization. In

addition to thermal processing, other factors that could influence starch and protein digestibility

have also been investigated.

It has been observed that moisture content, texture, and particle size may also affect the

digestibility of starch or protein. A decrease in moisture content could limit starch gelatinization

and hinder the in vitro starch digestibility (Hera et al. 2014). On the contrary, an increase in

water content likely can lead to an increase in the digestibility of starch and protein due to the

weakening or dissolution of the structure (Drechsler and Bornhorst 2018; Eyaru, Shrestha, and

Arcot 2009). In addition, processing can change the particle size of food products which leads to

a change in starch digestibility. It has been observed that the decrease of particle size would

increase the starch digestibility, due to an increase in the relative surface area. It has been

previously reported that the rate of starch digestion was inversely related to the square of particle

size (Mahasukhonthachat, Sopade, and Gidley 2010; Tinus et al. 2012). For example, Hera et al.

(2014) studied the influence of dough hydration level and particle size distribution of rice flour

on gluten-free bread quality and in vitro starch hydrolysis. It was found that slowly digestible

starch and resistant starch increased in coarse flour bread due to the decrease of the surface area

exposed to digestive enzymes. However, there is a lack of information about the impact of particle size of chickpea puree on starch and protein digestibility.

Overall, the effect of certain processing methods on the physicochemical properties and nutrient digestibility of chickpea and chickpea-based products has been determined. It has been indicated that the water content increases after soaking and boiling, which leads to a decrease in the hardness of the chickpeas. In addition, the digestibility of starch and protein increases after thermal processing due to starch gelatinization and protein denaturation, and the relationship between particle size with starch and protein digestion has been determined in specific food materials. However, there is a knowledge gap in the understanding of the water content and particle size difference of chickpea-based food products after processing and the interaction of water content and particle size on particle breakdown and starch and protein digestibility during digestion.

1.2 Objectives

The overall goal of this research was to develop a quantitative understanding of the influence of particle size and water content on particle breakdown, gastric emptying and the resulting modifications in the hydrolysis of starch and protein during dynamic in vitro digestion of chickpea products.

The specific objectives were as follows:

1. Determine the influence of initial particle size and water content on the rate of breakdown, pH and gastric emptying dynamics of chickpea-based puree and cracker during in vitro gastric digestion using a dynamic model.

2. Determine the influence of initial particle size and water content on starch and protein

digestibility of chickpea-based puree and cracker during in vitro small intestinal digestion.

2. LITERATURE REVIEW

2.1 Digestion Processes

2.1.1 Mastication and Esophageal Transit

The first step in the food digestion process is oral digestion, where foods are ingested through the mouth and undergo chewing, or mastication. During mastication, food is physically broken down into smaller particles and mixed with saliva, an enzymatic digestion fluid (Gail M. Bornhorst and Singh. 2013). Mastication is necessary to increase the surface area of ingested food, to facilitate an increased rate of digestion, to expose the food to digestive enzymes, to lubricate the food particles to form a cohesive bolus, and to facilitate swallowing (Johnson 2018).

Chewing food in the mouth stimulates the secretion of saliva (Edgar and Dawes 2004). Saliva is composed of about 99% water and a variety of electrolytes (sodium, potassium, calcium, magnesium, bicarbonate, and phosphates), immunoglobulins, proteins, enzymes, mucins, and nitrogenous products (urea, ammonia) (Humphrey and Williamson 2001). The normal pH of saliva is 6 to 7 (Humphrey and Williamson 2001; Johnson 2018). The residence time in the mouth, or chewing time, depends on the type of food and typically ranges from 10 s up to 60 s for solid foods, with an average time of around 30 s (Mulet-Cabero et al. 2020).

As food particles are broken down during mastication, their particle size will depend on the type and properties of the food. Previous studies have shown that the median particle size (d50) during mastication ranged from 0.82 to 3.04 mm for foods such as peanuts and gherkins (Jalabert-Malbos, M. L., Mishellany-Dutour, Woda, and Peyron 2007). In addition to physical breakdown, digestive enzymes also help to break food down into smaller particles. The most

characteristic enzyme of saliva is α-amylase, which breaks carbohydrates down to maltose and maltotriose from amylose, or maltose, glucose, and dextrins from amylopectin by cleaving the α-1-4 glycosidic bond. If the carbohydrate is starch, it is hydrolyzed mainly to alpha-dextrin by salivary amylase. Another salivary digestive enzyme is lingual lipase which breaks down a small fraction of dietary triglycerides (Pedersen et al. 2002; Welcome 2018).

In addition to helping in breaking down the ingested food, the water in saliva moistens the food particles, and the mucins help to bind food into a coherent and easily swallowed food mass, called a bolus. The amount of saliva secreted has been found to depend on the type and composition of the food being chewed. In general, greater amounts of saliva are secreted for dry food than for moist food. For example, the amount of saliva secreted per gram of Melba toast was significantly higher than cake because it contained a lower percentage of water compared to the cake (Gavião, Engelen, and Van Der Bilt 2004).

After mastication, the bolus is swallowed and passes through the esophagus. Swallowing of a food bolus may be triggered by both food particle size and lubrication, after a certain time (Hutchings and Lillford 1988). After swallowing, the bolus is transported through the esophagus via peristaltic muscular contractions and arrives to the stomach (Ravinder Mittal 2011).

2.1.2 Gastric Digestion

Gastric digestion is a complex process including both chemical reactions and mechanical processes. It is controlled by a variety of food-related factors such as buffering capacity, physical properties, and breakdown rate as well as physiological factors such as gastric secretion rate, gastric emptying rate, and gastric motility parameters (Gail M. Bornhorst 2017).

When a food bolus enters the stomach, a J‑shaped latex chamber, gastric digestion begins. The stomach can be functionally separated into the proximal and distal regions. The proximal region, about one-third of the stomach body, acts as a food reservoir to contain boluses before they can move into the distal region. When food boluses enter the distal region, they are physically broken down via peristaltic muscular contractions that act to crush and grind food particles (G.M. Bornhorst, Rutherfurd, et al. 2014; Somaratne et al. 2020).

In addition to physical breakdown, the low pH and enzymes in the stomach also work to chemically break down ingested foods. During gastric digestion, chemical breakdown is facilitated through mixing of the food bolus with gastric secretions. Gastric secretions contain electrolytes, enzymes (pepsin, gastric lipase), mucus, intrinsic factor, and HCl. In the stomach, the fasting pH (before a meal) is around 1.5 to 2 (Gail M. Bornhorst et al. 2016). As a result of gastric mixing, the pH gradually decreases to about 2, resulting in inactivation of salivary α‑amylase and simultaneous activation of gastric enzymes. Pepsin has optimal activity at pH 2 and is inactivated at pH $>$ 5.5; gastric lipase has optimal activity at pH 4 - 5.4 and is stable in acidic conditions until being inactivated at pH $>$ 7 (Yamile A Mennah-Govela, Singh, and Bornhorst 2019; Sams et al. 2016). The pH drop of the meal during gastric digestion will depend on the buffering capacity of the food and the rate of gastric mixing to mix secretions with the food (Yamile A Mennah-Govela, Singh, and Bornhorst 2019). Due to the combined physical and chemical breakdown in the stomach, as particles decrease in characteristic dimension to approximately < 1 mm, they are emptied from the pylorus at the distal end of the stomach into small intestine (Gail M. Bornhorst et al. 2016; Ehrlein and Schemann 2005; Welcome 2018).

2.1.3 Small Intestinal Digestion and Large Intestinal Fermentation

Small intestinal digestion is responsible for approximately 80% of fluid and electrolyte absorption. The small intestine can be divided into the duodenum, jejunum, and ileum (Gail M. Bornhorst et al. 2016). In the duodenum, the digested food that has exited the stomach, or chyme, is mixed with pancreatic secretions. Pancreatic secretions are comprised of enzymes including trypsin, chymotrypsin, lipase, phospholipase A, amylase, carboxypeptidase, elastase, and bile salts. These secretions are mixed with the chyme via muscular contractions of the intestinal wall including both peristaltic and segmentation contractions (Cotten 2020). The initial pH in the duodenum is about 6 and gradually increases to 7.4 by the end of the ileum (Fallingborg 1999). During small intestinal digestion, proteins, fats, and carbohydrates are broken down into smaller molecules in preparation for absorption into the network of capillaries and lymphatic vessels. For example, for carbohydrates, cleavage products of salivary amylase activity, such as alpha-dextrin, are digested by glucoamylase (alpha-dextrinases) to maltose and maltotriose. For proteins, after digestion in the stomach, the undigested residues of varying lengths or partially digested protein residues are further broken down to smaller units (amino acids and peptides of varying length—di-, tri-, and oligopeptides) by trypsin, chymotrypsin, and carboxypeptidases. Nutrient absorption mainly takes place in the duodenum and upper jejunum (Greenwood-Van Meerveld, Johnson, and Grundy 2017; Langerholc et al. 2011; Welcome 2018).

The large intestine is the final stage of digestion and is the body's fermenter. Reabsorption of water and short-chain fatty acid by-products occurs and waste material is converted into feces (K. S. Schulze 2015). During this process, the large intestine acts as a fermentation bioreactor, due to its large microbial population (approximately 10^{10}

microorganisms/g intestinal content), where fermentable sugars from remaining food particles are converted into other by-products (G.M. Bornhorst and Paul Singh 2014; C. Chen et al. 2012).

2.2 Digestion models

In order to obtain a mechanistic understanding of food breakdown in the gastrointestinal environment, in vivo and in vitro methods have been developed. In vivo methods are ideal for providing information on the actual digestion environment in animals or humans. However, in vivo methods are not always ethically and financially possible, and it may be difficult to take certain types of samples to monitor the digestion process. Simple in vitro digestion models simulating the gastrointestinal tract have been proposed as alternatives to in vivo experiments due to the lower cost and ethical considerations.

2.2.1 In Vivo Methods

In vivo digestion methods employing either human or animal subjects are still identified as the "gold standard" for conducting digestion-related research studies (Yao, Gibson, and Shepherd 2013). A variety of techniques exist for studying digestion in humans such as gastric emptying scintigraphy (GES), magnetic resonance imaging (MRI), and blood sampling (plasma analysis).

Gastric emptying scintigraphy (GES) using a solid meal is often recommended for the evaluation of gastroparesis by monitoring gastric emptying. Gastroparesis is one of the two most common sensorimotor disorders of the stomach, which is characterized by delayed gastric emptying (J.-P. Guo et al. 2001; Sachdeva et al. 2013). In GES, test meals are commonly labeled with a radionuclide marker such as Tc sulfur-colloid and the isotope presence is measured at various locations in the GI tract by taking images to analyze the percentage of food remaining in

the stomach at different time points (Christian et al. 1983; Wise et al. 2020). For example, Sachdeva et al. (2013) used GES together with a wireless motility capsule to compare gastric emptying and contractility of a standardized egg-white sandwich (EWS) solid meal to liquid nutrient meal (LNM). Gastric emptying was analyzed as the percentage of radioactivity retained in the stomach and contractility was characterized as the motility index of the wireless motility capsule. Gastric emptying half-time and gastric contractility of the LNM was similar to that of the EWS meal. Due to its higher fat content, the LNM emptied without a lag phase and took slightly longer to empty from the distal stomach.

Another in vivo method is magnetic resonance imaging (MRI). MRI is a non-invasive measurement using magnetic resonance imaging techniques to visualize organs of the GI tract, and quantify gastrointestinal function as well as to characterize luminal contents (Deng et al. 2020; Steingoetter et al. 2015). By using MRI, images of the stomach contents can be obtained to measure gastric emptying. For example, it is possible to investigate the change in gastric content volume and other intragastric processes, such as phase separation, as well as mixing and macroscopic changes in the texture of the chyme, such as gelling or coagulation (Smeets et al. 2020).

Blood sampling, or plasma analysis, is another non-invasive and widely-used analysis as part of in vivo human digestion studies. Blood samples are taken at certain time intervals after consumption of a test meal to determine the release of nutrients such as glucose, insulin, C-peptide, triacylglycerols, and nonesterified fatty acids, into the bloodstream after meal consumption. Gut hormones such as peptide YY (PYY) and glucose-dependent insulinotropic polypeptide (GIP) can also be measured in plasma samples and used to determine gastrointestinal tract response to various meals (C.H. Edwards et al. 2015).

An example of a study that used both magnetic resonance imaging and blood sampling was performed by Steingoetter et al. (2015). The objective of this study was to characterize the effect of the intragastric stability of fat emulsions on their dynamics of gastric processing and structuring to understand the influence on the gastrointestinal motor and secretory functions. In this study, 18 healthy subjects underwent MRI and blood sampling before and for 240 min after the consumption of the 4 different fat emulsions (with different particle size and either acid stable or unstable emulsions). According to the MRI data of the gastrointestinal tract and blood triglycerides, intragastric emulsion instability was related to changes in gastric emptying. Acid-unstable emulsions exhibited biphasic and faster emptying profiles than the acid-stable emulsions. Using MRI, it was possible to visualize large fat particles during gastric processing for the unstable lipid emulsion, with 0.32 μm solid fat particles. Blood triglycerides showing the flat triglyceride-absorption profile because of slow digestion confirmed this observation.

Besides human models, in order to improve efficiency and due to their relatively lower cost, animal models have been commonly used as part of in vivo digestion studies. Depending on the specific study purpose, typically pigs or rats are used as animal model systems to study food digestion (Deglaire and Moughan 2012). During a study, blood sample collection and digesta collection from various locations in the gastrointestinal tract are the most commonly used methods, but it is also possible to take tissue or other samples as well. Blood samples can be taken before euthanization to analyze nutrients such as α-tocopherol, glucose, and total triacylglycerols, or through using catheterization techniques to monitor the nutrients or hormones over time of animals that have in-dwelling catheters surgically installed. By using catheterization, absorption kinetics and apparent absorption of compounds such as

monosaccharides, amino acids, short-chain fatty acids and lignans can be investigated on conscious animals (Knudsen et al. 2006).

Besides analyzing the blood samples, measurement of digesta properties can be used to directly characterize the digestion process. For the collection of digesta, slaughter and cannulation techniques are often used. The slaughter technique involves the slaughtering of animals at a given time point after a meal and the collection of samples at various sites of the gastrointestinal tract (e.g. stomach, small intestine, and caecum). If cannulation techniques are used, animals have one or more cannulas surgically implanted in the gastrointestinal tract (e.g. stomach, duodenum, jejunum, ileum, or caecum) to get direct quantitative and qualitative information of digestion such as gastric emptying, digesta composition, and enzyme secretion (Knudsen et al. 2006).

An example in vivo study using digesta from the stomach of pigs was performed by Bornhorst, Roman, et al. (2014) with growing pigs fed either medium diced raw or hot-air roasted almonds. The objective of this study was to measure the changes in physical properties of a meal of raw or roasted almonds during gastric digestion. In this study, each pig was fed either raw or roasted almonds and euthanized at a specific time after finishing the meal. After euthanization, gastric chyme samples were taken from the proximal and distal stomach regions and analyzed for rheological properties, textural properties, and particle size. It was observed that after mastication and only a limited amount of time in the stomach, notable structural breakdown occurred. After 480 and 720 min of digestion, in the distal stomach, there was an accumulation of very large almond particles, but few small particles due to the effect of gastric sieving, where large particles go down to the bottom of the stomach, but are not emptied as they require further breakdown, while smaller particles are emptied from the distal stomach. It was also observed

after 720 min, changes in textural and rheological properties of the in vivo gastric chyme did not show consistent trends. By determining the concentrations of glucose, triacylglycerols, and α-tocopherol in peripheral plasma, Gail M. Bornhorst et al. (2013) also found that roasting almonds did not influence plasma glucose or triacylglycerols levels. However, postprandial plasma α-tocopherol levels were on average 33% greater after consumption of raw almonds, most likely as a result of the higher concentration of α-tocopherol in raw almonds compared to roasted almonds. The overall gastric emptying process did not show a difference between roasted and raw almonds. However, there were differences in the distribution of protein in the stomach and the gastric emptying of protein between raw and roasted almonds. The protein in the raw almonds emptied more rapidly than the protein in the roasted almonds. Compared to raw almonds, roasted almonds contained more protein (2.0% - 20.5%) in the stomach. These differences may have been caused by the variations in the amount of pepsin and/or gastric acid present after each of the meals, or may be attributed to the differences in almond protein structure which could alter their rate of emptying. These results show the different type of information that can be gained through analysis of digesta and blood samples using an animal model.

In vivo methods using human models can provide knowledge about the actual digestion environment, and common non-invasive methods such as GSE, MRI, and blood sampling are often used to investigate the gastric emptying processes and subsequent nutrient release. However, the disadvantages with human studies include low efficiency, differences between subjects, technical difficulty, cost, and being limited by ethics. Compared with human models, using animals and collecting their blood samples and/or digesta (slaughter or cannulation), can improve efficiency as well as reduce the cost and can directly investigate the physicochemical

properties of digesta at different digestion time points. However, the difficulties within in vivo

animal methods include differences from the human digestion environment, differences between

subjects, and a long experiment period (complexity). However, despite their disadvantages, in

vivo methods can still provide valuable information on the digestion process and as a basis for

developing in vitro digestion models.

2.2.2 In Vitro Methods

In vitro digestion methods are widely employed in pharmaceutical and nutritional science

to study the structural changes, digestibility, and release of food and pharmaceutical components

under simulated digestion conditions. They are considered to be cheaper, faster, and simpler to

perform than in vivo experiments. In vitro digestion methods typically have better

reproducibility and less inter-individual variability, and are therefore better to measure a

relatively large number of samples in parallel for screening purposes (L. Egger et al. 2017;

Minekus et al. 2014). Various in vitro digestion models have been developed to simulate oral,

gastric, small intestinal, and large intestinal digestion by taking into account the digestive

enzymes, pH, digestion time, and other factors such as transport of the digested meal (Minekus et

al. 2014).

In vitro gastric digestion methods can be divided into two categories, static and dynamic

models. For this review, static in vitro models are defined as those that only contain chemical

mechanisms that influence food breakdown while dynamic in vitro models are defined as those

that involve both physical and chemical changes in the food. Both static and dynamic models

have been applied in many foods, nutrition, and health studies (Somaratne et al. 2020). Static

models are the most simple and widely-used in vitro digestion models, which can be performed

in a water bath or a bioreactor (Somaratne et al. 2020). According to a recently proposed

harmonized static digestion protocol (Lotti Egger et al. 2019; Minekus et al. 2014), an in vitro

digestion may involve mixing samples with simulated saliva followed by mixing with simulated

gastric juice and incubation for different time points. After the gastric phase is completed,

simulated intestinal juice is added and samples are incubated for additional time points. All

incubations are performed at 37 °C on a rotating wheel. In addition to a rotating wheel, a shaking

water bath is another commonly used incubator for static in vitro digestion experiments.

