RELEASE PROPERTIES OF PEANUT NUTRIENTS AND BIOACTIVES DURING SIMULATED DIGESTION AS AFFECTED BY PROCESSING AND PARTICLE SIZE

by

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(Under the Direction of Fanbin Kong)

ABSTRACT

In order to better evaluate the health benefits of peanuts, significant contributions regarding digestion properties of peanuts need to be made. One objective of the proposed research was to employ static *in vitro* gastric and intestinal digestion methods to investigate the effect of processing and particle size on release properties of selected nutrients and bioactives in peanuts. Increased release properties were observed in smaller particles. After gastric digestion, raw peanuts showed increased release properties, while roasted had increased properties following intestinal digestion. Another objective of this research was to employ static and dynamic *in vitro* digestion methods to evaluate how physiological aspects of dynamic digestion compare to static models with constant pH and volume. Dynamic models showed higher release values for protein than static models but not for TPC and antioxidant power. In both types of model, decreased pH contributed to increased release properties of soluble solids, protein, and TPC.

INDEX WORDS: Peanut, *in vitro* digestion, release, processing, particle size

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DEDICATION

To the fantastic support system that has believed in me and loved me unconditionally, I could not have made it without the four of you.

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CHAPTER 1

LITERATURE REVIEW AND INTRODUCTION

Importance of Nut Consumption

In recent years, extensive research has been done to examine health benefits related to nut consumption. Although termed a nut, the peanut is an edible plant species belonging to the Leguminosaea family. The peanut is gastronomically similar to nuts, therefore many studies include peanuts when discussing tree nuts and other nuts (Higgs, 2003). Peanuts are high in protein and fiber, cholesterol-free, low in saturated fat and contain 13 essential vitamins and minerals. The peanut seed contains on average 50 percent fat and 25 percent protein (Pattee & Young, 1982). Additionally, one and one-half ounces of tree nuts or peanuts provides more than 10% of the recommended dietary allowance (RDA) for adult males of protein, iron, magnesium, phosphorous, zinc, copper, thiamin, and vitamin E. This same serving size of peanuts also accounts for more than 10% of the adult male RDA for niacin, pantothenic acid, and total folate (King, Blumberg, Ingwersen, Jenab, & Tucker, 2008).

Studies have shown that the consumption of nuts has a cholesterol-lowering effect and may also be inversely related to the risk of having a myocardial infarction (Higgs, 2003). As a result, in 2003 a qualified health claim (QHC) was approved by the FDA for nuts and cardiovascular diseases that read, “Scientific evidence suggests but does not prove that eating 1.5 ounces (42 g) of most nuts, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease”(King et al., 2008). In addition to nutritional components, peanuts contain bioactive components that provide additional protection from coronary heart disease. Such

components include dietary fibre, plant protein, micronutrients such as vitamin E, plant sterols and phytochemicals (Higgs, 2003). High doses of vitamin E, greater than (100IU/day) have been associated with reduced risk of CHD by inhibiting LDL oxidation (Higgs, 2003). Peanuts contain flavanoids and other phytochemicals, chemicals produced in plants to protect them from disease and attack. Studies suggest that flavonoids may protect against cardiovascular disease and reduce risk of cancer (Kris-Etherton, Zhao, Binkoski, Coval, & Etherton, 2001). Although it is not fully understood how each constituent protects health, it is reasonable to conclude that nuts and peanuts may contribute greatly to human health.

Peanut Background

Information on evolutionary genetics and diversity of the genus *Arachis* suggest that the primary center of origin of peanuts is in Southern Bolivia and Northwest Argentina where the climate is humid. According to archeological reports, peanuts are believed to have prehistorically been cultivated around 2500 B.C. in Peru (Krishna, 2008).

The peanut, also called the "groundnut" is unique in that it flowers above ground but grows underground. Peanut seeds are planted in April and May when soil temperatures are between 18°C and 21°C. Seeds are planted about two inches deep, and one to two inches apart in rows. An oval-leafed plant about 18 inches tall develops and yellow flowers appear (National Peanut Board, 2015). After fertilization, flower petals fall off and a budding ovary called a "peg" or gynophore grows down and away from the plant penetrating the soil (Maiti & Wesche-Ebeling, 2002). The growth cycle of the peanut from plant to harvest is four to five months, depending on the type and variety (National Peanut Board, 2015). The peanut grows well in tropical and subtropical regions, between 25-28°C and with 500 to 700 mm rainfall in open soil (Maiti & Wesche-Ebeling, 2002).

The genus *Arachis* can be divided into four botanical types: Virginia, Peruvian Runners, Valencia, and Spanish (Krishna, 2008). Each seed type and variety is differentiated by their flavor, oil content, size, shape, and disease resistance. Runners are grown predominantly in Georgia, Alabama and Florida as they need a warm climate and sandy, well-drained soil. About 50 percent of Runners are used in peanut butter (New Georgia Encyclopedia, 2006), as their flavor and uniform kernel size make them ideal for roasting (National Peanut Board, 2015). They are also used as snack nuts and in candies (New Georgia Encyclopedia, 2006).

The United States is the third largest producer of peanuts in the world (National Peanut Board, 2015). China is the leading peanut producer in the world, producing almost twice the amount of peanuts as India, the world's second largest peanut producer (Agricultural Marketing Resource Center, 2013). A total of 15 states in the United States grow peanuts commercially. The top peanut producing states can be divided into three geographical regions: the Southeast region (Georgia, Florida, Alabama and Mississippi), the Virginia-Carolina region (Virginia, North Carolina and South Carolina) and the Southwest region (Texas, Oklahoma, New Mexico and Arkansas) (National Peanut Board, 2015). In 2013, the Southeast region grew approximately 72% of peanuts in the United States (National Peanut Board, 2015).

Peanuts are one of the top five cash crops in Georgia. Having produced 1.64 billion pounds in 2008, Georgia is the top producer of peanuts in the U.S. providing over 45 percent of the nation's peanut crop each year (UGA Extension, 2011). The predominant type of peanut grown in Georgia is the Runner peanut, accounting for approximately 99 percent of acreage used to grow peanuts in the state (New Georgia Encyclopedia, 2006).

In the United States, peanuts are consumed primarily as a food source, as opposed to other parts of the world where peanuts are crushed and used for oil or meal (Pattee & Young,

1982). The majority of peanuts in the U.S. are shelled meaning that the shell is removed. Peanuts are not commonly consumed raw as the majority of these shelled peanuts are then processed and used in peanut butter, snack foods, candy, and other products (Wittenberger & Dohlman, 2010). Therefore, in order to better understand the potential health benefits of peanuts, the effect of processing and particle size on the bioaccessibility of nutrients and selected bioactives during peanut digestion must be understood.

Nutrient Bioaccessibility and Bioavailability

Recent studies have shown that peanuts may offer significant health benefits due to their nutritional composition and bioactive compounds. However, such benefits are dependent upon the "bioaccessibility" and "bioavailability" of macronutrients and micronutrients of the food. The total concentration of a nutrient or non-nutrient in a food does not provide information about its bioaccessibility or bioavailability (Cabañero, Madrid, & Cámara, 2004). Only a proportion (sometimes highly variable depending upon the food matrix, processing and storage) of these food components is absorbed and utilized (Cabañero et al., 2004). Therefore, in order to accurately make conclusions about health benefits of a food, the bioaccessibility and bioavailability of nutrients and non-nutrients should be investigated.

Bioaccessibility can be defined as the amount of a consumed nutrient that is available for absorption, and is dependent on digestion and release from the food matrix (Etcheverry, Grusak, & Fleige, 2012). It has been shown that bioaccessible values obtained by *in vitro* methods can be well co-related with that of human subjects and many animal models (Kulkarni, Acharya, Rajurkar, & Reddy, 2007). Such methods have been used for bioaccessibility studies in fish, in the meals of school going children, and in the medicinal supplements (Kulkarni et al., 2007). Bioaccessibility values should be considered relative indices of bioavailability meaning that the

method used provides a good basis for establishing tendencies, comparisons and the determination of effects caused by different factors (Camara, Amaro, Barbera, & Clemente, 2005).

Bioavailability, the amount of an ingested nutrient that is absorbed and available for physiological functions, however, is a more complex process (Etcheverry et al., 2012). In addition to digestion and release from the food matrix, bioavailability is also dependent on absorption by intestinal cells, and transport to body cells (Etcheverry et al., 2012). To estimate bioavailability, an accurate estimate of bioaccessibility must first be determined.

There has been increasing interest in the use of *in vitro* methodologies to study the human oral bioavailability of compounds from food (Versantvoort & Rompelberg, 2004). Methods to assess bioaccessibility and bioavailability provide information on possible interactions between nutrients and/or food components, the effect of luminal factors such as pH and enzymes, food preparation and processing practices (Etcheverry et al., 2012). Factors such as food processing and particle size can have both positive and negative effects on the bioaccessibility of nutrients and bioactives and may differ depending on the type of simulated *in vitro* digestion performed.

Well-established *in vitro* methods have been developed and used to assess bioaccessibility. The results from most *in vitro* methods are based on the formation of digestion products that are soluble and not precipitated by precipitating agents, or dialyzable (Boisen & Eggum, 1991). In this way, the bioaccessible fraction is determined, the maxiumum concentration soluble in simulated gastrointestinal media that is available for subsequemt processes of absorption into the intestinal mucosa (Ruby et al., 1999). Numerous research studies focus on determining the concentration of a nutrient or non-nutrient in the bioaccessible fraction so that bioaccessibility and ultimately bioavailability of foodstuffs can be determined.

As part of an ongoing study of mercury and selenium bioaccessibility from the human diet, a study by Cabañero et al. (2004) carried out Se and Hg quantification and speciation in fish samples via in vitro enzymolysis to broadly simulate human gastrointestinal digestion. In this study, the bioaccessible fraction is considered to be the soluble Se and Hg contents (μg/g fish). The bioaccessibility of these contents for fish is defined as the proportion of Se or Hg in fish available for absorption and was calculated using the following formula:

$\frac{\text{Se or Hg in bioaccessible fraction}}{\text{Se or Hg in fish}} \times 100$. Total Se and Hg quantification in the bioaccessible fraction was determined (by HG-AFS and HPLC-ICP-MS) for samples at the end of gastric and intestinal digestion phases.

Kulkarni et. al (2007) use a similar formula to evaluate the bioaccessibility of essential elements from the shoots of wheatgrass of 8-10 days old after subjection to *in vitro* gastric and gastrointestinal incubation (Kulkarni et al., 2007). In this study, the concentration of elements (μg/g) in the digest was estimated by instrumental neutron activation analysis (Kulkarni et al., 2007). Based on the concentration of elements in gastric and pancreatic digests, the percent bioaccessibility (%B) of the element from each food item was calculated. The following formula was used: $\%B = \frac{[GD] \text{ or } [PD]}{[T]} \times 100$, where [GD] is the concentration of an element in gastric digest, [PD] is the concentration of an element in pancreatic digest and [T] is total elemental content in the food product.

Similarly an *in vitro* digestion model was used to estimate the bioaccessible fraction of inorganic arsenic species in raw and cooked seaweed (Laparra, Velez, Montoro, Barbera, & Farre, 2004). In this study, bioaccessibility is defined as the percentage of total arsenic (t-As) or inorganic arsenic (i-As) solubilized after the gastrointestinal stage with respect to the total t-As or total i-As contents in seaweed (Laparra et al., 2004).

The food matrix plays an important role in bioaccessibility. The matrix affects the fraction of a nutrient or compound that is released into digestive fluid during transit through the gastrointestinal tract after digestion (Versantvoort & Rompelberg, 2004). Only those molecules released from the matrix in the small intestine are considered to be available for absorption in the intestine (Versantvoort & Rompelberg, 2004). Studies using an *in vitro* digestion model have shown that a considerable fraction of soil contaminants remain intact with the matrix therefore lowering the bioaccessible fraction and thus lowering internal exposure (Versantvoort & Rompelberg, 2004). Contrastingly, some studies have shown that the effects of the food matrix such as fat content in cocoa samples can increase the release of some phenolic compounds during duodenal digestion (Ortega, Reguant, Romero, Macià, & Motilva, 2009). The possible mechanism for this increase in bioaccessibility is probably related to the ability of the fat fraction to interact with certain polyphenolic compounds following a better micellarization of the digestion phenols (Ortega et al., 2009). Such results suggest that the food matrix may have varying effects on bioaccessibility.

The second key factor affecting bioaccessibility is digestion. Digestion can be described as the chemical breakdown of food by enzymes in the mouth, stomach and intestines (Johnson, 2001). This process is necessary so that the body has an adequate source of energy for daily activity as well as materials for the construction of new cells and tissues. As with most nutrients (except water, minerals, and some vitamins), the body breaks down the nutrient into its fundamental building blocks, transports the digested nutrient intro circulatory and lymphatic systems, and eventually uses these nutrients in the cells of the body for either energy or metabolic processes (Windelspecht, 2004).

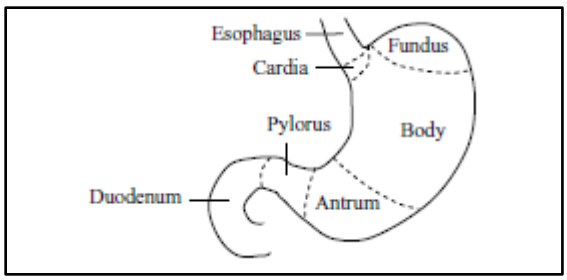
Human Digestive System

Digestion occurs in the gastrointestinal (GI) tract, a continuous tube that stretches from the mouth to the anus and serves as a portal where nutrients and water can be absorbed into the body (Barett, Barman, Boitano, & Brooks, 2010). The organs of the GI tract include the mouth or oral cavity, esophagus, stomach, small intestine, and large intestine also referred to as the colon. For reference purposes, the GI tract can be divided into two major sections: the upper and lower GI tract. The upper GI tract consists of the oral cavity, esophagus and stomach while the lower portion of the tract refers to the small intestine and large intestine. The human digestive system does not rely on gravity to move nutrients through it. Instead, the two muscle layers rhythmically move food by a series of coordinated contractions called peristaltic action (Windelspecht, 2004).

The mouth and teeth admit food into the GI tract, cut and break large pieces of food, chop, grind and moisten what can be chewed, and prepare a smooth, round bolus that can be swallowed and passed on to the system (Keshav, 2009). Within the mouth, three pairs of salivary glands secrete 1000 to 1500 mL of saliva per day (Barett et al., 2010). Saliva performs a number of important functions including but not limited to: facilitating swallowing, keeping the mouth moist, and serving as a solvent for the molecules that stimulate the taste buds (Barett et al., 2010). Saliva is 99.5 percent water and the remaining 0.5 percent of the volume contains important ions such as potassium, chloride, sodium, and phosphates which serve as pH buffers and activators of enzymatic activity. The amount of enzymatic digestion that takes place within the oral cavity is small in comparison to the activity of the lower GI tract. Nonetheless, some initial digestion of carbohydrates and lipids does occur at this point in the GI tract (Windelspecht, 2004).

Saliva contains salivary amylase, which belongs to a class of enzymes that digest complex carbohydrates such as starch into monosaccharides. Salivary amylase is mixed with the food by the tongue and cheeks and continues to break down the starches in food for approximately an hour until it is deactivated by the acidic pH of the stomach. A second enzyme, lingual lipase is also secreted in the oral cavity. This enzyme works to break down triglycerides in the food into monoglycerides and fatty acids. However, the action of this enzyme is minor and does not make a major contribution to overall lipid digestion (Windelspecht, 2004).

After the bolus exits the oral cavity, it enters the esophagus. The esophagus is not a major digestive organ though, as the only enzymes active in this part of the GI tract are the salivary amylase and lingual lipase from the oral cavity. The next major digestive organ the food comes in contact with is the stomach. The stomach is recognized as a muscular 'J' shaped sac that functions as a holding site for food prior to entering the small intestine. The stomach is primarily an organ of mechanical digestion and functions to thoroughly mix incoming food material with gastric juice to form a semi-solid mixture called chyme. This process occurs in three stages: (1) the filling of the stomach with food and the temporary storage of food, (2) the mixing of food with gastric juice, and (3) the emptying of the stomach. There are five distinct regions comprising the stomach: (1) the cardia immediately adjoining the esophagus, (2) the dome-shaped fundus extending to the left of the cardia, (3) the body or corpus, (4) the antrum, and (5) the pylorus which forms a tight sphincter separating the stomach from the duodenum (Keshav, 2009). Below is a diagram illustrating the different regions of the stomach (Kong, 2008).



An empty human stomach may have a volume of as little as 0.05 quarts (50 millimeters) while a full stomach may contain almost 1.06-2.11 quarts (1-2 liters) of food, depending on the individual. In one day, the stomach produces about 2.12 quarts (2 liters) of gastric juice which has a normal pH of around 2.0 (Windelspecht, 2004).

The prime nutrient target of enzymatic digestion in the stomach is protein by enzymes called proteases. Similarly to carbohydrates, there is no absorption of peptides or amino acids through the lining of the stomach. Pepsinogen, the enzyme precursor involved in the chemical digestion of proteins, is secreted by chief cells found predominantly in the corpus in response to a meal and low gastric pH (Johnson, 2001) and (Windelspecht, 2004). Parietal cells, found in glands throughout the fundus, corpus and antrum have an important role of manufacturing hydrochloric acid (Keshav, 2009) and (Windelspecht, 2004). They also secrete the glycoproteins intrinsic factor and gastroferrin, which aid in the absorption of vitamin B_{12} and iron respectively (Keshav, 2009).

Hydrochloric acid has a pH of 2.0 and is 100,000 times more acidic than water. Hydrochloric acid denatures the structure of proteins so that they are easier to digest and also acts as a deterrent against bacterial contaminants in the food. Pepsinogen is initially inactive to protect cells in the gastric pit from unintentional digestion. Once in contact with hydrochloric acid in the lumen or main cavity of the stomach, the acidic nature of hydrochloric acid activates

pepsinogen. The active form of this enzyme, pepsin then acts to break down some proteins into smaller peptide fragments for further digestion later in the small intestine (Windelspecht, 2004).

The lower gastrointestinal tract consists of the small intestine and large intestine. The small intestine is the main organ of digestion and absorption in the human body, and is the site where the bulk of nutrient processing is performed. The large intestine is involved in the reabsorption of water and salts back into the body, and the preparation of the fecal material for excretion. In the stomach the incoming food was mixed with gastric juice to form chyme. The chyme is then moved along the length of the small intestine by two different types of contractions, peristalsis and segmentation. While the peristaltic contractions in the small intestine are similar to those found in the stomach and esophagus, they are much lower in intensity than the ones in the upper GI tract (Windelspecht, 2004). Pancreatic juice is alkaline and almost 1500 mL of pancreatic juice is secreted per day. Bile and intestinal juices are also neutral or alkaline, and along with pancreatic juice act to neutralize the gastric acid by increasing the pH of duodenal contents from 6.0 to 7.0 (Barett et al., 2010).

There are six general classes of nutrients: carbohydrates, fats, proteins, water, vitamins and minerals. Carbohydrates, fats, and proteins are organic (or carbon-containing) molecules that are responsible for providing our bodies with the majority of the energy needed for daily metabolic reactions. Other components of the nutrient classes such as water and certain vitamins play integral roles in energy reactions within the body, however, the body does not get energy directly from these nutrients. Carbohydrates, fats, and proteins contain energy in the carbon-carbon bonds of their molecules, called the calorie. The calorie, a heat measurement, is the amount of energy required to raise 1 gram of pure water by 1 degree Celsius at sea level. For nutritional analysis, the term kilocalorie which is equivalent to 1,000 calories is often used.

Carbohydrates and proteins provide 4 kcal of energy per gram each, while fats provide 9 kcal of energy per gram (Windelspecht, 2004).

Most metabolic reactions of the body do not occur spontaneously and instead require enzymes to occur. Enzymes are catalysts that accelerate the rate of reaction to a point that is efficient for the cells of the body. Almost all enzymes are proteins, and have a three-dimensional shape that allows them to interact with other molecules. These molecules or substrates are specific to the enzyme with which they interact. Enzymes increase the efficiency of metabolic reactions by lowering the amount of energy needed to initiate the action. They are not consumed or destroyed during an enzymatic reaction, and can therefore be reused repeatedly for the same process (Windelspecht, 2004).

A variety of mechanisms and factors regulate the activity of an enzyme. All enzymes have a specific environment in which they are most efficient. The temperature and pH of this environment play a significant role in the activity of the enzyme. The temperature of the digestive system of humans remains fairly constant at 37°C, therefore pH is the primary factor that regulates digestive enzymes. The level of compartmentalization in the different parts of the human digestive system work to establish zones of enzyme activity. The digestive system uses a large number of enzymes to break down the nutrients within food into units small enough to be utilized by the body. In general terms, enzymes that aid in the processing of lipids, proteins, and carbohydrates are called lipases, proteases and amylases, respectively (Windelspecht, 2004).