Static models have been commonly used to perform measurements and analyses. In many

previous studies (Gail M. Bornhorst and Singh. 2013; Y.A. Mennah-Govela and Bornhorst 2016;

Minekus et al. 2014; Swackhamer et al. 2019), after simulated oral digestion, samples were

mixed with simulated gastric juice and placed in a shaking water bath at 37 °C. Samples were

removed at different time points and analyzed for physicochemical properties such as pH,

moisture content, texture, solid loss and fatty acid bioaccessibility. For example, Y.A. Mennah-

Govela and Bornhorst (2016) used a static in vitro gastric digestion method in a shaking water

bath to determine the acid and moisture uptake and its influence on macro- and micro-structural

changes during in vitro gastric digestion as a result of varying cooking treatments of sweet

potatoes. In this study, after mixing with simulated saliva for 30 s, boiled or steamed sweet

potato cubes were mixed with simulated gastric juice and placed in a shaking water bath (37 °C,

100 rpm). Acidity, moisture content, and solid loss were measured after 9 digestion times (15 to

240 min). Hardness and microstructure were completed before and after digestion. Results

showed that cooking method, cooking severity, and digestion time significantly influenced

moisture uptake. Acid uptake was significant influenced by digestion time, and both macro- and

microstructural changes were influenced by cooking treatment and gastric digestion time.

In addition to the use of static digestion models, several dynamic in vitro digestion methods have been developed to mimic more complex and realistic digestion conditions such as the TNO Gastro-Intestinal model (TIM) and Human Gastric Simulator (HGS). The TNO Gastro-Intestinal Model (TIM) is a multi-compartmental model, designed to realistically simulate digestive conditions in the lumen of the gastrointestinal tract. These digestive conditions include controlled parameters such as gastric and small intestinal transit, flow rates of secretions and composition of digestive fluids, pH values, and removal of water and metabolites (Kleiveland, Verhoeckx, and Cotter 2015).

While the TIM model can mimic the stomach and small intestine, other models have been developed to specifically focus on the gastric digestion phase. The Human Gastric Simulator (HGS) was designed to reproduce the fluid mechanical conditions driving the disintegration and mixing of gastric contents during gastric digestion. The HGS is comprised of a flexible outer vessel that simulates the gastric compartment. Gastric contractive motility is mimicked by a series of rollers that compress the compartment wall with increasing amplitude, moving toward the distal stomach (Gail M. Bornhorst and Singh 2012; Kong and Singh 2010).

The HGS has been used in many previous studies to measure physical and chemical changes during digestion. For example, Hayes et al. (2020) used the HGS to evaluate whether the physical properties of pearl millet couscous affected particle breakdown and starch hydrolysis during gastric digestion. In this study, three types of millet couscous and two types of wheat were put into HGS after simulated oral digestion to simulate gastric digestion. Digesta was collected every 30 min until 180 min. Particle size and starch hydrolysis were investigated at each time point. Results showed the number of particles per gram of dry mass significantly increased over digestion time for millet couscous ($p<0.05$) and had a significantly lower starch

hydrolysis per unit surface area of particles compared with wheat couscous (p<0.05). Besides particle size and starch hydrolysis, other processes that occur during gastric digestion have been assessed, such as protein hydrolysis and bioaccessibility of β-carotene (Roman, Burri, and Singh 2012; Somaratne et al. 2020). Based on the aims of the study, the characteristics of different models should be taken into consideration.

Static digestion models have been used to address diverse scientific questions such as the digestibility and bioaccessibility of pharmaceuticals, mycotoxins, and macronutrients such as proteins, carbohydrates and lipids due to their relative simplicity and ability to carefully control digestion conditions (G.M. Bornhorst, Roman, et al. 2014; Kopf-Bolanz et al. 2012; Paz-Yépez et al. 2019; Swackhamer et al. 2019). Compared to static in vitro models, dynamic in vitro models can provide a more accurate simulation of in vivo conditions. For example, gastric juice secretion rate can be controlled and motility of digesta can be mimicked. Similar to static digestion, physical and chemical changes during digestion have been analyzed. Overall, in vitro digestion models can be used to simulate digestion and characterize food breakdown by analyzing food property changes during digestion.

2.3 Impact of Processing on Food Properties

2.3.1 Moisture Content, Water Activity, and Water Holding Capacity

Water content, or moisture content, is the quantity of water present in a material. The water content in different foods ranges from a few percent in dried commodities (e.g. milk powder), around 15% in grains, 20% in honey, and 75% in meat, to about 90% in many fruits and vegetables (Sikorski 2006). The moisture content can be easily influenced by processing methods. There are various processing methods used to preserve foods by removing moisture,

such as conventional drying (hot air) and freeze-drying (Ratti 2001). For example, chickpeas (similar to other food legumes) are normally stored for significant time periods before use. Prior to long-term storage, chickpeas are commonly dehydrated (moisture content around 14%) to prevent changes in physicochemical properties such as color and texture, biological reactions such as the transformation of starch to sugars, as well as spoilage of the product (Moreira, Vazquez, and Chenlo 2002). The moisture content is an important parameter not only for evaluating storage conditions but is also relevant to determine appropriate drying conditions (Moreira, Vazquez, and Chenlo 2002). Besides drying, other processing methods such as frying, boiling, and wet roasting (soak and roast) also have an influence on moisture content. Debnath, Bhat, and Rastogi (2003) investigated the effects of pre-drying on the kinetics of moisture loss and oil uptake during deep frying of chickpea flour-based snack foods. They found that during frying, the moisture content of the fried product decreased and as the frying temperature increased, the equilibrium moisture content decreased. Bulbula and Urga (2018) found that there were significant differences among boiling, wet roasting, dry roasting, germination and fermentation on the moisture content of chickpeas. The maximum and minimum moisture content values were observed after wet roasting (9.37%) and dry roasting (5.91%), respectively.

Besides moisture content, water activity is another key parameter to ensure food quality and safety since it is related to sensorial quality (such as crispness), microbiological stability, and physical characteristics (such as texture) (Lertworasirikul and Tipsuwan 2008). Water activity has been defined as the partial pressure of water vapor in or around the food divided by that of pure water at the same temperature (Sikorski 2006). Similar to water content, water activity can also be easily influenced by processing methods. For example, heating, freeze drying, freeze concentration, and osmotic concentration methods are commonly used to reduce the water

activity of foods (Osman Erkmen and T. Faruk Bozoglu 2016). A decrease in water activity is

usually accompanied by a decrease in water content. For example, drying involves a decrease in

both water activity and moisture content by the removal of water. However, many fruit products

such as fruit juice concentrates, jams, and jellies have a low water activity together with high

water content for preservation. The low water activity depends on the high concentration of

sugars and other water binding compounds (Baker et al. 2005). The relationship between them is

complex. The relationship between water activity and moisture content at a given temperature is

called a moisture sorption isotherm. Various models have been proposed for sorption isotherms

of chickpea to specify the storage conditions for the seeds. It has been reported that the sorption

capacity of chickpea seeds decreased with an increase in temperature at constant water activity

(Menkov 2000).

Another important measurement in food processing applications that is related to the

moisture content and is often used to describe the hydration properties of pulse flours and

fractions such as chickpea, is the water holding capacity (Farooq and Boye 2011). Water holding

capacity (WHC) is defined as the amount of water that can be held per gram of sample. The

effect of processing on water holding capacity has been attributed to protein denaturation and

unfolding (previously hidden peptide bonds and polar side chains are exposed to hold more

water) as well as the content of carbohydrates such as starch, which gelatinizes, and dietary fiber,

which absorbs water (Aguilera et al. 2009). According to previous studies, water holding

capacity in legumes (chickpea and lentil) significantly increased during cooking and dehydration.

Lentil, chickpea, and pea flours exhibited high WHC values after boiling compared to raw and

roasted samples which may due to physical structural changes allowing greater porosity and

starch gelatinization as well as swelling of crude fiber (Ma et al. 2011).

In summary, moisture content, water activity, and water holding capacity are critical properties for food and are influenced during processing. Drying, roasting and other processing methods that can remove the water from food products can cause a decrease in water content and water activity. In addition, adding sugar or other water-binding compounds can also lead to a decrease in water activity. Boiling, dehydration, and other processing methods that can cause protein denaturation and starch gelatinization can increase the water holding capacity.

2.3.2 Texture

Food texture was originally defined as a sensory property deriving from the structure of the food and is a multi-parameter attribute that can be detected by several senses, including vision, hearing, touch, and kinesthetics (Szczesniak 2002). A variety of parameters have been developed to describe and quantify food texture including hardness, elasticity, adhesiveness, cohesiveness and others. These characteristics are usually evaluated using a texture analyzer by puncturing, compressing or breaking food materials (Lee et al. 2005; Sousa et al. 2007). Texture parameters can be determined because they are represented by different sections of the resulting texture cure (i.e. peak force, slope, area under the curve, etc.). According to numerous previous studies, the texture of food can be influenced by processing. For example, it has been widely observed that drying can increase the hardness of food, in contrast, soaking can decrease it. Debnath, Bhat, and Rastogi (2003) observed that the hardness of a fried chickpea snack increased quickly; breaking strength increased from ~7 to 9 N when the moisture content was reduced from 0.4 to 0.1 g water/ g sample. In another study (Clemente et al. 1998), chickpea showed a decrease in hardness due to hydration during the soaking process. Besides correlating texture with water content or water absorption during cooking, Alifakı and Şakıyan (2016) determined the effect of baking time and ingredients (chickpea flour concentration) on the

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hardness of chickpea cake. They found that an increase in baking time significantly increased the hardness, and that as chickpea flour content increased, the hardness of cakes also increased.

Texture is another important property and can be influenced by processing. Frying, baking and other processing methods that can reduce the moisture content can cause an increase in hardness. Additionally, soaking can decrease the hardness. Therefore, the texture is commonly used as a parameter in the food industry to characterize the quality of processed food and improve sensory properties for customers.

2.3.3 Starch and Protein

The main components found in foods include water, carbohydrates, proteins, lipids and minerals. Carbohydrates in food materials range from about 1% in meat and fish, 18% in potatoes, 15 to 21% in sugar beets, to more than 50% in legumes such as 54% in chickpea, 52% in pea and 56% in lentil (de Almeida Costa et al. 2006; Sikorski 2006). Carbohydrates are present in plants as starch. Starch is the most common storage carbohydrate in plants and the largest source of carbohydrates in human food. Starch is comprised of two types of molecules, amylose and amylopectin. Amylose is predominantly a straight chain polyglucan comprised of approximately 1000, α-d-(1–4) linked glucose units; amylopectin, which has a highly branched structure, is comprised of approximately 4000 glucose units with α-d-(1–6) linked branches. There are two crystalline structures of starch (an 'A' and 'B' type), based on differing proportions of amylopectin. A-type starches are usually found in cereals, B-type starches are often found in tubers and amylose-rich starches, while a third type called 'C-type' is identified as a mixture of both A and B forms and is found in legumes (Fuentes-Zaragoza et al. 2010). Although legume starch contains both amylose and amylopectin, they have been

characterized by high amylose contents (20.9% – 48.7% amylose for cowpea, 20.7% – 42.2% amylose for chickpea, and 22.0% – 49.6% amylose for yellow pea starches) (Huang et al. 2007).

When starch and water are subjected to heat, the intermolecular bonds of starch molecules will break down, known as starch gelatinization. As a result, processing methods, especially thermal processing, can easily affect the starch in foods. In a previous study by Aguilera et al. (2009), the available starch content (reducing sugars) of raw chickpea flour, soaked, soaked and cooked, dehydrated (after soaking and cooking) chickpea were compared. They found soaked chickpea had significantly increased available starch content (reducing sugars) and decreased resistant starch content compared to raw chickpea flour. A similar trend was reported by Frias et al. (2000); they demonstrated that soaking plus cooking brought a larger decrease in available carbohydrates (23–24%) compared to unprocessed chickpea. They also found that dry heating after soaking and cooking caused the largest losses (26% reduction) in starch content due to starch structure modification by high temperature and pressure during dry heating.

In addition to starch content, protein is another key nutrient that is present in chickpeas. The protein content in foods is present mainly as crude protein and ranges from around 1% of the weight of fruits and 2% of potatoes, and 12 to 22% of wheat grains to 25 to 40% of legumes. For example, chickpea contains around 24% protein, lentils contain around 26% protein and green pea contains around 25% protein (Iqbal et al. 2006; Sikorski 2006). Processing methods may cause protein denaturation due thermal tratment and moisture changes. After denaturation, the physical and chemical properties of protein are modified, and this may result in increased solubilization of the protein (Papadopoulos 1989). Ahmadian-Kouchaksaraei et al. (2014) investigated the effects of sodium bicarbonate concentration in soaking water, roasting

temperature and blanching time on physicochemical properties (pH, total solids, protein, fat, ash, lipoxygenase activity, stability, specific gravity, viscosity, color features) of sesame milk. They found roasting, soaking and blanching all resulted in decrease in the total protein of sesame milk (reduction of 0.06-0.37 g/100g protein). In another study, Y. Aguilera et al. (2009) compared protein solubility under different pH conditions between raw chickpea, lentils, and processed chickpea and lentils flours (soaked, cooked, dehydrated, freeze-dried, and sieved), and found that a low degree of protein solubility (< 20%) was observed in processed flours compared to raw

chickpea and lentil (30%-100%) along a pH gradient from 2 to 12. They hypothesized that thermal processing may have denatured the protein, causing reduced solubility. Ma et al. (2011) studied the effect of thermal processing on the functional properties and microstructure of lentil, chickpea, and pea flours. It was observed that protein solubility was significantly reduced by boiling compared to the raw and roasted pulse flours.

Starch and protein are important components of diet to provide energy (Lemon, Yarasheski, and Dolny 1984; Raben et al. 2003). However, their nutritional quality is limited by the occurrence of antinutritional factors (ANF). Broadly, the ANFs have been divided into proteins such as lectins and protease inhibitors and others such as phytate, tannins, and alkaloids (Martín-Cabrejas et al. 2009). ANF have been reported to obstruct the bioavailability of minerals and compromise the digestibility of protein (Martín-Cabrejas et al. 2009; Rathod and Annapure 2016). Uncooked (raw) legumes contain a high level of ANFs. For example, there is around 11.9 TIU/mg protein trypsin inhibitor (TIU: trypsin inhibitor unit), 6.22 HU/mg sample haemagglutinin activity (HU: haemagglutinin unit), and 4.85mg/g sample tannins in chickpea (Alajaji and El-Adawy 2006). Many methods have been investigated to reduce or remove ANFs such as boiling, autoclaving, and microwave cooking. Alajaji and El-Adawy (2006) found that

cooking treatments (boiling, autoclaving, and microwave cooking) could significantly ($p < 0.05$)

decrease trypsin inhibitor activity (reduction: 80.5-83.87%), tannins (48.04-50.10%), and

eliminate haemagglutinin activity, which increased the protein digestibility.

As two important nutrients in chickpeas, starch and protein can be easily influenced by

processing. For both starch and protein, processing, especially thermal processing, can cause a

decrease in available starch (reducing sugars) and protein solubility because of starch

gelatinization and protein denaturation, respectively, as well as elimination of ANF. Therefore,

the effect of processing of starch and protein in the food can affect the digestion of the starch and

protein and its nutritive value.

2.4 Impact of Processing on Properties and Behavior During Digestion of Legumes

In vitro digestion models are effective tools to study the impact of food processing on

food breakdown processes. The complete digestion process includes oral, gastric, small

intestinal, and large intestinal digestion phases. However, during in vitro digestion studies, it is

common to only focus on one or two phases because of complexity and depending on the aims of

the specific study. This section only considers gastric breakdown because the gastric phase is the

second most dominant phase for the physical breakdown of food after oral digestion, and the

breakdown and release of nutrients during the gastric phase will impact those nutrients available

in the intestinal phase for absorption.

Several factors influence the gastric breakdown of the food, including food

physicochemical properties, food composition and the processing of the food. Besides food

properties, the differences in digestion parameters may also influence breakdown, but discussion

of the impact of digestion parameters on food breakdown is outside the scope of this review. In

addition to food breakdown, the release of nutrients during digestion plays an important role in the physiological response after food consumption. During gastric digestion, nutrient release consists of multiple processes including the diffusion of gastric juice into the food, its simultaneous enzymatic degradation and mechanical breakdown by the peristaltic contractions of the stomach (Somaratne et al. 2020). Therefore, studying food breakdown and enzymatic hydrolysis can help to better understand the digestion process.

2.4.1 Starch and Protein Hydrolysis

Understanding starch hydrolysis is important to modulate starch digestion and glucose absorption (Fernandes et al. 2020). Through the action of various enzymes such as salivary amylase and pancreatic amylase, starch is broken down into smaller molecules such as maltose and glucose. To assist in the classification of the different fractions of starch that are released and hydrolyzed at different rates, starch has been classified into rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS). To determine the amount of each of these fractions of starch, an in vitro model is used to simulate the stomach and intestinal conditions. Samples are ground and incubated with pancreatic amylase and amyloglucosidase at 37 ℃ in shaking water bath. Glucose release is measured at different times, and these three starch fractions are defined as: rapidly digestible starch is the amount of glucose release after 20 min, slowly digestible starch is the amount of glucose released between 20 and 120 min, and resistant starch is the portion of starch that cannot be hydrolyzed by the enzymes in the small intestine after 120 min and would ferment in the large intestine (Englyst, Kingman, and Cummings 1992; Singh, Dartois, and Kaur 2010). During processing, starch molecules undergo physical changes and the processing method that may impact the rate of starch hydrolysis. For example, the effect of processing method including soaking, boiling and pressure cooking on the

starch of two legumes, red kidney bean (*Phaseolus vulgaris*) and yellow and green peas

(Pisum sativum) was studied by Roopa et al. (Roopa and Premavalli 2008). They found that

boiling could significantly increase rapidly available starch as well as decrease slowly digestible

starch compared with raw seeds due to the gelatinization of starch granules and increased

viscosity of flour.

Processing can also affect protein hydrolysis in different ways, depending on the specific

processing treatment and the interaction with the food matrix. On one hand, protein digestibility

can be increased by protein denaturation that occurs during thermal processing. During

denaturation, the tertiary and secondary structure of the protein is modified which result in

increase in protein hydrolysis. Conversely, thermal processing may also alter amino acid side

chains and form cross-links within or between molecules. These structural changes may result in

a delay of the action of certain digestive enzymes, resulting in decreased protein digestibility

(Sagum and Arcot 2000). For example, an in vitro study conducted by Alajaji and El-Adawy

(2006) investigated the effects of microwave cooking, boiling and autoclaving on chickpea

protein digestibility and ANF using an in vitro method where samples were ground and

incubated at 37 °C with a trypsin-pancreatin enzyme mixture for 10 min. It was found that the in

vitro protein digestibility was significantly higher in cooked chickpea compared to raw chickpea

(4.91-6.35% improvement), which may be attributed to the denaturation of protein, destruction

of the trypsin inhibitor, or reduction of tannins and phytic acid during cooking.

Extensive research has been done to study the influence of processing on starch and

protein digestibility. For example, the effects of thermal processing on protein and starch

digestibility of food legumes such as chickpea, lentils, red kidney bean, and black grams

subjected to different heating conditions were investigated by Rehman and Shah (2005) using an

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in vitro method where legumes were ground (40 mesh screen) and incubated with pepsin-HCl solution and pancreatic alpha amylase in 0.1 M phosphate buffer at 37 ℃. They found that starch digestibility of the uncooked food legumes (chickpea, lentils, red kidney bean, white kidney beans, and black grams) was 36.8-42.0% which improved to 70.0-77.3% after boiling and 87.4-91.0% after pressure cooking (121°C for 10 min). Similarly, the protein digestibility of the uncooked food legumes was 33.8-37.6% which increased to 63.0-72.0% after ordinary cooking and 68.0-76.0% after pressure cooking. In another study, the effect of various domestic processing methods including soaking, soaking and dehusking, ordinary cooking, sequential cooking (every 40 min, the cooking water was drained and boiling water was added during 130 min total boiling time), autoclaving, dry heating and germination on antinutrients and in vitro protein and starch digestibility of white and black varieties of *Mucuna pruriens*, a tropical legume, was examined by Siddhuraju and Becker (2001).They found that all of the treatments were effective in reducing the resistant starch content. Ordinary cooking, sequential cooking as well as autoclaving significantly improved the digestibility of protein (13.0−18.6 and 10.3−18% improvement in the white and black varieties, respectively) and starch (17.3−37.2% improvement in both varieties) compared to germination and dry heat treatment. Soaking samples followed by cooking was more effective to reduce the RS content and antinutrient content, including the total phenolics, phytic acid, L-dopa, as well as trypsin and chymotrypsin inhibitor activity (41.6−59% and 57−71% reduction in the white and black varieties) compared with raw and soaked samples due to the increased leaching out of phenolic substances under thermal conditions.

Starch and protein hydrolysis can be influenced by processing or cooking method. It has been found that cooking such as soaking, boiling, and pressure-cooking could cause a decrease in

RS and an increase in RDS due to gelatinization of starch and disruption of the starch structure during cooking, making the starch more vulnerable to α-amylase and pancreatic amylase hydrolysis. Cooking could also cause an increase in protein digestibility by denaturation of protein which increases the accessibility of the protein to enzymatic attack and also by inactivation of antinutritional factors such as tannins and phytic acid.

2.4.2 Particle Size Reduction

Particle size of food reduction begins during mastication where food is broken down by the teeth, then the particle size of remaining food will reduce in the stomach by the peristaltic muscle contractions of the gastric antrum. The initial particle size of food and its resistance to break down is important in gastric digestion which is one of the factors that influence the enzymatic digestion of starch and protein, as it increases the surface area available for diffusion and enzymatic hydrolysis (Gail M. Bornhorst, Kostlan, and Singh 2013; De La Hera, Rosell, and Gomez 2014). During gastric digestion, particle size distribution can be used to quantify food breakdown and may be measured using laser diffraction or image analysis techniques depending on the type of food. For example, Swackhamer et al. (2019) used image analysis to quantify the size reduction of particles that occurred during simulated gastric digestion in a static (shaking water bath) versus dynamic (Human Gastric Simulator) digestion model. By using this method, they found that the Human Gastric Simulator in vitro model of digestion led to the breakdown of almond particles during gastric digestion, which increased the release of fatty acids.

In addition to understanding the reduction in particle size during digestion, there have been several studies focusing on the relationship between particle size reduction before digestion and the digestibility of starch and protein. For example, a previous study examined the influence of physical form on starch digestion using a reciprocating (85 rpm) water bath with either

hammer-milled or cryo-milled sorghum of 10 particle sizes ($\cong$120–560 µm) (Mahasukhonthachat, Sopade, and Gidley 2010). It was observed that the smaller the particle size, the more starch was digested, due to an increase of relative surface area. After hammer milling, more starch was digested compared to cryomilling, likely due to frictional heat during hammer milling which could weaken the starch-protein matrix or endosperm structure to enhance swelling and digestion. This study showed that both the particle size and milling process (frictional heat) affected the sorghum starch breakdown kinetics. The smaller the particle size, as well as the more frictional heat generated during grinding of sorghum, resulted in more starch digestion. For cryo-milled sorghum with a particle size of 119 µm, there was 46 g/100 g dry starch digested starch after 240 min digestion which had more digested starch than particle size of 339 µm (34 g/100 g dry starch). For hammer-milled sorghum, 256 µm sample digested 14 g/100 g dry starch more starch than 444 µm sample after 240 min digestion.

A similar study conducted by Tinus et al. (2012) investigated the kinetics of starch and protein digestion in hammer- and cryo-milled cowpea (size range: 70-370 µm, 11 milling conditions). They found the *in vitro* protein digestibility of the cowpea was independent of the milling conditions. There was no relationship between the particle size and the rate of protein digestion for the hammer-milled cowpea. In contrast, the rate of starch digestion was inversely related to the square of the particle size, where more starch was digested as the particle size decreased. The protein digestion proceeded at a rate about 100× faster than the starch digestion. For cryo-milled samples with average particle size of 119 µm, after 240 min digestion, the digested starch and digested protein was more than 233 µm samples (digested starch was 20 g/g dry starch more; digested protein was 1.4 g/g dry protein more). Cathrina H. Edwards et al. (2021) found similar results for dry-milled chickpea and wheat with different particle sizes

during in vitro digestibility. They found the larger particles had the lowest starch digestibility due to more cell-wall-encapsulated starch. For particle size fraction of <0.21 of chickpea, the starch digestibility was 82.5 ± 1.5 % after 220 min digestion while the starch digestibility was only 33.0 ± 0.9 % for particle size fraction of 1.85 mm.

During gastric digestion, the foods are broken down into smaller particles and the particle size is related to starch and protein digestibility. As particle size decreases and digestion time increases, more starch and protein are digested due to an increase in surface area, but specific relationships between particle size and digestibility have not been determined. In addition, the influence of different processing methods should also be taken into account, because it may change the structure of starch and protein as well as decrease the antinutrients leading to higher digestibility.

2.5 Summary

Food properties can be divided into physical or structural properties and chemical composition. Chemical composition can be defined as the amount of components initially present in the food such as water, starch, and protein content. Physical composition or structure can be regarded as a particular arrangement of two or several components to provide specific mechanical properties to the food material. In a food system, the physical properties are related to texture and functional behavior (Sikorski 2006). Since it is an important property, food texture is commonly used as a parameter to estimate the quality of food after processing. Different processing methods were widely employed in the food industry which could increase the shelf-life of food and make it more nutritive and convenient for consumers. These processing methods have been developed that result in changes in temperature, pressure, and other factors such as

sugar concentration, that result in changes in food initial properties and modify their breakdown during gastric digestion.

Many studies have examined the effect of processing methods on initial food properties such as moisture content, water activity, water holding capacity and texture, as well as the digestibility of protein and starch during digestion. Processing methods, such as drying and soaking can significantly influence the moisture content of chickpeas. Processing such as boiling and autoclaving can significantly improve the digestibility of protein and starch compared to dried chickpea due to starch gelatinization, protein denaturation, and destruction of antinutrients. In addition, the particle size also has been shown to influence the digestibility of starch and protein of chickpea. However, previous studies have not systematically investigated the impact of water content and particle size of chickpea-based food products after processing and the effect of water content and particle size on particle breakdown and starch and protein digestibility during digestion. Improving our understanding of how water content and particle size influence digestion may help advance functional food development of nutrient-rich legumes, such as chickpeas.

3 MATERIALS AND METHODS

3.1 Raw Material and Processing Methods

Dried chickpeas (*Cicer arietinum L.*) were purchased from a local grocery store (Davis, CA). One, 22 kg (Jack Rabbit, Denver, CO, USA) bag was purchased and used for all experiments in this study to minimize biological variation. Chickpeas were prepared as a boiled chickpea puree or dried cracker with varying puree particle size. Steps required for the preparation of the four experimental treatments are shown in Figure 1.
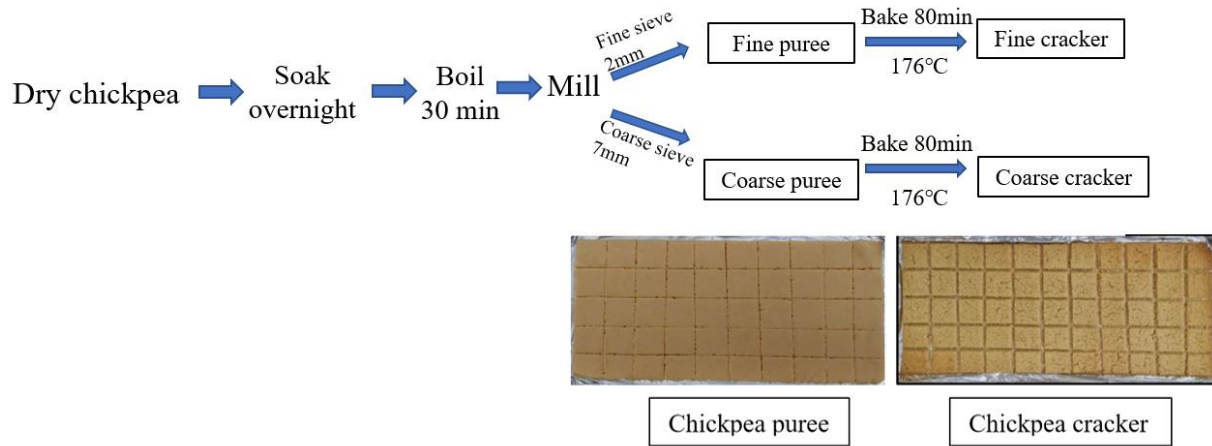
Figure 1. Steps required for preparation of the four experimental treatments: chickpea puree of small and large particle size (referred to as fine puree and coarse puree) and chickpea crackers made with puree of small and large particle size (referred to as fine cracker and coarse cracker).

For each experimental replicate, puree and crackers with the same initial particle size (either large or small) were prepared using the same batch of boiled chickpeas (Figure 1). Batches of 1.2 kg dried chickpea were soaked in 3 L Milli-Q Water (obtained from a Milli-Q Water Purification System (Merck Millipore, Billerica, MA, USA)) overnight at room temperature (at least 10 hours) as per package directions. The chickpeas were drained and rinsed

with tap water for 2-3 minutes. 6 L of Milli-Q water were heated up to 100 ºC in a large pot on a laboratory hotplate. After the water was boiling, the soaked chickpeas were immersed in the water and boiled for 30 minutes. The chickpeas were drained and cooled for 10 minutes. The boiled chickpeas were milled using a rotary food mill (Pantula, model: 8541971011, Blaine, WA, USA). One of two interchangeable stainless-steel grinding discs were utilized to obtain chickpea puree with two different particle sizes (fine: 2 mm opening; coarse: 7 mm opening). Chickpea puree was either stored in a sealed plastic bag at room temperature (to prevent increase of the resistant starch caused by storage at low temperature (Yadav, Sharma, and Yadav 2009) for digestion the next day or was used to prepare chickpea crackers.

To prepare chickpea crackers, batches of 1 kg chickpea puree (either small or large particle size; Figure 1) were placed on a rimmed baking pan (USA Pan, Pittsburgh PA, USA) and smoothed to a thickness of 5 mm (Figure 1). The puree sheet was scored into 2×2 cm squares and baked in a kitchen oven (Kitchen Aid, Benton Harbor MI, USA) at 176 ℃ for 80 minutes. The baking sheet of crackers was cooled for 10 min prior to separating into 2×2 cm crackers. Crackers were stored in a sealed plastic bag in a desiccator at room temperature prior to initial property analysis the following day and digestion two days later.

Chickpea puree and cracker with small and large particle size are referred to as fine puree and fine cracker as well as coarse puree and coarse cracker throughout (Figure 1).

3.2 Dynamic In Vitro Digestion

All chemicals were purchased from Thermo Fisher Scientific (Waltham, MA, US), with the exception of mucin and $CaCl_2(H_2O)_2$ (Sigma,-Aldrich, St. Louis, MO, US), α-amylase (MP

Biomedicals, Solon, OH), pancreatin (Sigma,-Aldrich, St. Louis, MO, US), and pepsin (MP Biomedicals, Solon, OH).

3.2.1 Simulated Digestion Fluids

Simulated digestion fluids were prepared following Minekus et al. (2014), with minor modifications. For simulated saliva, Milli-Q water was mixed with 1 g/L mucin (Type II from porcine stomach), 1.13 g/L KCl, 0.5 g/L KH_2PO_4, 1.14 g/L $NaHCO_3$, 0.03 g/L $MgCl_2(H_2O)_6$, 0.01 g/L $(NH_4)_2CO_3$ and 0.221 g/L $CaCl_2(H_2O)_2$. Finally, the pH was adjusted to 7.

For simulated gastric juice, Milli-Q water was adjusted to pH 0.8 and mixed with 1.5 g/L mucin, 0.515 g/L KCl, 0.122 g/L KH_2PO_4, 2.1 g/L $NaHCO_3$, 2.761 g/L NaCl, 0.024 g/L $MgCl_2(H_2O)_6$, 0.048 g/L $(NH_4)_2CO_3$ and 0.022 g/L $CaCl_2(H_2O)_2$.

For simulated intestinal juice, Milli-Q water was adjusted to pH 6.9 and mixed with 0.507 g/L KCl, 0.109 g/L KH_2PO_4, 7.14 g/L $NaHCO_3$, 2.25 g/L NaCl, 0.067 g/L $MgCl_2(H_2O)_6$, 0.088 g/L $CaCl_2(H_2O)_2$ and 10 g/L bile extract.

Immediately prior to digestion, the simulated saliva, gastric juice, and intestinal juice were heated to 37 °C, re-adjusted pH to 0.8 (gastric juice) or 7 (saliva and intestinal juice), if necessary, and 1.18 g/L α-amylase (190 BAU/mL), 2 g/L pepsin (2000 U/mL) or 2.4 g/L pancreatin (100 TAME units/mL) was added to the simulated saliva, gastric juice, or intestinal juice, respectively.

3.4 In Vitro Oral Digestion

Mastication of the chickpea crackers was simulated by using a food processor (Spectrum Brands, Inc. Food Processor Model: FP2500B, Middleton WI, USA) to reduce particle size, which has been conducted in previous in vitro studies (Hayes et al. 2020; Hwang et al. 2019).

Batches of ~ 300 g crackers were processed at high speed for approximately 40 seconds. After processing, particles were sieved, and the fraction with sizes ranging from 0.85 - 2.0 mm was retained to represent the range of particle size as previously reported from in vivo mastication studies (Jalabert-Malbos, M. L., Mishellany-Dutour, Woda, and Peyron 2007). Due to limited breakdown during mastication, chickpea puree did not undergo an additional physical breakdown step to simulate mastication (J. Chen et al. 2013). For each digestion experiment, to compare an equivalent amount of dry matter, the initial weight of chickpea puree and cracker was calculated maintaining constant the dry matter of the sample, 120 g. Chickpea puree or masticated crackers were mixed with 120 mL simulated saliva (1 mL saliva/g dry matter) for 30 seconds (Gavião, Engelen, and Van Der Bilt 2004; Yamile A. Mennah-Govela, Bornhorst, and Singh 2015; Mulet-Cabero et al. 2020).

3.2.2 In Vitro Gastric Digestion

Immediately after oral digestion, the chickpea + saliva mixture was transferred to the artificial stomach bag of the Human Gastric Simulator (HGS) containing 70 mL preheated (37°C) simulated gastric juice (Q. Guo et al. 2015) to start gastric digestion (Figure 3). In vitro gastric digestion was conducted using the Human Gastric Simulator (HGS), which has been previously described (Dupont et al. 2019; Hayes et al. 2020; Swackhamer et al. 2019). In the HGS, the sample was exposed to a simulated gastric environment by adding simulated gastric juice at a rate of 3.5 mL/min, with peristaltic contractions at a rate of 3 contractions per minute (K. Schulze 2006), and temperature control at 37°C. During the gastric digestion phase, around 105 g of sample was taken from the bottom of the stomach bag (simulated pylorus) after 30, 60, 90, 120, 150, and 180 min to simulate an average gastric emptying rate of 3.5 mL/min (Gail M. Bornhorst, Chang, et al. 2013; Hayes et al. 2020). Digesta mass and pH were measured and the

gastric digesta was sieved (0.6 mm mesh) to separate the solid and liquid phase. The ratio of solid and liquid was calculated and used to separate six aliquots of approximately 3 g of gastric digesta for small intestinal digestion (maintaining the same solid: liquid ratio of the gastric digesta emptied from the HGS). Immediately after the intestinal sample separation, to stop the pepsin activity and neutralize the pH to 7, an NaOH solution was added to each aliquot of gastric digesta reserved for small intestinal digestion as follows: 0.1 mL of 1 M NaOH for 30 and 60 min gastric digestion; 0.1 mL of 2 M NaOH for 90-120 min gastric digestion and 0.15 mL of 2 M NaOH for 150-180 min gastric digestion. The volume and concentration of NaOH solution were determined in preliminary experiments. Samples were immediately frozen in liquid nitrogen and stored at -18℃. Small intestinal digestion was completed within one week of gastric digestion for each replicate.