***In vitro* Digestion: Static and Dynamic Models**

In vivo feeding and digestion methods using animals or humans is the desired method of experimentation as it provides the most accurate results for food science and nutritional studies (Hur, Lim, Decker, & McClements, 2011). Consequently, the ideal method to determine

bioaccessibility or bioavailability should be by *in vivo* measurements performed in humans (Dyck, Tas, Robberecht, & Deelstra, 1996). However, human trials are time consuming, costly, resource intensive, and ethically disputable (Minekus et al., 2014). Although animal assays are less expensive than human assays, one limitation of *in vivo* studies in animals is the uncertainty with regards to differences in metabolism between animals and humans (Camara et al., 2005).

Therefore, the use of simple, rapid and inexpensive *in vitro* digestion methods has increased. *In vitro* digestion methods simulating gastrointestinal (GI) conditions are being utilized more as they are rapid, safe, and do not have the same ethical restrictions as *in vivo* methods (Liang et al., 2012). *In vitro* digestion models allow for the digestion process in the GI tract to be simulated in a simplified manner by applying physiological based conditions such as chemical composition of digestive fluids, pH and residence time periods typical for each compartment (Versantvoort & Rompelberg, 2004).

In vitro gastrointestinal models allow several digestion parameters to be altered and investigated such as peristalsis, churning and body temperature (Etcheverry et al., 2012). Digesta samples can also be collected at any step and time during the digestion process (Etcheverry et al., 2012). However, such models may be expensive and there are few validation studies to test accuracy of the models (Etcheverry et al., 2012).

The ideal *in vitro* digestion method would provide highly accurate results in a short time (Coles, Moughan, & Darragh, 2005). When using *in vitro* gastrointestinal models, potential endpoints to be measured are bioaccessibility, and bioavailability when paired with intestinal cells (Etcheverry et al., 2012). A three-step *in vitro* digestion process is often performed to simulate the human digestive system (Etcheverry et al., 2012). This three-step process simulates digestion conditions that occur in the mouth, stomach, and then intestine (Etcheverry et al.,

2012). In each compartment, a temperature of 37°C is achieved for a time relevant to the compartment (Versantvoort & Rompelberg, 2004). Saliva, gastric juices and intestinal fluids are added to simulate the digestive processes in the mouth, stomach and small intestine respectively (Versantvoort & Rompelberg, 2004). Accounting for the absorption of digestive juices during transit, a volume ratio of 1.5 (food intake): 1 (saliva): 2 (gastric juice): 2 (pancreatic juice): 1 (bile) is proposed for *in vitro* digestion methods (Versantvoort & Rompelberg, 2004). Modifications to this proposal may be made depending on the food sample, components to be analyzed and other experimental conditions.

Hur (2011) surveyed over 80 studies conducted within the last 10 years related to *in vitro* digestion models for food (Hur et al., 2011). Results from the survey showed that the most common food samples tested were plant-based foods (45%), meats (18%), dairy foods (9%), marine foods (9%), and emulsions (9%). The most common parameters measured in the studies were digestibility/degradation > bioaccessibility > sample stability > structural changes.

The most commonly used enzymes and biological molecules in these studies were pepsin, pancreatin, trypsin, chymotrypsin, peptidase, α-amylase, lipase, bile salt, and mucin. Some studies used enzymes collected from human subjects, while others used those from animal or plant sources (Hur et al., 2011). The type of enzymes used in each study depended on the major food components being investigated. For example, lipases were used for lipid digestion, proteases for protein digestion, and amylases for starch digestion. The digestion temperature for all 80 *in vitro* digestion model studies was 37°C and length of incubation times of samples reflected digestion times in humans.

Despite the similarities among food studies using *in* vitro digestion models, a number of differences were also observed. Key differences between surveyed studies were related to the

specific food component being analyzed, the nature of the food matrix, and the sophistication of the *in vitro* digestion model used. Additionally, *in vitro* digestion models differed in the number and type of steps included in the digestion sequence, the composition of the digestive fluids used in each step, and the mechanical stresses and fluid flows of each digestion step (Hur et al., 2011).

It is important to consider that bioaccessibility and bioavailability, which have a physiological or metabolic endpoint cannot be measured entirely by any *in vitro* method (Etcheverry et al., 2012). One reason for this disadvantage is the inability of *in vitro* assays to account for host factors that can possibly influence nutrient absorption such as intestinal microorganisms, nutrient status, age, genotype, physiological state (e.g., pregnancy, lactation and obesity), chronic and acute infectious disease states, secretion of hydrochloric acid, gastric acid, and/or intrinsic factor (Etcheverry et al., 2012) and (Guerra et al., 2012). Another major challenge of *in vitro* digestion is the inability to accurately simulate the peristalsis and realistic shape and motility of gastrointestinal compartments (Guerra et al., 2012). *In vitro* digestion models should also reproduce the biphasic gastric emptying curves observed *in vivo*, where emptying of solid food components presents a linear pattern starting after a lag phase and emptying of liquids begin immediately in an exponential manner (Siegel et al., 1988).

A major concern of *in vitro* digestion models is accuracy compared to *in vivo* studies. Data comparisons of *in vivo* and *in vitro* studies have been inconsistent. *In vitro* solubilisation data correlated well with the *in vivo* data for lipid-based drug samples (Fatouros & Mullertz, 2008). However in an *in vivo* feeding study by Armando et al. (1997) large differences in the microstructure of emulsions are seen as they pass through the GI tract compared to *in vitro* studies (Armand et al., 1997). The availability of accurate *in vivo* data is needed to assess the potential of *in vitro* methods (Coles et al., 2005) and can be done using *in vivo-in vitro*

correlations (IVIVC). The IVIVC reliably associates *in vitro* and *in vivo* data (Guerra et al., 2012) and is needed so that more realistic *in vitro* models can be developed to screen the bioavailability and digestibility of foods (Hur et al., 2011).

Presently, it is assumed that when using *in* vitro digestion models, a degree of accuracy and ease of utilization is often sacrificed for rapid results, and emphasis is placed on the assay's ability to rank digestibility of foods relative to one another (Coles et al., 2005). Despite the limitations of *in vitro* methods, it is still a reasonable and valuable research tool. In principle, *in vitro* digestion models provide a useful alternative to animal and human models by functioning as a screening, ranking, or categorizing tool (Etcheverry et al., 2012).

In vitro digestion models can be divided into two types: static and dynamic (Kong & Singh, 2008). Most models reported in literature are static, meaning there are constant ratios of meal to enzymes, salts, bile acids and other biological materials at each step of digestion (Minekus et al., 2014) The gastric phase is simulated in static models by pepsin hydrolysis of homogenized food at 37°C with a pH of 1-2 for 1 to 3 hours (Guerra et al., 2012). Potentially following this step, occurring in the same vessel is an intestinal phase characterized by pancreatic enzymes with or without bile with a pH of 6-7 (Guerra et al., 2012). However, static models do not mimic the physical and physiological processes that occur *in vivo* such as pH change and peristaltic movements (Kong & Singh, 2008).

To overcome limitations of static models, dynamic models were developed. Dynamic models can simulate transport of digested meals, variable enzyme concentrations and pH changes over time (Minekus et al., 2014). *In vitro* models with dynamic aspects also simulate the physical processing and physiological events of *in vivo* conditions such as peristalsis, gastric emptying (Guerra et al., 2012) and the effects on food (Moreno, 2007). The TNO Nutrition and

Food Research intestinal model (TIM) developed in the Netherlands is a commercial dynamic *in vitro* model designed with aforementioned characteristics of a dynamic system (Souliman, Blanquet, Beyssac, & Cardot, 2006). This model is composed of four compartments simulating the stomach, the duodenum, the jejunum and the ileum (Souliman et al., 2006). The TIM has been used in a variety of food studies such as evaluating the bioaccessibility of folate in fortified milk (Verwei et al., 2003) and phenolic compound release from food matrices such as orange juice, strawberries and strawberry jam (Gil-Izquierdo, Zafrilla, & Tomás-Barberán, 2002).

Although the TIM and other dynamic *in* vitro digestion models have been applied in a wide array of food science studies, the complexity of accurately simulating gastrointestinal conditions is still a limitation of dynamic models. Nonetheless, in order to more accurately simulate *in vivo* conditions, dynamic models should be used rather than static models (Minekus et al., 2014). However, when access to dynamic models is not an option, it is important to understand and utilize optimal static *in vitro* digestion conditions that will offer the most reflective results. When deciding the best type of model to use during experimentation, it is important to consider food sample, outcomes and endpoints to be measured. Additionally, further research needs to be done so that the accuracy of both static and dynamic *in vitro* models can be verified.

Effect of Processing on Nutrient Release

Processing whether during manufacturing or cooking can modify physical and chemical properties of food (Kong & Singh, 2008). Therefore, when assessing bioaccessibility and bioavailability of nutrients and non-nutrients, it is important to understand the effect of such processing on food structure, food disintegration and the release of nutrients from the food matrix.

While the majority of past gastric digestion studies focused on clinical or medical purposes, increasing research has been performed by food scientists interested in gaining an understanding of the role of microstructural properties in the physiology of human digestion and nutrition (Chen et al., 2011). The primary objective of a study conducted by Chen et al. (2011) was to test the feasibility of an *in vitro* digestion model for routine studies of food gastric digestion using roasted and non-roasted peanut particles. The status of peanut digestion was examined by the changes in particle size distribution and the mean particle size. The change in particle size over time of digestion was measured for both roasted and non-roasted peanuts. Results showed that for non-roasted peanuts, there was little change in the particle size after the first half hour of gastric digestion. However, after one hour of digestion, particle size began to decrease and there was a 25% reduction in $d_{43,}$ mean particle size. For roasted peanut particles, a different trend was observed. There was a rapid increase in particle size during the first half hour of digestion followed by a gradual decrease in size for the remainder of the digestion process that was first observed after 1.5 h. A 25% decrease in d_{43} was not observed until after 3 h of gastric digestion.

Also observed in the study by Chen (2011) was the effect of processing, specifically roasting on digestion that was confirmed by differences in surface microstructures in the roasted and non-roasted peanut particle. Prior to digestion, both types of peanuts showed naturally fractured surfaces with no particular morphological features. However, changes to plant walls were detected after digestion. The surfaces of non-roasted peanut particles appeared to be smoother and the plant cell appeared to contain an abundance of tightly packed granules. Contrastingly, in roasted peanuts, particle surfaces were hollow and few granules were seen at the surface.

Perhaps one of the most relevant studies to investigate the effect of processing on digestion properties on peanuts is that by Kong et. al (2013) (Fanbin Kong, Oztop, Paul Singh, & McCarthy, 2013). In the mentioned study, the influence of boiling, roasting and frying on the digestion of peanuts in a simulated gastric environment was investigated by measuring the following parameters: weight retention, dry solids loss and moisture change. Results showed that processing improved the gastric disintegration of peanuts. Processed peanuts disintegrated faster than raw peanuts, as can be seen by the following disintegration rate trend: fried > roasted > boiled > raw. Approximately 70% of initial weight in fried peanuts was lost, while there was almost no loss of the total weight in raw peanuts after 5 h of gastric digestion. Data of dry solids loss, an important parameter correlating directly with nutrient release and absorption showed a similar trend as that of weight retention. Fried peanuts lost the most solids, followed by roasted peanuts, and then raw and boiled which lost the least amount of solids. In regards to moisture change, raw peanuts absorbed the most water, followed by roasted and fried peanuts which absorbed similar amounts of water, and boiled peanuts absorbed the least amount of moisture due to its high initial moisture content. A possible explanation mentioned for the lower water absorption capacity in processed peanuts than raw peanuts is the protein denaturation and structural changes resulting from processing. Hydrophobic properties of oil in fried peanuts may further explain why the least amount of water was absorbed in the respective peanut sample (Kong et al., 2013). Similarly, roasting significantly improved the disintegration rates of almonds and increased loss of solids during simulated digestion. This finding is well correlated with the decrease in the rigidity of almonds after absorbing gastric juice (Kong, 2009).

The effect of processing on carotenoids is discussed by Etcheverry et al. (2012). In the food matrix, carotenoids are usually associated with proteins. Carotenes and lycopene are found

complexed to proteins in chromoplasts, whereas lutein is located in chloroplasts. Food processing conditions such as cooking, microwaving, and pasteurization, and enzymatic processes during digestion that soften or break cell walls disrupt the protein-carotenoid complexes favoring carotenoid release and bioavailability. Although cooking will increase carotene release and bioaccessibility from the food matrix, it can also lead to a reduction in carotene concentration, due to destruction of the molecule. Similarly, reduction in particle size perhaps from homogenization, grinding, or milling will favor carotenoid absorption. The presence of food components such as lipid droplets in dietary fat may also favor carotenoid absorption (Etcheverry et al., 2012).

It is well-known that thermal processing of foods can result in either the release or the degradation of bioactive compounds, therefore affecting the bioaccessibility and bioavailability of such components (Chukwumah, Walker, Vogler, & Verghese, 2011). In addition to processing effects, the chemical modification of compounds by digestive enzymes can affect their bioaccessibility and bioavailability by altering their absorption properties (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004).

An increasing amount of research in recent years to find potential health benefits of tree nuts has led to an increased need to determine their nutrient bioaccessibility and bioavailability. While numerous studies have been conducted to fulfill this need, more research still needs to be done in this area.

The objective of a study by Mandalari et al. (2013) was to evaluate the bioaccessibility of bioactives from pistachios which had not previously been evaluated. In that study, the release of polyphenols (among other bioactives such as xanthophylls and tocopherols) from pistachios during simulated human digestion was quantified (G. Mandalari et al., 2013). Pistachios in the

following forms were used: raw pistachios, roasted salted pistachios and muffins made with raw pistachios. Results showed that more than 90% of the polyphenols were released during gastric digestion, with almost 100% release of polyphenols during the intestinal phase. Roasting had no effect on polyphenol bioaccessibility as there were no significant differences observed between raw, shelled and roasted, salted pistachio which contained more total phenols than the pistachio muffins. This study demonstrated that bioactives from pistachios become rapidly accessible in the stomach, thus maximizing the possibility of absorption in the upper small intestine. Such findings (confirmed by human clinical studies) contribute to the beneficial relation between pistachio consumption and health-related outcomes.

Similarly, the evaluation of the bioaccessibility of almond nutrients is incomplete. As a result, a study was performed to quantify the release of lipid, protein, and vitamin E from almonds during digestion, which also provided detail about the role played by cell walls in the bioaccessibility of intracellular nutrients (Giuseppina Mandalari et al., 2008). Almond samples subjected to simulated gastric and gastrointestinal digestion in this study included natural almonds, blanched almonds, finely ground almonds and defatted finely ground almonds. Results showed that finely ground almonds were the most digestible with 39, 45, and 44% of lipid, vitamin E, and protein released after duodenal digestion, respectively. This study provided insight regarding the bioaccessibility of nutrients in almonds and confirms that physically intact almond cell walls play primary roles in influencing nutrient bioaccessibility.

In addition to bioaccessibility studies of various tree nuts, a few studies have investigated the bioaccessibility and bioavailability of compounds in peanuts. A study by Chukwumah et al. (2011) investigated *in vitro* polyphenol bioavailability using Caco-2 cells. The study assessed the absorption of resveratrol from boiled and roasted peanuts. Digests of roasted peanuts showed

higher resveratrol transport opposed to boiled peanuts even though bioaccessibility results were higher for boiled peanuts than roasted peanuts. It was found that resveratrol from boiled peanuts performed poorly in its transport across the intestinal epithelia. This finding supports the idea that a higher bioaccessibility of a compound does not necessarily imply higher bioavailability (Chukwumah et al., 2011).Results from this study imply that resveratrol from roasted peanuts is better absorbed. It also shows that enzymatic hydrolysis improves the absorption of resveratrol from peanuts (Chukwumah et al., 2011).

It is fairly common for peanuts to be contaminated with aflatoxins at concentrations higher than maximum safe levels for human consumption or animal feedings (Versantvoort & Rompelberg, 2004). The bioaccessibility of aflatoxin B1 from peanut slurries was determined to test the feasibility of the *in vitro* digestion test procedure (Versantvoort & Rompelberg, 2004). Specifically, the effect of contamination level, addition of a standard meal and amount of ingested food on the bioaccessibility of aflatoxin B1 from peanut slurries was investigated. Results showed a linear dose proportional relationship between the amount of aflatoxin B1 in the peanuts and the amount of aflatoxin B1 mobilised from the peanut slurry after digestion. Additionally, the addition of a standard meal or the amount of food had no significant effect on the bioaccessibility of aflatoxin B1 from peanut slurries.

Limitation of Past Research

While there have been a few studies to evaluate the bioaccessibility and bioavailability of nutrients and bioactives in tree nuts and peanuts, this information is not comprehensive. One reason for this may be due to the lack of reliable and accurate *in vitro* digestion models. Mandalari (2013) states that a major challenge in evaluating the role of individual health-promoting components in the pistachio is the lack of information on their behavior in the GI tract

and in particular on factors that influence their bioavailability (G. Mandalari et al., 2013). Similar challenges may also be applied to research regarding the bioaccessibility and bioavailability of nutrients and bioactives in peanuts.

Objectives

It is well known that a number of factors may have a major impact on the release of nutrients and bioactives in peanuts. In order to better evaluate the health benefits of peanuts, significant contributions regarding the extent of such factors on release properties needs to be made. There are two main objectives of the proposed research. This study will employ *in vitro* gastric and intestinal digestion methods to investigate the effect of processing and particle size on the release properties of selected nutrients and bioactives in peanuts. Another objective of this research is to evaluate how physiological aspects of dynamic digestion compare to static digestion conditions with constant pH and volume.

Research Framework

The framework for this research project can be divided into three main sections. The first section employed static digestion methods to evaluate the effect of particle size and processing on the release of nutrients and bioactives in peanuts during gastric digestion. The second section employed static digestion methods to evaluate the effect of processing on release properties of peanuts during intestinal digestion. Results from this section were compared to those in the first section. The third section of this project employed static and dynamic digestion models to compare the difference in release properties in peanut.

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CHAPTER 2

RELEASE PROPERTIES OF PEANUT NUTRIENTS AND BIOACTIVES DURING GASTRIC DIGESTION AS AFFECTED BY PROCESSING AND PARTICLE SIZE[1]

[1] Williams, B.S. and F. Kong. To be submitted to *Journal of Food Science*.

Abstract

The goal of this research was to employ static *in vitro* digestion methods to investigate the effects of processing and particle size on digestion properties of peanuts during the gastric phase. Raw and processed peanuts (roasted and boiled) were separated into four particle size ranges (0.85-1.0mm, 1.0- 1.4mm, 1.4-2.0mm, 2.0- 2.36mm) before exposed to simulated gastric digestion conditions. Release characteristics of peanuts were evaluated. During *in vitro* gastric digestion, as particle size decreased, the release of all measured nutrients and bioactives increased. The release of soluble protein and TPC, as well as protein bioaccessibility were higher in raw peanuts followed by roasted and then boiled peanuts. FRAP of roasted peanuts was higher than that of raw peanuts and least in boiled peanuts.

Introduction

In vivo digestion methods are the preferred method of experimentation as such methods provide the most reflective conditions of human digestion. However, because *in vivo* methods are time consuming, expensive, and ethically disputable, *in vitro* digestion methods have gained popularity (Hur, Lim, Decker, & McClements, 2011). In order to best utilize *in vitro* models, it is necessary to evaluate and compare accuracy of such models with others that are currently being used. As the application of *in vitro* digestion models increases, bioaccessibility of various foodstuffs such as peanuts can be determined to assess potential health benefits offered.

Digestion begins with the mastication of food in the mouth. The main role of mastication or chewing is to transform a mouthful of food into a bolus that can be swallowed (Peyron, Mishellany, & Woda, 2004). First, the teeth and tongue break food into smaller pieces. Incisors cut food, cuspids or canines tear and shred food, and molars crush and grind food. Although a short process, this step is important because of the influence mastication has on the overall

digestive process, specifically the gastric emptying rate (Guerra et al., 2012). The mechanical process of chewing creates smaller particles allowing for easier transportation of food through the digestive system. Chewing also increases the surface area of the food which may increase digestibility in a number of ways. The larger surface area increases contact between the food and digestive enzymes, and enhances salivation which can increase digestion of food in the mouth and stomach. Following mastication, the tongue passes the bolus into the esophagus. Peristaltic action, a series of coordinated contractions then move the food bolus from the esophagus to the stomach where the digestion process continues (Windelspecht, 2004).