The remaining solid sample was rinsed with 100 g or 200 g 0.5 M Na_2CO_3 (based on sample amount), to separate large digesta particles, and the resulting solid and liquid phases of digesta were weighed, kept on ice, and used for analysis the same day as the digestion. Digestions for each treatment were completed in triplicate with a new batch of chickpeas used for each replicate.

3.2.3 In Vitro Small Intestinal Digestion

For each gastric digestion time point, 6 gastric samples were used for in vitro intestinal digestion (36 intestinal samples for each gastric digestion). Each gastric sample was thawed in a shaking water bath (TSSWB27, Thermo-Fisher, Waltham, MA, USA) at 37 ℃, then 4 mL of preheated simulated intestinal juice per g gastric sample (ratio 4:1, v/w) was added, and the pH was adjusted to 7. Samples were incubated in a shaking water bath at 37 ℃, 100 rpm, for 30, 60, 90, 120, 150 or 180 min (Figure 2) (Roman, Burri, and Singh 2012). After intestinal digestion,

the tubes were immediately removed from the water bath, and digestion was stopped by adding 10 mL of 0.5 M Na_2CO_3 to each sample, and placing them on ice for 5 minutes. Samples were then centrifuged at 4122 xg for 8 min to separate liquid and solid phase to be weighed and used for analysis.
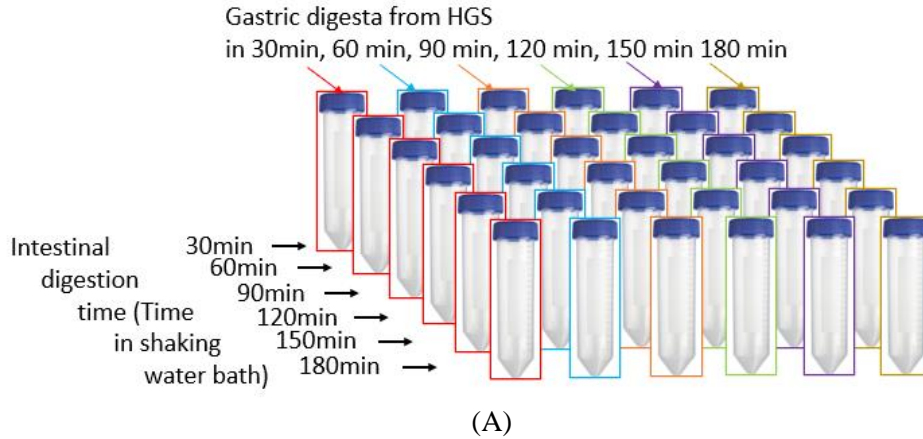
(A)

Total digestion time(min)

		Gastric digestion time					
		30min	60min	90min	120min	150min	180min
	30min	60	90	120	150	180	210
Intestinal digestion time	60min	90	120	150	180	210	240
	90min	120	150	180	210	240	270
	120min	150	180	210	240	270	300
	150min	180	210	240	270	300	330
	180min	210	240	270	300	330	360

(B)

Figure 2. Gastric and intestinal time points (A) and the total digestion time (B) for each combination of gastric and intestinal time that was utilized in the analysis of gastrointestinal starch and protein hydrolysis.

3.3 Digesta Sample Preparation for Analysis

For gastric digesta analysis, after removing aliquots for intestinal digestion and moisture content measurement, and rinsing the remaining solids with 0.5 M Na_2CO_3, 2 g solid were mixed with 7 mL 0.5M Na_2CO_3 for analysis of protein and starch hydrolysis (Figure 3).

The solution of the liquid from the gastric digesta mixed with the Na_2CO_3 used to rinse the solid fraction was used for particle size analysis (Section 3.5.3). An aliquot of this solution (~ 80 mL) was centrifuged at 4122 xg for 6 min to separate the small particles suspended in the liquid (referred to as suspended solids throughout) from the liquid. All of the phases (solid, suspended solid, and liquid) were carefully weighed.

After centrifugation, the liquid phase was diluted in sodium bicarbonate (300 μL liquid:1000 μL 0.5 M Na_2CO_3) and frozen at -18℃ for analysis of starch hydrolysis and protein hydrolysis (Figure 3). The suspended solids in the liquid phase, which precipitated during centrifuging, were analyzed for moisture content, and the remaining 2 g of suspended solids were separately mixed with 7 mL 0.5 M Na_2CO_3.

Each soild or suspended solid sample mixed with Na_2CO_3 was homogenized at 10,000 rpm (Ultra Turrax T18 digital with S18N-19G disperser, IKA Works, Wilmington, NC, USA) for 10 s. 200 mg of this mixture were reserved for analysis of protein hydrolysis. The homogenized suspended solids were centrifuged at 4122 xg for 6 min. 300 μL of the supernatant was diluted with 1000 μL 0.5M Na_2CO_3 for analysis of starch hydrolysis (Figure 3).

For intestinal digesta, after centrifugation (4122 xg for 8 min), the supernatant was weighted and 300 μL supernatant was diluted with 1000 μL of 0.5M Na_2CO_3 for starch and protein hydrolysis measurements (Figure 3). The solid fraction of the 180 min intestinal digestion time was also analyzed for remaining starch and protein content (Section 3.5.4 and 3.5.5, respectively).

All samples for starch and protein hydrolysis measurements were frozen at -18℃ and analyzed within one week of the digestion.

In vitro oral digestion	→	In vitro gastric digestion	→	In vitro intestinal digestion

Initial Properties
- Moisture content
- Fat content
- Water activity
- Water holding capacity
- Buffering capacity
- Texture
- Starch
- Protein

- pH
- Moisture content
- ❑ Solid phase (Separated by sieving)
  - Particle size
  - Starch Hydrolysis
  - Protein Hydrolysis
- ❑ Suspended solid phase (Separated by sieving & centrifuging)
  - Particle size
  - Starch Hydrolysis
  - Protein Hydrolysis
- ❑ Liquid phase (Separated by sieving & centrifuging)
  - Starch Hydrolysis
  - Protein Hydrolysis

- ❑ Liquid phase (Separated by centrifuging)
- Starch Hydrolysis
- Protein Hydrolysis

Figure 3. Analyses conducted at each stage of in vitro digestion on the solid, suspended solid, and liquid phases of digesta.

3.4 Initial Properties of Chickpea Crackers and Puree

Moisture content of the chickpea puree and crackers was determined gravimetrically by drying in a vacuum oven at 110°C for 20 h (Gail M. Bornhorst, Ströbinger, et al. 2013). Fat content of pre-dried chickpea crackers and puree was measured by Soxhlet extraction with n-hexane after 5 hours (Latimer 2012). Water activity of chickpea crackers and puree was measured using an Aqualab Series 3 water activity meter (Decagon Devices Inc., Pullman, WA, USA). Water holding capacity (WHC) was determined following Elhardallou and Walker (1993) with minor modifications. 2.5 g chickpea puree or masticated cracker was mixed with 30 mL milli-Q water in a 50 mL centrifuge tube and incubated in a shaking water bath at 37 °C, 50 rpm for 4 h, followed by centrifugation (4122 xg for 20 min). Then samples were taken, the water was removed, and the remaining sample was weighed (weight of wet sample) following by

drying in a vacuum oven at 110°C for 20 h and weighing (weight of dried sample). The WHC

was calculated as:

$$WHC_{diet}\left(\frac{g\ H_2O}{g\ DM}\right) = \frac{weight\ of\ wet\ sample}{weight\ of\ dried\ sample} - 1$$

Buffering capacity was measured following Y. A. Mennah-Govela, Singh, and Bornhorst (2019).

10 g chickpea puree or masticated chickpea cracker was mixed with aliquots of 0.5 mL of 0.16

M HCl using a spatula and the pH was measured with a pH meter (Dual pH Technology, IQ

Scientific Instruments) until reaching an endpoint of pH 1.5. Total buffering capacity was

determined as μmol H+ / (g sample × total pH change).

The texture of the chickpea puree and crackers was characterized using a TX-XT2

Texture Analyzer (Texture Technologies Corp., Hamilton, MA, USA). Chickpea crackers were

compressed at a speed of 1 mm/s to using a 3 mm cylindrical puncture probe until fracture

occurred (Kim et al. 2012). 20 g of chickpea puree was put inside a 50 mm diameter cylindrical

cup and was compressed at 1 mm/s to 33% strain using a 45 mm diameter cylindrical plunger

(Gail M. Bornhorst, Ferrua, and Singh 2015). Results for both chickpea crackers and puree were

expressed as hardness in N (peak force during compression) and Young's modulus E (slope up to

2% strain). Total starch was measured using a total starch analysis kit (K-TSTA, Megazyme,

Bray, Ireland) following the AACC method 76.13 (Flores-Silva et al. 2014).

Moisture content, water activity, water holding capacity, buffering capacity and texture

were measure in triplicate for each treatment. Fat content, starch, and protein content were

measured in duplicate for coarse and fine puree samples.

3.5 Digesta Analysis

3.5.1 pH

The pH of the gastric digesta was measured using a pH meter (Thermo Fisher Scientific, Waltham, MA, US). pH of digesta from each gastric time point was measured in duplicate.

3.5.2 Moisture Content

Moisture content was determined as described in section 3.4. Moisture content of each sample was measured in triplicate.

3.5.3 Particle Size

3.5.3.1 Solid Phase: Image Analysis

The particle size distribution of the chickpea puree, masticated chickpea cracker, and solid phase of gastric digesta was determined following the procedure by Swackhamer et al. (2019). Briefly, aliquots of 1 g of initial puree sample and digested sample were spread on a petri dish immersed with RO water as well as initial cracker sample were spread without adding RO water. After manually separating the particles in each dish to ensure particles were not touching, each dish was placed under a Canon EOS Rebel SL1 digital camera (18-megapixel, APS-C CMOS sensor, Canon USA, San Jose, CA) and illuminated from beneath using a lightbox (AGPtek HL0163, color temperature 6000°K). The camera settings were: no flash, 35 mm focal length, aperture F8.0, ISO 100, and shutter speed 0.1 second, 47 mm distance to the particles. An image of each dish was captured.

All the images were analyzed using MATLAB R2018b (MathWorks, Natick MA), as previously described (Hayes et al. 2020; Swackhamer et al. 2019) (Figure 4). Each image was converted into a binary image and the total number of particles as well as the area of each

particle were determined. Particle per gram of sample was defined as the ratio of the number of

particles in each sample to the dry mass of that digesta sample. The particle size distribution was

determined by fitting the cumulative area percentage of the particles to the Rosin-Rammler

model:

$$C_{area} = 1 - e^{\left(-\left(\frac{x}{x50}\right)^{b} \times ln(2)\right)}$$

(1)

where C_{area} is the cumulative area percentage of particles (0 to 100%); x_{50} is the median

particle area (mm^2); and b is a constant representing the broadness of the distribution

(dimensionless). Image analysis of each sample was done on duplicate aliquots.
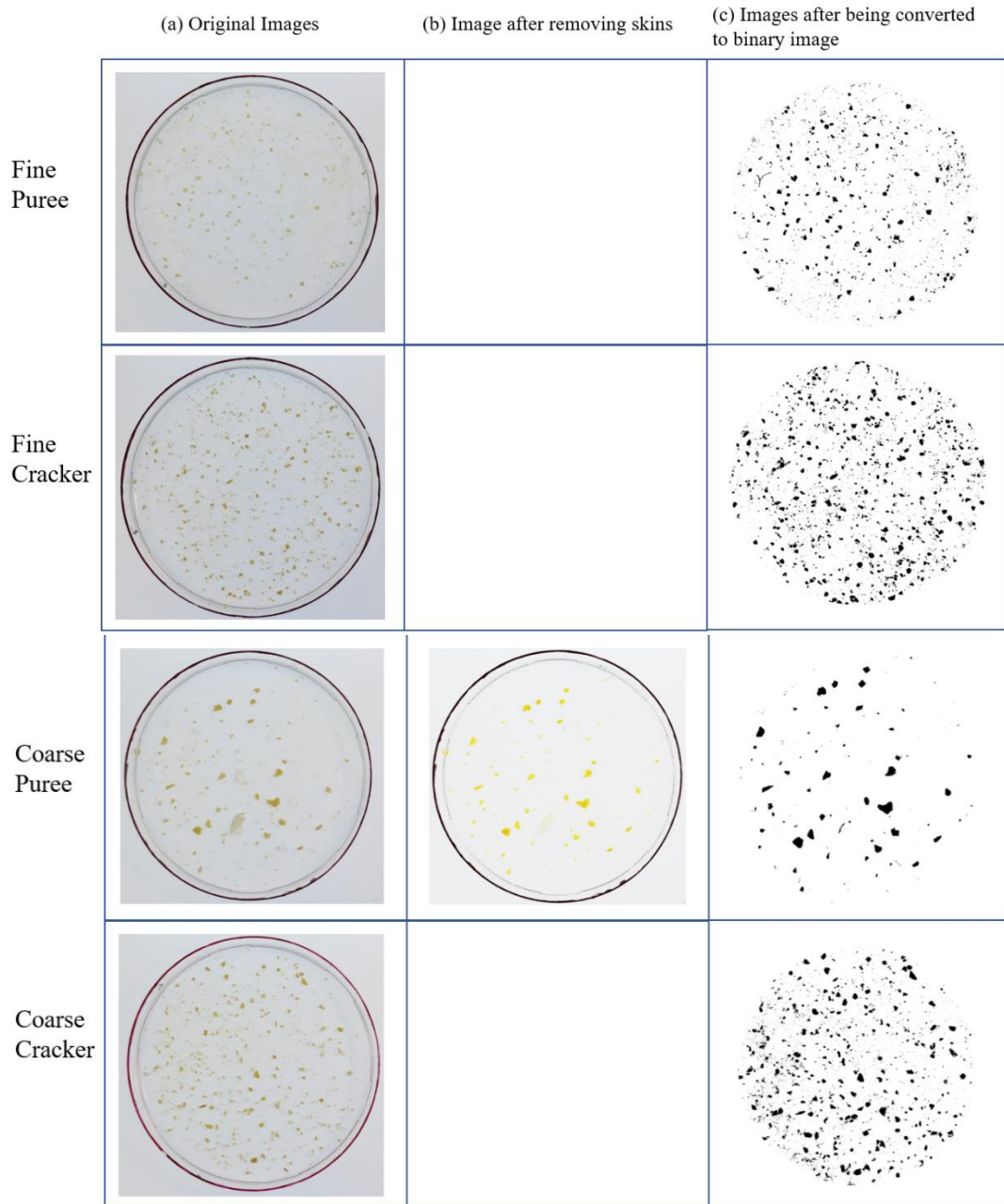
Figure 4. Example images from each of the four treatments (fine puree, fine cracker, coarse puree, and coarse cracker) from 30 min gastric digesta samples, showing the original image and the image masks after image processing.

3.5.3.2 Suspended Solids: Mastersizer

The particle size distribution of the suspended solids in the liquid phase was determined using the Mastersizer 3000E (Malvern Instruments Ltd, Worcestershire, UK) with the following settings: non-spherical particles, refractive index 1.47 (Yamile A. Mennah-Govela et al. 2020). Water was used as dispersant and a time period of 10 s was used for each measurement. Results were expressed as median particle size (d50), d90 and d10. Measurements were done in duplicate for each sample.

3.5.4 Starch Hydrolysis (RDS)

The starch degree of hydrolysis was measured based on the determination of reducing sugars (RDS) following the 3,5-Dinitrosalicylic acid (DNS) assay (Miller 1959) with maltose used as standard. Frozen samples were defrosted in the water bath (TSSWB27, Thermo-Fisher, Waltham, MA, USA) at 37 ℃. This assay is light sensitive, therefore it was done in a closed room using red lights. 400 µL of diluted samples were added to 400 µL DNS solution and incubated at 90 ℃ for 20 min in a water bath and were placed on ice. After cooling, 225 µL of each sample was pipetted to a well of a 96-well microplate (in triplicate) and absorbance read at 540 nm on a microplate reader (BioTek Instruments, Inc., Winooski, USA).

3.5.5 Protein Hydrolysis (OPA)

The protein degree of hydrolysis was determined with the o-phthaldialdehyde (OPA) method according to Nielsen, Petersen, and Dambmann (2001) with glycine used as a standard. After defrosting, 30 µL of the liquid samples were pipetted into each well on a microplate. 300 µL OPA solution (40 mg o-phthaldialdehyde (OPA) + 1 mL methanol + 1.25 mL SDS (20%) + 100 µL 2-Mercaptoethanol + 1 mL Methanol, brought up to 50 mL with Na-tetraborate solution

(19.05 g of $Na_2B_4O_7$ brought up to 500 mL with Milli-Q water, pH 9.3)) or No-OPA solution (same composition as above, only without OPA) was added to the microplate wells. After 4 min, the absorption was measured at 340 nm on a microplate reader.

For the solid samples, 200 mg of defrosted solid samples were added to the extraction buffer (4.77 g $Na_2B_4O_7.10H_2O$ + 10 mL SDS (20%), brought up to 1 L using Milli-Q water), and incubated for 60 min on an orbital shaker at ambient temperature. Following incubation, samples were centrifuged (4000 xg for 10 min). The liquid phase after centrifugation was analyzed in the same way as above for liquid samples.

3.6 Data and Statistical Analysis

3.6.1 Saturation Ratio

Saturation ratio (SR) of fine and coarse puree before digestion were calculated following Martens et al. (2019) as:

$$SR = \frac{Diet \ or \ digesta \ moisture \ content \ (g \ H_2O/g \ DM)}{WHC_{diet} \ (g \ H_2O/g \ DM)} \tag{2}$$

3.6.2 Gastric Emptying of Dry Matter

Gastric emptying was determined as the ratio of remaining dry matter at each gastric digestion time point (DM_t) to the initial dry matter used for the digestion (DM_0). The gastric emptying was fit to a modified power-exponential model (Gail M. Bornhorst, Chang, et al. 2013; Siegel et al. 1988):

$$\frac{DM_t}{DM_0} = 1 - \left(1 - e^{-kt}\right)^{\beta} \tag{3}$$

where k is the emptying rate (min^{-1}), t is gastric digestion time (min), and β is the theoretical y-intercept (dimensionless). The estimated time to empty 50% of the sample in the stomach was calculated as:

$$t_{50} = \frac{\ln\left(1 - 0.5^{1/\beta}\right)}{-k}$$ (4)

where t_{50} is the emptying half rate (min), and k and β are the parameters determined in Equation 3.