The stomach is a J-shaped organ and can be divided into five distinct regions: (1) the cardia immediately adjoining the oesophagus, (2) the dome-shaped fundus extending to the left of the cardia, (3) the body or corpus, (4) the antrum, and (5) the pylorus which forms a tight sphincter separating the stomach from the duodenum (Keshav, 2009). The stomach is primarily an organ of mechanical digestion whose main function is to transport digesta to the duodenum in a regulated manner to optimize intestinal digestion (Minekus et al., 2014). The stomach acts as a reservoir for food and the location where the bolus is mixed with gastric juice and partially digested by mechanical processes (Windelspecht, 2004). Gastric juice consists mostly of pepsin and lipase enymes which aid in the digestion of proteins and lipids, respectively (Guerra et al., 2012). Hydrochloric acid, secreted by parietal cells in the stomach also mix with the meal. Hydrochloric acid contributes to a gradual decrease in pH, approximately from 6.5 to 1.5 thus promoting protein hydrolysis. In the lower part of the stomach, the antrum, the meal is mixed and digested with secreted enzymes and hydrochloric acid, ground by antral movements, and gradually emptied into the duodenum (Minekus et al., 2014). The pylorus acts as a screening device and only allows small particles (chyme) to be emptied into the duodenum (Guerra et al.,

2012). Larger particles that are not passed on to the duodenum remain in the stomach to be further degraded and broken down. The prime nutrient target of enzymatic digestion in the stomach is protein (Windelspecht, 2004).

In order to assess the bioaccessibility and bioavailability of nutrients and non-nutrients, it is necessary to understand the disintegration of food in the human stomach (Chen et al., 2011). Several studies have employed *in vitro* gastric digestion to study allergenic food proteins. The effect of simulated gastric and intestinal digestion on temporal stability and immunoreactivity of peanut protein allergens was investigated in a study proposed by Toomer and others (Toomer, Do, Pereira, & Williams, 2013).

It is well known that processing of foods may have a significant impact on their digestion properties. The majority of peanuts consumed in the United States are processed and incorporated into other products prior to being consumed. As a result, an objective of this study is to employ *in vitro* gastric digestion methods to evaluate the effect and extent of processing and particle size on the bioaccessibility of nutrients and selected bioactives in peanuts.

Materials and Methods

Materials

Raw Runner peanuts (Southern Grace Farms) shelled, with skins on were obtained from a Georgia peanut shelling plant in Enigma, Georgia. All chemicals and digestive enzymes used were of reagent-grade and purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Deionized water was used throughout the study.

Sample Preparation

Prior to digestion trials, a variety of processing methods were used to process peanuts. Peanut samples analyzed in this study consisted of raw (skins removed), dry-roasted, and boiled

peanuts. Runner peanuts were roasted and boiled using modified standard industrial practices (Chukwumah, Walker, Vogler, & Verghese, 2007).

Dry-Roasting. One thousand grams of Runner peanuts were roasted with skins on in a commercial convection oven at 170 ± 5°C for 20 min. Peanuts were spread evenly on an aluminum pan and agitated every 5 min to ensure uniform roasting. The temperature was monitored using a digital thermocouple from Omega (Stamford, CT). Following the roasting period, peanuts were cooled to room temperature and skins were manually removed. Peanuts were then placed in plastic storage bags and stored at -20°C.

Boiled. Peanuts with skins (65 g) were added to a medium stockpot containing 500 mL of boiling deionized water for 30 minutes. After boiling, peanuts were strained, cooled and skins were manually removed. Peanuts were then placed in plastic storage bags and stored at -20°C.

Separation by Particle Size

After processing, peanuts were removed from -20°C and immediately ground in 5-second pulse intervals using a Ninja food processor. Ground peanuts were then sorted under refrigeration temperatures (below 4°C) into four particle size ranges using sieves. USA Standard Testing Sieves from VWR Scientific with the following mesh opening sizes were used: 0.85 mm, 1.00 mm, 1.40 mm, 2.00 mm and 2.36 mm. Particle size ranges used for simulated gastric digestion were: 0.85-1.0mm, 1.0- 1.4mm, 1.4-2.0mm, 2.0- 2.36mm.

Static In Vitro Gastric Digestion

Simulated static *in vitro* gastric digestion was performed according to a modified protocol (Minekus et al., 2014). Simulated saliva and gastric juices were prepared on the day of the experiment, according to Table 1.1. The target pH of prepared simulated juices was achieved using 1M NaOH and 1M HCl. Ground peanut samples (5.0 g) was added to a 125-mL

Erlenmeyer flask. Flasks were covered with Parafilm and placed in a Model 290400S water bath (Boekel Scientific, Feasterville, PA) set at 37°C with orbital agitation of 120 rpm. The sample was digested as follows: mouth—addition of 20 mL of salivary juice and mixing for 2 min; stomach—addition of 40 mL of gastric juice and mixing for 2 hours. Throughout the gastric digestion phase, 1 M HCl was added to the flasks to maintain a pH range of 3.0±0.5. Aliquots (5mL) were collected after 5, 10, 30, 60, 90 and 120 minutes of gastric digestion. Gastric digesta samples were neutralized to a pH of 6.5±0.5 using 0.5 M $NaHCO_3$ to slow down or inhibit further enzymatic action. Digesta samples were then filtered *in vacuo* through Whatman No. 1 filter paper and the filtrate was kept in frozen storage of -20°C before further analysis.

Total soluble solids

Total soluble solids of digesta samples were determined by gravimetric analysis. Prior to drying, 1 mL of the collected digesta sample was added to a small aluminum pan. Samples were placed in the NAPCO Vacuum Oven (Model 5831) at 65°C for 16-18 hours. Dried samples were removed from the oven and cooled in a desiccator for 30 minutes before weighing. An additional drying process was repeated for 1 hour at the same temperature to ensure complete moisture loss. Total soluble solids in digestive juices were determined used the same methods and subtracted from the total soluble solids of digesta samples to determine total soluble solids only from the peanut sample.

Soluble protein concentration

Quick Start Bradford protein assay was performed to determine soluble protein concentration (Bradford, 1976) of digesta samples. To a test tube, 100 µL of digesta sample and 5 mL of room temperature1x dye reagent were added. Samples were vortexed using a Digital VWR Mini Vortexer for 15 seconds and incubated at room temperature for a minimum of 5

minutes. Absorbance was then measured using an Evolution 300 UV-Vis Spectrophotometer by Thermo Scientific (Waltham, MA). at 595 nm using a 1 cm cuvette. Bovine serum albumin (BSA) was used to create a standard curve with protein concentrations ranging from 125-2,000 μg/mL. Soluble protein concentration results were reported in units of μg/mL.

Determining protein bioaccessibility

Soluble protein concentration at the end of the static *in vitro* gastric digestion phase (120 m) was used to determine protein bioaccessibility. Soluble protein concentration was multiplied by the total volume of gastric digesta (60 mL) to determine the mass of protein released during gastric digestion. Protein bioaccessibility was then calculated using the following equation:

$$Protein\ Bioaccessibility\ (\%) = \frac{mass\ protein\ released\ from\ sample}{theoretical\ mass\ of\ protein\ in\ sample} \times 100$$

The theoretical mass of protein in the peanut sample was determined by multiplying the percent composition of protein in the appropriate peanut sample by the total sample mass of peanuts used (5 g). The percent composition of protein in peanuts was obtained from the USDA National Nutrient Database.

Total phenolics content (TPC) assay

The TPC of digesta was determined using a modified Folin-Ciocalteu method (Singleton & Rossi, 1965). Peanut digesta samples were diluted 50-fold. A total of 6.5 mL of deionized water, 0.5 mL of Folin-Ciocalteu's phenol reagent, and 1 mL of saturated sodium carbonate solution were added to test tubes containing 2.0 mL of the diluted sample. The mixture was vortexed for 15 s using a Digital VWR Mini Vortexer, followed by an incubation period of 40 min at room temperature to allow optimal color development. Absorbance was measured at 750 nm in a 1 cm cuvette using an Evolution 300 UV-Vis Spectrophotometer (Thermo Scientific). A

standard curve was generated with gallic acid and TPC was expressed as g GAE equivalents/mL and used to determine TPC concentration of digesta.

Ferric reducing antioxidant power (FRAP) assay

A modified FRAP assay was utilized (Benzie, 1996). The FRAP reagent was prepared daily and consisted of 2.5 mL of 2,4,6-tri(2-pyridyl)-*s*-triazine solution (10 mM in 40 mM HCl), 2.5 mL of ferric chloride hexahydrate (20 mM) and 25 mL acetate buffer (300 mM, pH 3.6). Fresh reagent was held in a shaking water bath at 37°C. To a 1 cm cuvette, 100 μL of sample and 1 mL of FRAP reagent were added using a pipette. The mixture was allowed to react for 4 min before absorbance was read at 593 nm using an Evolution 300 UV-Vis Spectrophotometer from Thermo Scientific (Waltham, MA). Results were determined using a standard curve prepared using 100 μL of various ferrous sulfate heptahydrate solutions (0.1 to 1.0 mM) and presented as mM Fe^{2+} equivalents.

Table 1.1: Compositions of simulated digestive juices

Saliva stock solution	**Gastric stock solution**	**Duodenal stock solution**	**Bile stock solution**
500 mL DI water	500 mL DI water	500 mL DI water	500 mL DI water
0.0585 g NaCl 0.0745 g KCl 1.05 g $NaHCO_3$	2.752 g NaCl 0.824 g KCl 0.266 g NaH_2PO_4 0.399 g $CaCl_2 \bullet 2H_2O$ 0.306 g NH_4Cl	7.012 g NaCl 0.564 g KCl 3.388 g $NaHCO_3$ 0.08 g KH_2PO_4 0.05 g $MgCl_2$	5.29 g NaCl 0.376 g KCl 5.785 g $NaHCO_3$
0.2 g urea	0.085 g urea	0.1 g urea	0.25 g urea
	6.5 mL HCl	180 μL HCl	150 μL HCl
Simulated Saliva	**Simulated Gastric Juice**	**Simulated Duodenal Juice**	**Simulated Bile Juice**
Amount: 100 mL stock	Amount: 100 mL stock	Amount: 100 mL stock	Amount: 100 mL stock
0.1 g mucin 0.2 g α-amylase	0.5 g pepsin 0.6 g mucin	1.8 g pancreatin 0.3 g lipase	6 g bile
pH: 6.8±0.2	pH: 1.30±0.02	pH: 8.1±0.2	pH: 8.2± 0.2

Results and Discussion

In this experiment, the effect of particle size and processing on release properties of peanut nutrients and bioactives during static gastric digestion was investigated. When evaluating the effect of particle size, only raw and roasted samples were compared because there were samples for each of the following ranges: 0.85 to 1.0 mm, 1.0 to 1.4 mm, 1.4 to 2.0 mm, and 2.0 to 2.36 mm. However, boiled samples cannot be directly compared according to particle size as the only particle size for boiled samples is < 2 mm. When evaluating the effect of processing, boiled samples (< 2 mm) were compared to raw and roasted samples between 1.4 to 2.0 mm. This particle size range was selected because it is the closest range to < 2 mm and prevents overestimating the difference in raw and roasted samples by selecting a smaller range.

Figure 1.1 shows the change in soluble protein concentration for all samples during static gastric digestion trials. Soluble protein concentration increased over time and as particle size decreased. Soluble protein concentration of digesta from the largest raw peanut sample increased from 1.22 mg/mL to 1.45 mg/mL over the duration of gastric digestion. For raw peanuts of the smallest particle size range, soluble protein concentration increased from 1.85 mg/mL to 3.24 mg/mL over the 2 hour gastric digestion phase. The same trend can be seen for roasted peanuts. Digesta from roasted peanuts sized 2.0-2.36mm increased in soluble protein concentration from 3.70 mg/mL to 1.22 mg/mL during gastric digestion and from 1.05 mg/mL to 1.48 mg/mL for roasted peanuts with particle sizes between 0.85 and 1.0mm. Based on the type of processing, the general trend of soluble protein concentration is as follows raw> roasted> boiled.

In Figure 1.2, the effect of particle size and processing on protein bioaccessibility (%) after gastric digestion for two hours is shown. Coinciding with results from soluble protein concentration, protein bioaccessibility increased as particle size decreased. The variation of

protein bioaccessibility among the different particle size ranges is greater in raw peanuts than roasted peanuts. Protein bioaccessibility (%) for raw peanuts increased from 6.9 to 15.5 when comparing the largest particle size to the smallest particle size, respectively as opposed to an increase of 6.1 to 7.4 in respective roasted peanuts. Protein bioaccessibility of the smallest raw peanut is 124% greater than that of the largest raw peanut. However, protein bioaccessibility of the smallest roasted peanut is only 21% greater than the largest roasted peanut sample. Protein bioaccessibility is also greater in raw peanuts than roasted peanuts for all respective samples. Protein bioaccessibility for the smallest raw peanuts is more than twice the value of the respective roasted peanut sample.

These results can be compared to results from a 2008 study by Mandalari that investigated the release of macronutrients and vitamin E from almond seeds during digestion. The percent loss of protein, lipid, and vitamin E from almonds due to *in vitro* gastric digestion was highest in finely ground (raw) almonds, followed by blanched almond cubes, and the least in natural, raw almond cubes (Giuseppina Mandalari et al., 2008). During roasting, proteins in peanuts are denatured and aggregate or bind together. Although more proteins may have actually been released from roasted peanuts than raw peanuts, this aggregation of proteins in roasted peanuts may have been too large to pass through during filtration which may explain why total soluble protein concentration values were higher in roasted peanut digesta than raw peanut digesta. The relationship between total phenolic content of digesta and particle size after gastric digestion is displayed in Figure 1.3. In our study, total phenolic content is expressed as grams of gallic acid equivalents (GAE)/mL of digesta, and increased as particle size decreased. In raw peanut samples, TPC increased from 1.53 g GAE/mL in the largest particle size range to 3.09 g GAE/mL in the smallest range, a 102% increase. TPC content increased from 1.38 g GAE/mL to

2.36 g GAE/mL in the largest and smallest roasted peanuts, respectively, an overall increase of 71.0%. Similar to protein bioaccessibility, the change in TPC among different particle sizes is greater in raw peanuts than roasted peanuts.

Figure 1.4 shows the relationship between total phenolic content and processing. Total phenolic content is greater in raw digesta samples than roasted and boiled digesta samples. The TPC release in raw peanuts is 23.1% greater than respective roasted peanuts and 47.4% greater than that of respective boiled peanuts. This finding agrees with a 2013 study by Mandalari which found that the total amount of flavanoids and phenolic acids was slightly higher in natural, raw shelled pistachios compared to roasted, salted pistachios. This study also concluded that roasting did not have a significant effect on polyphenol bioaccessibility in pistachios (G. Mandalari et al., 2013). Mandalari et al. (2010) found that after simulated human digestion, total phenolic content was lower in blanched almond skins compared to natural almond skins (G. Mandalari et al., 2010). This is due to their partial solubilisation into the blanching water. A similar explanation can be used to explain the decreased TPC in boiled peanut digesta. The release of polyphenolic content of boiled peanuts in our study may be lower than that of other studies because in our study, peanuts were not boiled in the hull. Several studies have shown that peanut hulls are rich in polyphenolic compounds that increase with peanut maturity (Yen, Duh, & Tsai, 1993).

Figure 1.5 displays FRAP values of digesta after gastric digestion as affected by particle size. Similar to the previous trends, FRAP increased as particle size decreased. In the largest particle size range, FRAP was 0.29 mmol of Fe(II) equiv/L in raw peanuts and 0.44 mmol of Fe(II) equiv/L in roasted peanuts. For raw and roasted peanuts of the smallest particle size range, FRAP was 0.69 and 0.76, respectively. The change in FRAP values between the largest and

smallest particle size range is also greater in raw peanut samples compared to roasted samples, an increase of 138% and 73% respectively.

Figure 1.6 displays FRAP values as a result of processing. In contrast to previous findings for protein bioaccessibility and TPC, FRAP values are higher in roasted digesta samples than raw samples and boiled samples. FRAP values for roasted digesta samples are 8.6% greater than that of the corresponding raw digesta sample and 152% greater than the corresponding boiled digesta sample. Related research studies show that the antioxidant capacity of nut extracts such as almond skins is higher due to roasting. This may be due to the fact that other processes such as blanching may result in the degradation of polymerized polyphenols (Garrido, Monagas, Gómez-Cordovés, & Bartolomé, 2008).

Figure 1.7 shows the change in total soluble solids over time during gastric digestion. The percent of total soluble solids in digesta increased as particle size range decreased. Additionally, the release of total soluble solids was generally higher in raw peanut digesta compared to processed (roasted and boiled) digesta samples. For example, raw peanuts of the smallest particle size range released 3.27% of soluble solids at the end of digestion and the respective roasted peanuts released 1.88% of soluble solids at the same end point. During the 2 hour gastric digestion phase there was an 80.1% increase in %TSS of raw peanuts of the smallest particle size range and a similar increase of 79.0% in the corresponding roasted peanut sample.

Kong et al. (2013) studied the effect of processing on disintegration of peanuts in simulated gastric environment. Their results showed that processing improved the gastric digestion of peanuts, and the disintegration rate was as follows fried > roasted > boiled > raw peanuts (Kong, Oztop, Paul Singh, & McCarthy, 2013). Brittle surfaces of processed peanuts may have resulted in the increased disintegration rate. While results from Kong (2013) do not

coincide with results in the current study, it is important to note that the total loss of solid particles used to evaluate disintegration rate may include insoluble solid particles as well as soluble solid particles.

In our study, a portion of nutrients and bioactives were probably lost during the boiling process due to the difference in the concentration gradient and uptake of water by the peanut. This explains the decreased release of phenolic content and decreased FRAP as such components were lost during processing, prior to digestion.

Processing has a major impact on food texture such as hardness in roasted peanuts which may decrease the solubility of solid particles. It is possible that a crispier, harder texture may make it more difficult for components of a roasted peanut to solubilize in the digesta. A softer, less brittle texture of raw peanuts could increase solubility of raw peanut solids released during digestion, accounting for greater release properties of raw peanuts during the gastric digestion phase. In addition to increased solubility, digestive enzymes during the gastric phase may have been better able to penetrate the cell wall and matrix of raw peanuts compared to roasted peanuts.

A reduction in particle size can be achieved by chewing (mastication) or processing such as grinding. Smaller particles have an increased surface area for digestion and absorption compared to larger particles (Kong, 2008). Particle size resulting from mastication depends on characteristics of the food such as texture, dryness, hardness and size. Dental health status and degree of hunger of the individual may also affect the size of food after chewing (Hoebler, 2000). Such factors are important due to the major influence of particle size on the digestive process.

Research studies have shown that the structure of cell walls play a major role in bioaccessibility (Ellis et al., 2004; Giuseppina Mandalari et al., 2008). Cell walls that are intact act as a physical barrier by trapping nutrients and reducing the rate and extent from which they are released from the food matrix. Contrastingly, cell walls that have been damaged or ruptured by means of chewing or processing allow for a more rapid release of nutrients that are available for digestion and absorption. This is also a possible explanation for why bioaccessibility and release values are higher in digesta from peanuts of smaller particle sizes compared to larger particles.

Conclusions

The effects of processing and particle size on bioaccessibility and release properties of peanuts during gastric digestion were investigated in this experiment. During *in vitro* gastric digestion the release properties of all measure parameters of peanuts were shown to increase as particle size decreased. Measured parameters were higher in raw peanuts compared to processed peanuts except for FRAP. A variety of factors including but not limited to particle size and processing have an impact on digestion properties. In order to fully understand the effect of digestion on bioaccessibility and the release of nutrients and non-nutrients, all phases of digestion (gastric and intestinal) need to be examined.

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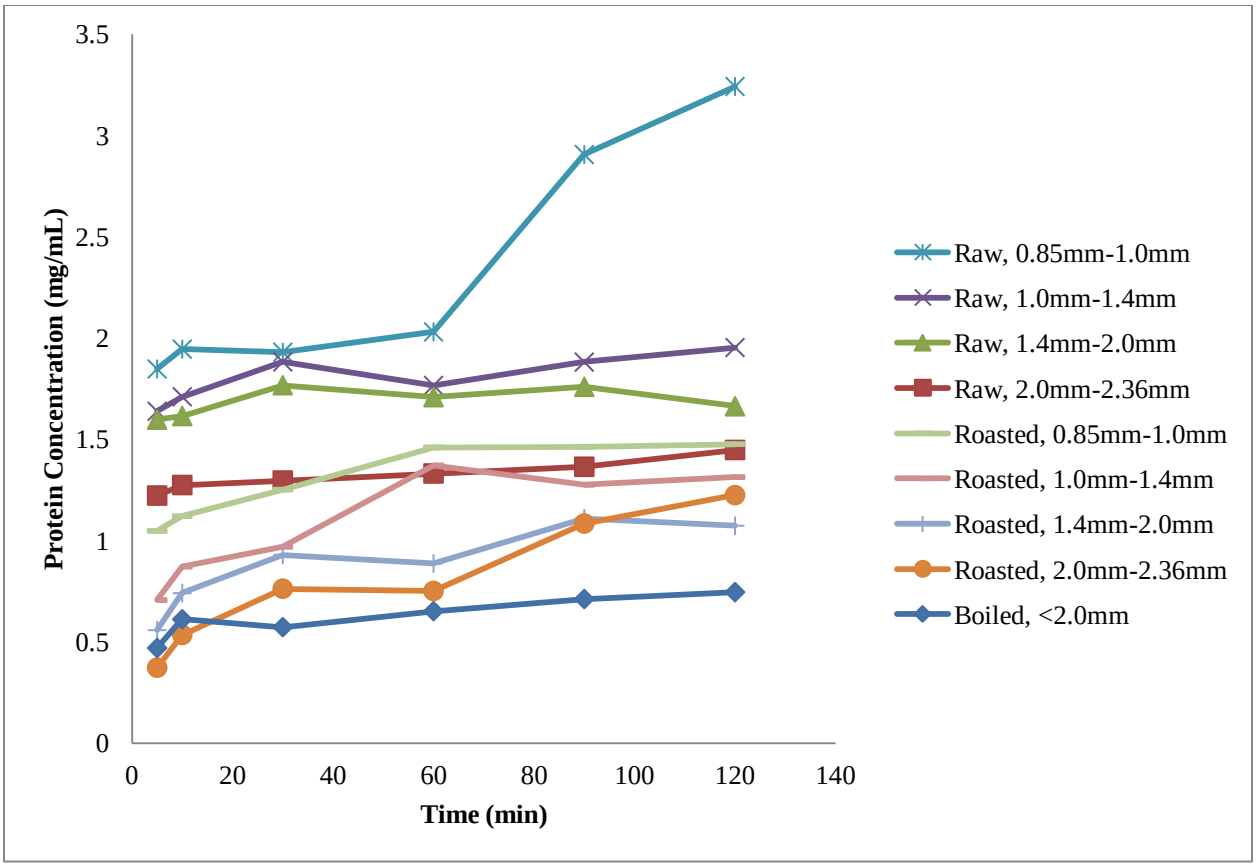

Figure 1.1.Soluble protein concentration during gastric phase of digestion as affected by particle size and processing.

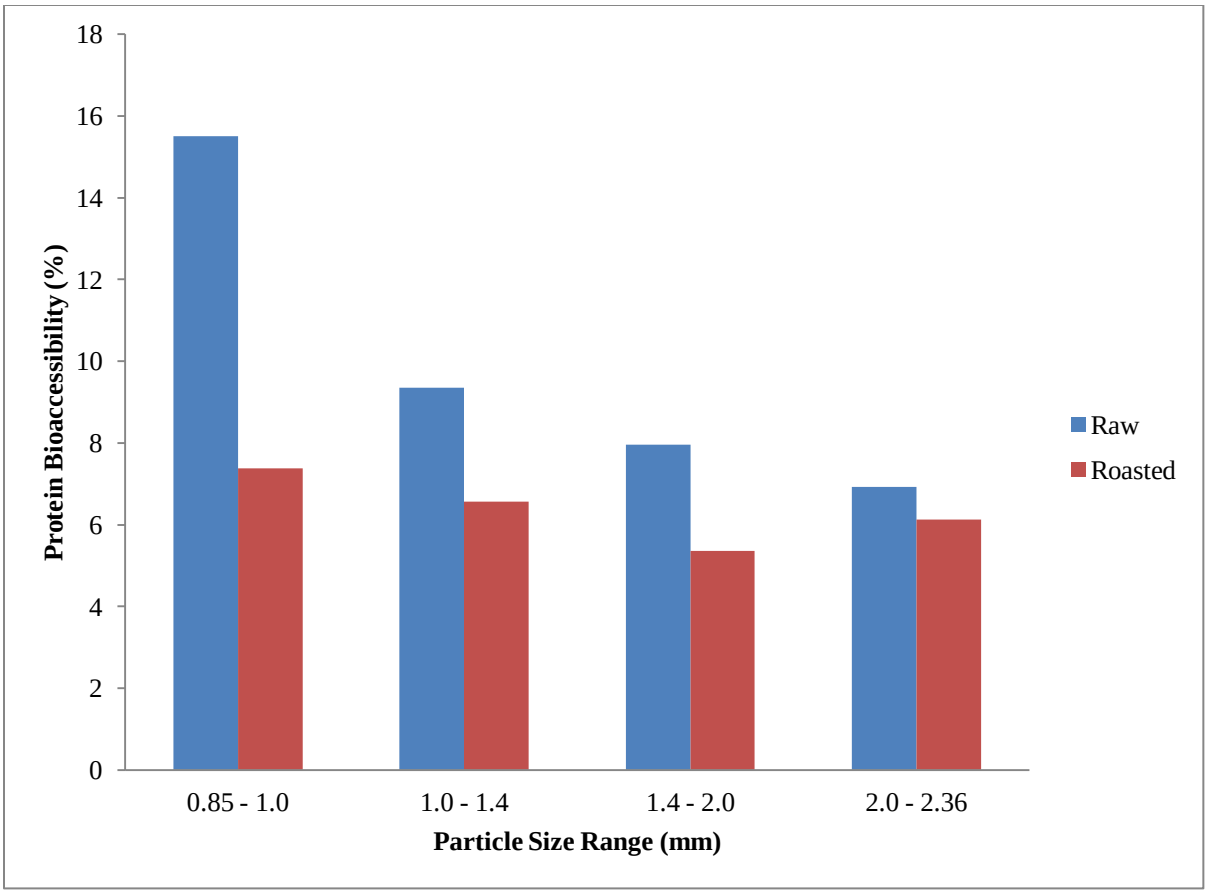

Figure 1.2.Protein bioaccessibility (%) after gastric digestion as affected by particle size.

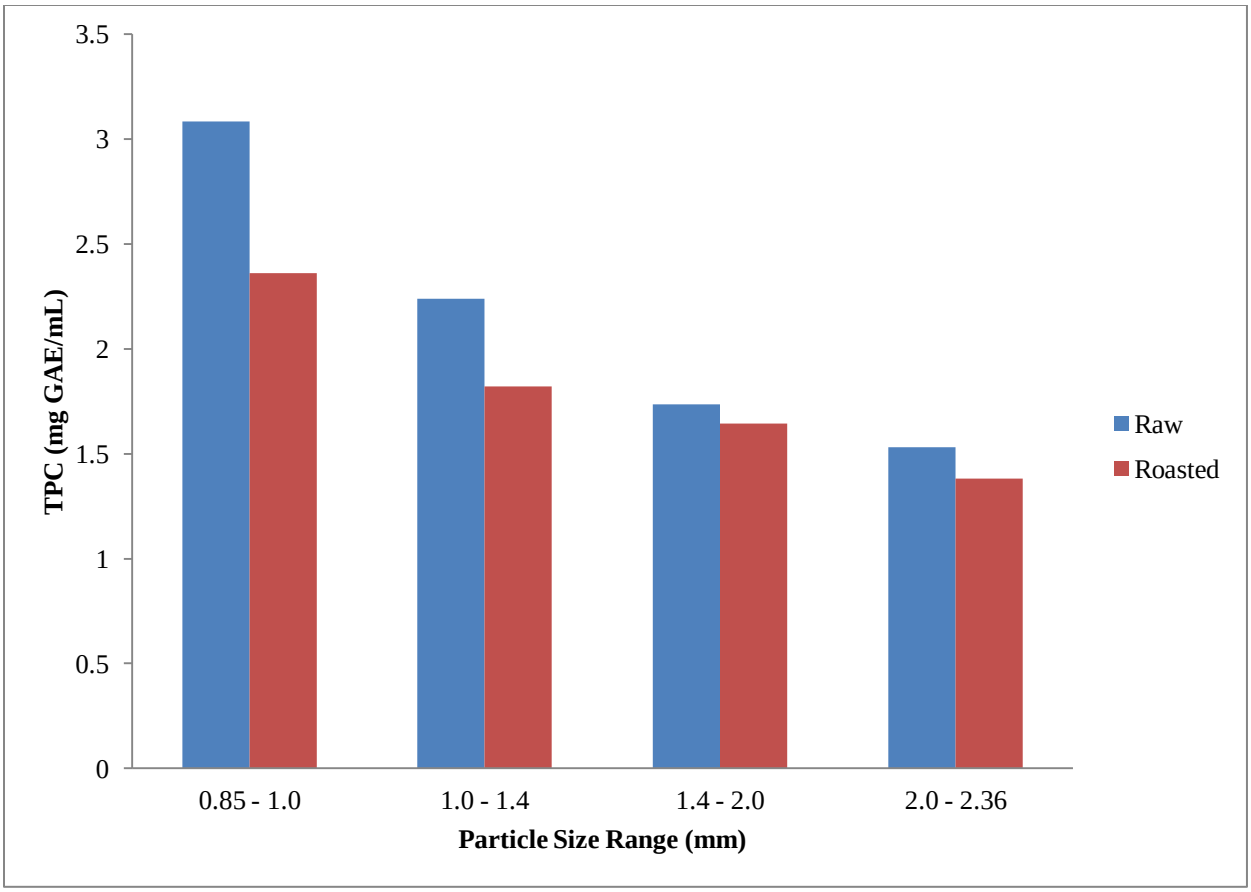

Figure 1.3.Total phenolic content of digesta after gastric digestion as affected by particle size.

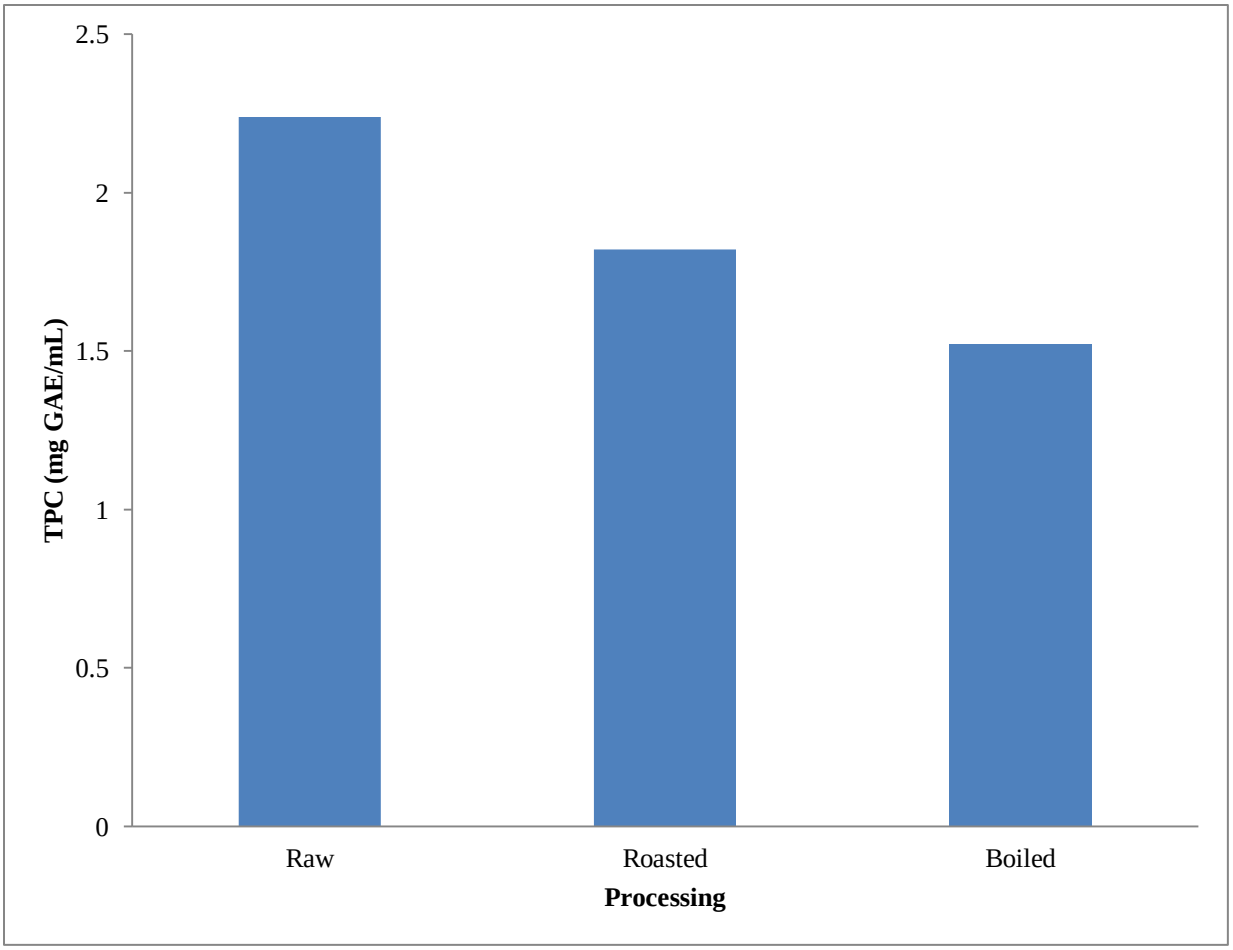

Figure 1.4. Total phenolic content of digesta after gastric digestion as affected by processing.

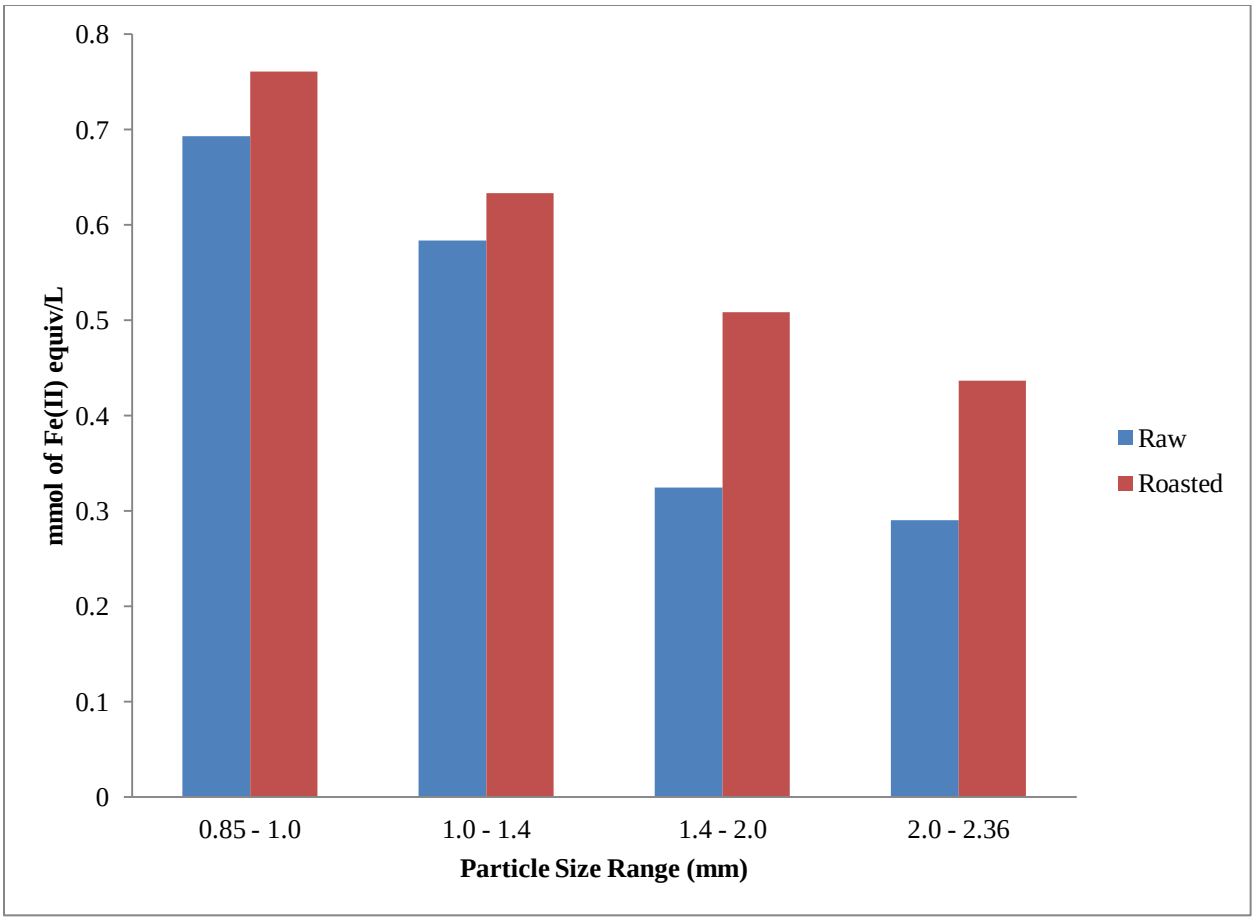

Figure 1.5.FRAP values of digesta after gastric digestion as affected by particle size.

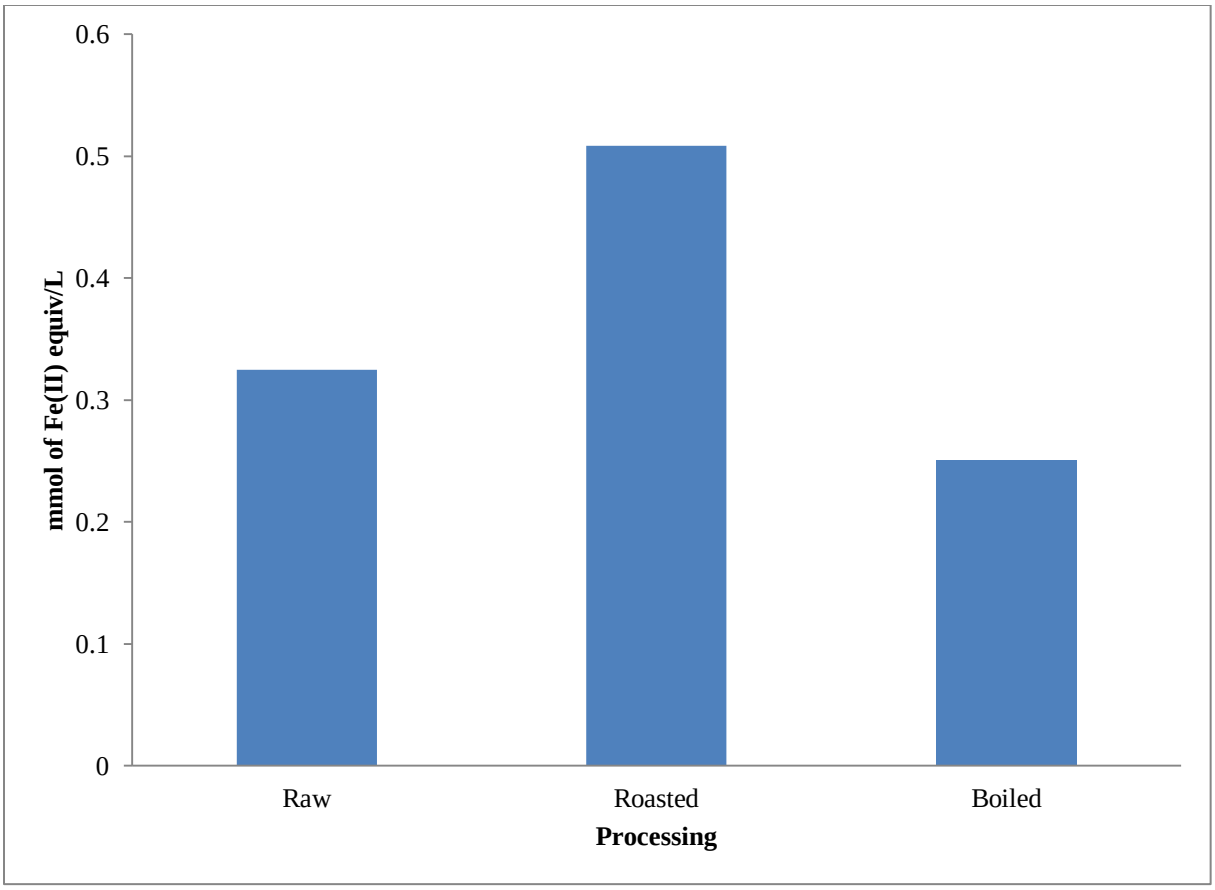

Figure 1.6. FRAP values of digesta after gastric digestion as affected by processing.