3.6.3 Starch Hydrolysis

Starch hydrolysis during 180 min gastric digestion was determined as the ratio of reducing sugar in the liquid, suspended solid or solid phase to the initial starch content. The total starch hydrolysis during 360 min digestion (gastric + intestinal) was determined as the increase in reducing sugar in the liquid phase after each 30 min time point compared to the previous time point. The total cumulative starch hydrolysis during 360 min digestion was determined as the cumulative sum of the increase in reducing sugar content in the liquid phase to at each total digestion time point. In addition, the percent of starch hydrolysis was calculated using the total starch content in the samples prior to digestion.

The total cumulative starch hydrolysis (%) during 360 min digestion was fit to a first-order equation model (Isabel Goñi, Garcia-Alonso, and Saura-Calixto 1997):

$$C = C_\infty\left(1 - e^{-kt}\right)$$ (5)

where C is the starch hydrolysis at time t, C_∞ (%) is the equilibrium concentration of starch in the simulated gastro-small intestinal digestion process, and k is the kinetic constant (min^{-1}) and t is time (min).

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The Hydrolysis Index (HI) was calculated as the area under the hydrolysis curve (AUC) divided by the AUC of white bread (dimensionless). The AUC was calculated using the first order equation (Annor et al. 2015):

$$\text{AUC} = C_\infty(t_f - t_0) - (C_\infty/k)\big(1 - e^{-k(t_f - t_0)}\big) \tag{6}$$

where t_f is the final time (360 min) and t_0 is the initial time (0 min).

The estimated Glycemic Index (eGI) was calculated from HI values (dimensionless), following equation proposed by Isabel Goñi, Garcia-Alonso, and Saura-Calixto (1997):

$$\text{eGI} = 0.862\ \text{HI} + 8.198 \tag{7}$$

3.6.4 Statistical Analysis

Analysis of variance (ANOVA) was conducted using SAS Enterprise 7.1 (SAS Institute, Cary NC). The initial properties (moisture content, water activity, texture, water holding capacity, saturation ratio, and buffering capacity) and the gastric emptying model parameters (k, β, t_{50}) were analyzed using a mixed model, two-factor ANOVA to determine the effect of initial particle size (fine or coarse) and drying (puree or cracker) on each property. Particle size and drying were fixed effects. The fat, starch, and protein content were analyzed using a one-way ANOVA to determine the impact of particle size (fine or coarse).

A mixed model, three-factor ANOVA was used to determine the effect of initial particle size (fine or coarse), drying (puree or cracker), and gastric digestion time (30, 60, 90, 120, 150, 180 min) on the properties of the emptied gastric digesta including pH, moisture content, the particle size of the liquid and suspended solid (d50, d10, d90) as well as the particle size of solid phase (x50, x10, x90) and the number of particles /g dry matter. The initial particle size (fine or

coarse) and drying (puree or cracker) were fixed effects and the digestion time was a repeated effect within each digestion replicate. For starch and protein hydrolysis of liquid, solid and suspended solid phase, the digesta fraction was an additional factor, nested within the repeated effect of digestion time. If the main effects were significant, the Tukey-Kramer test was used to compare means at a significance level of $p < 0.05$.

4 RESULTS AND DISCUSSION

4.1 Initial Properties

The moisture, water activity, hardness, and Young's modulus (Table 1) were not significantly influenced by particle size ($p > 0.05$), but were significantly influenced by drying ($p < 0.05$). For both particle sizes, the water activity of the chickpea puree (average of 1.01 ± 0.005) was almost 10 times greater than the crackers (0.11 ± 0.025). Drying led to crackers with lower moisture content compared to puree, which resulted in an increase in hardness. Previous studies observed similar trends for bread crumbs (De La Hera, Rosell, and Gomez 2014) and for chickpea snacks (Debnath, Bhat, and Rastogi 2003), where decreasing the water content increased the hardness.

Water holding capacity and saturation ratio were significantly different between the puree and cracker with fine particle size ($p < 0.05$), where the fine cracker had a greater water holding capacity and lower saturation ratio compared to the fine puree. However, the water holding capacity and saturation ratio were not significantly influenced by drying within the products with coarse particles ($p > 0.05$). The water holding capacity observed in this study is different from those reported by other researchers (Aguilera et al. 2009; Ma et al. 2011). For example, the water holding capacity for boiled chickpea flour was around 1.8 mL H_2O /g sample and 0.8 mL H_2O /g

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sample for chickpeas flour that was roasted in a previous study (Ma et al. 2011) compared to the average 3.28 g H_2O/g DM for chickpea puree and average 3.5 g H_2O/g DM for chickpea cracker in this study. The divergence between the present study and the previously reported values might be due to differences in the processing method and thermal processing conditions, where the previous study boiled and roasted dehulled chickpea flour and the current study soaked, boiled, and dried the whole chickpea resulting in swelling and enlargement of some of the starches and had larger particles as well as included pieces of chickpea skin. The chickpea skins were the most prevalent in the large puree, due to the coarse sieve (7 mm) used after milling, which allowed skins to pass into the sample. These large pieces of chickpea skin may have increased the ability of the coarse puree to absorb water (Chenoll, Betoret, and Fito 2009), resulting in the significantly greater (p<0.05) water holding capacity of coarse puree compared to fine puree (3.53 ± 0.23 vs. 3.02 ± 0.04 g H_2O/g DM, respectively). When comparing the fine cracker and puree, it is likely that drying and milling caused protein denaturation and unfolding that exposed more peptide bonds and polar side chains resulting in higher water holding capacity (Aguilera et al. 2009). Buffering capacity was significantly influenced by drying and initial particle size (p<0.05), where the buffering capacity values were: fine puree > coarse puree, fine cracker > coarse cracker. This is in general agreement with previous studies, where it has been observed that samples with a higher surface area had higher buffering capacity (Y.A. Mennah-Govela et al. 2020).

Table 1. Initial properties of chickpea puree and cracker with fine and coarse particles. Values for fat content and starch represent the average (n=2) ± standard deviation and represent the average (n=3) ± standard deviation for others. Letters identify significant differences (p<0.05) between treatments (ab) across each row.

	Fine puree	Fine cracker	Coarse puree	Coarse cracker
Moisture (g H$_2$0 / g sample mass)	0.66 ± 0.02^a	0.01 ± 0^b	0.65 ± 0^a	0.04 ± 0^b
Water activity	1.0 ± 0.001^a	0.1 ± 0.03^b	1.0 ± 0^a	0.1 ± 0.02^b
Hardness (N)	4.35 ± 10.6^a	27.83 ± 86.82^b	4.47 ± 4.89^b	32.48 ± 53.03^a
Young's modulus (N/m^2)	0.35 ± 0.08^a	11.85 ± 2.26^b	0.34 ± 0.09^a	13.29 ± 2.70^b
Fat content (g fat/100 g dry matter)	2.52 ± 0.18		2.25 ± 0.03	
Starch (g starch/100 g dry matter)	45.57 ± 0.05		46.34 ± 1.68	
Water holding capacity (g H$_2$O/g DM)	3.02 ± 0.04^a	3.58 ± 0.08^b	3.53 ± 0.23^b	3.44 ± 0.01^b
Saturation ratio (dimensionless)	0.25 ± 0^a	0.22 ± 0^b	0.22 ± 0.01^b	0.23 ± 0^b
Buffering capacity (µmol HCl/g sample×ΔpH)	50.93 ± 0.21^a	48.61 ± 0.26^b	49.32 ± 0.73^b	42.95 ± 1.50^c

4.2 pH

The pH of the gastric digesta (Figure 5) was significantly influenced by gastric digestion time (p<0.0001) and drying (p=0.0022), but not by particle size (p>0.05). The pH of the four treatments ranged from 3.38-4.00 after 30 min and decreased to 1.61-1.14 after 180 min gastric digestion. These values are similar to previous studies, where the mean pH for roasted almonds

decreased from 5.0-6.5 to 3.0-5.5 after 180 min in vivo digestion from pigs (Gail M. Bornhorst, Roman, et al. 2013) and for whey protein gels, the pH decreased from 7.5 ± 0.0 before digestion to 1.3 ± 0.1 after 180 min of in vitro gastric digestion (Yamile A. Mennah-Govela and Bornhorst 2021).

The pH for fine puree from 60-150 min gastric digestion was significantly ($p<0.05$) higher than the fine cracker, coarse puree, and coarse cracker. The higher pH in the fine puree may be attributed to the fine puree buffering capacity, which was significantly ($p<0.05$) higher than the other treatments. Due to the greater buffering capacity, there was a slower decrease in pH, as proposed in other previous studies (Yamile A Mennah-Govela, Singh, and Bornhorst 2019). Similarly, the pH of coarse puree decreased directly, instead of increasing from 30-60 min in the other treatments. This may be due to its lower buffering capacity compared with fine puree and fine cracker.
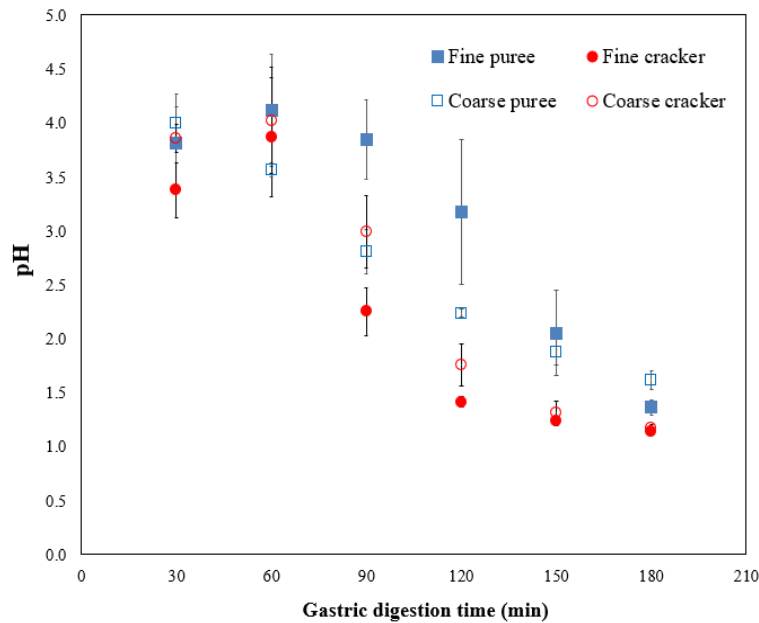

Figure 5. pH of emptied gastric digesta from the HGS for fine puree (■), fine cracker (●), coarse puree (□), and coarse cracker (○). Data points represent the average values (n=3) with error bars as the standard deviation.

4.3 Gastric Emptying

The ratio of solid, suspended solid, and liquid phase in the emptied digesta and their trends during gastric digestion varied between fine puree, fine cracker, coarse puree, and coarse cracker. These ratio and trends were used to indicate the relative state of the meal that was emptied from the HGS, and provide insight on the relative solid emptying and mixing with the simulated gastric fluids (Figure 6). For fine puree and coarse crackers, the ratios of three phases were significantly ($p<0.05$) influenced by digestion time, where the liquid phase increased with the decrease of solid and suspended solid phase at longer digestion times. For example, the ratio of liquid for fine puree was $2.07 \pm 0.01\%$ after 30 min and reached $58.93 \pm 0.23\%$ after 180 min gastric digestion, and the ratio of liquid for coarse cracker increased from $15.53 \pm 0.07\%$ after 30 min to $52.79 \pm 0.16\%$ after 180 min gastric digestion. The lower increase in the ratio of liquid emptied from the HGS in the cracker was likely due to hydration, where the crackers absorbed a large amount of the simulated gastric fluids at earlier time points, leaving less free liquid emptied. Besides, the fine cracker had higher hydration compared with the coarse cracker due to smaller initial particle size (De La Hera, Gomez, and Rosell 2013). The hydration was in agreement to the rapidly increased moisture content from 0 min (0.04 ± 0 g H_2O/g DM) to 30 min (3.45 ± 0.23 g H_2O/g DM) for coarse crackers. Compared with coarse puree and cracker, the ratio of emptied liquid from the HGS for the fine puree and cracker was lower (avg. 1.59% for fine puree and cracker vs. avg. 22.52% for coarse puree and cracker at 30 min), likely due to increased hydration in the fine particle size compared to the coarse particle size. This result was consistent with the findings of Z. Farooq and Boye (2011) who found that for rice, smaller particle size had higher swelling power because of higher surface area.
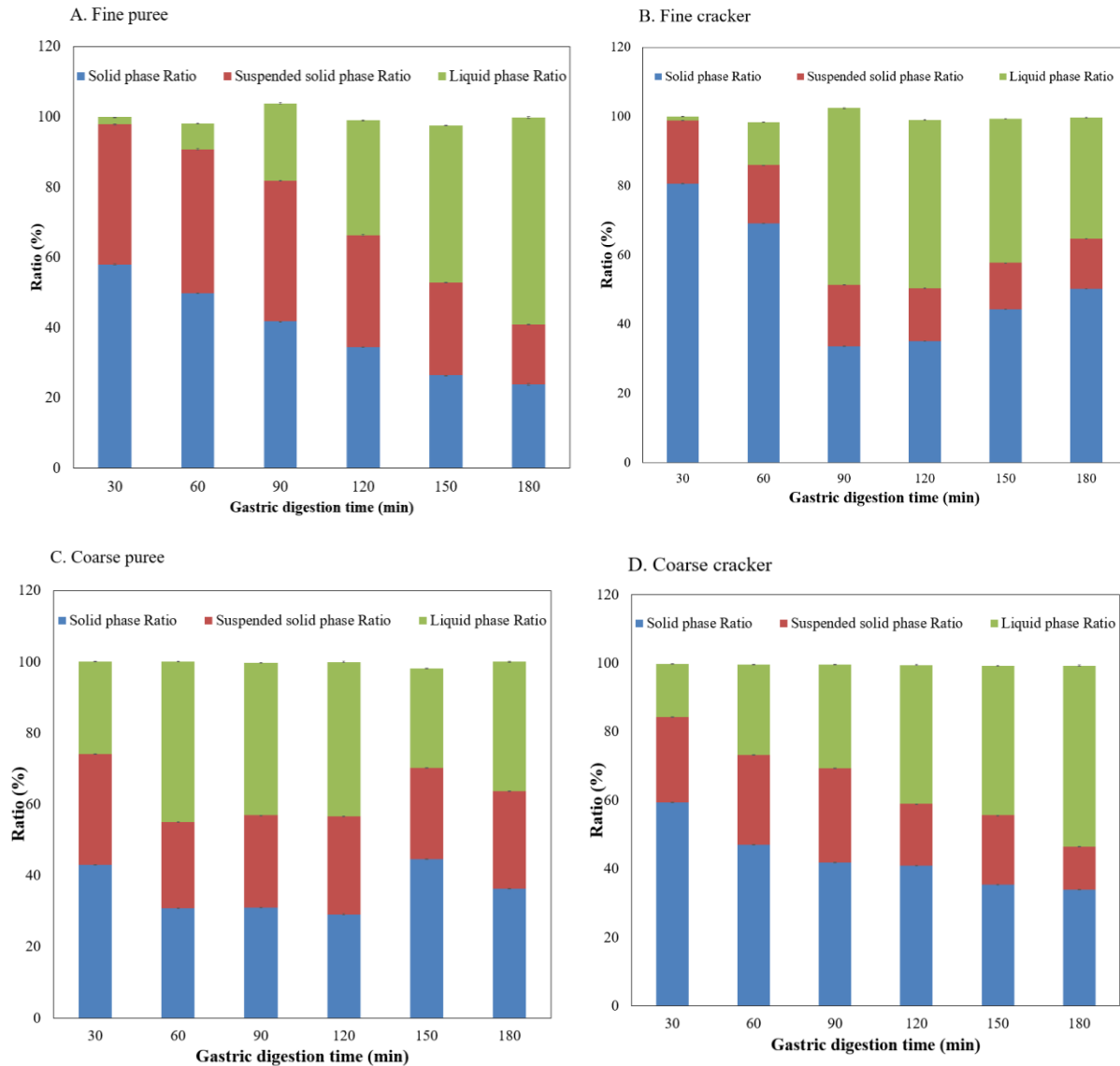
Figure 6. Ratio of solid, suspended solid, and liquid phase (% of total mass) for fine puree (A), fine cracker (B), coarse puree (C), and coarse cracker (D) during gastric digestion. Values are the average from triplicate digestions for each treatment and error bars represent the standard deviation.

For the fine crackers, the ratio of suspended solids was not significantly influenced by digestion time (p>0.05), but the ratio of liquid phase increased significantly (p<0.05) from 60 min (12%) to 90 min (51%) with the significant decrease of the solid phase. This is in agreement with the gastric emptying rate (Figure 7, Table 2) that after 60 min, the rate of gastric emptying

of dry matter decreased; a similar trend was also observed with the number of particles/g dry matter in the fine crackers (Figure 10B) and the moisture content (Figure 8), that the number of particles/g dry matter decreased after 60 min, indicating fewer solids emptied at longer digestion times and the moisture content increased after 60 min, indicating more liquid were released.

Table 2. Gastric emptying model parameters calculated based on Equations 3 and 4. Values represent the average (n=3) ± standard deviation. Within each row, letters identify significant differences (p<0.05) between treatments (xyz).

	Fine puree	Fine cracker	Coarse puree	Coarse cracker
$k \times 10^3 (\text{min}^{-1})$	18.27 ± 0.39^x	13.12 ± 0.75^y	14.56 ± 0.8^y	18.04 ± 0.57^x
β (dimensionless)	1.89 ± 0.06^x	1.39 ± 0.06^y	2.08 ± 0.07^z	1.99 ± 0.03^{xz}
t_{50} (min)	65 ± 11	72 ± 7	87 ± 9	68 ± 6
R^2	0.99 ± 0.01	0.99 ± 0.01	0.98 ± 0.07	0.99 ± 0.01

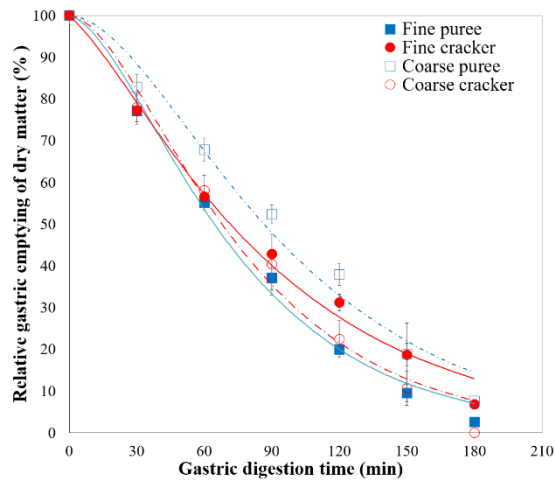**Figure 7. Relative gastric emptying of dry matter from the HGS for fine puree (■), fine cracker (●), coarse puree (□), and coarse cracker (○). Data points represent measured average values (n = 3) with error bars as the standard deviation. Lines represent predicted lines using the parameters in Table 2 from Equation 3.**

For the coarse puree, the ratio of suspended solids was also not significantly influenced by digestion time (p>0.05), but the ratio of the liquid phase increased significantly from 30 min to 60 min with the significant decrease of the solid phase (p<0.05). After 120 min gastric digestion, the ratio of the liquid phase also decreased with the increase of the solid phase. The lower ratio of solid content in the coarse puree compared to the other treatments is aligned with the overall slower gastric emptying rate of dry matter in the coarse puree (Figure 7, Table 2) and similar moisture content (Figure 8) from 30-180 min (average of 4.41 ± 0.402 g H_2O/g DM).