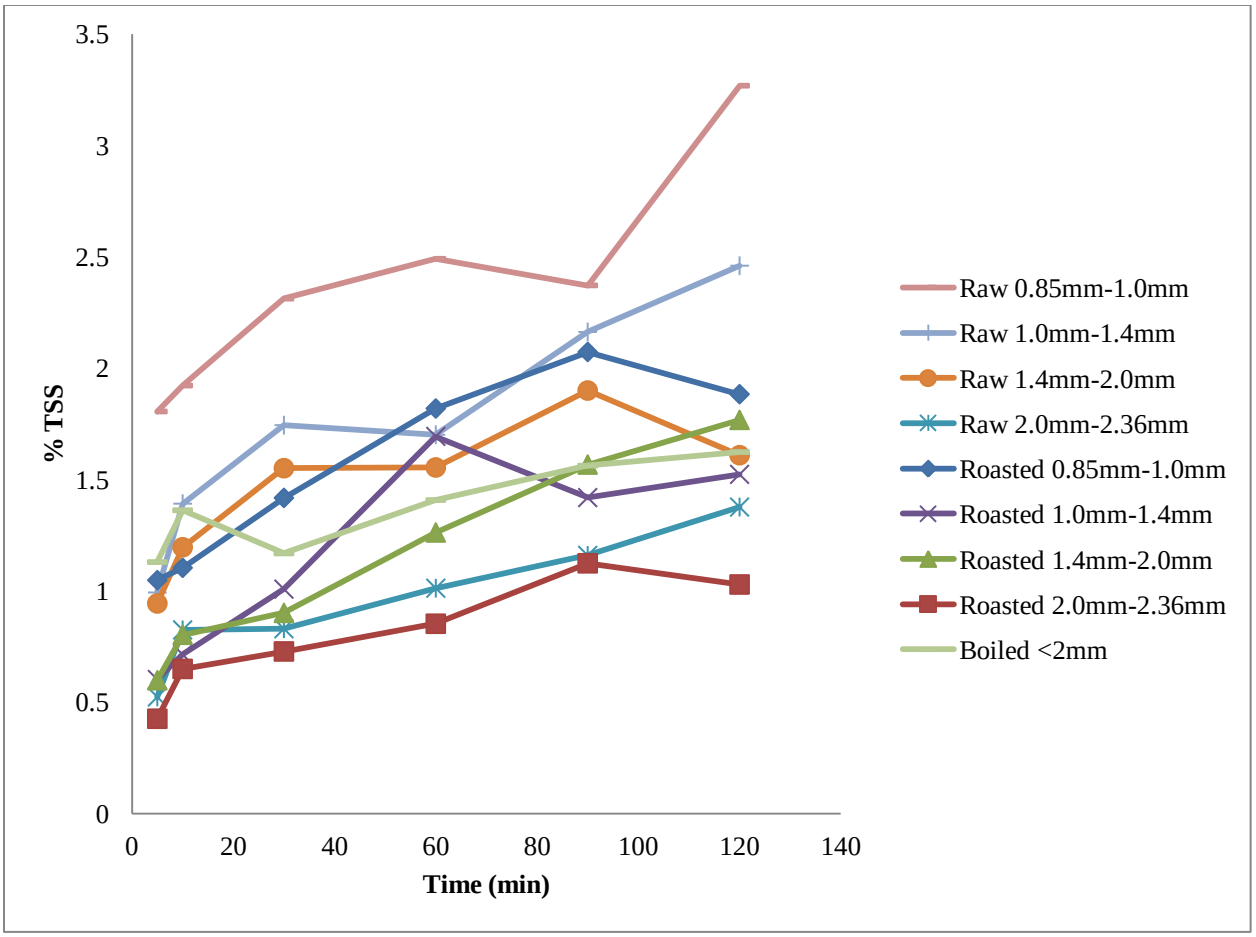

Figure 1.7.Total soluble solids (%) of digesta after gastric digestion as affected by particle size and processing.

CHAPTER 3

RELEASE PROPERTIES OF PEANUT NUTRIENTS AND BIOACTIVES DURING INTESTINAL DIGESTION AS AFFECTED BY PROCESSING[2]

[2] Williams, B.S. and F. Kong. To be submitted to *Journal of Food Science*.

Abstract

The objective of this research was to evaluate the effect of processing on digestion properties of peanuts during the intestinal phase. Raw, roasted, and boiled peanuts underwent gastric and intestinal digestion before evaluating release characteristics and calculating bioaccessibility. During *in vitro* intestinal digestion, soluble protein concentration, protein bioaccessibility and total soluble solids of roasted peanuts was higher than raw samples. Raw peanuts had higher release amounts of total phenolic content and FRAP values. Bioaccessibility and released content values were higher after intestinal digestion compared to gastric digestion, confirming the importance and role of the small intestine during digestion.

Introduction

Recent studies suggest that nut consumption may have positive effects on health such as the reduced risk of heart disease, cancer, obesity and type II diabetes (Higgs, 2003; King, Blumberg, Ingwersen, Jenab, & Tucker, 2008).Such potential benefits are a result of the nut components that operate via a variety of mechanisms. While it is accepted that peanuts fall into this category, it is important to understand that the digestion process of food has a major impact on the true health benefits. An important measurement to consider when evaluating such health benefits is bioaccessibility, the amount of a consumed nutrient or bioactive that is available for absorption (Etcheverry, Grusak, & Fleige, 2012).

In vivo digestion methods are considered the gold standard when investigating digestion properties of food. However, a variety of limitations of *in vivo* methods such as cost and ethical restrictions have led to the increased utilization of *in vitro* methodology and models (Hur, Lim, Decker, & McClements, 2011). One characteristic that *in vitro* digestion models for food differ in is the number and type of steps included in the digestion sequence, *e.g.* mouth , stomach, small

intestine, large intestine (Hur et al., 2011). However, it is important to consider all steps of digestion to accurately simulate *in vivo* conditions.

Digestion can be separated into a 3-step process: mouth, stomach, and intestines. The small intestine is the primary organ of digestion and absorption as the bulk of nutrient processing occurs there (Windelspecht, 2004). In an adult, the small intestine averages only about 1 inch in diameter and can be over 10 feet in length. Only smaller particles, roughly less than 1mm enter the small intestine by passing through the pylorus valve which separates the stomach and small intestine (Hur et al., 2011). The small intestine functions as the main organ of digestion and absorption in the human body as the bulk of nutrient processing is done here.

Mandalari (2013) states that, lack of information regarding pistachio behavior in the GI tract is a challenge when determining bioaccessibility and bioavailability. Similarly, most research regarding peanut digestion is limited as most studies focus on allergenicity and fail to address nutrient bioaccessibility. The ideal *in vitro* experiment pairs gastric digestion with intestinal digestion to most accurately simulate human digestion and is a valuable addition to the lack of research in this area. Therefore, the objective of this study was to simulate both gastric and intestinal phases of digestion to obtain a more complete representation of the release of peanut nutrients and bioactives affected by processing.

Materials and Methods

Materials

Materials used in this section of the study were the same as those presented in Chapter 2: Release Properties of Peanut Nutrients and Bioactives during Gastric Digestion as Affected by Processing and Particle Size.

Sample Preparation

Sample preparation for this portion of the research was the same as mentioned in Chapter 2. The pylorus of the stomach only allows smaller particles to be passed on to the small intestine. Therefore, only the smallest particle size range, 1.0mm-0.85mm was used for intestinal digestion experiments.

Static In Vitro Gastric and Gastrointestinal Digestion.

Simulated static *in vitro* intestinal digestion was performed according to a modified protocol (Minekus et al., 2014). Simulated saliva, gastric, duodenal, and bile juices were prepared on the day of the experiment, according to Table 1.1 presented in the previous chapter. The target pH of prepared simulated juices was achieved using 1M NaOH and 1M HCl. Ground peanut samples (5.0 g) were added to a 250-mL Erlenmeyer flask. Flasks were covered with Parafilm and placed in a Model 290400S water bath (Boekel Scientific, Feasterville, PA) set at 37°C with orbital agitation of 120 rpm. The sample was digested as follows: mouth—addition of 20 mL of salivary juice and mixing for 2 min; stomach—addition of 40 mL of gastric juice and mixing for 2 hours; and intestines—addition of 40 mL of duodenal juice and 20 mL of bile for an additional 2 hours. The pH of gastric digestion was maintained at 3.0±0.5 while the pH of intestinal digestion was maintained at 7.0±0.5. Aliquots (5mL) were collected after 5, 10, 30, 60, 90 and 120 minutes of intestinal digestion. Intestinal digesta samples were placed in a boiling water bath of 100°C for 10 minutes to slow down or inhibit further enzymatic action. Digesta samples were then filtered *in vacuo* through Whatman No. 1 filter paper and the filtrate was kept in frozen storage of -20°C before further analysis.

Total soluble solids

Total soluble solids of digesta samples were determined by gravimetric analysis. Detailed methodology for this measurement can be found in Chapter 2.

Soluble protein concentration

Quick Start Bradford protein assay was performed to determine soluble protein concentration (Bradford, 1976) of digesta samples. Methods are the same as those in Chapter 2. Roasted intestinal digesta samples were diluted 2-fold. Soluble protein concentration results were reported in units of μg/mL.

Determining protein bioaccessibility

Soluble protein concentration at the end of the static *in vitro* intestinal digestion phase (120 m) was used to determine protein bioaccessibility. Soluble protein concentration was multiplied by the total volume of intestinal digesta (120 mL) to determine the mass of protein released during intestina; digestion. Protein bioaccessibility was then calculated using the following equation:

$$Protein\ Bioaccessibility\ (\%) = \frac{mass\ protein\ released\ from\ sample}{theoretical\ mass\ of\ protein\ in\ sample} \times 100$$

The theoretical mass of protein in the peanut sample was determined by multiplying the percent composition of protein appropriate peanut sample by the total sample mass of peanuts used (5g). The percent composition of protein in peanuts was obtained from the USDA National Nutrient Database.

Total phenolics content (TPC) assay

The TPC of digesta was determined using a modified Folin-Ciocalteu method (Singleton & Rossi, 1965). Details for this assay are the same as those in Chapter 2 and expressed as g GAE equivalents/mL of digesta.

Ferric reducing antioxidant power (FRAP) assay

A modified FRAP assay was utilized (Benzie, 1996). This procedure is the same as that previously presented in Chapter 2. Results were determined using a standard curve prepared using 100 μL of various ferrous sulfate heptahydrate solutions (0.1 to 1.0 mM) and presented as mM Fe^{2+} equivalents/L.

Results and Discussion

The effect of size during the intestinal phase of digestion cannot be determined because only peanut particles sized 0.85-1.0mm were used in this phase (with the exception of boiled peanuts <2mm). This is due to the selectivity of the pylorus valve which only allows small particles (<1mm) to pass from the stomach into the small intestine. In this section, intestinal digestion refers to intestinal digestion coupled with gastric digestion.

Figure 2.1 shows the change in soluble protein concentration of peanut samples during intestinal digestion trials. Soluble protein concentration increases over the period of intestinal digestion for all samples and can be summarized by this trend: roasted > raw > boiled at the end of the intestinal digestion phase. This trend differs from that seen at the end of gastric digestion where raw > roasted > boiled. Protein aggregates that may have been too large to pass through filtration during the gastric digestion phase may have broken down and been reduced in size during the intestinal digestion phase and were now filtered. Figure 2.2 displays the protein bioaccessibility (%) after intestinal digestion and is higher in roasted peanuts than raw peanuts. This differs from gastric digestion where the trend for protein bioaccessibility is raw > roasted. Protein bioaccessibility values after gastric + intestinal digestion for roasted and raw peanuts are 44.0%, and 34.8% respectively. The gastric + intestinal phase of digestion removed the majority of the accessible protein for all samples. The gastric + intestinal phase of digestion accounted for 83.2% of the total protein released in roasted samples and 55.5% in raw samples.

Results from Mandalari's study (2008) show that finely ground raw almonds released more protein, lipid, and vitamin E than natural almonds and blanched almonds after duodenal digestion (Mandalari et al., 2008). Gastric digestion removed most of the accessible protein (38%) and only 9% more of additional protein was released after the duodenal phase. In that

study, the duration of gastric digestion was 2 hours, and intestinal digestion only 1 hour. This differs from methodology of our study with each phase of peanut digestion lasting 2 hours, for a total digestion time of 4 hours.

In Figure 2.3, the total phenolic content of digesta after the gastric + intestinal phase is shown and can be described by the following trend raw > roasted > boiled. Raw TPC values were only 7.2% higher than roasted TPC values, but 44% greater than boiled TPC values. There was an increase in the amount of total phenolics released following the gastric + intestinal phase for all samples compared to the gastric phase. From gastric phase to intestinal phase, there was a 30% increase in TPC in raw digesta, 59% increase in roasted digesta, and 89 % increase in boiled digesta.

FRAP values after gastric + intestinal digestion are shown in Figure 2.4. Results coincide with those for TPC and are highest in digesta from raw peanuts, followed by roasted peanuts and then boiled peanuts. There was a much greater increase in FRAP during gastric + intestinal digestion compared to gastric digestion for all samples. The FRAP value of raw digesta after gastric + intestinal digestion is more than 8 times the FRAP value after gastric digestion. In roasted and boiled samples, there is a 611.8% and 952.0% increase of FRAP respectively, between intestinal and gastric phases.

Polyphenols occur in nature in free or bound forms. Therefore, some processing methods such as boiling or heating may result in an increase in polyphenolic content of foods (Chukwumah, Walker, Vogler, & Verghese, 2007). For example, roasted peanuts had a numerically larger amount of total polyphenols then raw peanuts but the difference was not significant (Chukwumah et al., 2007). Researchers found that blanching produced a significant loss (88.9%) in total polyphenol contents of peanut skin, while roasting did not significantly

reduce (4.6%) the concentration of these compounds (Yu, Ahmedna, Goktepe, & Dai, 2006). In another published study, processing resulted in significantly higher phenolic content in roasted almonds compared to blanched almonds (Garrido, Monagas, Gómez-Cordovés, & Bartolomé, 2008). Talcott (2005) found that when assessing total soluble phenolics by HPLC and the Folin-Ciocalteu assay, poor correlation to antioxidant activity was observed. This was due to contributions from soluble proteins that were only corrected for when detecting phenolic concentrations using HPLC.

Figure 2.5 displays the change in total soluble solids of digesta over the duration of the intestinal digestion phase. Total soluble solids (%) increased over time and maximum values were seen the end of the digestion period. At the start of the intestinal digestion phase, %TSS was highest in digesta from raw peanuts and lowest in digesta from boiled peanut samples. However, at the end of the digestion phase, %TSS shifts and was higher in roasted peanut digesta than raw and boiled digesta samples. This is well correlated with change of protein contents, as protein is the major portion of the TSS. Additionally, more soluble solids were released during intestinal digestion than gastric digestion.

Results show that boiled peanuts released the least amount of all measured indices (this does not include protein bioaccessibility). It is known the boiling caused significant loss of soluble solids and nutrients (Kong et al. 2012).These soluble solids contain nutrients such as proteins as well as bioactive compounds of the peanut. In our experiment, peanuts were not boiled in the hull or shell and were only enclosed by the peanut skin. This is because peanuts purchased and used in all other parts of this experiment already had the shell removed. Related studies contribute higher total polyphenolic content and high antioxidant capacity of boiled peanuts to the presence of peanut hulls (Chukwumah et al., 2007). However, the absence of the

hull in our study may explain decreased release values of nutrients and bioactives as such components were soluble in the surrounding water and were removed from the peanut during the boiling process.

Interestingly, the majority of nutrients and bioactives for all samples in this study were released during the intestinal digestion phase as opposed to the gastric digestion phase. One possible explanation may be due to the cell structure of the peanut. Perhaps, the 2 hour gastric digestion phase and concentration of enzymes were not sufficient enough to cause a substantial increase in the release of cellular components. After additional contact with digestive enzyme during the intestinal phase, proteins and polyphenolic compounds were unbound and free, resulting in a greater release during this phase of digestion.

During gastric digestion, more soluble solids and proteins were released from raw peanuts than roasted peanuts. The increased release of soluble solids and proteins in raw peanuts may be explained by the textural differences in raw and roasted peanuts and aggregation of proteins in roasted peanuts. Texture analysis shows that roasted peanuts are harder, crisper, and more brittle than raw peanuts (Kong, Oztop, Paul Singh, & McCarthy, 2013). Harder pieces may have required a longer digestive period to breakdown and become more soluble in digestive fluids. Results also show that during intestinal digestion, more soluble solids and proteins are released from roasted peanuts rather than raw peanuts. A reasonable explanation for this finding is that proteins previously aggregated in roasted peanut digesta were broken down increasing solubility and ability to be filtered.

Conclusions

During the intestinal digestion phase, there was an increase in soluble protein concentration, with the highest concentration in roasted peanuts compared to any other sample. This finding is well correlated with findings for %TSS. TPC and FRAP however, were lower in processed peanut digesta than in raw peanut digesta. The intestinal digestion is the last step of a three-step digestion process following the mouth and the stomach. Therefore, it is important to understand and consider what occurs during this final step. Compared to gastric digestion, intestinal digestion results in higher bioaccessibility and release values, giving a more complete and final illustration of biaccessibility and release of nutrients and non-nutrients.

In recent years, there has been an increase in health consciousness and growing interest in the role of food maintaining and improving human well-being and consumer health. Years ago, research suggested that processing resulted in nutrient loss and reduced food quality. In response to these findings, the food industry has improved traditional processing and increased research such as our own has been done to better evaluate the influence of processing on content, activity and bioavailability of nutrients and bioactive compounds. Roasting had the greatest improvement on protein bioaccessibility and similar effects on TPC and FRAP compared to raw peanuts. However, boiling resulted in the lowest release properties of nutrient and bioactives compared to any other processing type. Therefore, it is important to understand that the processing type may have varying effects on the nutritional content of a food.

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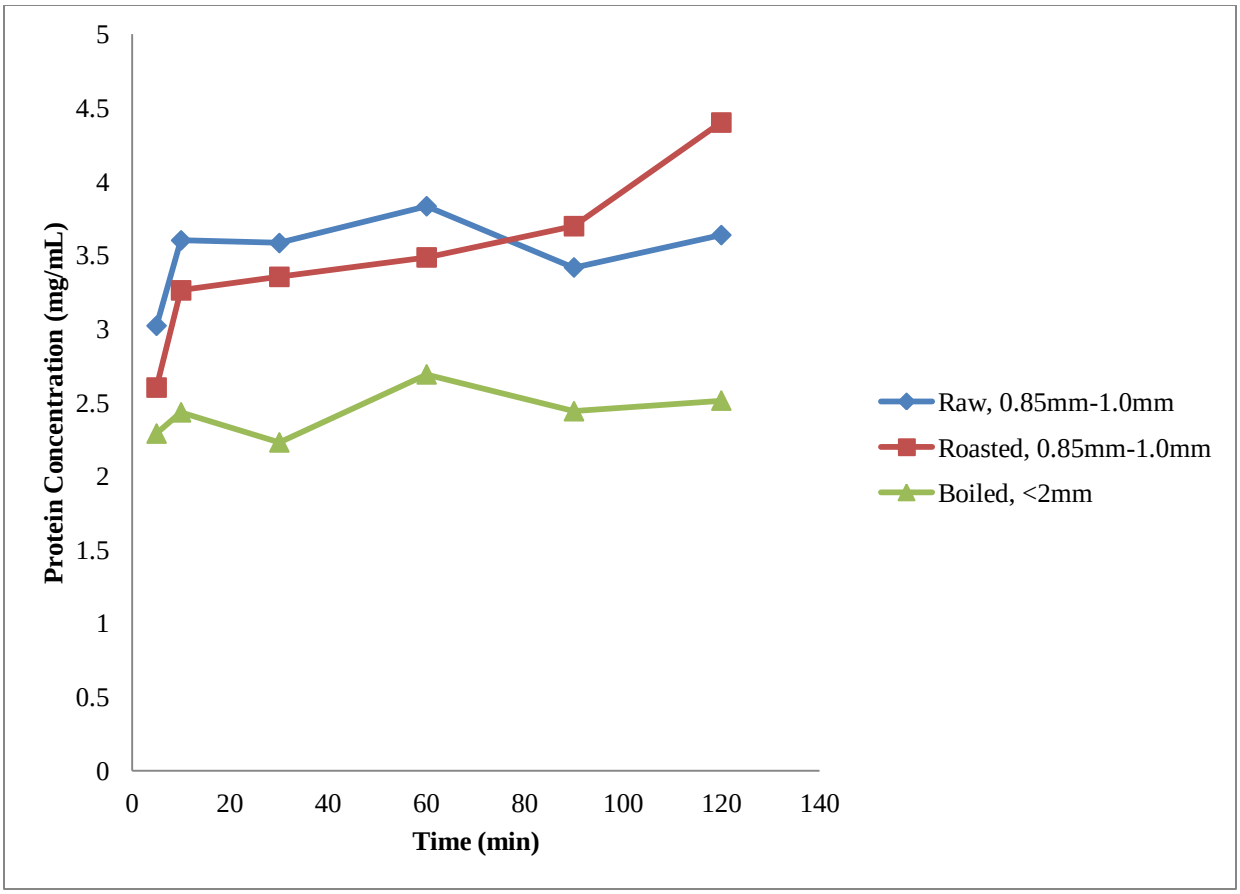

Figure 2.1.Soluble protein concentration during intestinal phase of digestion.

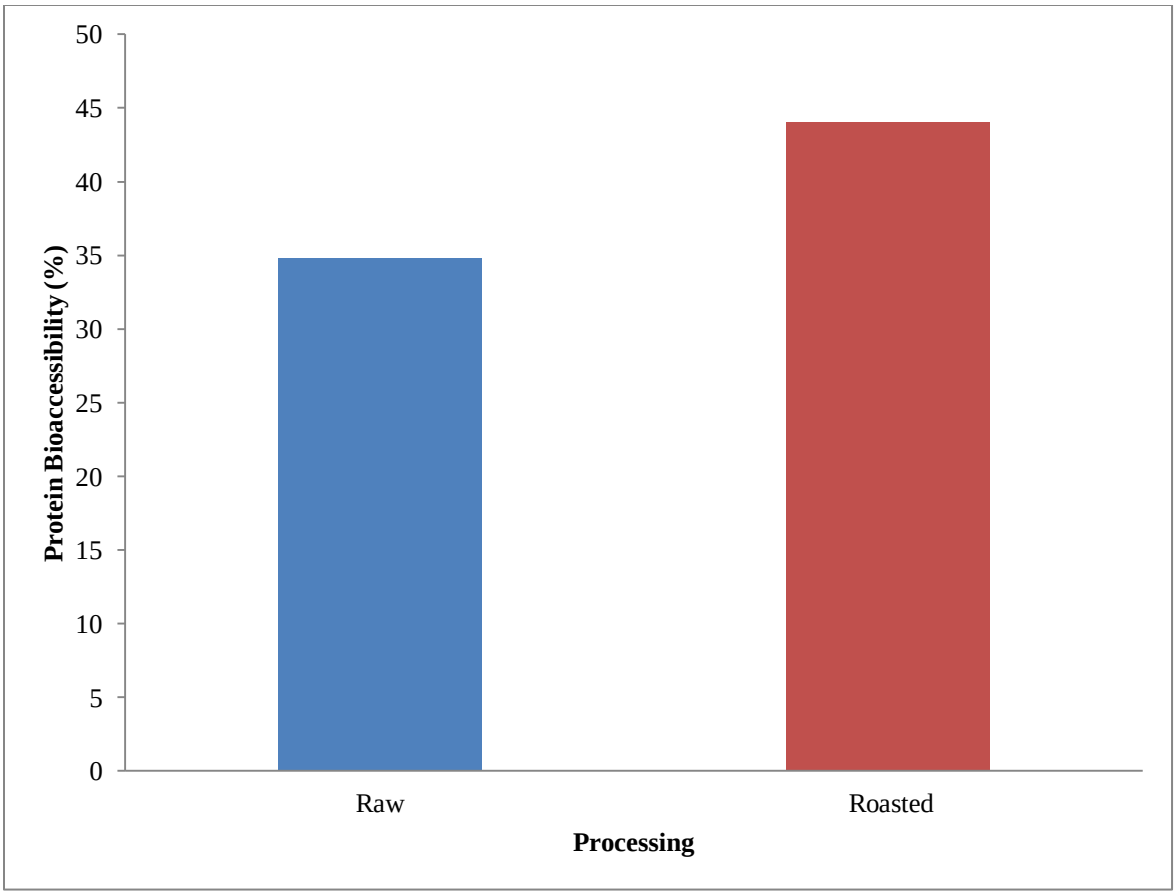

Figure 2.2.Protein bioaccessibility (%) after intestinal digestion as affected by processing.

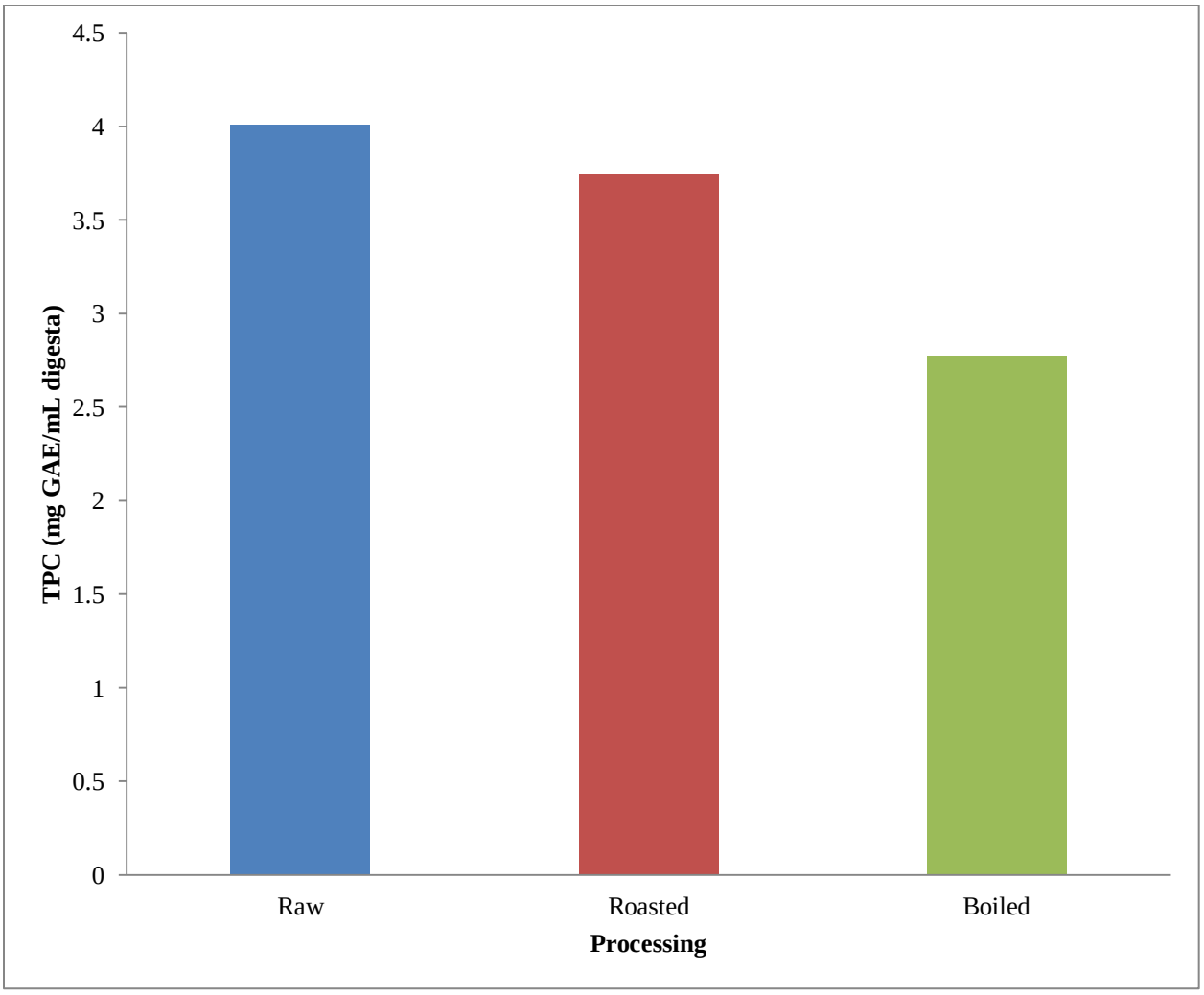

Figure 2.3.Total phenolic content of digesta after intestinal digestion as affected by processing.

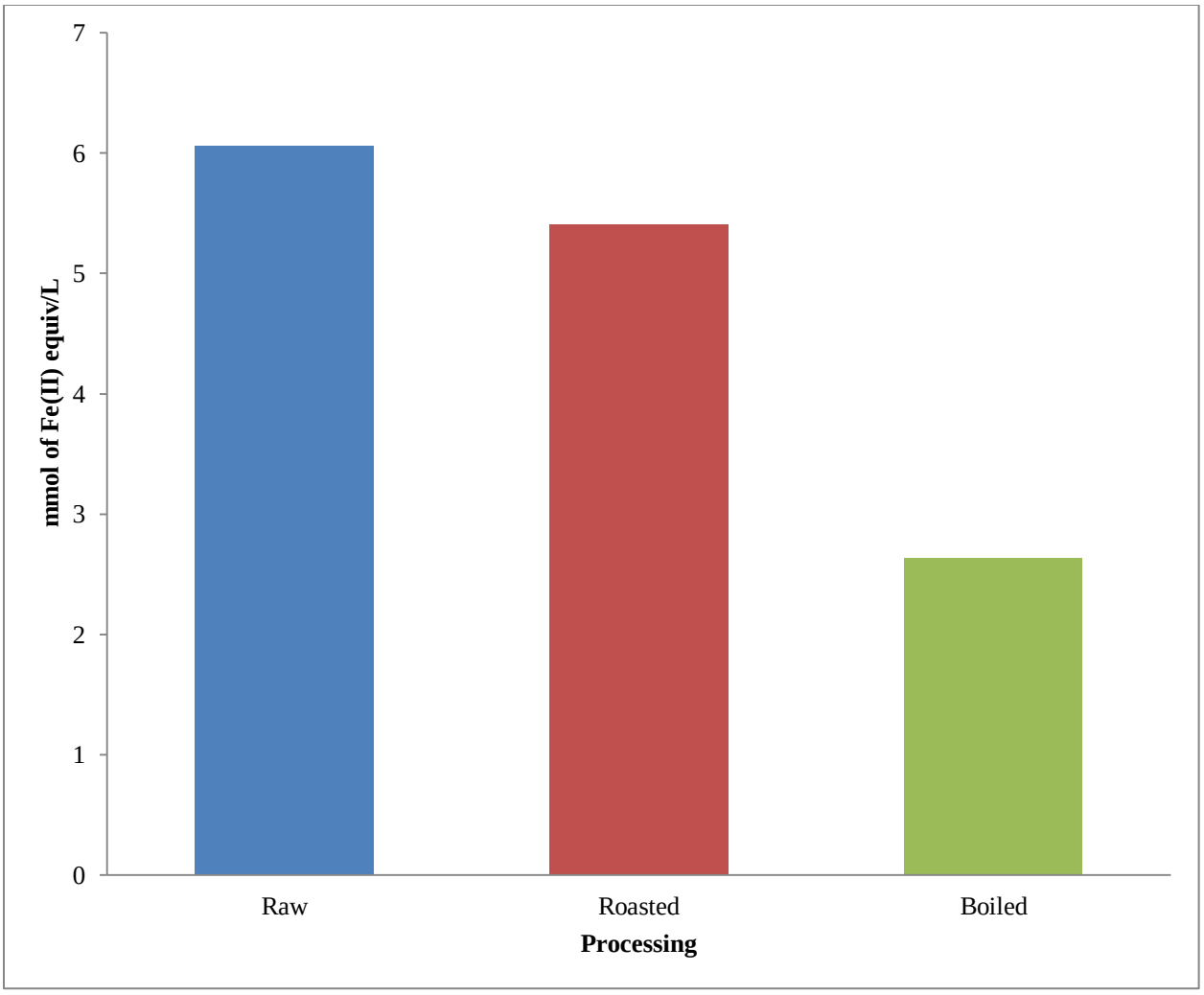

Figure 2.4.FRAP values of digesta after intestinal digestion as affected by processing.

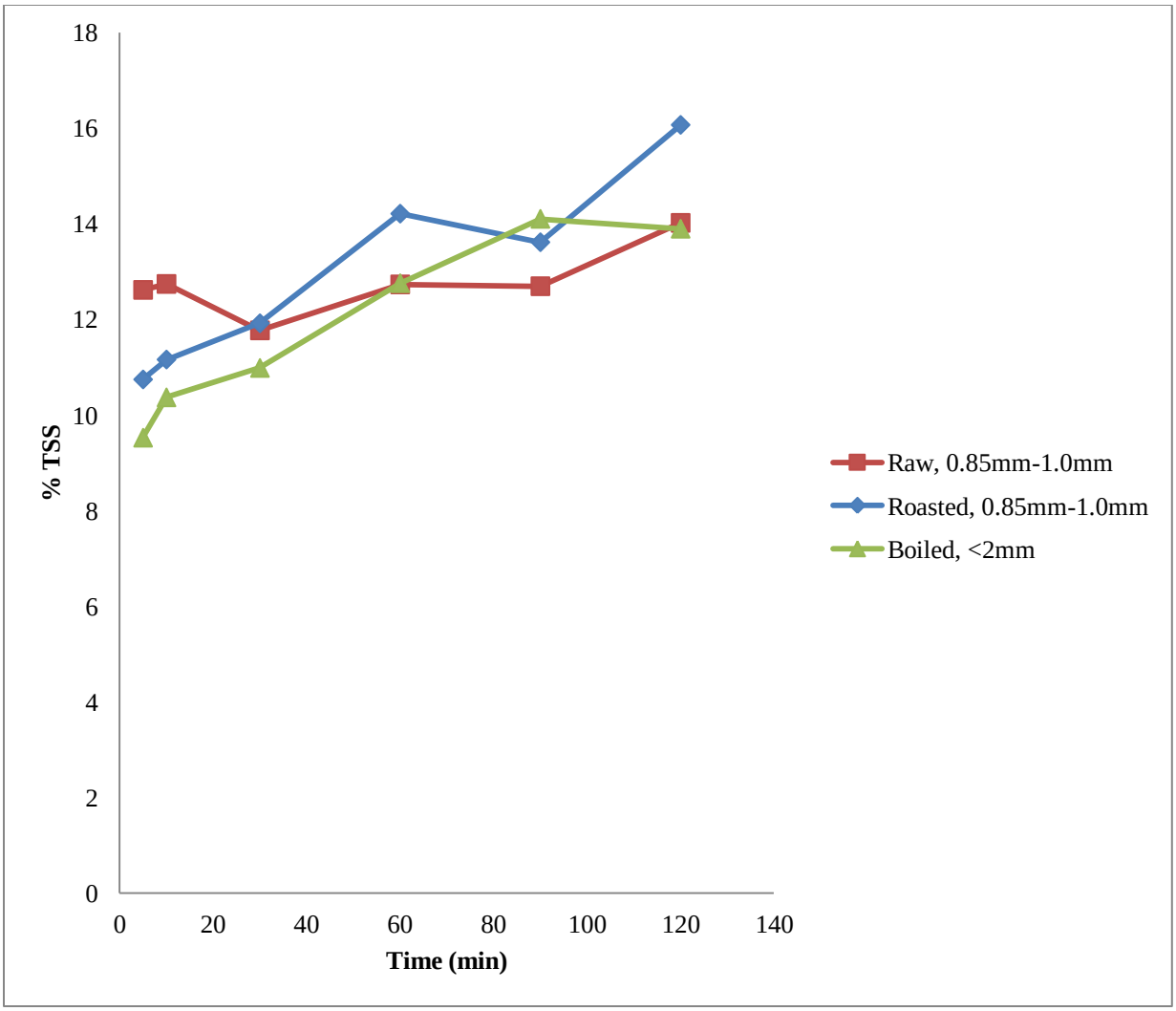

Figure 2.5.Total soluble solids (%) of digesta after intestinal digestion as affected by processing.

CHAPTER 4

COMPARISON OF RELEASE PROPERTIES OF PEANUT NUTRIENTS VIA STATIC AND DYNAMIC IN VITRO DIGESTION MODELS[3]

[3] Williams, B.S. and F. Kong. To be submitted to *Journal of Food Science*.

Abstract

Several nutritional and epidemiological studies have suggested that nut consumption can reduce the risk of cardiovascular disease, cancer, and diabetes. In order to exert such health benefits, food components of peanuts must first be biaccessible by digestion in order for potential uptake by the body. The complexity of human digestion has resulted in the need for *in vitro* models that best resemble the digestion process. While most developed models are static, dynamic models are more accurate and better mimic this process. The objective of this experiment was to employ static and dynamic digestion models to evaluate and compare the release properties of peanut nutrients and bioactives. Additionally, optimal pH conditions of static digestion were determined in order to best resemble conditions and results from dynamic digestion. The effect of static and dynamic gastric digestion on the release properties of roasted peanuts between 1.0-1.4mm was evaluated in this experiment. When simulating gastric digestion using a dynamic model, final soluble protein concentration, protein bioaccessibility, total phenolic content and the percentage of total soluble solids to dry solids were greater than when using static digestion models for all tested pH values. As pH of static digestion trials decreased, the release of peanut nutrients and components increased, with the exception of FRAP value at pH 3.0 which is higher than FRAP values of any other digestion trial. Results from this experiment suggest the when determining whether to use a static or dynamic model, it is necessary to consider advantages and limitations of both systems, as well as the released substance of interest.

Introduction

As interest in the health effects of nut consumption increases, so does the need for reliable and accurate data pertaining to nut digestion. As a result, there is a growing demand for

digestion models that simulate the complex physiochemical and physiological events that occur in the human GI tract (Hur, Lim, Decker, & McClements, 2011). *In vivo* feeding methods using humans or animals usually provide the most accurate results, but they are time consuming, expensive, use large amounts of resources, and raise ethical concerns. Additionally, the metabolism of animal subjects used for *in vivo* studies may be different from human metabolism. Such drawbacks have led to an increase in the development of alternative *in vitro* digestion models.

There are two main types of *in vitro* digestion models, static and dynamic. Due to their simplicity and ease of use, the majority of *in vitro* tools used to study digestion are static. Static trials are characterized by the constant ratio of meal to enzymes, salt, bile acids, etc. and a fixed pH at each step of digestion. However, static digestion models lack physiological relevance and complexity of the digestive system (Menard et al., 2014). A solution to this problem has been the development of *in vitro* dynamic digestion models that allow for the modification of pH, dynamic flows of food and concentration of digestive enzymes in the different compartments of the GI tract (Menard et al., 2014).

In static digestion models, pH and digestive enzyme concentrations can be adjusted to have major impacts on digestion properties. The pH of an environment has a significant influence on enzyme activity and was therefore selected as the factor to be altered in static gastric digestion trials. Following the consumption of food, pH within the stomach increases to 5 or higher as a result of the buffering capacity of the meal. As hydrochloric acid is secreted by cells in the stomach, the pH is lowered to just below 2 where optimal enzyme activities can be carried out. Optimal gastric lipase activity occurs in slightly acidic conditions ranging from pH 4 to 6. An optimal pH range between 2 and 4 is required for pepsin activity. Based on such values,

Minekus and others suggest a static value of pH 3.0 to be used when simulating a gastric digestion phase of 2 hours (Minekus et al., 2014). However, it is well known that enzymatic activities in the human body take place over a pH range rather than just one pH value. Therefore, when simulating static digestion, it is worthwhile to conduct experiments over multiple ranges of pH values to determine the influence of pH on the bioaccessibility of ingested nutrients and bioactives of interest.

Two main processes occur simultaneously during human digestion: (1) mechanical transformations that reduce the size of food particles; and (2) enzymatic transformations where macromolecules are hydrolyzed into smaller units that are absorbed into the bloodstream (Guerra et al., 2012). The mouth and stomach are the main sites of food disintegration while enzymatic digestion and absorption of nutrients and water primarily occurs in the small and large intestine (Guerra et al., 2012). Dynamic gastric models are unique in that they account for the region specificity and forces of digestive organs such as the amplitude, intensity, and frequency of peristaltic movements of the stomach. Such factors contribute greatly to the two aforementioned processes of human digestion and should be accounted for when appropriate.

While *in vivo* studies are considered to be the gold standard for addressing diet related questions, a number of limitations make *in vitro* digestion models the more practical choice. Dynamic *in vitro* models better mimic physiological and physiochemical events occurring in the human digestive system compared to static models and serve as a reliable alternative to *in vivo* studies. However, the majority of *in vitro* tools are static as they are reproducible and easy to use. Therefore, it is necessary to optimize conditions of static *in vitro* models so that conditions best reflect physiological conditions of human digestion and appropriate results may be obtained. The objective of this research was to employ static and dynamic *in vitro* digestion models to evaluate

and compare the effect of physiological processes of dynamic models and constant pH and volume of static trials on release properties of peanut nutrients.

Materials and Methods

Raw Runner peanuts (Southern Grace Farms) shelled, with skins on were obtained from a Georgia peanut shelling plant in Enigma, Georgia. All chemicals and digestive enzymes used were of reagent-grade and purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). The ratio of peanut sample to saliva to gastric juice is the same in static and dynamic digestion trials (0.25:1:2).

Sample Preparation

Prior to analysis and processing, raw peanut kernels were removed from frozen storage at -20°C and allowed to reach room temperature. For analysis, peanut samples consisted of roasted peanuts within the particle size range of 1.0-1.4mm. Raw Runner peanuts were roasted using modified standard industrial practices (Chukwumah, Walker, Vogler, & Verghese, 2007). One thousand grams of Runner peanuts were roasted with skins on in a commercial convection oven at 170 ± 5°C for 20 minutes. Peanuts were cooled and skins were manually removed.

In vitro gastric digestion

Static Digestion. Simulated static gastric digestion was performed according to a modified protocol (Minekus et al., 2014). Simulated saliva and gastric juices were prepared on the day of the experiment, according to Table 1.1. The target pH of prepared simulated juices was achieved using 1M NaOH and 1M HCl. Ground peanut sample (5.0 g) was added to a 125-mL Erlenmeyer flask. Flasks were covered with Parafilm and placed in a Model 290400S water bath (Boekel Scientific, Feasterville, PA) set at 37°C with orbital agitation of 120 rpm. The sample was digested as follows: mouth—addition of 20 mL of salivary juice and mixing for 2

min; stomach—addition of 40 mL of gastric juice and mixing for 2 hours. Gastric digestion trials differed in the target pH maintained throughout the duration of each trial: 2.0±0.5, 2.5±0.5, and 3.0±0.5 and was achieved by adding appropriate amounts of1 M HCl to the flasks. Aliquots (5mL) were collected after 5, 10, 30, 60, 90 and 120 minutes of gastric digestion. Gastric digesta samples were neutralized to a pH of 6.5±0.5 using 0.5 M $NaHCO_3$ to inhibit further enzymatic action. Digesta samples were then filtered *in vacuo* through Whatman No. 1 filter paper and the filtrate was kept in frozen storage of -20°C before further analysis.