Gastric emptying of dry matter from the HGS was significantly influenced (p<0.05) by particle size, drying and digestion time (Table 2). The predicted gastric emptying curves (Eqn. 3) fit well to the measured gastric emptying data (Figure 7) with an average $R^2 > 0.98$. The emptying rate (k) was significantly higher (p<0.05) for fine puree and coarse cracker (avg. 18.16 min^{-1}) compared to the fine cracker and coarse puree (avg. 13.84 min^{-1}). The higher emptying in the fine puree and coarse cracker was in agreement with the rapid decrease observed in the dry matter emptying profile. The higher emptying rate of fine puree may be due to higher pH where α-amylase was not be inactivated and helped soften the matrix (Gail M. Bornhorst et al. 2016) and lower water holding capacity which led to faster breakdown when it was not able to accommodate water (Gail M. Bornhorst and Singh. 2013). Besides, the small initial particle size may also lead to fast emptying (Hornby et al. 2021). For coarse cracker, the higher gastric emptying rate may be correlated with the damage of fiber during baking and milling compared with coarse puree (Wood and Grusak 2007). In contrast with the coarse cracker, the lower emptying rate for coarse puree may be caused by the coarse pieces of chickpea skin which contain dietary fiber that likely results in a slower breakdown and slower gastric emptying due to increased viscosity (Benini et al. 1995; Liu et al. 2016; Vincent et al. 1995; Wood and Grusak

2007). When the crackers made with the coarse puree were masticated prior to gastric digestion, it is likely that many of the intact skin pieces (present in the coarse puree) were further broken down, and did not slow the gastric emptying for the coarse crackers.

For the β parameter (predicted y-intercept), only the fine cracker was significantly ($p<0.05$) lower than the other treatments, in agreement with the clearer lag phase observed in the dry matter gastric emptying profile of fine crackers (1.39 for fine cracker vs. 1.98 averaged across the other three treatments). Although the k and β values were different between certain treatments, the resulting t50 (Eqn. 4), or the time estimated to empty 50% of the stomach content was not significantly different between the four treatments (73 min, averaged across all four treatments). Compared to a rice meal and crushed whole almonds, the t50 of chickpea puree and cracker were smaller, the t50 of cooked white rice and brown rice was 227 and 229 min during in vivo gastric digestion of growing pig (Gail M. Bornhorst, Chang, et al. 2013), and of crushed whole almonds during in vivo gastric digestion of growing rat was 194 min (Sophie Gallier et al. 2014). However, the t50 of chickpea puree and cracker were similar to a plain salted wheat cracker which was 90 min during in vivo human gastric digestion (Moser et al. 2018), and also similar to a meal consisting of chicken and roasted vegetables during in vivo human gastric digestion which was 77 min (Marciani et al. 2012).

4.4 Moisture Content

The moisture content of digesta (g H_2O/g DM) (Figure 8) was significantly influenced by gastric digestion time ($p<0.05$), but not by drying, initial particle size, or their interaction ($p>0.05$). Across the four treatments, the moisture ranged from 2.85-4.05 (g H_2O/g DM) after 30 min and increased to 5.01-11.31 (g H_2O/g DM) after 180 min gastric digestion. Hayes et al. (2020) found that , for wheat couscous, the moisture content increased from 2.66-2.93 g H_2O/g DM after 30 min to 4.09-5.16 g H_2O/g DM after 180 min gastric digestion, Dalmau et al. (2017) found similar results; for raw apple, the moisture content increased from 6.8-6.9 g H_2O/g DM after 30 min to 7.1-7.2 g H_2O/g DM after 180 min gastric digestion. From 30- 60 min gastric digestion, the moisture content of coarse puree and cracker was higher than the fine puree and cracker, which was in accordance with the highest liquid ratio in the emptied digesta observed in the coarse puree and crackers (4.3 Gastric Emptying). For the fine puree, the moisture content increased to a greater degree than the other treatments. After 180 min gastric digestion, the moisture content of fine puree was more than 3 times larger than the moisture content after 30 min gastric digestion (3.09 vs. 11.31 g H_2O/g DM after 180 and 30 min, respectively). Coupled with the higher gastric emptying rate observed in the fine puree, which indicated that greater amounts of solids emptied from the HGS in the earlier time points, there were more gastric fluids remaining in the HGS at the later digestion times, evidenced by the large liquid ratio and high moisture content of the emptied digesta (4.3 Gastric emptying). The fine puree had the lowest water holding capacity of the four treatments, so as the gastric juice mixed with the meal, it led to an increase in moisture content which was not able to be accommodated in the food matrix, resulting in rapid disintegration and emptying of solids (Gail M. Bornhorst and Singh. 2013).
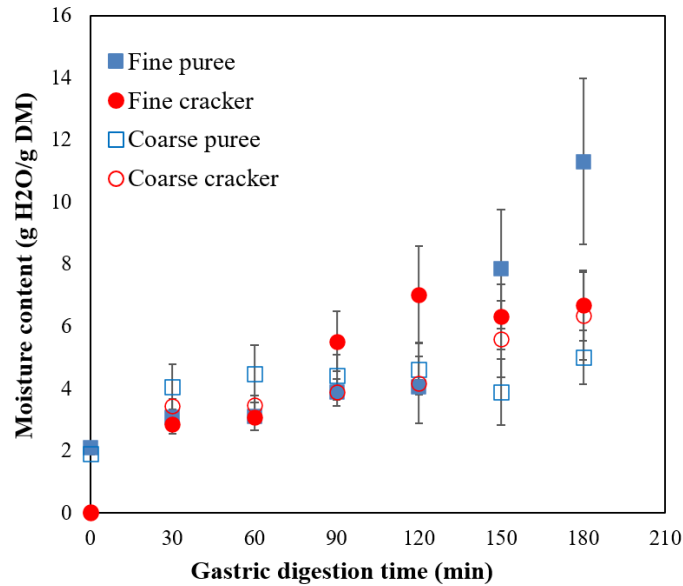
Figure 8. Moisture content (g H₂O/g DM) of emptied gastric digesta from the HGS for chickpea fine puree (■), fine cracker (●), coarse puree (□), and coarse cracker (○). Data points represent the average values (n=3) with error bars as the standard deviation.

4.5 Particle Size Distribution

Particle size distribution was measured in both the solid and suspended solid phases. The particle size distribution of the suspended solid phase was measured by laser diffraction (Table 3, Figure 9). Overall, d50 and d90 values in the suspended solid phase were significantly influenced by drying ($p<0.05$) but not by initial particle size or digestion time ($p>0.05$). d10 values were not significantly influenced by drying or initial particle size ($p>0.05$). For d50 values, 180 min was the only time point that was significantly different from 30-90 min within fine cracker and significantly different from 90 min within coarse cracker. For d10 values in the fine crackers, the d10 value decreased significantly ($p<0.05$) with a longer digestion time. The d10 at 150-180 min (avg. 67.35–62.87 µm) were significantly different ($p < 0.05$) from 30-120 min (avg. 75.94–69.9

µm). For the coarse crackers, d50 and d10 at 90 min was the only time point that was significantly different from the rest of the time points (p<0.05). The larger particle size of coarse cracker compared with fine cracker at 90 min may be attributed to more hydration which was in agreement with higher moisture content (4.4 Moisture Content) and less ratio of liquid as well as a higher ratio of the suspended solid phase (4.3 Gastric Emptying) as well as higher pH (4.2 pH). For example, Figure 9 shows the representative trend of the particle distribution of the suspended solid phase, including the difference between 30 and 180 min for fine cracker and the difference between 30 and 90 min between coarse cracker as well as fine puree, fine cracker, coarse puree and coarse cracker at the same digestion time point (180 min). The maximum peak for fine cracker at both time points (30 and 180 min) is similar (at ~120-160 µm), however, the peak height increased after 180 min of digestion indicating more smaller particles and fewer larger particles are seen (Figure 9A). The coarse crackers had a similar maximum peak after 30 min and 90 min gastric digestion. However, the peak height decreased after 90 min of digestion and another peak appeared, indicating more larger particles were seen (~400-600 µm). When all treatments were compared after 180 min digestion, the maximum peak of coarse puree was larger than other treatments (~200-230 µm) showing more larger particles were present in the soluble solid phase. For the fine puree, the peak height was higher than the fine cracker showing more smaller particles were emptied from the HGS in the soluble solid phase.

Table 3. Particle size distribution of the suspended solid phase measured by laser diffraction of emptied gastric digesta from the HGS for puree and cracker with fine particles and coarse particles. Values represent the average (n=3) ± standard deviation. Letters identify significant differences (p<0.05) between treatments at same digestion time (within each row: xy) and between digestion time within same treatment (within each column: ab).

	Gastric digestion time (min)	Fine puree	Fine cracker	Coarse puree	Coarse cracker
d50 (μm)	0	128.33±2.12	N/A [1]	126.33±2.12	N/A [2]
	30	123.67±0.48[x]	154.06±2.71[ay]	127.83±1[x]	151.17±9.54[aby]
	60	122.67±0.19[x]	152.17±6.21 [ay]	130.17±1.07[x]	155±0.87[aby]
	90	122.17±0.19[x]	152±0.5[ay]	128.5±0.29[x]	170.33±9.31[ay]
	120	123.33±0.1[x]	143±5.41[abxy]	128.67±1.36[x]	157.83±16.46[aby]
	150	123.44±0.32[x]	138.67±6.51[abxy]	127.33±1[x]	156.67±19.44[aby]
	180	124.17±0.1[x]	128±6.14[bxy]	129.61±0.9[xy]	148.83±11.18[by]
d10 (μm)	0	70.97±1.84	N/A [3]	72.53±1.2	N/A [4]
	30	69.72±0.15	75.94±1.37[a]	74.43±1.1	77.03±4.81[ab]
	60	68.62±0.08[x]	73.12±1.65[axy]	75.38±1.78[xy]	78.83±1.96[aby]
	90	68.57±0.13[x]	73.65±1.52[axy]	75.25±0.28[xy]	81.75±2.93[ay]
	120	68.67±0.05	69.9±1.69[ab]	75.3±0.44	74.83±7.86[ab]
	150	68.91±0.22	67.35±3.04[b]	75.22±0.44	75.7±7.06[ab]
	180	69.6±0[xy]	62.87±6.05[bx]	75.87±0.47[y]	72.15±4.44[bxy]
d90 (μm)	0	322.67±9.33	N/A [5]	254.67±16.84	N/A [6]
	30	282.17±11.74[x]	431.67±41.19[ay]	253±6.6[x]	400.83±30.88[ay]
	60	277.83±0.19[x]	451±42.64[ay]	300.67±32.68[x]	446±38.11[by]
	90	268.5±1.15[x]	446.67±10.1[aby]	259±11.27[x]	522.83±37.22[aby]
	120	296.17±0.67[x]	393.17±53.86[abxy]	273.33±5.68[x]	466±55.16[aby]

150	341.22±4.17[xy]	396.33±66.3[abx]	253.17±3.15[y]	451±51.94[abx]
180	333.17±16.55	307.33±52.35[b]	300.72±36.01	431.67±79.84[ab]

[1] [2] [3] [4] [5] [6] N/A The particle size of fine cracker and coarse cracker at 0 min were measured by image analysis
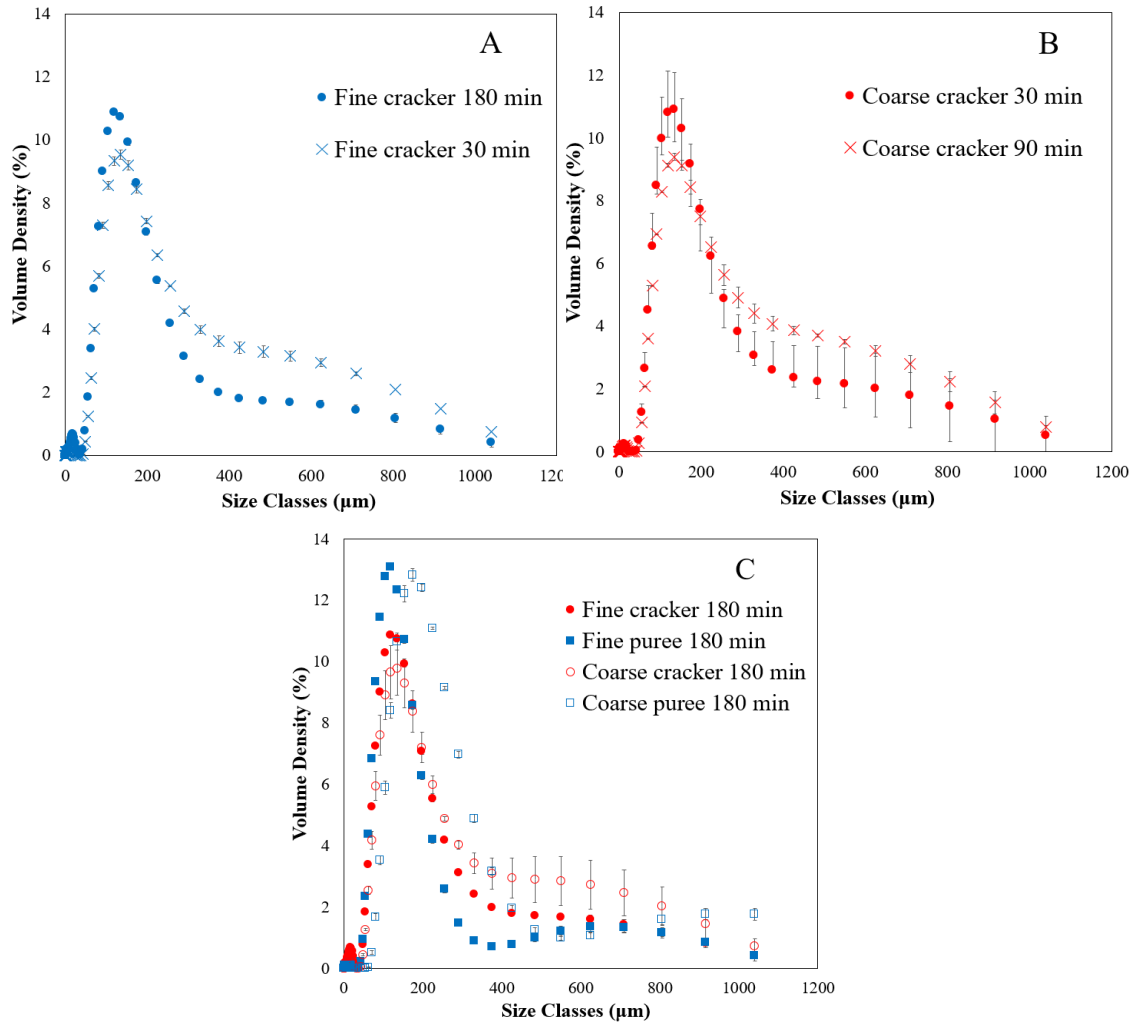
Figure 9. Particle size distribution of the suspended solid phase measured by laser diffraction showing (A) fine cracker after 30 min (x) and 180 min gastric digestion (●); (B) coarse cracker after 90 min (x) and 180 min gastric digestion (●); and (C) fine cracker (●), fine puree (■), coarse cracker (○), and coarse puree (□) after 180 min gastric digestion. Data points represent measured average values (n = 3) with error bars as the standard deviation. Error bars are included but are too small to be seen for many data points.

The particle size distribution of the solid phase was measured by image analysis (Figure 10, Table 4). Overall, x50, x10 and x90 values for coarse puree were significantly higher than the other treatments (p<0.05). For fine cracker and coarse cracker, the x50 and x10 values significantly decreased (p<0.05) from the initial (0 min, avg. 4.89 ± 0.49 mm^2 (x50) and 1.73 ± 0.02 mm^2 (x10)) to 30 min (avg. 2.56 ± 0.62 mm^2 (x50) and 0.31 ± 0.02 mm^2 (x10)), indicating particle size reduction in the fine and coarse crackers in the first 30 min of gastric digestion in the HGS. Similar results have been found for wheat couscous during in vitro digestion in HGS that the particle size of wheat decreased from 2.09 to 0.75 mm^2 from 0.5 to 30 min digestion (Hayes et al. 2020). However, this trend was not observed in fine puree, which had an initial average x50 of 2.03 mm^2 and 1.77 mm^2 after 30 min gastric digestion, likely because fine puree already had a small initial particle size. This result was similar to the almonds during in vivo digestion of pigs in that the particle size for roasted almonds almost kept the same in both proximal (around 1.6 mm^2) and distal (around 2.2 mm^2) during 720 min digestion (G.M. Bornhorst, Roman, et al. 2014). For the coarse puree, x50 values were higher than other treatments after 30 min digestion (Figure 10A) and increased from 5.96 mm^2 at 30 min to 7.03 mm^2 after 180 min gastric digestion. This increase in particle size during digestion may be attributed to the skin pieces that were present in the coarse puree (Figure 11) but were broken down in the fine puree (during milling) and in the fine and coarse cracker (during simulated mastication of the crackers). Considering the gastric half emptying time (t$_{50}$, 4.3 Gastric Emptying) where coarse puree had the highest value of t$_{50}$ (Table 2), these results suggest a gastric sieving phenomenon was present in the HGS where small particles were emptied first and larger particles were retained for further breakdown. Bornhorst, Chang, et al. (2013) observed similar results for cooked brown meals during in vivo digestion of pigs that brown rice showed

an accumulation of bran layer fragments that required additional breakdown compared to the inner starchy grain.
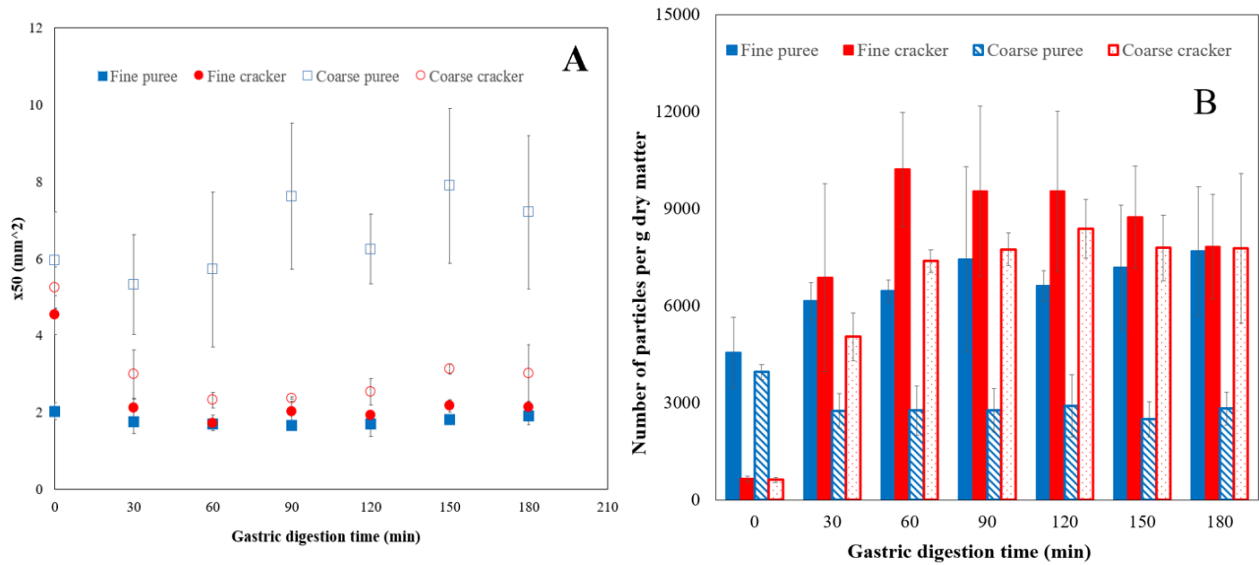
Figure 10. A. Median particle area (x50) given by the Rosin-Rammler model (Eqn 3) expressed in mm^2 over gastric digestion time of the solid phase measured using image analysis of fine puree (■), fine cracker (●), coarse puree (□), and coarse cracker (○). Data points represent the average values (n=3) with error bars as the standard deviation. B. Number of particles per g dry matter of the solid phase measured using image analysis during gastric digestion of fine puree (■), fine cracker (■), coarse cracker (□), and coarse puree (□). All bars represent the mean (n=3) ± standard deviation.

Figure 11. A. Coarse puree in the HGS after 150 min gastric digestion. B. Solid phase of coarse puree after 150 min gastric digestion.

Table 4. Parameters of the Rosin-Rammler model (Eqn 3) fit to particle areas of the solid phase measured by image analysis. All values represent means (n=3) ± standard deviation. Letters identify significant differences (p<0.05) between treatments at same digestion timepoint (within each row: xyz) and between digestion timepoint within same treatment (within each column: ab).

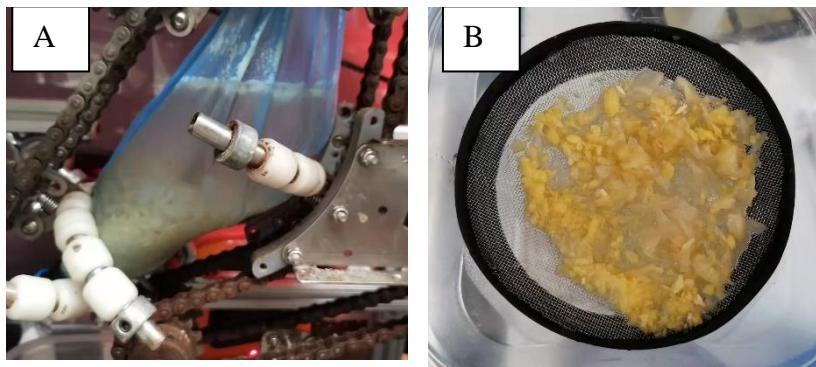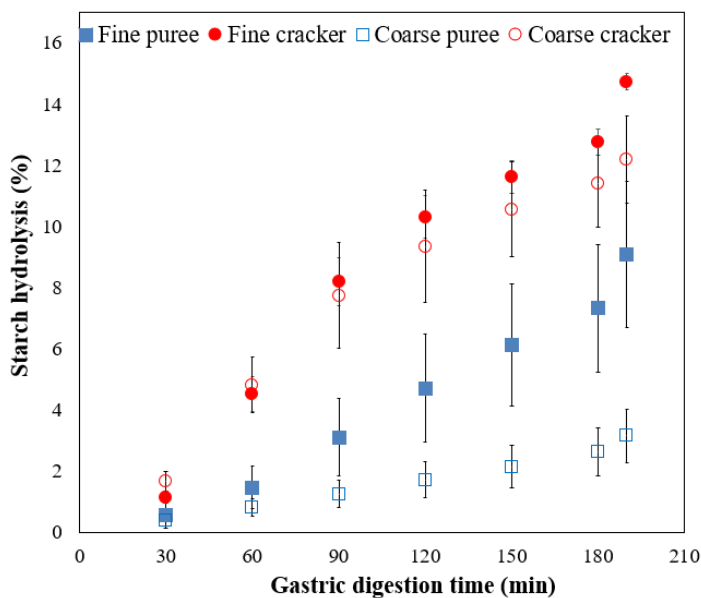|	Gastric digestion time (min)	Fine puree	Fine cracker	Coarse puree	Coarse cracker
x10 (mm²)	0	0.39 ± 0.13^{x}	1.71 ± 0.21^{ay}	0.35 ± 0.03^{ax}	1.74 ± 0.41^{ay}
	30	0.3 ± 0.13^{xy}	0.3 ± 0.06^{bxy}	0.44 ± 0.06^{ay}	0.32 ± 0.1^{bx}
	60	0.27 ± 0.04^{xy}	0.2 ± 0.04^{bxy}	0.54 ± 0.24^{aby}	0.19 ± 0.01^{bx}
	90	0.26 ± 0.03^{xy}	0.32 ± 0.05^{bxy}	0.62 ± 0.25^{aby}	0.22 ± 0.03^{bx}
	120	0.27 ± 0.04^{xy}	0.3 ± 0.01^{bxy}	0.58 ± 0.14^{aby}	0.23 ± 0.04^{bx}
	150	0.31 ± 0.02^{xy}	0.36 ± 0.04^{bxy}	0.82 ± 0.24^{by}	0.36 ± 0.06^{bx}
	180	0.39 ± 0.08^{xy}	0.38 ± 0.1^{bxy}	0.56 ± 0.07^{aby}	0.37 ± 0.13^{bx}
x90 (mm²)	0	6.1 ± 0.72^{x}	8.61 ± 1.57^{xy}	45.29 ± 18.57^{z}	10.81 ± 0.58^{y}
	30	5.56 ± 0.35^{x}	7.35 ± 0.39^{x}	23.93 ± 5.18^{z}	13.04 ± 3.68^{y}
	60	5.61 ± 0.8^{x}	6.86 ± 0.57^{x}	26.63 ± 11.94^{z}	11.43 ± 0.94^{y}
	90	5.51 ± 0.61^{x}	6.6 ± 0.8^{x}	42.76 ± 19.62^{z}	10.54 ± 0.35^{y}
	120	5.51 ± 1.43^{x}	6.4 ± 0.4^{x}	34.49 ± 13.29^{z}	11.71 ± 1.75^{y}
	150	5.64 ± 0.31^{x}	6.87 ± 0.48^{x}	49.15 ± 31.93^{z}	12.7 ± 1.34^{y}
	180	5.32 ± 0.66^{x}	6.43 ± 0.25^{x}	41.12 ± 22.67^{z}	11.77 ± 2.19^{y}

The number of particles per g dry matter (Figure 10B) provides complementary information to the particle area changes during digestion, as it allows for comparison of the total number of particles present in a sample, regardless of their size. The number of particles per g dry matter of fine puree was not significantly influenced by digestion time after 60 min (p>0.05),

with an average value of 6920.48 ± 1361.52 particles/g dry matter during 90 min -180 min digestion. However, the number of particles per g dry matter for fine and coarse crackers increased significantly ($p<0.05$) from the initial (651.3 ± 92.06 particles/g dry matter for fine cracker and 620.16 ± 72.7 particles/g dry matter for coarse cracker) until after 60 min gastric digestion (6864.18±2902.2 particles/g dry matter for fine cracker and 5041.02 ± 748.2 particles/g dry matter for coarse cracker). Coupled with the decrease in particle area in the fine and coarse crackers, the increase in the number of particles in the first 60 min of gastric digestion indicates breakdown of the cracker matrix, with more smaller particles emptied in the solid phase (Figure 10). At later digestion times, the number of particles per g dry matter decreased significantly ($p < 0.05$) between 60 min and 90 min for fine crackers, and after 120 min for coarse crackers. For the coarse puree, the number of particles per g dry matter decreased significantly during the first 30 min digestion ($p<0.05$), but then was not significantly influenced ($p > 0.05$) by digestion time (3960.13±234.1 N/g dry matter initial, 2751.2±530.4 particles/g dry matter 30 min, 2749.65±687.5 N/g dry matter average of rest of the times) due to skin pieces that retained to be further breakdown (Bornhorst, Chang, et al. 2013).
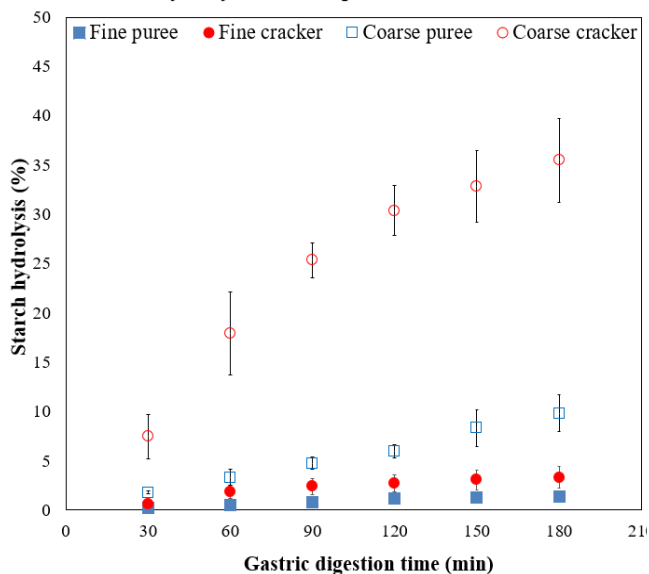
4.6 Starch Hydrolysis

Digestion time, drying, and particle size had a significant impact on the starch hydrolysis in the gastric phase ($p < 0.05$). Overall, the starch hydrolysis of the liquid phase during gastric digestion ranged from 0.93 % after 30 min to 9.79% after 180 min, averaged across the four treatments (Figure 12A). After 180 min gastric digestion, fine crackers had significantly higher starch hydrolysis ($p < 0.05$) in the liquid phase compared with coarse cracker (avg. 14.73% for fine cracker vs. avg. 12.17% for coarse cracker). Fine puree also had higher starch hydrolysis in the liquid phase compared with coarse puree after 180 min gastric digestion (avg. 9.08% for fine puree vs. avg. 3.15% for coarse puree) suggesting smaller initial particle size would lead to higher starch hydrolysis during gastric digestion. Similar results were observed for pasta (Cañas, Perez-Moral, and Edwards 2020) and bread (De La Hera, Rosell, and Gomez 2014). Starch digestibility was lower in samples prepared with large particles compared to small particles due to the lower surface area limiting the attack and hydrolysis by enzymes. According to the study of pasta, the starch hydrolysis of boiled small pasta (2 mm) was 40% after 80 min gastric digestion which was more than twice the amount of starch hydrolysis of the boiled large pasta (5mm, 20%) (Cañas, Perez-Moral, and Edwards 2020). In addition to variations in starch hydrolysis related to particle size, the crackers had significantly higher starch hydrolysis ($p < 0.05$) of liquid phase after 180 min gastric digestion compared with puree (avg. 42.22% for cracker vs. avg. 32.43% for puree). The higher increase in reducing sugar release of cracker compared with the puree during gastric digestion may have been caused by gelatinization of starch and disruption of the starch structure during drying (Rehman and Shah 2005; Roopa and Premavalli 2008) and hydration which facilitate the enzyme diffusion and accessibility within the starch granule surface (De La Hera, Gomez, and Rosell 2013).

A. Starch hydrolysis of liquid phase

B. Starch hydrolysis of solid phase

C. Starch hydrolysis of suspended phase
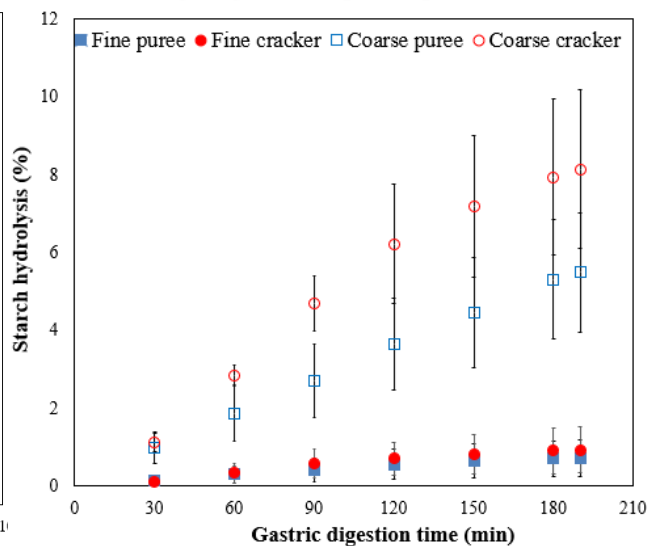
Figure 12. Starch hydrolysis (%) of liquid phase (A), solid phase (B), and suspended solid phase (C) for fine puree (■), fine cracker (●), coarse puree (□), and coarse cracker (○) over gastric digestion time. Data points represent the average values (n=3) with error bars as the standard deviation. Note that each graph has a different y-axis scale to facilitate viewing of results.

In addition to the reducing sugars released in the liquid phase, the reducing sugars remaining in the solid and suspended solid phase were also determined in the emptied gastric digesta (Figure 12B 12C). The amount of reducing sugars was lower in the solid phase and suspended phase compared to the liquid phase for fine puree and fine cracker after 180 min gastric digestion (avg. 11.91% for liquid phase vs. avg. 2.48% for solid phase and 0.81% for suspended phase of fine puree and cracker). However, the starch hydrolysis of the solid phase and suspended phase for coarse puree and coarse cracker was higher than that of liquid phase (avg. 7.67% for liquid phase vs. avg. 23.52% for solid phase and 6.81% for suspended phase) indicating that the coarse puree and coarse cracker had more starch hydrolysis in the solid and suspended solid phases during 180 min gastric digestion that was not released from the matrix into the liquid phase. The higher starch hydrolysis of solid and suspended phase for coarse cracker compared to fine cracker may be attributed to fewer particles/ g dry matter released (Figure 11B), and for coarse puree compared to fine puree may be attributed retained of skins (4.5 Particle Size Distribution) and lower gastric emptying rate (4.3 Gastric Emptying).

The starch hydrolysis of the liquid phase during the combined gastric and small intestinal digestion was quantified as the reducing sugar increase in the liquid phase at each digestion time, as well as the cumulative starch hydrolysis in the liquid phase over the total digestion time (Figure 13B). The digestion time refers to the total gastric and intestinal time for each sample.
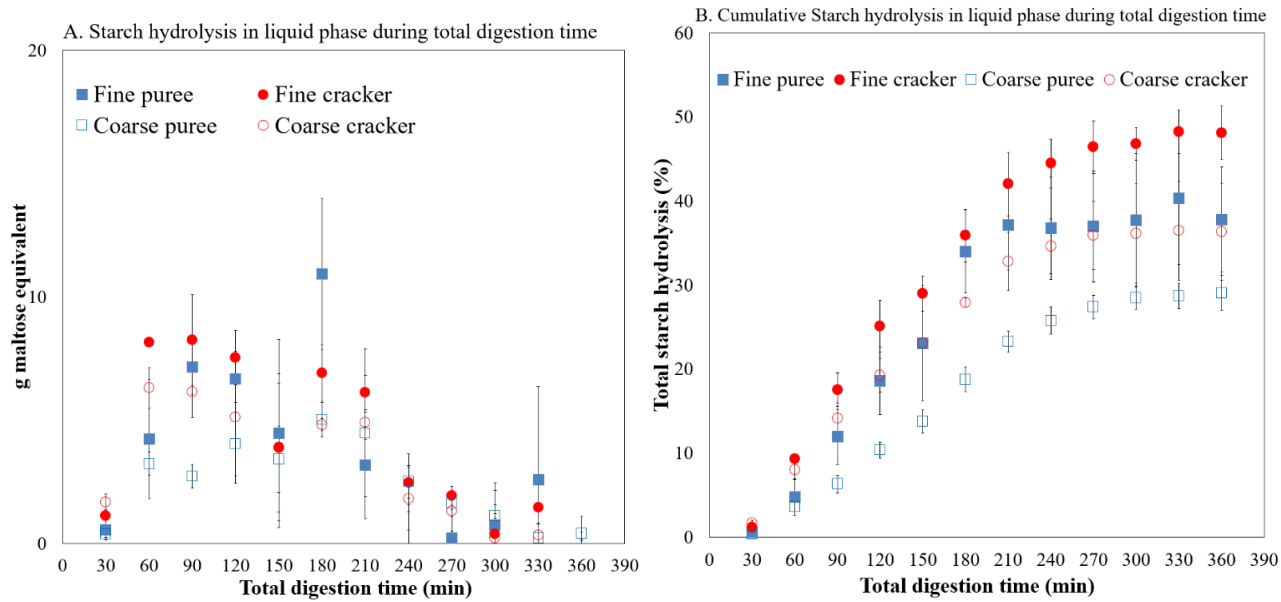
Figure 13. Total starch hydrolysis in liquid phase (A) and total cumulative starch hydrolysis in liquid phase (B) for fine puree (■), fine cracker (●), coarse puree (□), and coarse cracker (○) during gastric and small intestinal digestion. The total digestion time was determined by the sum of the gastric and small intestinal digestion times as described in Figure 2. Data points represent the average values (n=3) with error bars as the standard

The reducing sugar released into the liquid phase (Figure 13A) for fine cracker and coarse cracker reached a maximum after 60 min of total digestion time (fine cracker: 8.16 ± 0.18 g maltose equivalent, coarse cracker: 6.31 ± 0.82 g maltose equivalent), while the fine puree and coarse cracker reached a maximum after 180 min of total digestion time (fine puree: 4.23 ± 2.41 g maltose equivalent, coarse cracker: 3.23 ± 0.47 g maltose equivalent). Similar to the trend in gastric starch hydrolysis, the fine crackers released more reducing sugars compared to the other treatments due to large surface area and gelatinization of starch as well as disruption of the starch

structure by drying (Cañas, Perez-Moral, and Edwards 2020; Rehman and Shah 2005; Roopa and Premavalli 2008).