Dynamic Digestion. The Dynamic Gastric Simulated Model (DGSM) displayed in Figure 3.1 was utilized to perform dynamic *in vitro* digestion in the stomach. The model consists of two main components, an acrylic water insulated vessel and a polyvinyl chloride probe. The probe is connected to a TMS—Pro texture analyzer with a 500 N load cell. To simulate gastric emptying, plastic tubing with an inner diameter of 3.4mm was placed at the bottom of the insulated vessel to empty gastric contents. The insulated vessel simulates the gastric chamber and was surrounded by water at 37°C. The probe takes the shape of a cone attached to an elongated cylinder and compresses food by moving up and down into the insulated vessel. The probe keeps a 2mm gap between the end of the probe and the bottom of the vessel before moving back up and repeating this cycle. A 2mm gap was determined based on the fact that particles only less than 1-2 mm are emptied from the stomach into the small intestine (Hur et al., 2011). A mesh net was also placed inside the vessel to prevent larger particles of food from being emptied and clogging the gastric emptying tube.

For dynamic trials, a 15 g peanut sample was mixed with 180 mL of distilled water. To this sample, 60 mL of simulated saliva was added and mixed for 2 minutes. After mixing, contents were added to the gastric chamber of the dynamic model. Gastric secretion was initiated

at a constant flow rate of 1 mL /min using a Variable Flow Mini-Pump. Digesta contents were emptied through the plastic tubing using another mini pump at a continuous rate of 3 mL/min. Contents were emptied into a flask placed in a boiling water bath of 100°C to inactivate enzymes and inhibit further enzymatic reactions. Samples of 5 mL aliquots were collected at 0, 10, 30, 60, 90, and 120 minutes of the gastric digestion phase and neutralized to a pH of 6.5±0.5 using 0.5M sodium bicarbonate to slow down or inhibit further enzymatic action. Neutralized samples were filtered *in vacuo* through Whatman #1 filter paper and stored at -20°C prior to additional analysis.

Analysis of Digesta

Soluble protein concentration, total phenolic content, and ferric reducing antioxidant power were measured following methods as described in Chapter 2: Release Properties of Peanut Nutrients and Bioactives during Gastric Digestion as Affected by Processing and Particle Size. Static gastric digesta samples and dynamic gastric digesta samples were not diluted in any of the three assays.

Total soluble solids to dry solids ratio

The mass of soluble solids was calculated by determining total soluble solids (%) of digesta at the end of the gastric digestion period. The percentage of total soluble solids was determined gravimetrically following methods in Chapter 2. The mass of soluble solids contributed by both gastric juice and saliva juice at the end of the digestion phase were then subtracted from %TSS to give the mass of soluble solids contributed by the peanut sample. The mass of soluble solids from digestive fluids was calculated by multiplying the concentration of soluble solids in each respective fluid (salivary and gastric juice) by the volume of fluid. The mass of dry solids from peanuts was determined by multiplying the initial sample mass (5g in

static trials and 15g in dynamic trials) by the dry solids content of the sample. Dry solids content was calculated as the difference between 100 and the moisture content (99.45%) of the roasted peanut sample.

Calculating protein bioaccessibility

Protein bioaccessibility (%) was calculated for both static and dynamic models.

Static models. Soluble protein concentration at the end of the static *in vitro* gastric digestion phase (120m) was used to determine protein bioaccessibility. This value is used because the volume and concentration of digestive enzymes and fluids is constant and represents the total amount of protein released during this trial. Soluble protein concentration at t=120m was multiplied by the total volume of gastric digesta (60 mL) to determine the mass of protein released during gastric digestion. Protein bioaccessibility (%) was then calculated using the following equation:

$$Protein\ Bioaccessibility\ (\%) = \frac{mass\ protein\ released\ from\ sample}{theoretical\ mass\ of\ protein\ in\ sample} \times 100$$

The theoretical mass of protein in the peanut sample was determined by multiplying the percent composition of protein in the appropriate peanut sample by the total sample mass of peanuts used (5g). The percent composition of protein in peanuts was obtained from the USDA National Nutrient Database.

Dynamic models. The average soluble protein concentration of dynamic *in* vitro gastric digestion at sample collection points (t= 0, 10, 30, 60, 90, and 120) was used to determine protein bioaccessibility in dynamic models. Due to the changing volume and concentration of digestive enzymes and fluids over the duration of the gastric digestion period, the average value is used. The average of the soluble protein concentration determined was calculated and multiplied by the total volume of gastric digesta (360 mL) to determine the mass of protein

released during gastric digestion. Protein bioaccessibility was then calculated using the same equation presented above.

Results and Discussion

The changes in pH with time for static and dynamic trials are shown in Figure 3.2. In static trials, pH is fixed and is maintained within 0.5 pH units of the target pH. This differs from the pH of the dynamic model which constantly decreases with time due to gastric secretion and emptying. The decreased pH of the dynamic model as well as the decreasing pH between static trials is well correlated with the increased release of nutrients and bioactives of these trials to be presented in this section.

Continuous gastric secretion and emptying of gastric contents of the dynamic model result in a changing volume of digesta in the gastric chamber. Changes in the volume of gastric contents over time are shown in Figure 3.3. Digesta in the gastric chamber decreases with time as the emptying rate is greater than the rate of gastric secretion. This decrease in volume correlates with the decrease in pH over time as food is emptied from the stomach and gastric juice continues to be released.

Figure 3.4 displays soluble protein concentration during the gastric phase of static and dynamic digestion trials. Soluble protein concentration of static models generally increased over time while the concentration fluctuated using the dynamic model. At the end of the 2 hour gastric digestion phase, soluble protein concentration was highest when using the dynamic model and lowest when using the static model at pH 3.0±0.5. Soluble protein concentration trends for static trials with target pH values of 2.5±0.5 and 2.0±0.5 are very similar to each other. The final concentration of these trials (pH 2.5±0.5 and 2.0±0.5) are closer to that of dynamic trials compared to the static trial with target pH of 3.0±0.5. These findings can be explained by the

more acidic conditions of the dynamic trial and static trials with pH of 2.5 and 2.0 as well as simulated mechanical forces during dynamic digestion.

Figure 3.5 compares protein bioaccessibility (%) of static and dynamic digestion following the gastric phase. For static gastric digestion trials, as target pH decreased, protein bioaccessibility increased. Overall, protein bioaccessibility was highest at the end of the dynamic trial and is almost twice as much as the next highest bioaccessibility value, which is the static trial with the lowest pH of 2.0.

Pepsinogen, the precursor enzyme is converted to pepsin in gastric juice when the pH drops below 5 (Johnson, 2001). Once pepsin is formed, it can catalyze the formation of additional pepsin from pepsinogen. The enzyme pepsin is responsible for beginning the digestion of protein by splitting interior peptide linkages (Johnson, 2001). This mechanism may be a possible explanation for the increase in final soluble protein concentration of both static and dynamic trials as pH decreases. The movement toward acidic conditions and decreased pH may cause greater formation of pepsin and thus increased protein digestion. At the start of dynamic gastric digestion, pH is slightly below 7.0 and continues to decrease to just less than 2.0 after the 2 hour gastric phase is complete. Although the pH of the dynamic system does not reach low acidic levels of those seen in static trials until after 60 minutes of digestion, it continues to drop to less than 2.0 as opposed to static trials where this only occurs in the trial with pH 2.0±0.5. There are also additional factors such as force exhibited by the probe which may contribute to the increase in soluble protein concentration. Based on results from previous sections of our research, a decrease in particle size increased surface area exposed to digestive enzymes. The increase in surface area resulted in an increase of soluble protein concentration, and thus

increased protein bioaccessibility (%). As particles are broken down into smaller pieces, surface area is increased for digestion and absorption compared to larger particles (Kong, 2008).

The initial high protein concentration in the dynamic trial may be due to the highest content of substrate (peanut particles) released and the fast loss of solids from surface of the particles during the beginning stage of digestion. Despite a decrease in soluble protein concentration in the dynamic model after 10 minutes, protein concentration began to increase after 60 minutes had elapsed. This may be the point at which pH levels are optimal for pepsin formation. Additionally, based on gastric secretion and emptying rate, digesta volume after one hour has decreased from 360 mL to 240 mL and more gastric juice continues to be secreted, causing an increase in soluble protein concentration. In a 2011 published study, a comparison of a static and dynamic *in vitro* model is used to estimate the bioaccessibility of elements in seaweed and shellfish (Torres-Escribano et al., 2011). Bioaccessibility of cadmium and mercury are greater when using a dynamic digestion model compared to a static digestion model. A possible explanation for this finding was due to acidic conditions (pH of 2) of the stomach for over 2 hours which may have favored demetallation of metallothioneins and therefore solubilization of cadmium and mercury. Our study indicated the mechanical forces, changes in pH and volume of digesta and contents of substrate and enzymes caused by continuous gastric secretion and emptying are the important factors contributing to the different release values by dynamic model compared to static trials.

In Figure 3.6, the total phenolic content of digesta after the gastric phase of static and dynamic digestion is displayed. The total phenolic content was greatest in the dynamic digestion trials and least in the static digestion trial with pH of 3.0±0.5. The TPC of static trials increased as target pH decreased, but TPC of dynamic digestion was still more than double the amount of

the static trial with the highest TPC. Results from a recent study evaluating the biaoaccessibility of pistachio polyphenols show that release of phenolic acids increased with time during simulated dynamic gastric digestion which may be a result of the decreasing pH (Mandalari et al., 2013). Similarly, a published study compared five *in vitro* digestion models to study the bioaccessibility of soil contaminants (Oomen et al., 2002). The lowest bioaccessibility values were measured when the pH of gastric juice was 4.0, which was higher than other gastric juice pH values between 1 and 2. The relatively high pH was cited as the likely cause for the lower liberation of contaminations from the soil matrix. Similarly the higher pH of 3.0 in static peanut digestion trials may result in the lower release of phenolic content from the food matrix. More specifically, stronger enzymatic and acidic hydrolysis at low pH condition may have possibly promoted structure modification and breakdown of peanut particles facilitating release of nutrients and bioactives. The higher release of phenolics from dynamic trials is likely because of the more acidic conditions resulting from the constant secretion of gastric juice which assists in food digestion. Mechanical forces of the dynamic model may also contribute to the disintegration and breakdown of smaller particles which increases surface area exposed to digestive enzymes, and ultimately larger release of phenolic content.

In Figure 3.7, FRAP values of digesta after gastric phase of static and dynamic digestion are shown. Although the static trial with target pH of 3.0±0.5 had the highest FRAP value, there was no consistent relationship between FRAP and pH. When the pH of static trials dropped to 2.5, FRAP values decreased, but increased again when pH dropped to 2.0.However, FRAP values of dynamic digestion are lower than any other trial, possibly suggesting that acidic conditions decrease antioxidant properties. Results from a 2010 study evaluating *in vitro* bioaccessibility of grape polyphenols support the notion that as conditions become more

alkaline, as with the static trial of pH 3.0, antioxidant activity of digested compounds increases (Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010). Phenolic compounds are not stable in acidic environment and will lose antioxidant capacity at low pH.

The percentage of total soluble solids to dry solids is shown in Figure 3.8. As the pH of static digestion trials decreased, this ratio increased, and was the highest in the dynamic digestion trial. Forces exhibited in the DGSM likely contributed to the breakdown of smaller particles that were emptied from the gastric chamber and recovered as dry solids. This, in addition to the decreasing pH of the dynamic model may explain why the ratio of TSS: Dry Solids is greater than the ratio for static digestion trials. When evaluating static digestion trials, the decrease in pH likely contributed to the disintegration of solid particles over time. The acid and enzymes in the gastric juice enhance the release of solids due to hydrolysis and enzymatic reactions that reduce the cohesive forces that bond solid material within the food matrix (Kong, 2009).

Conclusion

Results from our study suggest that pH is the common factor influencing the release of nutrients and bioactives in both static and dynamic simulated digestion trials. A decrease in pH was strongly correlated to an increased release of soluble and dry solids, soluble protein and phenolic content in all trials. The acidic conditions encourage hydrolysis and enzymatic reactions that breakdown peanut particles and increase the release of nutrients and bioactives. Furthermore, simulated mechanical forces in dynamic digestion resulted in a higher release of peanut components compared to static trials. Although not as strong of a correlation, an increase in pH generally resulted in higher FRAP values among digestion trials as there is an increase of antioxidant activity in more alkaline conditions.

Increased utilization of *in vitro* digestion models have resulted in valuable contributions to the area of health and food science. Static models are easy to use and provide reproducible results while dynamic models include parameters such as stomach secretion and emptying rate and GI transit time which resemble true conditions of the human digestive system. *In vitro* models will never be able to fully replace *in vivo* experiments, but results show that both dynamic and static models can be sufficient alternatives when results from such models are carefully interpreted. To optimize *in vitro* static models, a series of experiments may need to be performed to determine the pH, ratio of substrate:enzyme and volume of digestive fluids to use in trials. Results from our study show that static trials performed in more acidic conditions (pH 2.0) best resemble conditions and results of dynamic digestion.

Despite recent advancements of static and dynamic models, they still fail to include aspects such as feedback mechanisms, resident microbiota, immune system, and specific hormonal controls. Continued research and developments needs to be made to further improve *in vitro* digestion models. When selecting the most appropriate digestion model to use, it is important to consider the advantages and disadvantages of each system, as well as the nutrient or bioactive to be released.

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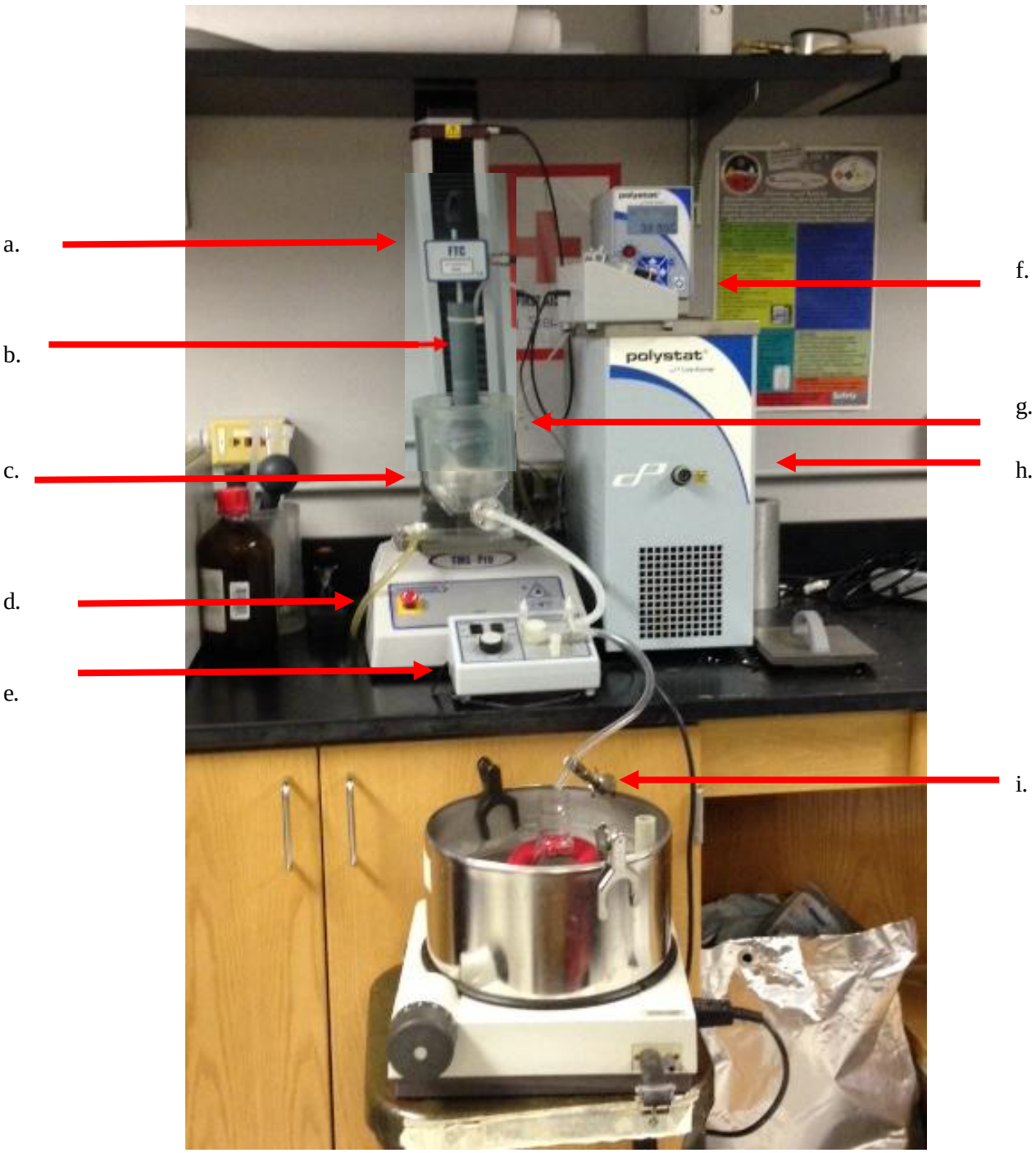

Figure 3.1. Dynamic Gastric Simulating Model: a) Texture analyzer, b) Probe, c) Gastric chamber, d) Water outlet, e) Emptying pump, f) Gastric secretion pump, g) Water inlet, h) Circulating water bath, i) Emptying tube.

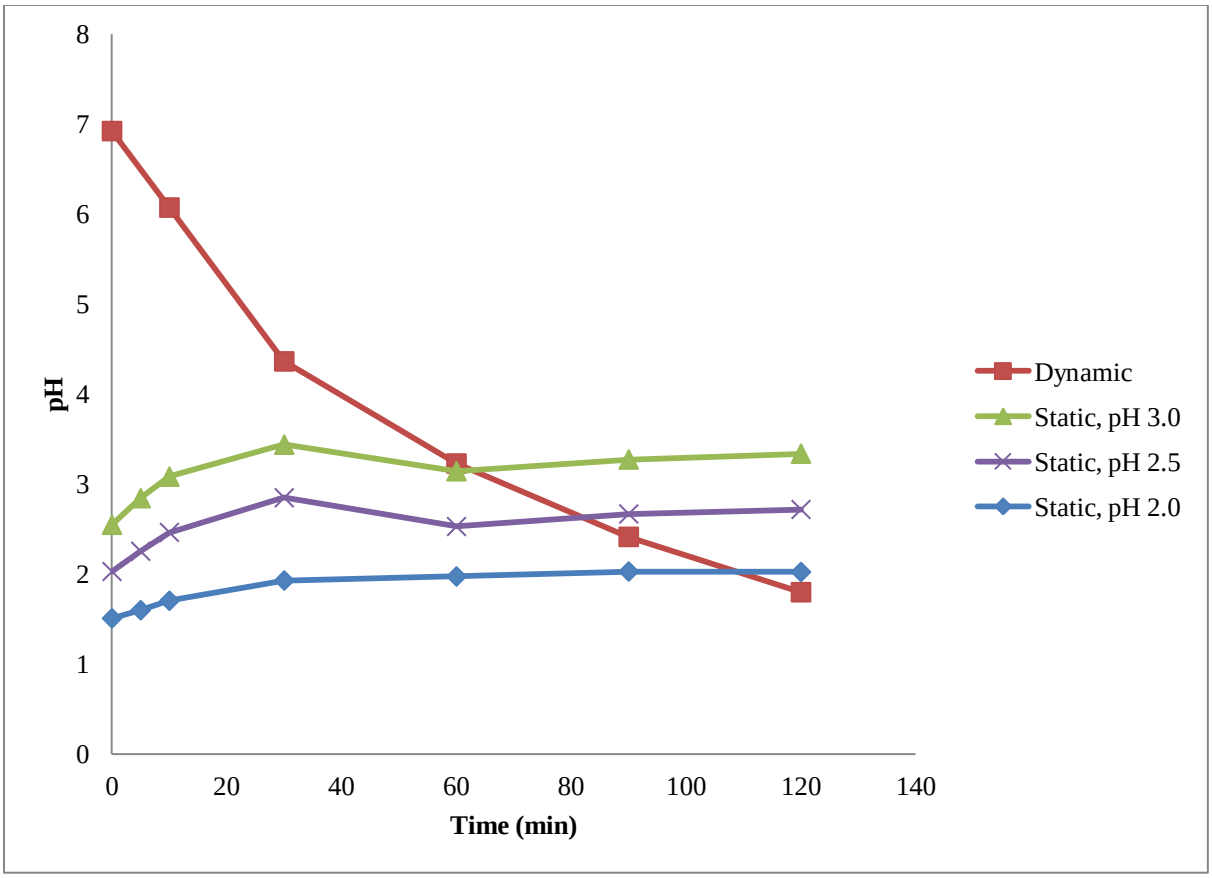

Figure 3.2. Changes in pH with time during gastric digestion in static and dynamic models.

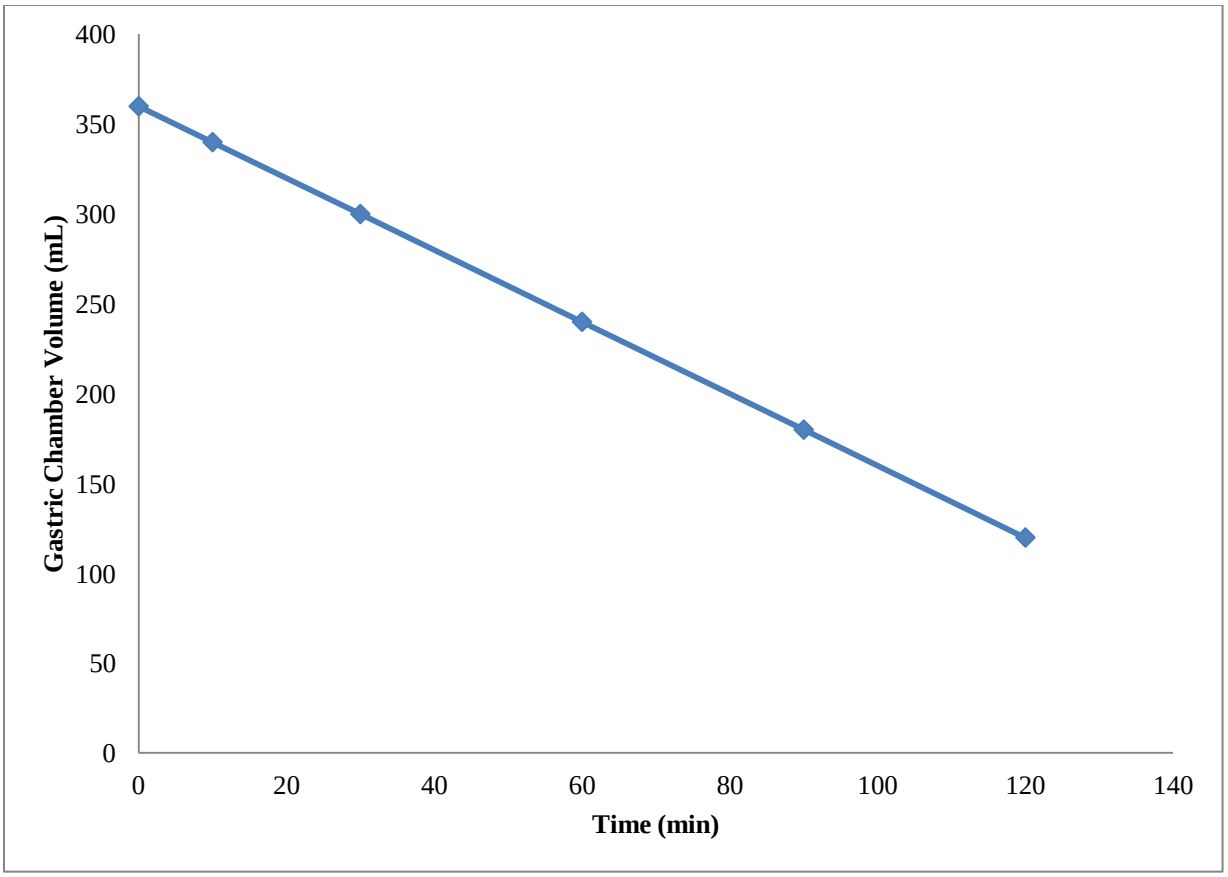

Figure 3.3. Changes in volume with time during gastric digestion using DGSM.

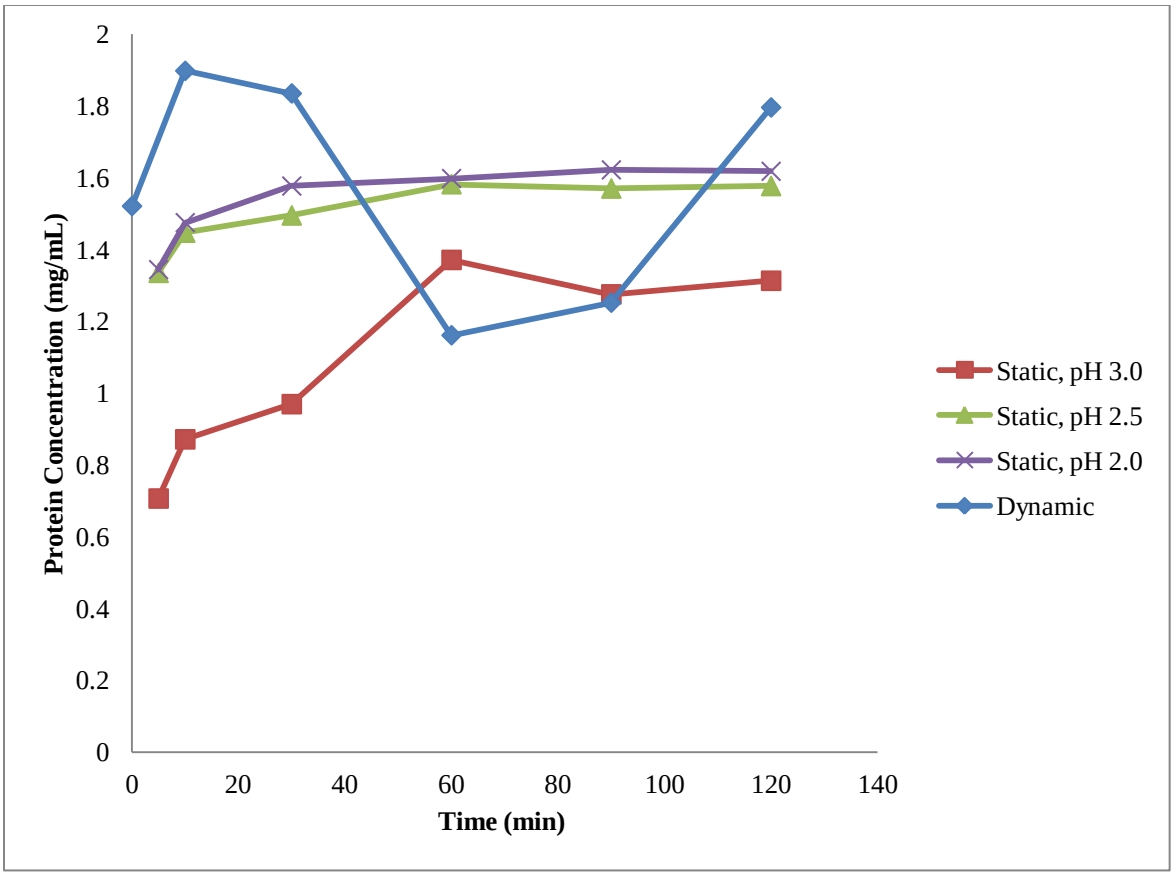

Figure 3.4. Soluble protein concentration during gastric digestion using static and dynamic models.

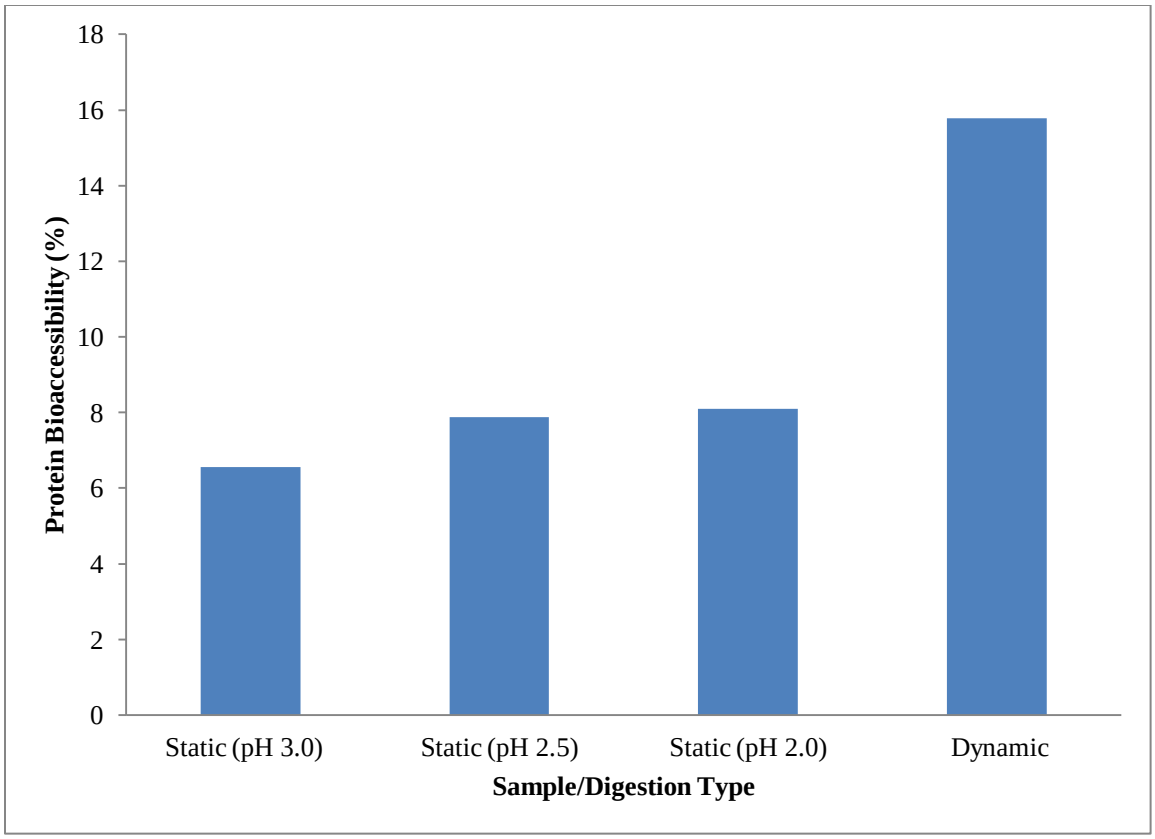

Figure 3.5. Protein bioaccessibility (%) after gastric phase of static and dynamic digestion.

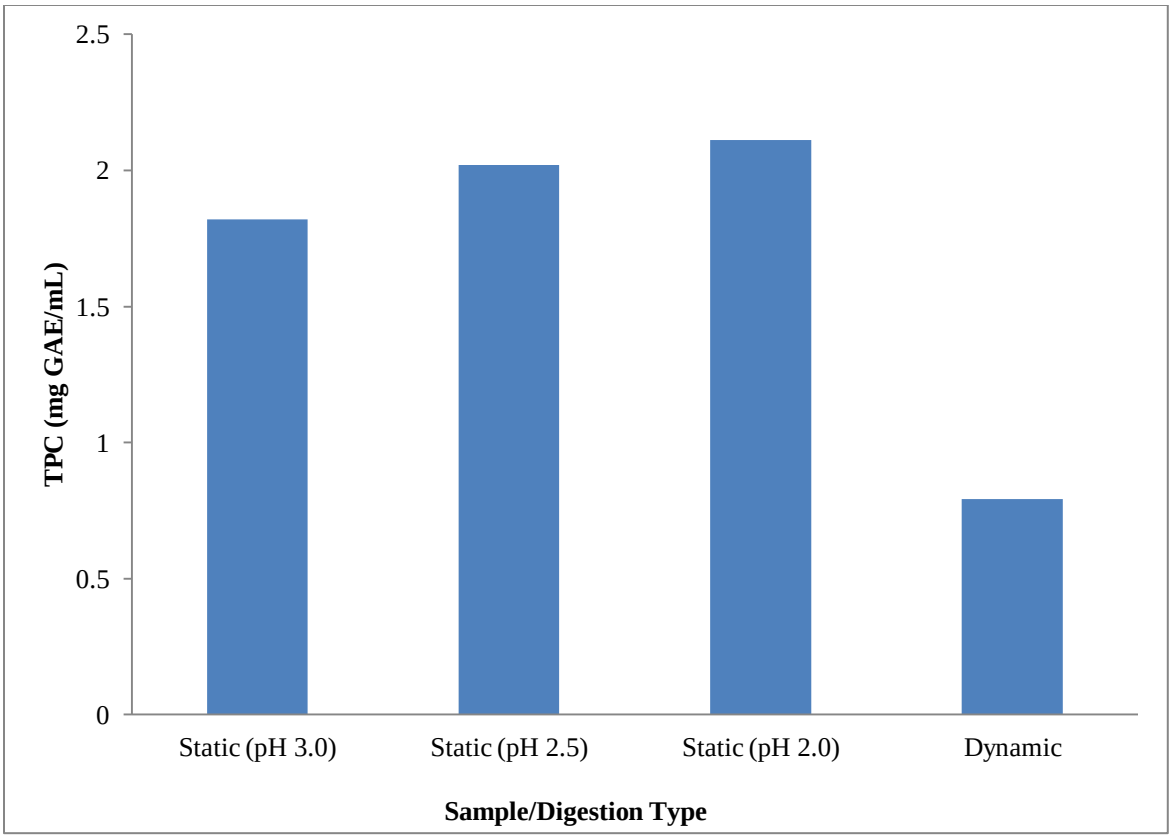

Figure 3.6.Total phenolic content of digesta after gastric phase of static and dynamic digestion.

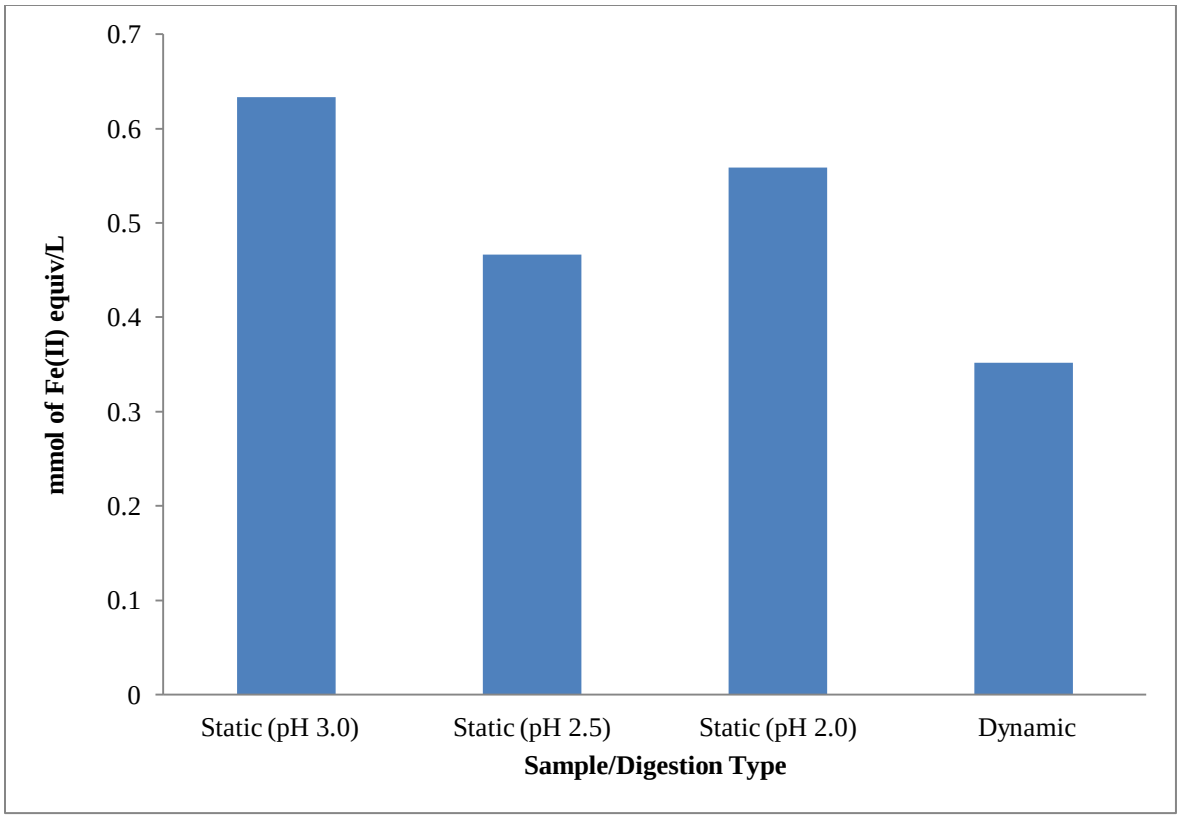

Figure 3.7.FRAP values of digesta after gastric phase of static and dynamic digestion.

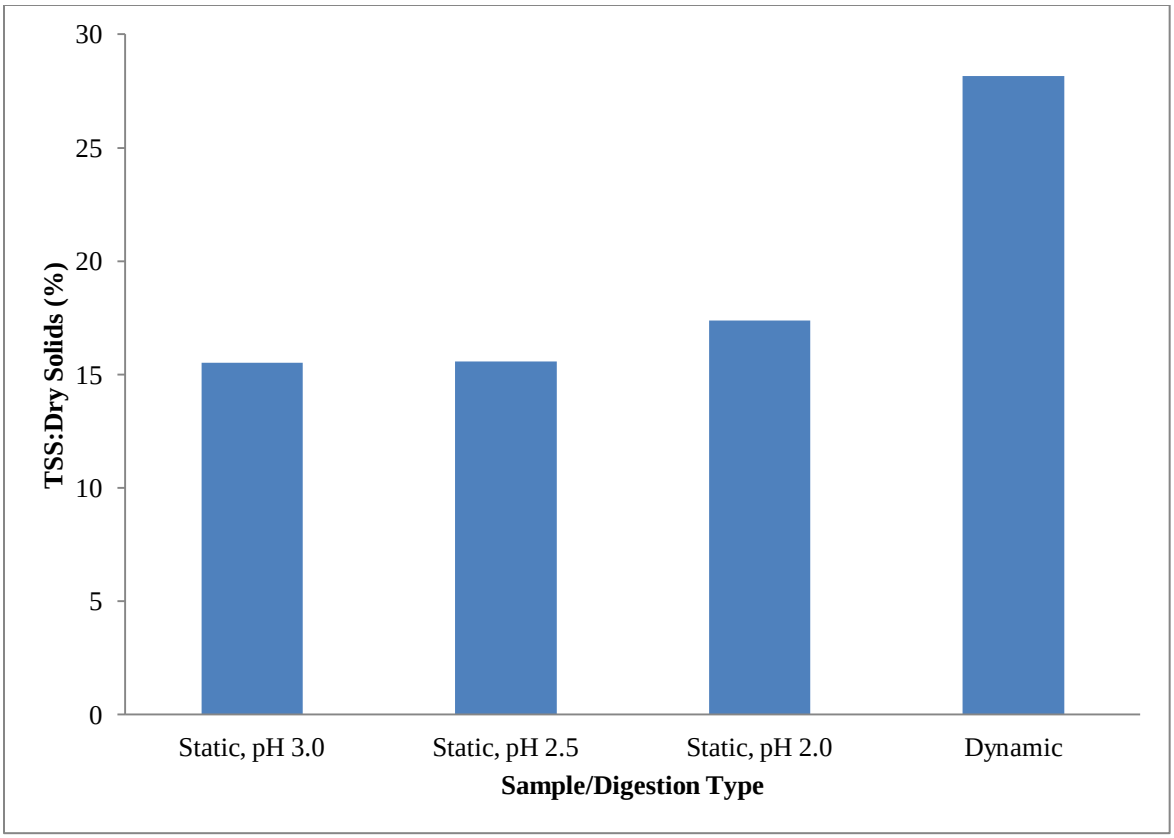

Figure 3.8.Total soluble solids to dry solids ratio after gastric digestion of static and dynamic digestion.

CHAPTER 5

OVERALL CONCLUSIONS

Results from this research allow for several conclusions regarding the release properties of peanuts during digestion to be made. Results from static digestion trials showed that particle size played a major role in peanut release properties during both gastric and intestinal phases of digestion. Smaller particles resulted in the greatest release properties. Despite different trends between phases, at the end of intestinal digestion there was a similar extent of release in raw and roasted peanuts for all parameters. Release properties of boiled peanuts were consistently lower than other samples. Employing the dynamic gastric model showed the highest protein bioaccessibility but not of other release properties such as TPC and FRAP, compared to when using the static digestion model. To optimize conditions of static digestion trials, a series of experiments needs to be done to determine conditions such as optimal pH and ratio of meal to enzyme. Additionally, when deciding the type of digestion model to use, careful considerations need to be made. Results from this research provide insight on peanut digestion, but may not be applicable for all other food samples.