The cumulative reducing sugar release (Figure 13B) was significantly impacted by initial particle size and drying (p<0.05). After 210 min of total digestion time, the amount of cumulative reducing sugar release was significantly higher (p<0.05) in fine crackers compared to the other treatments (42.01±3.78% vs. 31.07 ±2.41% starch hydrolysis after 210 mins for fine crackers and the average of fine puree and coarse crackers and puree, respectively). Tinus et al. (2012) found higher starch hydrolysis for cowpea during in vitro digestion that after 240 min incubation with artificial saliva, pepsin, pancreatin, and amyloglucosidase at 37 °C. In this study, the starch hydrolysis for hammer milled cowpea was 40-55 g digested starch/100 g dry starch compared to 40-60 g digested starch/100 g dry starch for cryo-milled cowpea, which may have been due to more smaller particles in the cryo-milled cowpea.

Using the cumulative reducing sugar release (Figure 13B), the kinetics of starch hydrolysis, hydrolysis index (HI), and estimated glycemic index (eGI) for fine puree, fine cracker, coarse puree, and coarse cracker were calculated (Table 5). The equilibrium starch hydrolysis (C_∞, %) of fine cracker was highest compared with other treatments while the coarse puree was the lowest, although a significant difference between fine cracker and fine puree was not found. The kinetic constants (k), the hydrolysis index (HI), and the estimated glycemic index (eGI) had similar trends. Tamura et al. (2016) reported that the estimated glycemic index of uncooked rice was 61.3, which was similar to the coarse puree (eGI:57.9 (dimensionless)), but was much lower compared with fully cooked rice (eGI:88.3 (dimensionless)). The highest equilibrium starch hydrolysis and estimated glycemic index of fine cracker may be attributed to the smallest initial particle size compared with coarse puree and coarse cracker which led to

higher surface area to be contacted and hydrolyzed by enzymes and more particles breakdown that occurred during digestion of the fine crackers (4.5 Particle Size Distribution). In addition, the gelatinization and structure of starch changes caused by drying helped the release of reducing sugars during digestion (Cañas, Perez-Moral, and Edwards 2020; Rehman and Shah 2005; Roopa and Premavalli 2008). For the coarse puree, the accumulation of skins in the HGS resulted in increased particle size (4.5 Particle Size Distribution) and the smallest half gastric emptying time (t_{50}) (4.3 Gastric Emptying) leading to the smallest equilibrium starch hydrolysis and estimated glycemic index. In a previous study of chickpeas, Ghavidel and Prakash (2007) found that dehulling after germination resulted in increases in starch and protein digestibility for chickpea compared with germinated chickpea may due to lowered levels of antinutrients, indicating that in chickpeas with more skins, there may be lower in vitro starch and protein digestibility.

Table 5. Kinetics of starch hydrolysis percentage, hydrolysis index (HI) and estimated glycemic index (EGI) for fine puree, fine cracker, coarse puree, and coarse cracker. Values represent the average (n=3) ± standard deviation. Letters identify significant differences (p<0.05) between treatments (within each row: xyz).

	Fine puree	Fine cracker	Coarse puree	Coarse cracker
C_∞ (%)	38.0 ± 6.2^x	50.3 ± 3.6^y	29.0 ± 2.1^z	37.1 ± 5.9^x
$k\times10^{-3}$(min-1)	7.69 ± 0.49^x	7.19 ± 0.37^y	6.83 ± 0.32^z	8.01 ± 0.52^x
HI	79.9 ± 8.5^x	102.7 ± 10.6^y	57.7 ± 7.8^z	79.4 ± 8.2^x
eGI	77.1 ± 7.7^x	96.7 ± 9.4^y	57.9 ± 6.3^z	76.6 ± 7.8^x

4.7 Protein hydrolysis

For protein hydrolysis, the digestion time, phase of digesta sample (solid, suspended solid, liquid), drying, and particle size had a significant impact ($p < 0.05$; Figure 14) on the cumulative free amino groups in the emptied digesta from the gastric phase. In contrast to the starch hydrolysis results in the gastric phase (Figure 12), the amount of cumulative free amino groups in the liquid phase was higher than in the solid phase and suspended phase. However, the trend in the liquid phase was similar to the starch hydrolysis results, where the free amino groups released (on average) followed the order: fine cracker > coarse cracker > fine puree > coarse puree. The free amino groups for fine puree were significantly higher than coarse puree after 150 min ($p < 0.05$; Figure 14A) due to the smallest initial particle size. In addition, the drying which caused more particle breakdown (4.5 Particle Size Distribution) and protein denaturation as well as hydration (Frias et al. 2000; Sagum and Arcot 2000). Similarly, Martín-Cabrejas et al. (2009) found drying after soaking and boiling promoted higher protein digestibility of chickpea (5% improvement). In the solid phase, the free amino groups were higher in coarse cracker than other treatments, following the trend: coarse cracker > fine cracker > coarse puree> fine puree (Figure 14B). There were no significant differences in the protein hydrolysis between the four treatments in the suspended solid phase ($p > 0.05$; Figure 14C).
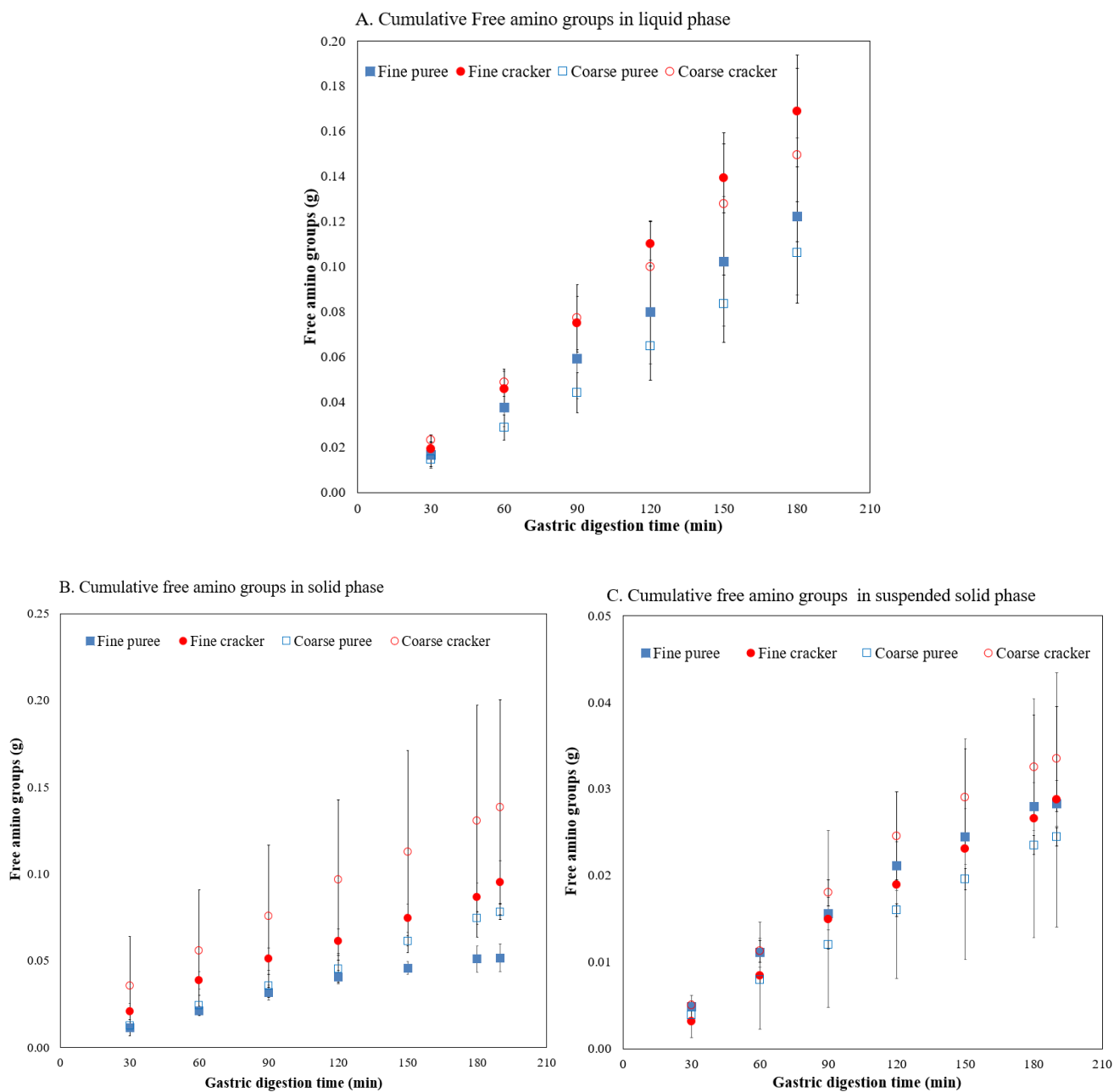
Figure 14. Cumulative free amino groups in liquid phase (A), solid phase (B), and suspended solid phase (C)for fine puree (■), fine cracker (●), coarse puree (□), and coarse cracker (○) over gastric digestion time. Data points represent the average values (n=3) with error bars as the standard deviation.

Similar to the starch hydrolysis results, the protein hydrolysis during gastric and small intestinal digestion was quantified as the free amino groups released into the liquid phase at each total digestion time (Figure 15A), as well as the cumulative protein hydrolysis over the total digestion time (Figure 15B). The release of free amino groups was not significantly influenced by particle size or drying ($p > 0.05$), and was only significantly influenced by digestion time ($p < 0.05$; Figure 15B). Siddhuraju and Becker (2001) found the globulin fraction of some legumes (comprised 50-70% of the total protein of dry seed) was resistant to be hydrolyzed by proteolytic enzymes which led small value of in vitro protein digestibility. The free amino group release reached a maximum value in the fine cracker and coarse puree after 120 min total digestion time (fine cracker: 0.24±0.093g, coarse puree: 0.24±0.107 g), and the maximum free amino group release in fine puree and coarse cracker was after 180 min total digestion time (fine puree: 0.23±0.049 g, coarse cracker: 0.22±0.040 g; Figure 15A).
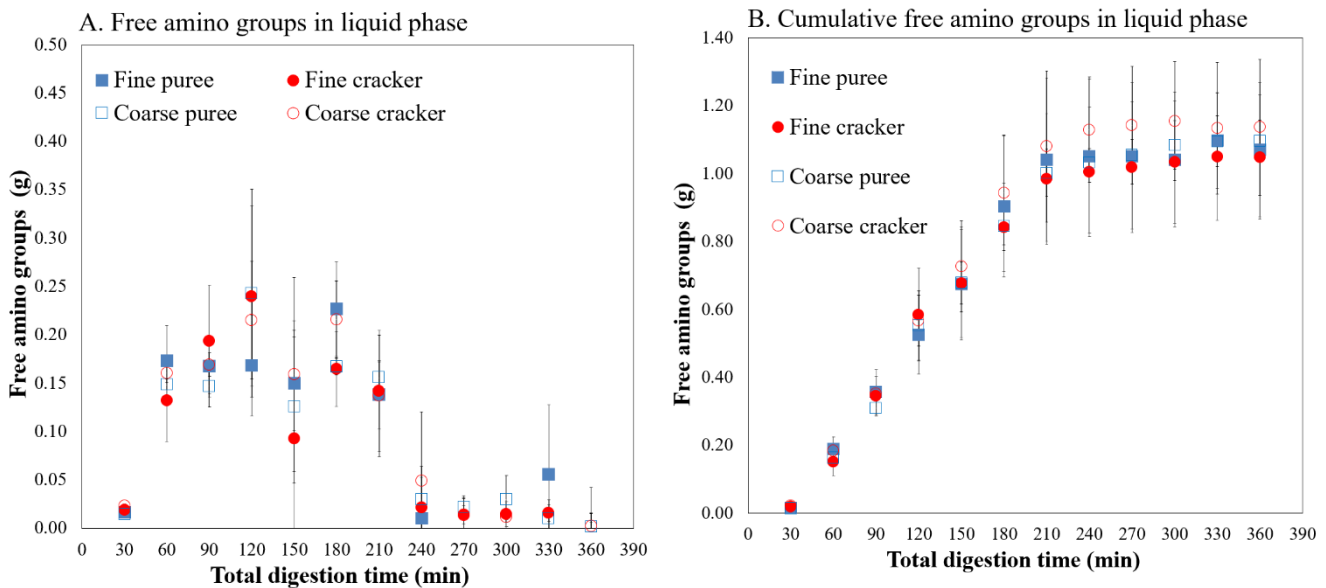

Figure 15. Total free amino groups released (A) and total cumulative free amino groups released (B) for fine puree (■), fine cracker (●), coarse puree (□), and coarse cracker (○) during gastric and small intestinal digestion. Data points represent the average values (n=3) with error bars as the standard deviation.

5. CONCLUSIONS

The following conclusions could be drawn after completion of this study:

1. For the four treatments, the initial fat content and starch content were similar. Both fine puree and coarse puree had higher moisture content, water activity, but lower hardness compared with fine cracker and coarse cracker due to the effect of drying. The fine puree had the smallest water holding capacity but the highest buffering capacity due to smaller initial particle size and higher moisture content.

2. The pH decreased for four treatments, but the pH of fine puree was highest from 60-150 min compared with other treatments as it had the highest buffering capacity.

3. Gastric emptying of dry matter during gastric digestion was significantly influenced (p<0.05) by particle size and drying. Fine puree and coarse crackers showed faster gastric emptying compared to the fine cracker and coarse puree. For the fine puree, it may be due to higher pH allowing α-amylase to increase breakdown, and lower water holding capacity which cannot hold more liquid compared with other treatments. For coarse cracker it may be correlated with the damage of fiber during baking and milling. The faster gastric emptying was supported by the higher emptying rate (k).

4. For fine puree, there was limited breakdown due to its small initial size and higher water content which limited hydration. The lack of changes in fine puree was supported by no change in x50 of particle size or number of particles/g DM during gastric digestion time. The coarse puree also showed limited breakdown may be due to the accumulation of chickpea skins. The highest x50 of particle size and smallest numbers of particles/ g DM were observed after 30 min gastric digestion. The fine cracker and coarse cracker broke down faster during 0-30 min

digestion due to drying which cause the structure changes compared to fine puree and coarse puree. Besides, the fine cracker broke down faster than the coarse cracker because of the smaller initial particle size. Rapid decreases of x50 and increase in the numbers of particles/g DM were observed for fine and coarse cracker between 0-60 times, indicating rapid initial breakdown during gastric digestion.

5. The starch hydrolysis of the liquid phase during gastric digestion was significantly influenced by the particle size and drying. Fine puree had higher starch digestibility than coarse puree, and fine cracker had higher starch digestibility than coarse cracker during gastric and total digestion due to small initial particle size which helped increase the surface area during digestion leading to faster breakdown. In addition, fine cracker had higher starch digestibility than fine puree and coarse cracker had higher starch digestibility than coarse puree due to drying which caused gelatinization and structural changes in the starch. The coarse puree had the lowest starch digestibility, which may be attributed to the accumulation of skins. This large amount of skins in the HGS increased the particle size, and thus slowed breakdown. In addition, chickpea skins may contain antinutrients such as trypsin inhibitors, tannins, and chymotrypsin inhibitors that prevented the release of reducing sugars.

6. The protein hydrolysis had a similar tread as starch hydrolysis during gastric digestion in the liquid phase. The fine cracker and puree had higher protein digestibility than coarse cracker and puree due to their smaller initial particle size. Also, the fine and coarse crackers had higher free amino groups release than fine and coarse puree due to the effect matrix modifications that occur during drying.

Overall, the study showed that the smaller initial particle size and drying would increase the rate of breakdown and starch digestibility. Due to the smaller initial particle size, the fine

puree and cracker had higher surface area leading to higher buffering capacity, starch hydrolysis as well as more free amino groups released in the liquid phase during in vitro gastric and total digestion compared with coarse puree and cracker. In addition, the drying may cause starch gelatinization and protein denaturation as well as a decrease in antinutrients. These resulted in both fine and coarse cracker having higher water holding capacity, rapidly decrease of particle size as well as the increase in the number of particles/ g dry matter, higher starch hydrolysis, and more free amino groups released in the liquid phase during in vitro gastric and total digestion compared with fine and coarse puree. Smaller initial size together with drying caused fine cracker releasing more reducing sugars and free amino groups in the liquid phase during in vitro gastric and total digestion compared with other treatments. In addition, the chickpea skins remaining in the coarse puree also had an influence on the breakdown and reducing sugars released which increase the particle size as well as slowed the gastric emptying, and resulted in the lowest reducing sugars and free amino groups releasing in the liquid phase during in vitro gastric and total digestion. This study investigated the interaction between particle size, water content, and modifications in nutrient release and digestibility which may help develop nutritious and slowly-digestible chickpea-based snacks.

6. FUTURE WORK

The present investigation demonstrates the effect of moisture content and particle size on the properties changes and starch and protein digestibility of chickpea-based snacks during simulated digestion; however, future work needs to be done to strengthen and complement these results. Some suggestions for future directions are:

a. Measure the crude protein and free amino acid of the initial puree and cracker to help determine the protein hydrolysis.

b. The influence of chickpea skins on the properties changes and starch and protein digestibility should be further investigated.

c. Measure the antinutrients such as dietary fiber fractions, protein trypsin inhibitor, phytic acid and tannin of four treatments before digestion and during digestion.

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